Human iPSCs-derived muscle cells as a new model for investigation of EDMD1 pathogenesis.

Authors: Marta Lisowska[#1], Marta Rowińska[#1], Aleksandra Suszyńska[1], Claudia Bearzi[2], Izabela Łaczmańska[3], Julia Hanusek[1], Amanda Kunik[1], Volha Dzianisava[1], Ryszard Rzepecki[1], Magdalena Machowska[1†], Katarzyna Piekarowicz[1†]

Corresponding Author**:** Katarzyna Piekarowicz, katarzyna.piekarowicz@uwr.edu.pl
[#]first joint Authors
[†]last joint Authors

Affiliations:

1 Laboratory of Nuclear Proteins, Faculty of Biotechnology, University of Wroclaw, Joliot-Curie 14a, 50-383 Wrocław, Poland
2 Institute for Biomedical Technologies, National Research Council, Via Fratelli Cervi, 93, 20054, Segrate, Milan, Italy
3 Department of Genetics, Wroclaw Medical University, Marcinkowskiego 1, 50-368 Wroclaw, Poland

Abstract

Emery-Dreifuss muscular dystrophy type 1 (EDMD1) is a rare genetic disease caused by mutations in the *EMD* gene, which encodes the nuclear envelope protein emerin. Despite understanding the genetic basis of the disease, the molecular mechanism underlying muscle and cardiac pathogenesis remain elusive. The scarcity of skeletal muscle cells hampers progress, therefore there is an urgent need for innovative, human-specific cellular models.

In this study, we present the generation and characterisation of induced pluripotent stem cell (iPSC) lines derived from EDMD1 patients carrying *EMD* mutations that lead to truncated or absent emerin, together with iPSCs from healthy control. The patient-specific iPSCs exhibit stable karyotype, maintain appropriate morphology, express pluripotency markers and demonstrate the ability to differentiate into three germ layers.

To model EDMD1, these iPSCs were differentiated into myogenic progenitors, myoblasts and multinucleated myotubes, which represent all stages of myogenesis. Each developmental stage was validated by the presence of specific phenotypic features and stage-specific marker expression, ensuring the accuracy of the model. We present the first iPSC-based *in vitro* platform that captures the complexity of EDMD1 pathogenesis during myogenesis. This model can significantly contribute to understanding disease mechanisms, and develop the targeted therapeutic strategies for EDMD1.

Keywords: emerin, *EMD* mutation, Emery-Dreifuss muscular dystrophy, skeletal muscles, stem cells, disease modelling, differentiation to skeletal muscle cells in vitro.

Introduction

Emery-Dreifuss muscular dystrophy type 1 (EDMD1, OMIM #310300) is a rare genetic disorder with a prevalence of 1 per 100,000 male births. It is an X-linked recessive disease caused by mutations in the *EMD* gene (*locus Xq28*) coding for nuclear envelope (NE) protein emerin[1]. The main symptoms of EDMD1 are observed in the skeletal muscles and heart, including muscle weakness and wasting, tendon contractures, and cardiac dysfunction[2]. The disease is incurable, with a substantial risk of sudden death in middle age caused by heart block. Understanding the still not well-known disease mechanisms is fundamental for therapy design, development, and evaluation.

Emerin is a transmembrane protein with a LEM domain that interacts with various partners in cell nucleus, including the highly conserved DNA-binding protein BAF (Barrier-to-Autointegration Factor), facilitating DNA tethering to NE. Emerin plays an important role for nuclear stability, gene regulation, chromatin organization, and cell cycle [3-5]. It is especially significant in muscle cells, where emerin is essential for muscle development and maintenance [6-8]. The absence or misexpression of emerin leads to muscle degeneration. Furthermore, emerin together with other NE protein lamin A/C appears critical for muscle cells differentiation [9]. Dysfunction of satellite cells (SCs), the progenitors of muscle cells, has also been hypothesized to contribute significantly to the progression of EDMD by impairing myofiber repair and regeneration [10]. Despite this, the role of emerin in precursor muscle cells remains poorly understood. One major limitation is the lack of appropriate models for study. Not enough data have been acquired with human, patient-derived muscle precursor cells, with investigation of patients-derived SCs mostly restricted to microscopic analysis of rare muscle biopsies [11-13].

The function and regulation of emerin are species-dependent, limiting the utility of mouse models and non-human cells. While emerin-null mice exhibit only mild phenotypic changes such as delayed skeletal muscle regeneration and repair, mild atrioventricular alterations, and motor coordination defects [14, 15], humans with emerin deficiency develop muscular dystrophy. Additionally, mutation-related phenotype in humans significantly depends on genotype background [16-18]. Human muscular *in vitro* models of EDMD1 are missing, and the availability of skeletal muscle cells and cardiomyocytes, which may serve as a model system for EDMD1 background investigations, is very limited. It is possible to preserve patient-specific genetic background using approach basing on MyoD overexpression in patient's fibroblasts, which was already applied in laminopathies research [19], but this model does not allow for studying myogenic precursors, as well as MyoD-related signaling pathways may be influenced. Therefore, patient-specific induced pluripotent stem cells (iPSCs) offer a promising alternative. Differentiated muscle cells derived from iPSCs could serve as a key model for elucidating disease mechanisms and the development of targeted therapies.

iPSCs can be differentiated into striated muscle progenitors and subsequently into myoblasts and multinucleated myotubes using a transgene-free protocol [20]. This approach enables the generation of unlimited material for research, facilitating experimental repeatability. Importantly, as the starting material is derived from patients, the model retains the patient-specific genetic background, providing a physiologically relevant system for studying disease mechanisms. The initial step of iPSCs differentiation procedure produces muscle progenitors, also called SCs. However, this method does not allow for obtaining separately quiescent and activated SC populations. Single-cell transcriptomic analyses of muscle progenitors obtained *via* iPSCs differentiation using various

chemical methods revealed that their expression profile closely resemble those of embryonic skeletal muscle progenitor cells (SMPCs) while also sharing substantial similarity with adult muscle SC population [21]. SCs are typically characterized by the expression of the transcription factors Pax3 and Pax7, which serve as key markers of this cell population.

iPSC-derived myogenic progenitors can be further differentiated to myoblasts, followed by differentiation to myocytes and the formation of multinucleated myotubes, which serve as an *in vitro* model for mature skeletal muscle cells. The differentiation process is closely associated with changes in transcriptional profiles. Myoblasts are usually characterized by the expression of transcription factors MyoD and Myf5, while typical markers of myotubes are myosin heavy chain (MHC), actinin, titin or elevated desmin level.

