Running head: Mesoangioblasts restore dystrophin in a DMD model

Skeletal muscle differentiation on a chip shows human donor mesoangioblasts efficiency in restoring dystrophin in a DMD model

E. Serena[1,2], S. Zatti[1,2], A. Zoso[1,2], F. Lo Verso[1,2], F. S. Tedesco[3], G. Cossu[4]*, N. Elvassore[1,2,]*

[1]Industrial Engineering Department, University of Padova, Padova, Italy

[2]Venetian Institute of Molecular Medicine, Padova, Italy

[3]Department of Cell and Developmental Biology, University College London, London, United Kingdom

[4]Institute of Inflammation and Repair Manchester, University of Manchester, Manchester, United Kingdom

* Corresponding authors

Correspondence information:

Elvassore Nicola

via Marzolo 9, 35131 Padova, Italy

Telephone: +39 049 827 5469, +39, fax: 049 827 5461, nicola.elvassore@unipd.it,

http://www.bioera.dipic.unipd.it

Author contributions

E. Serena: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing.

S. Zatti: conception and design, collection and/or assembly of data, data analysis and interpretation.

A. Zoso: collection and/or assembly of data, data analysis and interpretation

F. Lo Verso: collection and/or assembly of data

S. Tedesco: collection and/or assembly of data, data analysis and interpretation

G. Cossu: conception and design, financial support, revision and final approval of manuscript

N. Elvassore: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript

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ABSTRACT

Restoration of the protein dystrophin on muscle membrane is the goal of many research lines aimed at curing Duchenne muscular dystrophy (DMD). Ongoing pre-clinical and clinical trials suggest that partial restoration of dystrophin might be sufficient to significantly reduce muscle damage. Different myogenic progenitors are candidates for the cell therapy of muscular dystrophies but only satellite cells and pericytes have already entered clinical experimentation.

Our study aims at providing in vitro quantitative evidence of the ability of mesoangioblasts to restore dystrophin, in terms of protein accumulation and distribution, within myotubes derived from DMD patients using a micro-engineered model.

We designed an ad hoc experimental strategy, seeking to miniaturizing on chip the standard process of muscle regeneration, independently from variables such as inflammation and fibrosis. It is based on the co-culture, at different ratios, of human dystrophin positive myogenic progenitors and dystrophin negative myoblasts in a substrate with muscle-like physiological stiffness and cell micropatterns. Results showed that both healthy myoblasts and mesoangioblasts restore dystrophin expression in DMD myotubes. However, mesoangioblasts showed unexpected efficiency with respect to myoblasts in dystrophin production in terms of amount of protein produced (40% vs 15%) and length of the dystrophin membrane domain (210-240μm vs 40-70μm).

These results show that our microscaled in vitro model of human DMD skeletal muscle validate previous *in vivo* pre-clinical work and may be used to predict efficacy of new methods aimed at

enhancing dystrophin accumulation and distribution before they are tested *in vivo*, reducing time, costs and variability of clinical experimentation.

1. Introduction

Duchenne muscular dystrophy (DMD) is a genetic disease caused by mutations in the gene encoding the protein dystrophin[1]. Dystrophin is a critical component of the dystrophin-glycoprotein complex (DGC) in muscle that links the actin cytoskeleton to the extracellular matrix of myofibers. The lack of a functional dystrophin protein causes loss of proper localization of many of the DGC components at the sarcolemma of muscle fibers leading to membrane instability and myofiber degeneration[2]. DMD primarily affects skeletal muscles and results in progressive paralysis and premature death. At the moment, no successful treatments are available, but new drug, gene and cell therapy strategies are under clinical investigation[3,4].

Many of the therapeutic strategies and research lines for DMD are focused on the restoration of the protein dystrophin. Recently completed clinical trials showed that 15%[5] and 18%[6] of normal levels of dystrophin resulted in a moderate but significant clinical benefit during a 12 weeks study. Indeed reports indicate that dystrophin production as low as 30% of that found in healthy animals or individuals prevents muscular XLDC dystrophy in humans[7,8].

