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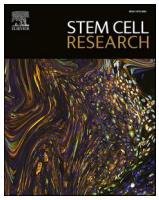

Lab Resource: Single Cell Line

Reprogramming of dermal fibroblasts from a Duchenne muscular dystrophy patient carrying a deletion of exons 45–50 into an induced pluripotent stem cell line (CCMi005-A)

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A B S T R A C T

Duchenne muscular dystrophy (DMD) is an X-linked syndrome that affects skeletal and cardiac muscle and is caused by mutation of the dystrophin gene. Induced pluripotent stem cells (iPSCs) were generated from dermal fibroblasts by electroporation with episomal vectors containing the reprogramming factors (OCT4, SOX2, LIN28, KLF4, and L-MYC). The donor carried an out-of-frame deletion of exons 45–50 of the dystrophin gene. The established iPSC line exhibited normal morphology, expressed pluripotency markers, had normal karyotype and possessed trilineage differentiation potential.

Resource Table:

Unique stem cell line identifier	CCMi005-A
Alternative name(s) of stem cell line	DMD4 C3
Institution	Centro Cardiologico Monzino-IRCCS
Contact information of distributor	Davide Rovina; davide.rovina@ccfm.it
Type of cell line	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 10 years old (at biopsy) Sex: Male Ethnicity if known: Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Associated disease	Duchenne Muscular Dystrophy
Gene/locus	*DMD* gene, Xp21.2-p21.1
Date archived/stock date	June 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/CCMi005-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, 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 No GTB12001 and GUP13013 respectively.

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1. Resource utility

This iPSC line carrying a *DMD*-causing mutation will be very useful in studying the pathophysiological mechanisms underlying dystrophin deficiency and discovering new therapeutic compounds.

2. Resource details

X-linked Duchenne muscular dystrophy is a neuromuscular disorder that affects both skeletal and cardiac muscle functions (D'Amario et al., 2018). Dystrophin localizes below the sarcolemma and links the actin cytoskeleton and plasma membrane to the extracellular matrix through the dystrophin-associated protein complex (DAPC) (Rovina et al., 2020). DMD is caused by mutations that lead to absence of full-length

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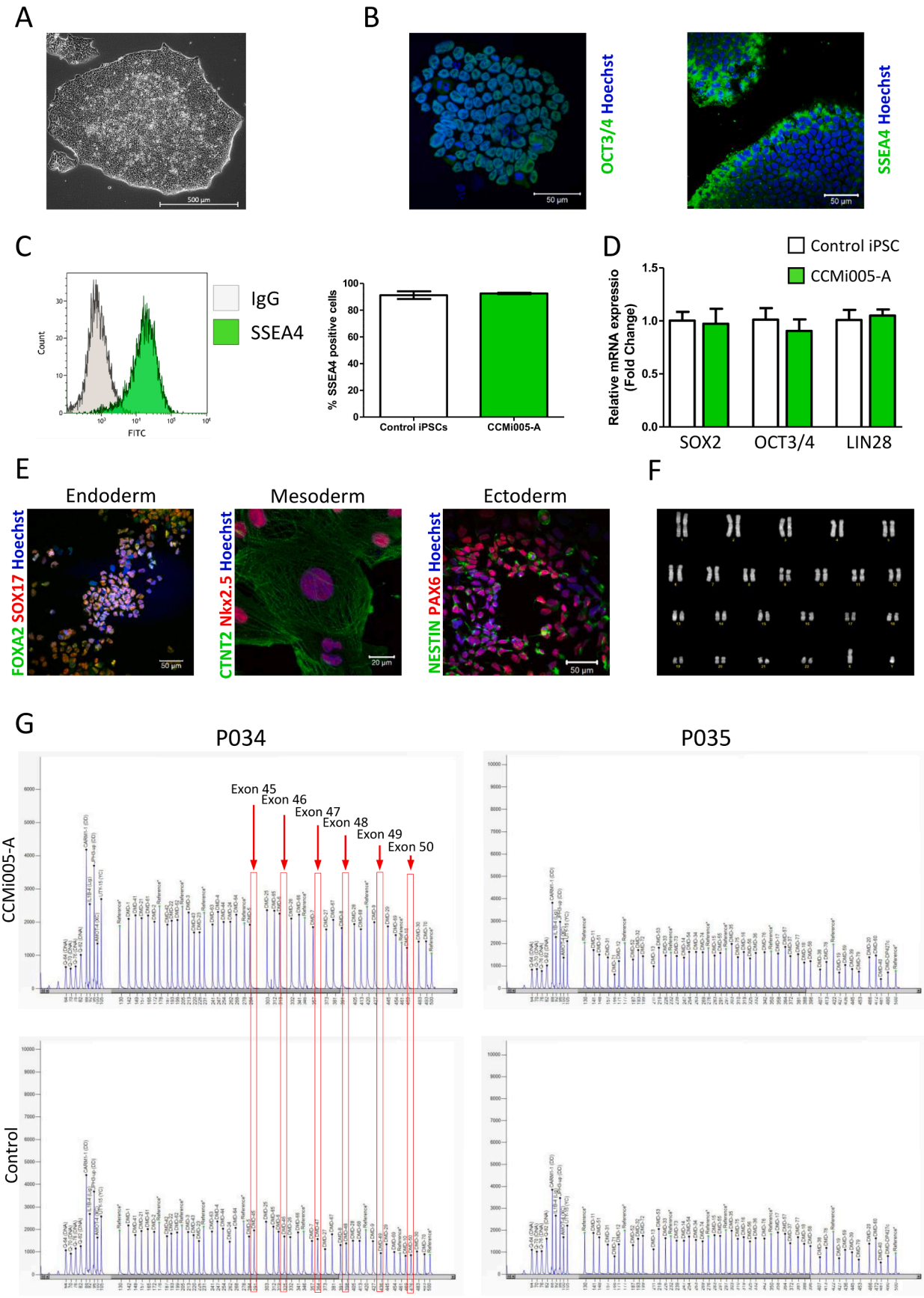