In this study, we generated and characterized five new iPSC lines from fibroblasts obtained from EDMD1 patients and a healthy donor. These included one clone from a patient bearing an *EMD* mutation c.153del and two clones from a patient with c.451dup mutation, both causing a frameshift and creation of a premature termination codon, resulting in the lack of protein or shorter protein, respectively.

Subsequently, we differentiated the newly established clones, along with four iPSC clones previously generated by reprogramming fibroblasts from two EDMD1 patients with c.153del mutation [22], into skeletal muscle cells. Using a transgene-free differentiation method, we successfully obtained cells representing three stages of skeletal muscle development: myogenic progenitors, myoblasts and myotubes. The differentiated muscle cells expressed proper stage markers. Additionally, we obtained myotubes, the final stage of *in vitro* muscle differentiation, showing sarcomere structure and multiple nuclei per cell, hallmark features of mature skeletal muscle cells.

This work establishes a novel, robust model of EDMD1, providing a valuable platform for investigating disease pathogenesis and developing targeted therapies. Furthermore, the generated iPSCs may also be used in the future for the generation of cardiac and nerve cells, as well as for isogenic controls preparation which could extend the platform.

Materials and Methods

iPSCs generation and expansion

Fibroblasts obtained from skin biopsies were cultured in a fibroblast medium containing DMEM with high glucose 4,5 g/L (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM Glutamax (Gibco) and penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco). For reprogramming, fibroblasts were plated on 35 mm dishes for 2 days and then transduced with a set of non-integrating Sendai viruses using the CytoTune 2.0 Sendai reprogramming kit (Invitrogen) according to the manufacturer's instructions. The medium was changed daily with the fresh fibroblast medium. On day 7, cells were plated on new dishes coated with Geltrex (Gibco). Starting the following day, the fibroblast medium was replaced with Essential 8 Medium (Gibco) every day. After 3-4 weeks individual iPSC colonies were manually picked and transferred to new Geltrex-coated 24-well plates. iPSCs were expanded in Essential 8 Medium on Geltrex-coated dishes, they were dissociated with 0.5 mM EDTA in DPBS and split every 4-5 days at a ratio 1:8-1:12 using Revitacell Supplement (Gibco). Fibroblasts and early passage iPSCs were cultured at 37°C in 5% CO_2 and frozen at passage 4-5.

Cells were expanded until at least passage 10 in 10% O_2, 37°C, 5% CO_2 and characterized.

Immunofluorescence (IF)

iPSCs were seeded on Geltrex-coated glass coverslips and cultured for 3-4 days until proper-sized colonies were formed. Then they were fixed in 4% paraformaldehyde for 15 minutes, washed with PBS, permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature (RT) and washed again with PBS. Preparations were blocked for 30 minutes at RT with 1% donkey serum (DS, Gibco) or 1% FBS (Gibco) in PBS for germ layer markers and other stainings, respectively. Then preparations were incubated with primary unconjugated antibodies overnight at 4°C, washed with PBS and incubated with secondary antibodies or primary antibodies conjugated with a fluorophore for 1 h at RT, and washed again with PBS. Antibodies for iPSC and germ layer markers were diluted in 1% DS in PBS (antibodies and dilutions used shown in Table 1). Coverslips were mounted on glass slides with the DABCO mounting medium (Fluka) with DAPI. Staining was visualized with a Zeiss Axiovert A1 fluorescence microscope using a 10x objective, using ZEN Software.

To obtain myotubes specimens, myoblasts were seeded on Geltrex-coated glass coverslips and cultured for 3-4 days until reaching 100% confluency. Then the medium was changed for DMEM-HS. After 4 days cells were fixed in 4% paraformaldehyde for 15 minutes, then washed with PBS, permeabilized with 0.5% Triton X-100 for 5 minutes at RT and washed again with PBS. Preparations were blocked with 1% FBS in PBS for 45 minutes at RT, then incubated with primary unconjugated antibodies overnight at 4°C, washed with PBS and incubated with secondary antibodies conjugated with a fluorophore for 1 h at RT, and washed again with PBS. Antibodies for muscle markers were diluted in 1% FBS in PBS (antibodies and dilutions shown in Table 1). Coverslips were mounted on glass slides with the DABCO mounting medium with DAPI. Staining was visualized with a Stellaris (Leica) microscope using a 63x objective.

Table 1: Antibodies used for immunofluorescence (IF) and western blotting (WB) staining.

	Antibody	**Dilution**	**Company, cat#**
Pluripotency Markers	Rabbit anti Oct4	1:100 IF / 1:1000 WB	Cell Signalling Technology cat# 2750
	Mouse anti SSEA4 DyLight550	1:100 IF	Invitrogen cat# MA1-021-D550
iPSCs differentiation markers	Goat anti SOX17	1:10 IF	R&D Systems cat# AF1924
	Goat anti Otx2	1:10 IF	R&D Systems cat# AF1979
	Goat anti Brachyury	1:10 IF	R&D Systems cat# AF2085
Skeletal muscle markers and reference	Mouse anti Pax3	1:1000 WB	DSHB clone C2 [D1]
	Mouse anti Pax7	1:200 WB (milk)	DSHB [D2]
	Rabbit anti Myf5	1:2500 WB (milk)	Abcam cat# ab125078
	Mouse anti MHC	1:10 IF / 1:500 WB	DSHB clone MF-20 [D3]
	Mouse anti desmin	1:2500 WB	SigmaAldrich cat# SAB4200707
	Mouse anti actinin	1:2000 WB (milk)	SigmaAldrich cat# A7811
	Mouse anti myogenin	1:50 WB	DSHB clone F5D [D4]
	Rabbit anti GAPDH	1:10000 WB (milk)	SigmaAldrich cat# G9545
	Mouse anti actin α	1:1000 WB	SigmaAldrich cat# A2228
	Mouse anti tubulin α	1:1000 WB (milk)	DSHB clone 12G10 [D5]
	Rabbit anti emerin	1:1000 WB (milk)	Cell Signalling Technology cat# 30853
	Rabbit anti lamin A/C	1:4000 WB (milk)	Cell Signalling Technology cat# 2032S
	Rabbit anti lamin B1	1:100 IF / 1:500 WB	Proteintech cat# 12987-1-AP
Secondary antibodies	Donkey anti-goat AF568	1:200 IF	Invitrogen cat# A11057
	Donkey anti-rabbit TRITC	1:50 IF	Jackson Immunoresearch cat# 711-025-152

	Donkey anti-rabbit AF488 Goat anti-rabbit HRP Donkey anti mouse HRP	1:200 IF 1:10000 WB 1:10000 WB	Jackson Immunoresearch cat# 711-545-152 Jackson Immunoresearch cat#111-035-144 Jackson Immunoresearch cat# 715-035-151

Antibodies deposited to the Developmental Studies Hybridoma Bank (DSHB) by [D1] C.P. Ordahl, [D2] A. Kawakami, [D3] D.A. Fischman, [D4] W.E. Wright and J. Frankel / E.M. Nelsen.