In this scenario, cell therapy has good potential. In particular, strategies able to restore the compartment of muscle stem cells are amongst the most promising, since they would not require continuous injections to sustain the muscle regeneration. However, cell therapy is a complex regenerative process which includes intra-arterial (or intramuscular) cell delivery, crossing the blood vessel wall, survival, migration and contribution to skeletal muscle regeneration by fusion with regenerating muscle fibers and by entering the satellite cell compartment[8,9]. In this perspective, pericytes are a promising cell source because of their peculiar characteristics: these cells surround the endothelium of small vessels and can differentiate into different mesoderm cell types, including skeletal muscle[8,10]. When delivered into the arterial circulation, mouse mesoangioblasts cross the blood vessel wall and participate in skeletal muscle regeneration, ameliorating signs of muscular dystrophy in animal models such as the α-sarcoglycan–null (Sgca-null)[11] and mdx[8] mice and the golden retriever muscular dystrophy (GRMD) dog[12]. When cells similar to mouse mesoangioblasts were isolated from human adult skeletal muscle they were shown to correspond to a subset of pericytes, expressing alkaline phosphatase[13]. Cells derived from *in vitro* expansion of human skeletal muscle vasculature pericytes, which we deemed mesoangioblasts, have recently been transplanted in DMD patients in a phase I/IIa clinical trial (EudraCT no. 2011-000176-33) whose results showed safety and limited efficacy[14].

Clinical trials are expensive and time-consuming processes required before drugs and therapies reach the market and the clinics: many drugs are withdrawn and many therapies fail during clinical trials. Moreover it may be extremely useful to test a large number of variables that may synergise to increase efficacy (e.g. combination of different drugs with cell/gene therapy) but it is in fact almost impossible because of logistic and economical reasons. In order to overcome this problem, the concept of "clinical trial on dish" or "clinical trial on chip" has been recently proposed[15]. Such complementary approaches could provide therapy efficiency information at early stage of protocol development. For instance, Liang and colleagues validate the capacity of a library of human induced pluripotent stem cell–derived cardiomyocytes to be used as a clinical trial in a dish model for accurate detection of patient-specific drug responses and drug-induced cardiotoxicity profiles[16]. The concept of "clinical trial on chip" is based on the advances in micro-technology, microfluidics and development of physiologically relevant 3D organs or tissues at the microscale. The attempt is to give a cheaper, faster and more accurate way to screen drugs or therapies for efficacy and toxicity. For a review of the potential of this approach see[15,17–19].

So far no study reports the use of skeletal muscle for clinical evaluation of therapy in a dish. However, the derivation of physiologically relevant skeletal muscle tissues *in vitro* is not straightforward. Physiological stimuli and interactions must be reproduced and finely controlled. In particular, the development of skeletal muscle model and a DMD assay requires the formation of a mature tissue with a high degree of differentiation and a proper expression of dystrophin, which is a late marker, not commonly detected in standard *in vitro* cultures. For this reason, primary myoblasts freshly isolated from patient should be used, since they can be expanded and successfully differentiated into mature myotubes, the functional unit of skeletal muscle. The major drawback of this cell source, the relatively low number of derived cells, could be overcome with the design of micro-scaled assay able to maintain physiological relevance.

In this scenario, we developed a human-based skeletal muscle tissue-on-chip derived from healthy and DMD donors [20,21]. We engineered the culture substrate, in terms of mechanical and topological properties, for optimizing human myoblasts differentiation and obtaining the expression of dystrophin *in vitro*[20].

In this work, we aim at exploiting the developed human-based skeletal muscle tissue-on-chip for testing a number of experimental variables which may subsequently benefit the design of a protocol for future clinical trials, leading in the end to more efficacious stem cell therapies. In this specific work we tested the ability of human mesoangioblasts, in comparison with human myoblasts, to restore

level of dystrophin expression and distribution along the myotube when co-cultured with myoblasts from DMD patients, using a micro-engineered DMD model of skeletal muscle. Wild-type (dystrophin-positive, Dys^+) cells were co-cultured with DMD cells (dystrophin-negative, Dys^-) at different ratios. The co-culture of Dys^+ with Dys^- cells induced the formation of myotubes containing nuclei encoding for dystrophin and DMD nuclei not encoding for this protein.
It is worth to underline that the mesoangioblasts used in this study were harvested from three healthy donors, who participated to a phase I/II clinical trial (EudraCT no. 2011-000176-33).

2. Materials and Methods

2.1 Cell Culture.

Throughout the manuscript we refer to myoblasts (Mbs) as the *in vitro* counterpart of satellite cell-derived myoblasts. We refer to mesoangioblasts (Mabs) as an *in vitro* expanded peri-vascular cell population sharing markers of skeletal muscle pericytes and likely corresponsing to a subpupolation thereof. Mesoangioblasts isolated from post-natal mammalian muscle express (>95%) markers common to MSC such as CD44, CD90, CD13 etc. but not endothelial or hematopoietic markers such as CD31, CD34 and CD45. They do not express the satellite cell marker CD56. As for "pericyte" markers they express, at variable extent in different preparations, PDGF Receptor beta, smooth alpha actin, NG2, Alkaline Phosphatase and CD146[22].

