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 R T I C L E  I N F O
Article history:
Received 4 April 2017
Received in revised form 17 October 2017
Accepted 26 October 2017
Available online 28 October 2017

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

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<th>Unique stem cell line identifier</th>
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<tr>
<td>Institution</td>
<td>Centro Cardiologico Monzino-IRCCS</td>
</tr>
<tr>
<td>Contact information of distributor</td>
<td>Aoife Gowran, <a href="mailto:agowran@ccfm.it">agowran@ccfm.it</a></td>
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<td>Method of reprogramming</td>
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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

https://doi.org/10.1016/j.scr.2017.10.018
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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](image_url)
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 in mTeSR1™ media with daily media changes. At 80–90% confluency, iPSCs were non-enzymatically passaged (every 3–4 days) with 0.25% trypsin, 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.

3.2. 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%), transferred into a single well of a 6 well plate pre-coated with Optichrome (28 °C, 42% RH) with methanol/acetic acid (3:1), Q-banded and photographed. Karyotype images were obtained at 100× magnification (Olympus BX...
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.

Acknowledgements

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

Appendix A. Supplementary material

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

References

Lian, X., et al., 2013. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. Nat. Protoc. 8 (1), 162–175.