Gene expression analysis

Total RNA was extracted from iPSCs using Universal DNA/RNA/Protein Purification Kit (EurX). 1 µg of total RNA was reverse transcribed to cDNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Real time quantitative PCR (qPCR) was performed using SYBR Green Master Mix (Applied Biosystems) and Quant Studio 5 Real-Time PCR System (Applied Biosystems); all samples were analyzed in four technical replicates. The reaction parameters were as follows: 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 64°C for 30 seconds. The reaction ended with 3 steps of the melt curve (95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds). Pluripotency markers' relative gene expression was normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) and expressed relatively to an established iPSCs line E1M40 1.7 (UWRBTi003-B [22]). The sequences of the designed primers are listed in Table 2.

For the analysis of muscle markers, the total RNA was extracted using Direct-zol RNA Microprep Kit. 500 ng of the isolated RNA was reverse transcribed as described above. qPCR was performed as described with samples being analyzed in two biological repeats, each in three technical repeats. Relative gene expression was normalized to *GAPDH* and Hypoxanthine phosphoribosyltransferase (*HPRT*). Primers' sequences are presented in Table 2.

Table 2. Primers

List of all primes used for qPCR, EMD sequencing and episomal vector presence detection.

Primers		
	Target	**Forward/Reverse primer (5'-3')**
Episomal Plasmids (PCR)	SeV (genomic sequence of Sendai virus) *KOS* (Human *KLF4*, OCT3/4, SOX2) *KLF4* *c-MYC*	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG
Pluripotency Markers (qPCR)	*NANOG* *OCT4* *SOX2* *LIN28* *hTERT* *REX-1*	CCTCCAGCAGATGCAAGAACTC/ CCTTCTGCGTCACACCATTGCTA AGT TTG TGC CAG GGT TTT TG/ ACT TCA CCT TCC CTC CAA CC TGCACATGAAGGAGCACCCG/GCTCGCCATGCTATTGCCGC GAGGCAGTGGAGTTCACCTTTAAG/ GATCTGCGCTTCTGCATGCTC GGAGCAAGTTGCAAAGCATTG/ TCCCACGACGTAGTCCATGTT GCTGAAACAAATGTACTGAGGCTG/ GCTGTCTTCAGCAAACACCTGC
Skeletal muscle markers (qPCR)	*PAX3* *PAX7* *MYF5* *MYOD*	CAAGCCCAAGCAGGTGACAAC/CACAGACCGCGTCCTTGAGTAA TTTGCCGACTTTGGATTCGTCCC/GGTGGACACTTCCAAAGGGAATC ATCGAGAGCCTGCAGGAGTTG/TACATTCGGGCATGCCATCAGAG GACGGCATGATGGACTACAGC/CGCCTCGTTGTAGTAGGCGC
House-Keeping Genes (qPCR)	*GAPDH* for qPCR *GAPDH* for PCR	CGGAGTCAACGGATTTGGTC/ CATGTAAACCATGTAGTTGAGGTC *CGGAGTCAACGGATTTGGTC/CATGTAAACCATGTAGTTGAGGTC*

	HPRT for qPCR	CCCTGGCGTCGTGATTAGTG/GCAAGACGTTCAGTCCTGTCC
Targeted mutation sequencing	*EMD* gene amplification Amplicon sequencing	CTCCCGCGGTTAGGTCCCG/TTCCCCAAAGACCTAGCTCTG CTCCCGCGGTTAGGTCC (for mutation c.153del) CATGACAGGGCCATGGTGG (for mutation c.451dup)

The episomal vectors' presence

Total RNA was isolated from iPSCs using Universal DNA/RNA/Protein Purification Kit (EurX). cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). PCR analysis of episomal vectors presence was performed using 5 pairs of primers shown in Table 2 (four pairs recognizing reprogramming vectors (sequences from CytoTune-iPS 2.0 Sendai Reprogramming Kit) and 1 pair for *GAPDH* as control). Reactions were performed with Taq polymerase (EURx), with 35 cycles of amplification using thermocycler TOne 96G (Biometra) with the following parameters: 95°C for 5 minutes, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 55°C (*c-MYC, GAPDH, SeV, KOS*) or 63°C (*KLF4*) for 30 s, and extension at 72°C for 30 s and 5 minutes final extension at 72°C. Normal human dermal fibroblasts (Lonza) were used as a negative control, cells collected shortly after Sendai virus transduction were used as a positive control.

Karyotyping

iPSC lines and fibroblasts were treated with 0.67 µg/mL colcemid (BioWest) for 2,5 hours, then dissociated by Trypsin-EDTA (Gibco) and centrifuged at 1400 rpm, 7 minutes. After incubation in hypotonic solution (Ohnuki's solution) and fixation in methanol:glacial acetic acid (3:1) mixture (Chempur), microscopic slides were prepared. G-banded metaphase analysis was performed according to the International System for Human Cytogenomic Nomenclature 2020 (ISCN 2020; AGT manual [23, 24]) employing the Imager.M1 (Zeiss) microscope and the Ikaros software (version 6.3, Metasystems DE). Up to 30 metaphases from iPSCs (resolution 400-450 bands) and at least 15 metaphases from fibroblasts (resolution 350-400) were karyotyped by an experienced cytogeneticist.

STR Analysis

The profiling of the human cell lines was performed by Microsynth and Eurofins companies using highly polymorphic short tandem repeats loci (STRs). STR loci were amplified using the PowerPlex 16 HS System (Promega). Fragment analysis was done on an ABI3730xl (Life Technologies) and the resulting data were analyzed with GeneMarker HID software (Softgenetics).

Eurofins performs genotyping according to ANSI/ATCC standard ASN-0002 testing 16 DNA markers using the Applied Biosystems AmpFLSTR Identifiler Plus PCR Amplification Kit system.

Sequencing

Genomic DNA was isolated using Universal DNA/RNA/Protein Purification Kit (EurX), then *EMD* gene was amplified with Phusion HSII DNA Polymerase (Thermo Scientific), with GC buffer and 3% DMSO and primers listed in Table 2 (previously published [16]). Reactions were performed with 35 cycles of amplification using thermocycler TOne 96G (Biometra) with the following parameters: 98°C for 5 minutes, followed by 34 cycles of denaturing at 98°C for 15 s, annealing at 62°C for 30 s, and extension at 72°C for 90 s and 10 minutes final extension at 72°C. PCR product was purified with PCR/DNA Clean-

Up Purification Kit (EurX) and sequenced by Microsynth company with sequencing primers shown in Table 2.