Human primary myoblasts were provided by the ''Telethon BioBank'' (Telethon Research Service, Istituto Nazionale Neurologico ''Carlo Besta'', Milano, Italy). They are derived from healthy donors (Dys^+) and from DMD affected donors (Dys^-). The mutation of DMD donor is the deletion of Exon 45. Myoblasts were expanded with proliferation medium: 60% High-Glucose Dulbecco's Modified Eagle's Medium (DMEM Glutamax, Gibco-Invitrogen, Waltham, Massachusetts), 20% Medium M199 (Sigma-Aldrich, St. Louis, Missouri), 20% fetal bovine serum (FBS, Gibco-Invitrogen, Waltham, Massachusetts), 10 ng/mL EGF (PeproTech, Rocky Hill, New Jersey), 2 ng/mL β-FGF (PeproTech, Rocky Hill, New Jersey), 10 μg/mL insulin (insulin from bovine pancreas, Sigma-Aldrich, St. Louis, Missouri) and 1% penicillin−streptomycin−glutamine mix solution (Gibco-Invitrogen, Waltham, Massachusetts); on standard 100 mm tissue culture Petri dishes previously coated with 0.5% gelatin solution (gelatin from porcine skin, Sigma-Aldrich, St. Louis, Missouri).
Human mesoangioblasts were derived from skeletal muscle biopsies of healthy subjects as described[22]. Biopsies were obtained from the Orthopedic Surgery Department of the San Raffaele

Hospital following authorization of the Institute Ethics Committee and signature of an informed consent by the patient or his/her parents/caregivers.

Briefly the biopsy was cleaned from fat and connective tissue, minced in small fragments that were plated on collagen coated dishes (6 or 9 cm depending on the size) and cultured with 1 or 2 ml (respectively) of Megacell medium (see below). More medium is gently added the next day. After a week, when cells have outgrown form the explant, loosely attached or floating cells are collected by gentle pipetting and sub-cultured as P1.

Human mesoangioblasts derived from healthy donors (Dys^+) were expanded in gelatin-coated dish with proliferation medium composed of MegaCell™ DMEM (Sigma-Aldrich, St. Louis, Missouri) supplemented with 5% heat inactivated fetal bovine serum (Gibco-Invitrogen, Waltham, Massachusetts), 2 mM L-Glutamine (Gibco-Invitrogen, Waltham, Massachusetts), 1% non-essential aminoacids (Gibco-Invitrogen, Waltham, Massachusetts), 0,1 mM β-mercaptoethanol (Gibco-Invitrogen, Waltham, Massachusetts), 5 ng/ml β-FGF (PeproTech, Rocky Hill, New Jersey), 1% penicilin-streptomicin (Gibco-Invitrogen, Waltham, Massachusetts). Mesoangioblasts were induced to differentiate into myotubes by changing proliferation medium with the same differentiating medium used for human primary myoblasts. Human primary myoblasts and mesoangioblasts were induced to form myotubes in differentiating medium: 98% DMEM Glutamax (Gibco-Invitrogen, Waltham, Massachusetts), 2% horse serum (Gibco-Invitrogen, Waltham, Massachusetts), 30 µg/mL insulin and 1% penicillin−streptomycin−glutamine mix solution (Gibco-Invitrogen, Waltham, Massachusetts). We used three batches of mesoangioblasts, three batches of myoblast from healthy individuals and one batch of myoblast from DMD patients.

2.2 Dys^+ and Dys^- co-culture on micro-patterned substrate

The engineered culture system was prepared as previously described[20,21]. Briefly, hydrogel with muscle-like stiffness were fabricated with acrylamide/bisacrylamide 29:1 40% solution (Sigma-Aldrich, St. Louis, Missouri) over a 25mm coverslip glass. The pre-polymer was diluted in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, Missouri) to the final concentrations of 10%. The photoinitiator (Irgacure 2959; Ciba Specialty Chemicals, Basel, Switzerland) was added to the acrylamide/bis-acrylamide solution with a final concentration of 20 mg/ml, and mixed thoroughly. Hydrogel polymerization occurred by exposing the prepolymer solution to UV light for 3 min (high-pressure mercury vapor lamp (Philips HPR 125 W) emitting at 365 nm with an incident light intensity of 20 mW/cm^2). Non-polymerized acrylamide was removed using distilled water. Hydrogel films were immersed in ultra-pure distilled water for 48 h to ensure complete removal of the unreacted monomeric units or photoinitiator and final sterilization occurred after 20 min exposure to UV light under a sterile hood. The hydrogel has a diameter of 18 mm.