Fig. 1. Characterization of the CCMi005-A iPSC line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	*Normal*	Fig. 1*A*
Phenotype	Qualitative analysis: immunocytochemistry	*Staining pluripotency marker: Oct4 and SSEA4*	Fig. 1*B*
	Quantitative analysis: Flow cytometry	*Positive cells for cell surface marker SSEA-4 (92%)*	Fig. 1*C*
	Real Time-PCR	*mRNA expression level of pluripotency markers: SOX2, OCT3/4 and LIN28*	Fig. 1*D*
Genotype	Karyotype (Q-banding) and resolution	*46 XY* *Resolution < 400*	Fig. 1*F*
Identity	Microsatellite PCR (mPCR) OR STR analysis	*Not performed* *18/18 loci matched*	*N/A* *submitted in archive with journal*
Mutation analysis (IF APPLICABLE)	Sequencing MLPA	*N/A* *Detection of deleted exons 45, 46, 47, 48, 49 and 50 of the dystrophin gene*	*N/A* Fig. 1*G*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR; Negative*	*Supplementary Fig. 2*
Differentiation potential	Directed differentiation	*Determined the expression of markers for each of the three germ layers*	Fig. 1*E*
List of recommended germ layer markers	Expression of these markers demonstrated at protein (IF) levels.	*Ectoderm:* PAX6 and Nestin Endoderm: SOX17 and FOXA2. Mesoderm: CTNT2 and Nkx2.5	*Immunofluorescence (IF) with specific antibodies*
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*Not performed*	*N/A*
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	*Not performed* *Not performed*	*N/A* *N/A*

dystrophin protein (427 kDa). Dystrophin deficiency induces disruption of the DAPC and sarcolemma making myofibres more sensitive to mechanical damage and develop deregulated signalling pathways that activate cell death (Farini et al., 2019).

After institutional ethical committee approval and informed consent obtainment, fibroblasts were isolated from a skin biopsy of a 10-year-old male with DMD. No identifying patient information are included to preserve donor privacy. Subsequent to skin biopsy the donor lost ambulation at 12 years and developed clinical signs of cardiomyopathy including electrocardiographic abnormalities, left ventricular dysfunction and reduced ejection fraction.

Induced pluripotent stem cells (iPSCs) were generated by electroporation of skin fibroblasts with vectors encoding human L-MYC, LIN28, SOX2, KLF4, OCT3/4 and cultured under feeder-free conditions.