Mycoplasma test

Mycoplasma PCR test was performed on culture supernatants (at least 48h without medium change and confluence at least 80%) using Kit Mycoplasma PCR Detection Kit (Abcam) according to the manufacturer's instructions.

In vitro differentiation to three germ layers

In vitro trilineage differentiation was carried out using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems) according to manufacturer's instructions. Briefly, iPSCs were trypsinized and seeded on Geltrex-coated coverslips in a 24-well plate, at a density of approximately $9*10^4$ cells per well in Essential 8 medium with Revitacell Supplement (Gibco).

After obtaining 60-70% confluence for mesoderm and 70-80% for ectoderm and endoderm (24h/48h), E8 medium was replaced with proper differentiation medium (for endoderm, ectoderm or mesoderm) and after 40 h for mesoderm and 72 h for ectoderm and endoderm coverslips were fixed with 4% PFA and stained for differentiation markers (as described in Immunofluorescence section).

Differentiation of iPSCs into skeletal muscle cells

To generate skeletal muscle cells, the iPSC lines were seeded in 60 mm dishes coated with Geltrex (Gibco), at a density of 2,500 or 5,000 cells per 1 cm^2, depending on the clone, in Skeletal Muscle Induction Medium (SKM01, AMSBIO) supplemented with penicillin-streptomycin (Gibco). For the first 24 h the RevitaCell Supplement (Gibco) was added to the medium. Then, the medium was changed every other day. Upon reaching full confluency (5-7 days), cells were collected using TrypLE Express Enzyme (Gibco) as SCs and additionally seeded at a density of 2,500 cells per 1 cm^2 of a 60 mm dish coated with Geltrex. Cells were seeded in the Myoblast Medium (SKM02, AMSBIO) supplemented with penicillin-streptomycin (Gibco) to induce differentiation into myoblasts. The medium was changed every 2-3 days. After the next 6-8 days, at the point of full confluency the medium was changed to SKM03+ (AMSBIO) or DMEM-HS (DMEM high glucose (4.5 g/L, Gibco) supplemented with 2% horse serum (Gibco)), GlutaMAX Supplement (Gibco) and penicillin-streptomycin for myotube formation, and the cells were collected as myotubes. Some clones needed additional culture time (with culture splitting) in SKM02 (up to 3 weeks) to be able to differentiate into myotubes. Cells were cultured in standard conditions (37°C, 5% CO_2). Upon each differentiation stage cells were photographed with ZEN Software on ZEISS AxioVert Microscope with 10x objective.

Western blotting (WB)

Cell pellets were resuspended in Laemmli hot lysis buffer (50 mM Tris-HCl pH 6.8; 10% glycerol; 2% SDS). Lysates were boiled for 5 minutes (96°C) and cooled. BCA assay (Thermo) was performed for 10x diluted samples, according to the manufacturer's instructions, in a 96-well plate format. After measurements, samples were supplemented with DTT to a final concentration of 50 mM and boiled again. 25 µg of protein extracts were separated via SDS-PAGE on 6%-12% gels, transferred to

nitrocellulose membranes, and blocked in 5% non-fat milk in PBS with 0.075% Tween 20 (PBST) for 1 h at RT. Primary antibodies were applied overnight at 4°C in PBST (or in 5% non-fat milk as marked in the Table 1), and secondary antibodies conjugated with horseradish peroxidase (HRP) were applied for 1 h at RT in 5% milk in PBST buffer (antibodies and dilutions shown in Table 1). The proteins were visualized using ECL substrate (Bio-Rad) and analyzed with ImageLab (Bio-Rad).

Results

For iPSCs generation, we used dermal fibroblasts obtained from a skin biopsy of two EDMD1 patients and a healthy donor. Donors were male, Caucasian ethnicity, unrelated, and born in Poland. The patients had confirmed mutation in *EMD* gene (*locus Xq28*) and exhibited typical EDMD symptoms [25, 26, 27].

We reprogrammed the fibroblasts to iPSCs using non-integrating Sendai virus (SeV) vectors encoding four Yamanaka factors: Oct, Sox2, Klf4, and c-Myc. We established two iPSCs clones for the healthy donor, two clones for the patient with *EMD* mutation c.451dup, and one clone for the patient with *EMD* mutation 153del (Table 3). Additionally, we previously generated four clones from two patients with *EMD* mutation c.153del, which were also used in this study (Table 3) [22]. None of the donors were related, even those sharing the same mutation.

iPSCs were expanded to at least passage 10, characterized and validated, as summarized in Table 4. Other iPSC clones, which did not pass all tests, were discarded due to the genomic instability detected in some cells. The morphology of colonies was typical for iPSCs (Figure 1A). We confirmed the expression of pluripotency markers using IF (Figure 1B) and qPCR (Figure 1C). All clones expressed Oct4, SSEA4, NANOG, hTERT, Lin28, Sox2 and REX-1.

Table 3: Summary of generated iPSC lines

The Table summarizes newly validated iPSC clones from three donors alongside previously reported clones from two donors. Emerin protein level was confirmed by WB analysis in myotubes (Figure 4A). All donors were unrelated.

iPSC line name	Unique stem cell lines identifier	Disease	Age of biopsy	Genotype (EMD gene)	Emerin expression
Newly established clones					
C1M35 L26	UWRBTi001-C	N/A	35	NM_000117.3(EMD), CCCCC**C**AGC, GG**G**AACGCC	normal
C1M35 1.6	UWRBTi001-D	N/A			
E1M40 1.4	UWRBTi003-A	EDMD1	40	NM_000117.3(EMD):c.153del p.(Ser52fs), CCCCC_AGCT, hemizygous, rs876661345	not detected
E1M19 1.5	UWRBTi005-A	EDMD1	19	NM_000117.3(EMD):c.451dup*, p.(Glu151fs), GGG**G**AACGCC, hemizygous	shorter protein
E1M19 1.8	UWRBTi005-B	EDMD1			
Previously generated clones					
E1M40 1.7	UWRBTi003-B	EDMD1	40	NM_000117.3(EMD):c.153del p.(Ser52fs), CCCCC_AGCT, hemizygous, rs876661345	not detected
E1M40 1.9	UWRBTi003-C	EDMD1			
E1M51 1.4	UWRBTi004-A	EDMD1	51	NM_000117.3(EMD):c.153del p.(Ser52fs), CCCCC_AGCT, hemizygous, rs876661345	not detected
E1M51 1.8	UWRBTi004-B	EDMD1			

*****Mutation c.451dup was previously reported also as c.450insG or c.450dup (e.g. www.umd.be/emd database).

Table 4: Characterization and validation of iPSCs

The Table contains all performed tests for iPSCs validation with reference to corresponding data.