Matrigel (2.5% v/v in DMEM) was used for the micropattern. Micropatterning geometry (parallel lanes, 1 cm^2) was optimized for myoblasts and pericytes, according to our previous work[21]. Dys^+ and Dys^- cells were co-cultured with the following ratio: 1:1, 1:2 and 1:9, respectively. 300 µL of the cell suspension (1×10^5 cells/mL) were dropped over the micropatterned hydrogel and the cultures kept at 37 °C, 5% CO_2. Co-cultures were maintained in a 1:1 mix of each specific proliferation medium for 1 or 2 days, followed by 8 days of culture in differentiating medium.

2.3 Western Blot

The cultures were treated ice-cold with 50 µl of lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM DTT, 10% Glycerol, 1 mM EDTA, 10 mM MgCl2, 2% SDS, 1% Triton X-100 1 mM PMSF, 1 mM NaV, 5 mM NaF, 3 mM β-glycerol (all Sigma, St. Louis, Missouri), and Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Lysis buffer were dropped directly onto the hydrogel surfaces and incubated at 4°C for 1 h. After 1 h treatment, lysis buffer was resuspended on the hydrogel in order to collect all the cellular contents. Cell fractions were sedimented by centrifugation at 13000 g for 20 min at 4°C, and supernatant collected. 10 µg per lane of protein extract were solubilized in loading buffer (Gibco-Invitrogen, Waltham, Massachusetts), 10% DTT (Gibco-Invitrogen, Waltham, Massachusetts) and heated for 10 minutes at 70°C. Proteins were resolved in 3-8% precast gels (Tris-Acetate NuPAGE, Gibco-Invitrogen, Waltham, Massachusetts) and then transferred on PVDF membranes (Gibco-Invitrogen, Waltham, Massachusetts) under a potential difference of 45V, 400mA for 6 h. Membranes were blocked with 5% nonfat dry milk (Bio-Rad, Hercules, California) in TBST (TBS, 0.05% Tween 20) and then probed with primary antibodies for dystrophin (Abcam, Cambridge, UK), myosin heavy chain II (Sigma-Aldrich, St. Louis, Missouri) and β-actin (Sigma-Aldrich, St. Louis, Missouri), and then with the proper HRP–conjugated secondary antibodies: goat anti-rabbit antibody (Gibco-Invitrogen, Waltham, Massachusetts) and goat anti-mouse antibody (BioRad, Hercules, California). Proteins were visualized by enhanced chemiluminescence (Gibco-Invitrogen, Waltham, Massachusetts) and dystrophin content was quantified by densitometry using ImageJ software (US National Institutes of Health). For each culture condition, we quantified the intensity of dystrophin and myosin heavy chain bands and normalized them by the housekeeping protein, β-actin.

2.4 Immunofluorescence

Primary antibodies used in this study were against myosin heavy chain II (Sigma-Aldrich, St. Louis, Missouri) and dystrophin (Abcam, Cambridge, UK). A standard immunohistochemistry protocol was used[20]. Nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, Missouri) and samples were

mounted with Elvanol, and viewed under a fluorescence confocal microscope (Leica, Wetzlar, Germany).

3. Results

3.1 Assay validation

The micro-engineered DMD model used in this study has been developed in our laboratory[20,21] and it can be placed in a well of a standard 6 multiwell plate. It allows reducing the amount of reagents and the number of cells per sample: the culture surface is 0.5 cm^2 and as few as 3×10^4 cells per sample can be used. In order to analyze the contribution of mesangioblasts derived from skeletal muscle vasculature and myoblasts in the restoration of dystrophin, we designed an experimental strategy based on the co-culture at different ratios of Dys^+ and Dys^- human cells in a micro-engineered *in vitro* model of human DMD skeletal muscle (Fig. 1). We named "Dys^+ cells" either myoblasts or mesoangioblasts derived from skeletal muscle vasculature from healthy subjects; while "Dys^- cells" refers to myoblasts harvested from DMD patients.

We recently demonstrated that human myoblasts (both Dys^+ and Dys^-) differentiated optimally in our system and gave rise to fully differentiated myotubes[20,21]. Sarcomeric striations of myosin heavy chain (MyHC) are visible (Fig. 2A) and dystrophin is expressed and located at the membrane (Fig. 2C). Also mesoangioblasts differentiate in our system: we obtained myotubes striated for MyHC (Fig. 2B) with membrane localized dystrophin (Fig. 2D). As expected, myotubes derived from myoblasts of DMD patients show sarcomeric organization of MyHC (Fig. 2E) but no dystrophin expression (Fig. 2F).