The iPSC line named CCMi005-A entered the characterization by analysing: iPSC pluripotent cell morphology (Fig. 1A) and expression of pluripotency marker OCT3/4 and SSEA4 (Fig. 1B). In addition, 92% of cells were positive for SSEA4 as observed by FACS analyses, with no significant difference compared to well-characterized iPSC cell lines derived from healthy individuals (Fig. 1C). Moreover, CCMi005-A expressed the pluripotency markers SOX2, OCT3/4 and LIN28 at levels comparable to the ones of a well characterised control iPSC line, as demonstrated by real time-PCR (Fig. 1D). CCMi005-A is able to differentiate *in vitro* into cells of the three germ layers as demonstrated by *in vitro* trilineage differentiation assays (endoderm: SOX17/FOXA2; mesoderm: cardiac troponin T type 2 CTNT2/Nkx2.5; and ectoderm: NESTIN\PAX6; Fig. 1E). Karyotype executed on approximately 20 metaphases, highlighted that CCMi005-A has a normal 46 XY karyotype (Fig. 1F). Multiple ligation probe amplification (MLPA) assay performed on genomic DNA extracted from CCMi005-A demonstrated the presence of a deletion of exons 45–50 of the dystrophin gene (Fig. 1G). Short Tandem Repeat (STR) analysis of 17 loci (LGC Standards and ATCC) demonstrated a match between the donor's primary fibroblasts and the derived CCMi005-A iPSC cell-line, with no match to any other cell line in the ATCC-STR database. The lack of genomic integration was verified by PCR (Supplementary Fig. 1). Mycoplasma-free iPSCs (Supplementary Fig. 2) were harvested and frozen in mFreSR™ (Stemcell Technologies) and transferred to long-term nitrogen storage.

3. Materials and methods

3.1. Reprogramming of fibroblasts

Donor dermal fibroblasts were electroporated (1650 V, 10 s, 3 pulse; Neon™, Invitrogen) with 1.25 μg of episomes (pCXLE-hUL, pCXLE-hSK, pCXLE-hOCT3/4-shp53-F; Addgene), transferred into one well of a 6-well plate pre-coated with human recombinant vitronectin (Life Technologies) and cultured at 37 °C with 5% CO_2. Following 3 days, cells were cultured in ReproTeSR™ (Stemcell Technologies) with daily media changes. When iPSC colonies reached approximately 1000 μm in diameter they were harvested by manual isolation and transferred into one well of a 12-well plate containing StemFlex™ (Gibco) with RevitaCell™ (Gibco). iPSCs cultured in StemFlex™, were non-enzymatically passaged with ReLeSR™ (Stemcell Technologies) when they reach 70–80% confluence (every 3–4 days), and plated on vitronectin (Life Technologies) coated plates as small aggregates suspended in StemFlex™ containing RevitaCell™. Stock vials of iPSCs were harvested in mFreSR™ (Stemcell Technologies) and stored in liquid nitrogen (Table 1).

3.2. Immunofluorescence

CCMi005-A at P14-18 were grown in vitronectin-coated chamber slides, fixed in 4% formaldehyde (10 min RT), permeabilized with 0.1% Triton-X 100 in PBS for 5 min and blocked with 3% BSA in PBS for 1 h. Antibodies were diluted in 0.3% BSA in PBS and incubated at 4 °C overnight (primary) and 1 h at RT (secondary) (Table 2). Nuclei were counterstained with Hoechst 33342 (1:500 in PBS; Invitrogen) for 15 min at RT. Slides were analysed with a confocal microscope (LSM710, Zeiss).

3.3. Flow cytometry

iPSCs were collected at P14 using ReLeSR™, washed using PBS/0.5 mM EDTA, fixed for 20 min on ice in BD Cytofix buffer (BD Biosciences) and stained with SSEA4 antibody (1 h, 4 °C) followed by a fluorescent secondary antibody (1 h, 4 °C). Antibodies were diluted in PBS containing 0.1% BSA/0.5 mM EDTA. CCMi005-A were analysed using a Gallios flow cytometer and Kaluza software (Beckman Coulter).