Parameter	Test results	Data
Colony morphology	Confirmation of normal colony morphology with transmission light microscopy.	Figure 1A
Phenotype	IF confirmation of pluripotency markers presence (Oct4, SSEA4).	Figure 1B
	RT-qPCR detection of pluripotency markers expression (*NANOG, hTERT, LIN28, OCT4, SOX2, REX-1*).	Figure 1C
Genotype	46,XY karyotype confirmed by G-band analysis.	Figure 2A, Supplementary Figure S1
Identity	STR analysis (16 loci, all matched).	Supplementary Table S1
Mutation analysis	DNA sequencing of amplified *EMD* confirmed wild-type gene or mutation (according to Table 3).	Figure 2B, Supplementary Figure S1
Presence of reprogramming factors	The PCR test for reprogramming factors was negative.	Figure 2C
Microbiology	PCR test for mycoplasma was negative	Figure 2D
Differentiation potential	Directed differentiation followed by positive staining for three germ layers: Sox17 (endoderm), Brachyury (mesoderm), Otx2 (ectoderm)	Figure 2E

The genomic stability of iPSC clones was confirmed by karyotypes analysis (Figure 2A) and STR profiling (16 loci, Supplementary Table S1), compared to parental fibroblasts (Supplementary Figure 1A). DNA sequencing of the *EMD* gene confirmed the wild-type sequence in control cells and the *EMD* mutations in EDMD1 cells (Figure 2B), matching the sequences found in the parental fibroblasts (Supplementary Figure S1B).

The absence of SeV vectors used for reprogramming was confirmed around passage 15 by PCR detecting four different sequences of vectors (Figure 2C). All cells were confirmed by PCR as free of mycoplasma (Figure 2D). Finally, we checked the ability of our clones to differentiate into three germ layers *in vitro* using dedicated growth media (as described in the *Materials and Methods* section). The lineage commitment was evaluated by IF staining for the following germ layers markers: Sox17 for endoderm, Brachyury for mesoderm and Otx2 for ectoderm (Figure 2E). All tests confirmed the pluripotency, purity and stability of the five iPSC clones.

As newly established clones were validated as functional iPSCs, we utilized them to obtain muscle cells *in vitro*. Using a transgene-free method based on growth media, we differentiated iPSCs into muscle precursors (SCs), myoblasts and multinucleated myotubes (Figure 3A, 3B). In addition to the iPSCs prepared in this study, we differentiated to muscle cells the previously published clones: E1M40 1.7, E1M40 1.9, E1M51 1.4, and E1M51 1.8 [22], which have the *EMD* mutation c.153del resulting in the absence of emerin (Figure 4A).

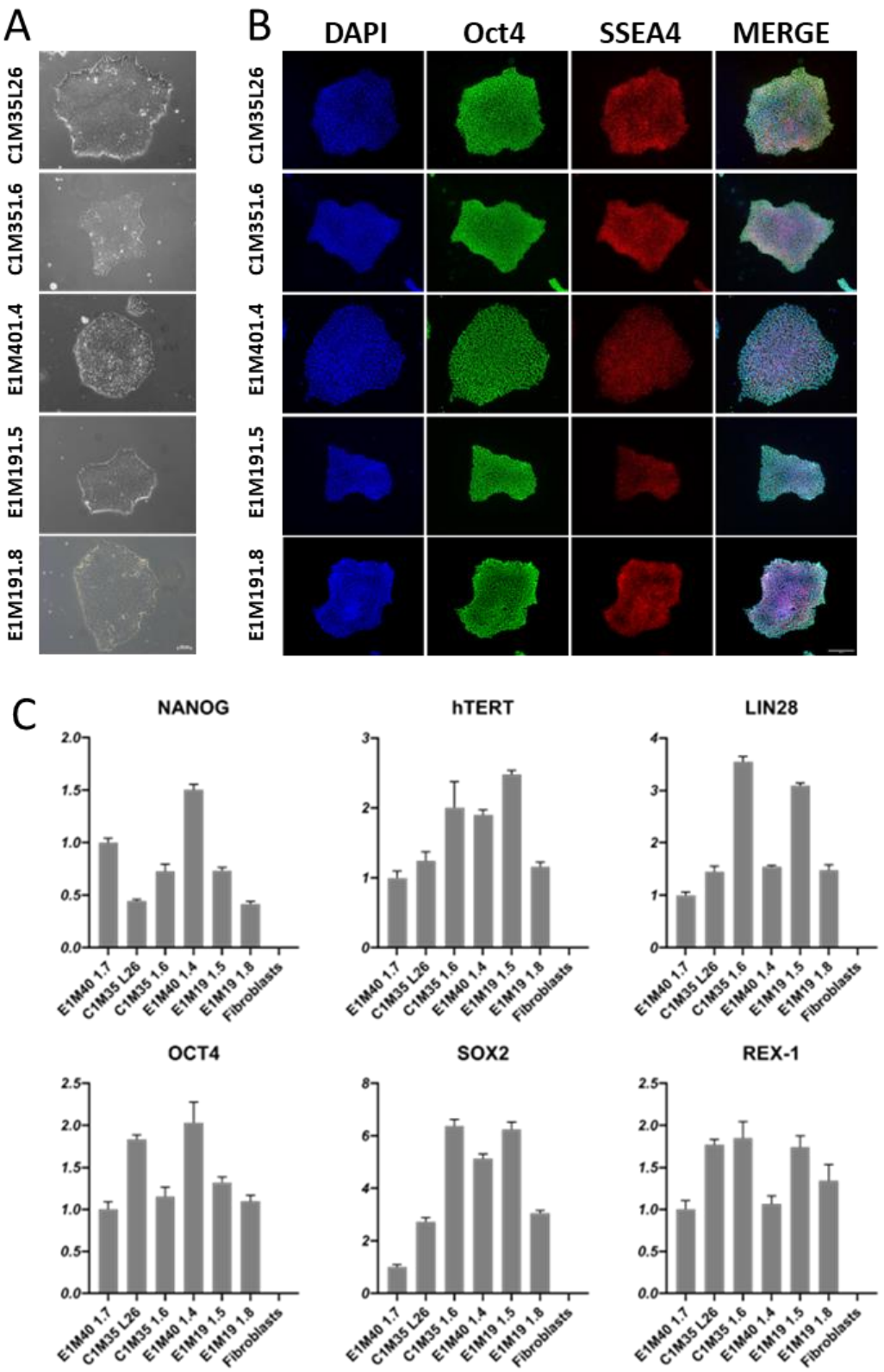

Figure 1. Morphology and markers expression analysis of iPSC lines.