Since this study is based on the co-culture of human Dys^- (myoblasts from DMD patients) and Dys^+ myogenic cells (myoblasts and mesoangioblasts from healthy individuals), we developed an assay to distinguish the two cell populations.

We tested a number of standard methodologies (Fig. 1S), but most failed for different reasons. The only methodology that allowed us to identify hybrid myotubes derived from fusion of Dys^+ with Dys^- cells is the use of lipophilic tracers. We marked Dys^+ cells with Dil (red) and Dys^- cells with DiO (green) and performed the co-culture experiments with two different ratio of cell from the two populations: 1 Dys^+ cell every 9 Dys^- cells (1:9) and 1 Dys^+ cell every 29 Dys^- cells (1:29) (Fig. 2G and 1S, D-F, n = 3 independent experiments).

We observed four types of myotubes: myotubes formed only by Dys^- cells, with a green fluorescence (Dys^- myotubes); myotubes formed only by Dys^+ cells, with a red fluorescence (Dys^+ myotubes);

myotubes without fluorescence (Not marked myotubes), since these tracers marked around 80% of the cells; and myotubes formed by Dys^+ and Dys^- cells, with an orange fluorescence (Dys^-/Dys^+ myotubes). We quantified that Dys^-/Dys^+ myotubes represent the 51% and 42% (in 1:9 and 1:29 co-culture, respectively) of myotubes expressing dystrophin (since Dys^- myotubes do not express dystrophin). We thus concluded that the fusion of Dys^- and Dys^+ cells contribute to 50% of the dystrophin signal in the co-culture experiments.

Unfortunately, tracking Dys^- and Dys^+ cell nuclei, which would help to provide better characterization of the systems, is not straightforward for long-term cell fusion experiments. Staining with Hoechst 33258 [22] (Fig 1S, A) resulted in a reduced cell viability after 5 days of culture. Human myoblasts infected with an adenovirus expressing the Green Fluorescent Protein (Fig 1S, B) showed low infection efficiency (around 50%). The use of a lentivirus encoding for a nuclear LacZ showed high efficiency (90%), however, the β-galactosidase was translocated to all the nuclei inside the myotube. After 10-12 days, we observed myotubes with all positive nuclei, myotubes with all negative nuclei and, in few cases, gradient of staining (Fig 1S, C). Thus, it was not possible to track clearly nuclei origin inside Dys^- and Dys^+ myotubes.

The expression and accumulation of dystrophin (Dys) was then analyzed through western blot. First of all we verified that the culture of Dys^+ cells performed in our micro-engineered model maintains the same ratio between dystrophin and MyHC of cultures performed in standard multiwell (48 wells) for both myoblasts and mesoangioblasts (Fig. 2H). The results showed that our micro-engineered model induces a differentiation consistent with standard culture substrate and increases the expression of dystrophin (Fig. 2H. n = 3 independent biological replicates). In addition, we determined the Dys/MyHC ratio *in vivo* (western blot of a biopsy of human muscle). The *in vivo* Dys/MyHC ratio is 0.08, roughly 1/10 of the ratio found in myotubes formed *in vitro* (1.07 for mesoangioblasts and 0.97 for myoblasts, on hydrogel) (data not shown). We expected this result because the average diameter of a myotube is 10 μm, while the diameter of a muscle fiber is 100 μm. Dystrophin covers only the periphery of the myotube/fiber, while MyHC occupies the whole fiber. Therefore, the Dys/MyHC ratio between *in vivo* and *in vitro* should be around 1/10.

In order to determine the lower ratio of Dys^+ and Dys^- cells with a detectable expression of dystrophin, we performed co-cultures of Dys^+ and Dys^- myoblasts at different ratio: 1:1, 1:2 and 1:9 respectively. We quantified the production of dystrophin and MyHC as the intensity of western blot bands. Figure 2I shows a representative western blot. The quantification of western blot bands are reported in figure 2L (n = 4 independent biological replicates). As expected, the production of dystrophin is directly proportional to the number of Dys^+ cells in culture. Based on these results, we decided to use the 1:9 ratio since the dystrophin band is still detectable.