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-Oct4	1:250	Abcam Cat#ab19857	RRID: AB_445175
	Mouse anti-SSEA4	1:100 for FACS 1:200 for IF	Abcam Cat# ab16287	RRID: AB_778073
Differentiation Markers	Rabbit anti-SOX17	1:300	Cell Signaling Inc. Cat# 81778	RRID: AB_2650582
	Mouse anti-FOXA2	1:200	Abcam Cat# ab60721	RRID: AB_941632
	Mouse anti-cardiac troponin type 2 (CTNT2)	1:300	Thermo Fisher Scientific Cat# MA-512960	RRID: AB_11000742
	Rabbit anti-Nkx2.5	1:200	Thermo Fisher Scientific Cat# PA5-49431	RRID: AB_2634885
	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) 1:300 (for NESTIN) 1:200 (for CTNT2)	Thermo Fisher Scientific Cat# A11059	RRID: AB_2534106
	Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:400 (for Oct4)	Thermo Fisher Scientific Cat# A11034	RRID: AB_2576217
	Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa®Fluor 594	1:300 (for PAX6)	Thermo Fisher Scientific Cat# A11037	RRID: AB_2534095
	Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 546	1:300 (for Sox17)	Thermo Fisher Scientific Cat# A11010	RRID: AB_2534077
	Primers			
	Target	Size of band	Forward/Reverse primer (5′-3′)	
Episomal plasmid (PCR)	L-MYC	122 bp	GGCTGAGAAGAGGATGGCTAC/ TTTGTTTGACAGGAGCGACAAT	
Episomal plasmid (PCR)	LIN28	251 bp	AGCCATATGGTAGCCTCATGTCCGC/ TAGCGTAAAAGGAGCAACATAG	
Episomal plasmid (PCR)	SOX2	111 bp	TTCACATGTCCCAGCACTACCAGA/ TTTGTTTGACAGGAGCGACAAT	
Episomal plasmid (PCR)	KLF4	156 bp	CCACCTCGCCTTACACATGAAGA/ TAGCGTAAAAGGAGCAACATAG	
Episomal plasmid (PCR)	OCT3/4	124 bp	CATTCAAACTGAGGTAAGGG/ TAGCGTAAAAGGAGCAACATAG	
Pluripotency Markers (qPCR)	SOX2	151 bp	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
Pluripotency Markers (qPCR)	OCT3/4	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
Pluripotency Markers (qPCR)	LIN28	129 bp	AGCCATATGGTAGCCTCATGTCCGC/ TCAATTCTGTGCCTCCGGGAGCAGGGTAGG	
House-Keeping Genes (qPCR)	GAPDH	89 bp	CCACCCATGGCAAATTCC/ TCGCTCCTGGAAGATGGTG	

3.4. RNA extraction, retro-transcription and real time-PCR

RNA was extracted at P18 using Total RNA Purification Kit (Norgen Biotek Corp) according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher). cDNA was amplified for SOX2, OCT3/4, LIN28 and GAPDH as housekeeping gene using iTaq™ Universal SYBR® Green Supermix (Biorad). The relative quantification of the pluripotency genes was determined with the $2^{-\Delta\Delta Ct}$ method. The primers used are reported in Table 2.

3.5. Trilineage differentiation potential assay

To differentiate iPSCs at P15 into cells of ectodermal or endodermal lineage, the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) was used according to the manufacturer's instructions. For mesodermal differentiation, cells were treated with small molecules targeting the Wnt pathway to differentiate into cardiomyocytes according to Lian et al. (2013). Differentiated cells were stained with specific antibodies (Table 2) and observed using confocal microscopy (LSM710, Zeiss).

3.6. DNA isolation, MLPA and PCR

Genomic DNA was extracted at passage 13, using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

MPLA was performed with the SALSA MLPA probe sets P034 and P035 (MRC Holland), products were run on the ABI PRISM 3130 genetic analyser (Applied Biosystems) and the data were analysed using Coffalyser software (MRC Holland).

The lack of genomic integration was verified by PCR using Go Taq G2 Flexi DNA Polymerase (Promega) and the primers reported in Table 2. Amplicons were visualised on 2% agarose gel.

3.7. Karyotyping

Cells at P12 treated with colcemid (10 μg/ml) overnight at 37 °C (5% CO_2, 95% rH) and with 0.05% Tryspin-EDTA, were incubated in hypotonic solution (KCl 0.56%, 6 min, RT), washed for 3 min with acetic acid 5% and fixed with methanol/acetic acid (3:1). Q-banded metaphases were photographed at 100× (Leica) and karyotyped using CytoVision software (Leica).

3.8. STR

STR was performed by the ATCC cell-line authentication service. Seventeen loci and a gender-determining locus, were amplified using the PowerPlex® 18D Kit (Promega). Samples were processed using ABI Prism® 3500xl genetic analyzer. Data were analysed with GeneMapper® ID-X v1.2 software (Applied Biosystems).

3.9. Mycoplasma analyses

Mycoplasma absence was verified using the EZ-PCR Mycoplasma Detection Kit (Biological Industries) on cells at P18 according to the manufacturer's instructions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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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.2022.102889.

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