(A) *Transmitted light images show properly formed iPSC colonies (10x objective). Scale bar is 100 µm.* ***(B)*** *IF staining of iPSCs shows proper localization and equal distribution of pluripotency markers Oct4 and SSEA4 in representative iPSC colonies. Scale bar is 500 µm.* ***(C)*** *qPCR analysis shows relative gene expression levels of iPSC pluripotency markers, normalized to the previously published E1M40 1.7 clone [22] and to GAPDH expression level. Error bars present standard deviations.*

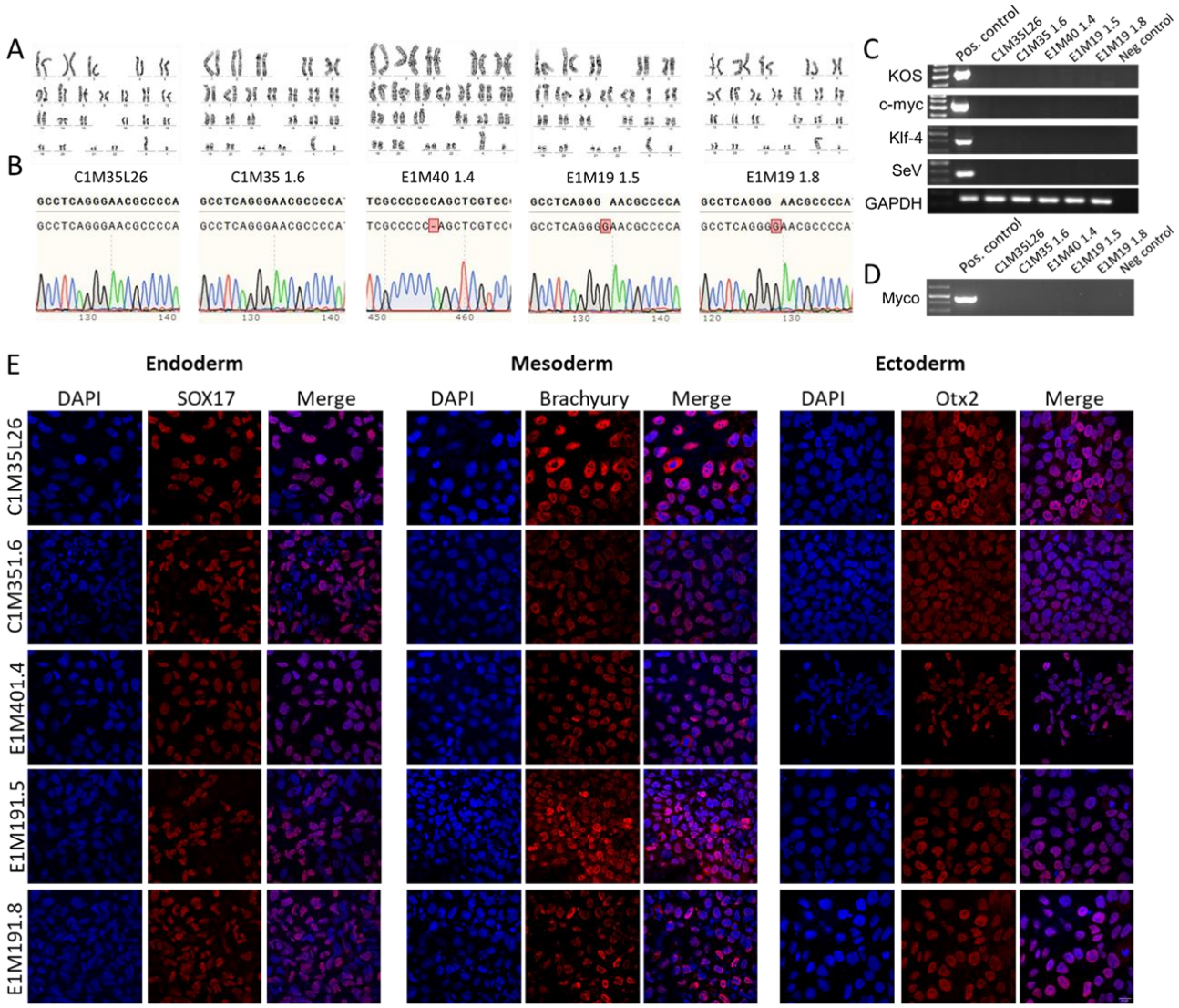

Figure 2. Characterization of iPSC lines.

(A) *G-banding analysis shows proper 46,XY karyotypes.* ***(B)*** *DNA sequencing chromatogram of fragment of amplified EMD gene confirmed wild-type sequence in control lines, cytidine deletion c.153del (CCCCC_AG) in E1M40 1.4 clone and guanine insertion c.451dup (GGG**G**AAC) in both E1M19 lines. The upper sequence shows the reference wild-type EMD, while the bottom sequence shows the result of DNA sequencing.* ***(C)*** *PCR verification of loss of each reprogramming vector (KOS, c-MYC, KLF4, SeV) in iPSC lines together with control GAPDH.* ***(D)*** *PCR analysis shows lack of mycoplasma in each iPSC clone.* ***(E)*** *IF staining of germ layers markers. Each iPSC clone was differentiated independently into three germ layers on coverslips, followed by cells staining for adequate markers: Sox17 for endoderm, Brachyury for mesoderm and Otx2 for ectoderm. Scale bar is 20µm.*