3.2 Dystrophin accumulation

We analyzed dystrophin expression by Dys^+ myoblasts and mesoangioblasts, when co-cultured with Dys^- myoblasts in a 1:9 ratio, through western blot analysis (Fig. 3A). The graph in figure 3B reports the percentage of dystrophin restoration versus MyHC intensity. The percentage of dystrophin restoration is defined as the intensity of dystrophin from the co-culture normalized by the intensity of dystrophin from samples of Dys^+ cells (myoblasts or mesoangioblasts, accordingly). Dystrophin restoration is represented as a function of MyHC expression because the latter represents the differentiation degree of the culture. Since dystrophin expression strictly correlated to the differentiation degree of the culture, we observed that, despite the differentiation timing was kept constant (8 days), cultures reached different levels of differentiation, in particular for Dys^- myoblasts. Therefore, in order to compare different samples, dystrophin restoration is reported in function of MyHC expression. The results showed that dystrophin restoration is always higher within mesoangioblasts co-cultures than myoblasts co-cultures (Fig. 3B, n = 3 independent biological replicates for each cell type).

3.3 Dystrophin expression domain on the myotube surface

In terms of dystrophin localization, immunofluorescence analysis indicated that Dys^+ myoblasts, co-cultured with Dys^- myoblasts (at 1:9 ratio), gave rise to myotubes where dystrophin is expressed in defined portion of the myotube (Fig. 4A, B). On the contrary, Dys^+ mesoangioblasts in the same conditions gave rise to myotubes expressing dystrophin for the majority of their length: the protein was expressed almost along the entire myotube (Fig. 4C, D). We analyzed the length of the myotube in which dystrophin is expressed, that we named dystrophin domain. The distribution of the dystrophin domain length is depicted in figure 4E (n = 3 (Mbs) and n = 2 (Mabs) independent biological replicates). The dystrophin restoration domain sustained by myoblasts had an average length of 40-100 μm, while mesoangioblasts nuclei contributed to restore dystrophin for 210-240 μm.

It is worth to underline that we analyzed the number of nuclei per myotube in the dystrophin domain intervals, in order to avoid that a longer dystrophin domain could be due to a higher number of nuclei per myotube (Fig. 1S). We observed that the average number of nuclei per myotube was comparable in myoblasts and mesoangioblasts co-cultures.

Discussion

This study shows that human mesoangioblasts, derived from skeletal muscle vasculature, restore dystrophin expression and distribution in an *in vitro* model of human DMD skeletal muscle tissue-on-chip. Unexpectedly they do so more efficiently than human skeletal myoblasts.

The human skeletal muscle tissue-on-chip used in this work is a versatile tool for studying human skeletal muscle differentiation in vitro. Recently, we demonstrated that human wild type and DMD myoblasts can differentiate optimally in this model thanks to the mechanical and topological stimuli exerted[20,21]. In addition, dystrophin is expressed at significant levels and can be detected by immunofluorescence analysis[20]. These two main characteristics make this model suitable for studying human skeletal muscle differentiation *in vitro*. In this work, the developed human skeletal muscle tissue-on-chip have been exploited for studying the ability of mesoangioblasts from skeletal muscle vasculature, in restoring dystrophin expression in hybrid myotubes formed with an excess of dystrophic myoblasts and this has been compared with satellite cell-derived myoblasts.

First, we verified that mesoangioblasts differentiate optimally in our micro-engineered model (Fig. 2). We observed that the substrate stiffness (15 kPa) and topology (parallel lanes) induced the differentiation of mesoangioblasts to functional myotubes (sarcomeric striation of MyHC) expressing dystrophin. Indeed the culture in our tissue-on-chip induced a higher expression of dystrophin if compared to the standard culture on dish: the ratio between dystrophin and MyHC is higher in the culture performed in our microengineered model than the one performed onto standard multiwell (Fig. 2H).Our model on chip also offers the additional advantage of an order topology of myotubes. In our model, myotubes grow only onto the micro-patterned area and are all oriented along the main direction of the patterning. Such spatial organization allows easy quantification of the dystrophin expression domain, which would be much more difficult in a standard culture of myotubes, where they are randomly oriented.

Human mesoangioblasts[9,13] are a promising cell source for DMD therapy because they overcome some of the limitations associated with myoblast intra-muscular injections[8]. In particular they can be delivered through intra-arterial injections since they cross the endothelium and migrate extensively in the interstitial space, show long-term survival and partially restore muscle structure and function in dystrophic mice and dogs[11,12], beside contributing to the muscle satellite cell pool[8]. Here we tested the ability of mesoangioblasts to restore dystrophin expression, in terms of protein accumulation and distribution along the surface of myotubes derived from DMD patients, using a micro-engineered DMD model.