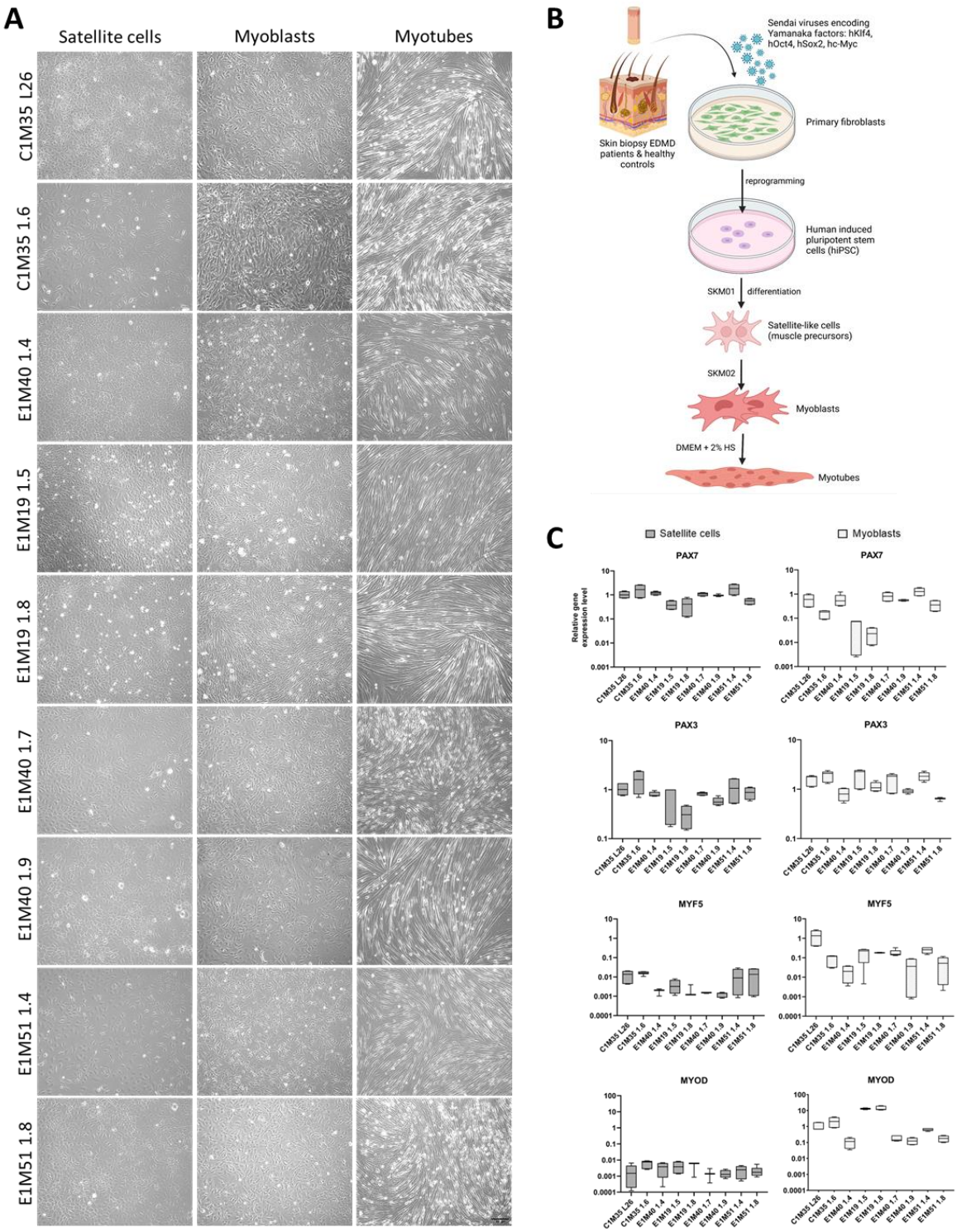

Figure 3. iPSCs differentiation into subsequent developmental stages of muscle cells.

(A) *Images of SCs (day 6-7), myoblasts (day 6-7) and myotubes (up to 5 days in HS), transmission light, 10x magnification, scale bar 300 µm.* ***(B)*** *Graphical representation of the reprogramming and differentiation processes, from the skin biopsy from the EDMD1 and healthy donors, through fibroblast reprogramming to iPSCs, to differentiation to subsequent developmental stages of muscle cells.* ***(C)*** *qPCR analyses of relative transcript levels of muscle early markers (Pax3, Pax7, Myf5, MyoD) in SCs and myoblasts (n=2). Boxes depict 25th to 75th percentiles of relative gene expression with mean value marked with horizontal line, error bars illustrate minimal and maximal values. Samples were normalized to GAPDH and HPRT.*

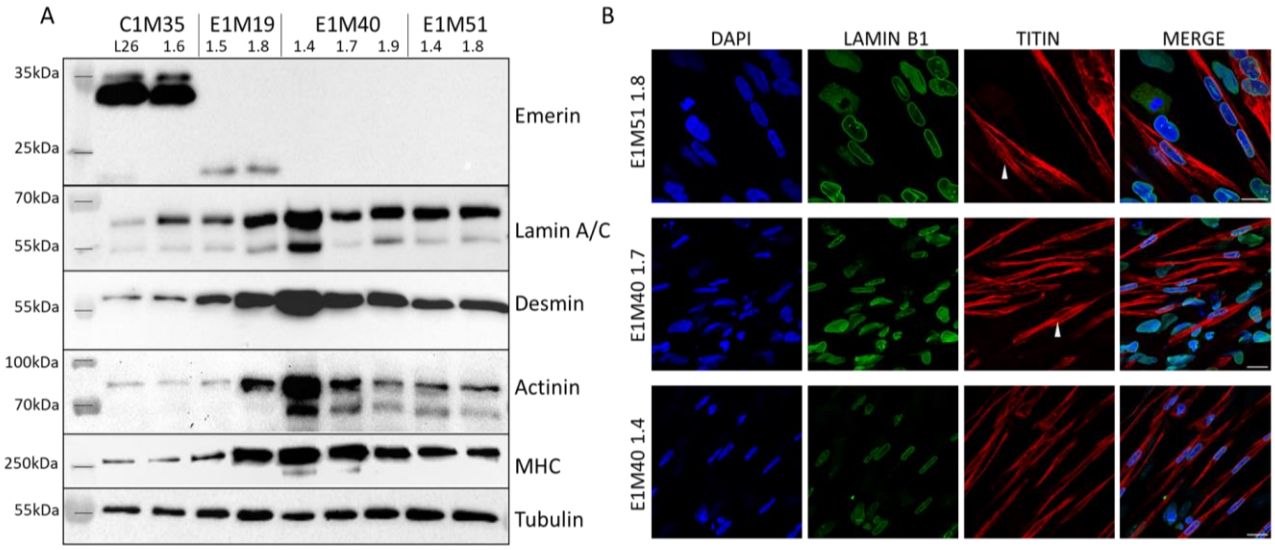

Figure 4. Myotubes analysis.

(A) *WB analysis of myogenesis markers and NE protein levels in myotubes. Full-length emerin was present in control cells, its shortened form in E1M19 clones while no emerin was detected in E1M40 and E1M51 cells. Lamin A/C, desmin, MHC and actinin were confirmed in all clones, while proteins' levels depend on clone properties and differentiation time. Tubulin was used as loading control.* ***(B)*** *IF images of patient's cells derived myotubes, triple stained for chromatin (DAPI), lamin B1 and titin. Titin staining shows sarcomere structure in myotubes (white arrows). Multinucleated myotubes were observed (with 2 or more nuclei per cell). Scale bar is 20µm.*

Using qPCR, we confirmed the expression of muscle markers of particular stages of myogenesis. SCs express transcription factors Pax3 and Pax7 (Figure 3C), but not pluripotency marker Oct4 (analyzed by WB; Supplementary Figure S2). At the myoblast stage, the expression levels of myoblasts markers Myf5 and MyoD were elevated in comparison to SCs (Figure 3C). For MyoD, and to a lesser extent Myf5, transcript level varied greatly between myoblasts obtained from particular donors. These muscle markers were also detected by WB (Supplementary Figure S2) showing that their expression level increased with time of differentiation.

Next, we differentiated myoblasts into myotubes using a medium supplemented with 2% HS (Figure 3A). Depending on the clone, cells required 1-3 weeks of proliferation in myoblast medium, followed by differentiation, to be able to produce multinucleated myotubes.

Myotubes had an elongated morphology as expected, with the fraction of multinucleated cells depending on a particular clone. Using WB, we confirmed the presence of typical myotube markers: desmin, actinin, and MHC (Figure 4A). All clones expressed lamins A/C, which is the main interaction partner of emerin, and its level increases during the differentiation. The full-length emerin (254 amino acids) was detected only in control cells. For both clones of E1M19 cells, we noted lower level of a faster migrating form of truncated emerin, resulting from a frameshift from Glycine 151, followed by a STOP codon after 208 amino acids. In turn, mutation c.153del results in the absence of emerin (all clones of E1M40 and E1M51 donors, Figure 4A). IF analysis of selected patient myotubes revealed a sarcomere structure shown by titin bands in some cells (Figure 4B). Additionally, lamin B1 staining

showed multiple nuclei in titin-positive cells, which confirms cells' fusion. In conclusion, we obtained mature myotubes from patients-derived cells.

Discussion

EDMD1, a rare genetic disorder belonging to laminopathies, still remains without a cure. Available treatments are limited to symptome-targeted therapies [28]. The precise molecular mechanisms underlying EDMD1 remain not fully understood. The development of an appropriate research model is crucial for advancing our understanding of the disease, which may also lead to the discovery of more effective therapies.

iPSCs-based models have been established for a range of muscular dystrophies and laminopathies, including Limb-Girdle muscular dystrophy [29], Duchene muscular dystrophy [30], Hutchinson-Gilford progeria syndrome or Dilated Cardiomyopathy [31], and also for EDMD type 2 (laminopathy caused by mutation in *LMNA* gene encoding lamin A/C) [32, 33]. These models are widely used for *in vitro* differentiation into various tissues such as skeletal muscles, cardiomyocytes, adipocytes, and vascular smooth muscle cells, which serve as a model of tissues affected by the particular disease. Their utility in studying molecular mechanisms, testing candidate therapies, and exploring novel therapeutics, is invaluable [29, 31, 34]. However, an iPSC-based model for EDMD1 has not yet been developed.

Here, we present the first cellular model of EDMD1 based on iPSCs generated from skin biopsies from EDMD1 patients, which was verified for proper differentiation to skeletal muscle cells. This model includes a collection of iPSC lines from three patients, two of whom are unrelated but share the same mutation. This is one of the most comprehensive iPSC-based models of laminopathies, ensuring replicates of clones and control cells reprogrammed simultaneously with the patient's cells. All clones, including those described here for the first time and previously published by our group [22], have been fully characterized and validated, demonstrating the expected functional properties of iPSCs. Additionally, the cell collection includes both emerin-null cells and truncated emerin, exhibiting abnormal locations described earlier [35], enabling the study of EDMD1 in the context of its genetic and phenotypic heterogeneity.

iPSCs may serve as a valuable starting point for various applications. In the case of EDMD1, the tissues the most affected by the disease are skeletal muscles and heart. In this work, we demonstrate the ability of iPSCs to robust differentiation into skeletal muscle cells through all stages of myogenesis, including SCs (progenitor cells), myoblasts, and myotubes. This model allows for in-depth analysis of myogenesis, which is difficult to achieve using other methods, particularly given the challenges associated with obtaining muscle biopsies from patients suffering from rare muscular dystrophies. The use of a transgene-free reprogramming approach minimizes potential artifacts from exogenous protein expression or genetic modifications, ensuring the disease mechanism remains unchanged.

An unexpected outcome was the sustained expression level of transcription factors Pax3 and Pax7 during the myoblasts stage, as compared to their expression in SCs. Although these proteins are typically considered SC markers, their persistence in myoblasts has been observed in other studies. Usually their level drastically decrease in myoblasts, but for example Pax7 is expressed in mouse myoblast cell line C2C12 [36], and in a subset of myoblasts differentiated from iPSCs [37, 38], as well as in primary myoblasts from mouse [39] and porcine [40] models. Elevated levels of both Pax3 and

Pax7 have also been reported in myoblasts and myotubes obtained by MyoD overexpression [19]. Therefore high levels of Pax3 and Pax7 in myoblasts or myotubes do not have to impair their functionality. In the model described here, the elevated levels of both Pax3 and Pax7 in myoblasts did not impair their differentiation to myotubes.

Additionally, we observed that myoblasts from different donors required diversified varying durations of exposure to myoblast medium (1-3 weeks) before they could successfully differentiate into myotubes. This variability in efficiency will be investigated further to determine whether it is linked to emerin expression.

In summary, to the best of our knowledge, we have established the first iPSC-based model of EDMD1, derived from three patients, that can successfully progress through multiple stages of myogenesis. These iPSCs also have the potential for differentiation into other tissues, including neurons, cardiomyocytes, and organoid skeletal muscle models. This new EDMD1 platform provides a valuable tool for advancing therapeutic development, including high-throughput pharmacological screening, as well as gene and cell therapy investigations.

Supplementary Materials

Supplementary Table S1. STR profile summary of iPSC clones and parental fibroblasts.

Supplementary Figure S1. The analysis of parental fibroblasts. (A) Karyotypes of parental fibroblasts. (B) Results of DNA sequencing of *EMD* gene in parental fibroblasts.

Supplementary Figure S2. The analysis of protein levels in iPSCs-derived SCs and myoblasts.

Authors' contribution

KP, MM and RR designed the study. CB and MM generated iPSCs. KP, ML and MR expanded iPSCs. ML, MR, IŁ and AS performed iPSCs validation tests. AS, KP, ML differentiated iPSCs to muscle cells. AS, KP, JH, AK and ML performed muscle cells analysis. VD was responsible for the resources. KP supervised the study. KP and MM wrote the original draft of the manuscript. All authors reviewed the manuscript. KP, MM and RR provided funding.

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Conflicts of Interest

The authors declare no conflict of interest.

Ethical approval

Patient cells (skin biopsies) were collected according to the ethical and medical standards set by Polish law and based on approval of the ethical committee (DOP-GMO.431.85.2018; DOP-GMO.431.79.2018) and after obtaining written consent from the patients.

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