Interesting, three different batches of mesoangioblasts showed the ability to restore a significant level of dystrophin, which was analyzed by immunofluorescence and western blot. In terms of dystrophin

accumulation, restoration of dystrophin by mesoangioblasts was higher than the hypothetic 30% of control (according to study on animal or patient with XLDC)[7,8], provided that a 1:9 ratio was achieved *in vitro*. The domain of dystrophin restoration due to healthy mesoangioblasts nuclei spanned almost along the entire myotube (with an average domain length of 240 μm).

It is worth to underline that the mesoangioblasts used in this study were used in a phase I/II clinical trial based on intra-arterial delivery of mesoangioblasts, in children affected by Duchene Muscular Dystrophy at the San Raffaele Hospital in Milan (EudraCT no. 2011-000176-33). Cell delivery *in vivo* is a complex process and reproducing *in vitro* all the phases (such as delivery, migration, fusion to host tissue) will be very challenging. However, these phases may be dissected in separate steps, for each of which an *in vitro* assay may be developed as a predictive tool of the corresponding *in vivo* performance. For example, Boiden chambers coated with endothelium, under various experimental conditions, may mimic crossing of the blood vessel *in vivo*[23].

However, how a mesoangioblast nucleus (or of other myogenic progenitors) contributes to dystrophin synthesis and consequently, which is the minimal ratio of delivered/survived and fused cells versus resident myofiber nuclei needed to rise dystrophin level above the 30% threshold, is a fundamental question in this context. Indeed, in any cell therapy protocol, the preparation of donor cells to be injected require their manipulation *ex vivo* and it is widely known how this step could be crucial for the obtainment of good results. In this scenario, we hypothesize that our microscaled *in vitro* model could be used as a quality control test of donor cell batches and could help the prediction of the clinical outcomes. In this respect it is interesting to note that preliminary results from the trial[14] indicated that, on the patient with highest engraftment, donor dystrophin (detected by Western Blot was) was expressed, albeit at a very low level, when donor DNA (analyzed by satellite micro-chimerism) was approximately 1% of total DNA in the biopsy.

Another important aspect to consider is the very large number of variables that may be tested to enhance the efficacy of cell therapy, such as for example, pre-treatment of donor cells and/or of host muscle cells with molecules that may enhance differentiation, protein synthesis or fusion. Obviously it would be impossible to test all these variables in patients and pre-clinical experimental studies on animal models may not reveal subtle differences between mouse and human cells[24,25]. Our model could be used as an *in vitro* standard for testing the extent of dystrophin restoration in parallel to pre-clinical and before clinical studies.

In conclusion, we demonstrate that the evaluation of a fundamental clinical outcome such as dystrophin expression, conducted on skeletal muscle-on-chip could be a valuable support during pre-clinical phases or clinical trials.

References

1. Hoffman, E. P., Brown, R. H. & Kunkel, L. M. Dystrophin: The protein product of the duchenne muscular dystrophy locus. *Cell* **51,** 919928 (1987).
2. Emery, A. E. The muscular dystrophies. *The Lancet* **359,** 687–695 (2002).
3. Benedetti, S., Hoshiya, H. & Tedesco, F. S. Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies. *FEBS J.* **280,** 4263–4280 (2013).
4. Goyenvalle, A., Seto, J. T., Davies, K. E. & Chamberlain, J. Therapeutic approaches to muscular dystrophy. *Hum. Mol. Genet.* **20,** R69–78 (2011).
5. Goemans, N. M. *et al.* Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy. *N. Engl. J. Med.* **364,** 1513–1522 (2011).
6. Cirak, S. *et al.* Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet* **378,** 595–605 (2011).
7. Neri, M. *et al.* Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human. *Neuromuscul. Disord. NMD* **17,** 913–918 (2007).
8. Tedesco, F. S. *et al.* Stem Cell-Mediated Transfer of a Human Artificial Chromosome Ameliorates Muscular Dystrophy. *Sci. Transl. Med.* **3,** (2011).
9. Dellavalle, A. *et al.* Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. *Nat Commun* **2,** 499 (2011).
10. Cappellari, O. & Cossu, G. Pericytes in Development and Pathology of Skeletal Muscle. *Circ. Res.* **113,** 341–347 (2013).
11. Sampaolesi, M. *et al.* Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. *Science* **301,** 487–492 (2003).
12. Sampaolesi, M. *et al.* Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* **444,** 574–579 (2006).
13. Dellavalle, A. *et al.* Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat. Cell Biol.* **9,** 255–267 (2007).

14. Cossu, G. *et al.* Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy. *EMBO Mol. Med.* **7,** 1513–1528 (2015).
15. van de Stolpe, A. & den Toonder, J. Workshop meeting report Organs-on-Chips: human disease models. *Lab. Chip* (2013). doi:10.1039/C3LC50248A
16. Liang, P. *et al.* Drug Screening Using a Library of Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes Reveals Disease-Specific Patterns of Cardiotoxicity. *Circulation* **127,** 1677–1691 (2013).
17. Engle, S. J. & Puppala, D. Integrating Human Pluripotent Stem Cells into Drug Development. *Cell Stem Cell* **12,** 669–677 (2013).
18. Lesko, L. J. & Schmidt, S. Individualization of Drug Therapy: History, Present State, and Opportunities for the Future. *Clin Pharmacol Ther* **92,** 458–466 (2012).
19. Luni, C., Serena, E. & Elvassore, N. Human-on-chip for therapy development and fundamental science. *Curr. Opin. Biotechnol.* **25,** 45–50 (2014).
20. Serena, E. *et al.* Soft substrates drive optimal differentiation of human healthy and dystrophic myotubes. *Integr. Biol. Quant. Biosci. Nano Macro* **2,** 193–201 (2010).
21. Zatti, S. *et al.* Micropatterning Topology on Soft Substrates Affects Myoblast Proliferation and Differentiation. *Langmuir* **28,** 2718–2726 (2012).
22. Tonlorenzi, R., Dellavalle, A., Schnapp, E., Cossu, G. & Sampaolesi, M. Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. *Curr. Protoc. Stem Cell Biol.* **Chapter 2,** Unit 2B.1 (2007).
23. Pomerantz, J. H., Mukherjee, S., Palermo, A. T. & Blau, H. M. Reprogramming to a muscle fate by fusion recapitulates differentiation. *J. Cell Sci.* **122,** 1045–1053 (2009).
24. Giannotta, M. *et al.* Targeting endothelial junctional adhesion molecule-A/ EPAC/ Rap-1 axis as a novel strategy to increase stem cell engraftment in dystrophic muscles. *EMBO Mol. Med.* **6,** 239–258 (2014).
25. Willmann, R. *et al.* Enhancing translation: Guidelines for standard pre-clinical experiments in mdx mice. *Neuromuscul. Disord.* **22,** 43–49 (2012).
26. Meng, J., Muntoni, F. & Morgan, J. E. Stem cells to treat muscular dystrophies – Where are we? *Neuromuscul. Disord.* **21,** 4–12 (2011).

Figure captions

Figure 1: Description of the experimental strategy. Clinical trial (light red box) on stem cell therapies are based on the isolation and expansion of stem cells from a healthy donor following GMP guidelines. These cells are then injected into the DMD patient. Clinical trial on chip (light blue box) is based on the exploitation of the skeletal muscle on a chip, which allow to perform multiparametric and highthroughput experiments *in vitro*. Dys^+ and Dys^- cells are co-cultured at different ratio within the skeletal muscle on chip. The proportion used for the core set of experiments was 1 Dys^+ and 9 Dys^- cells (1:9). Dys^+ and Dys^- cells fuse and result in myotubes composed of Dys^+ and Dys^- nuclei. The micro-engineered model induces the functional maturation of the myotubes, promoting MyHC sarcomeric organization and dystrophin expression within 8 days. The co-cultures are analyzed in terms of dystrophin accumulation and localization within the membrane. The knowledge acquired is then integrated within the clinical trial process.

Figure 2: Validation of the assay. We verified the differentiation of primary myoblasts and mesoangioblasts on our device and characterized the co-culture assay. A-F: functional maturation of human myoblasts (A, C) human mesoangioblasts (B, D) and human DMD primary myoblasts (E,F) was confirmed through immunofluorescence against MyHC (A, B, E) and Dystrophin (C, D, F). G: Graph representing the percentage of myotubes derived from Dys^- cells (green), Dys^+ cells (red), not marked cells (white), and Dys^- cells fused with Dys^+ cells (orange). H: Graph representing the ratio between the band intensity of dystrophin and MyHC in Dys^+ myoblasts (Mbs) and Dys^+mesoangioblasts (Mabs). The culture were performed in standard 48 multiwell (MW) and onto the micro-engineered skeletal muscle chip (HY). I: a representative western blot of the co-culture of myoblasts. Samples: 1:1, 1:2, 1:9 are the ratio of Dys^+ and Dys^- myoblasts, DMD are Dys^- primary myoblasts (affected by DMD), H are Dys^+ myoblasts (from healthy donor). J: quantification of the bands intensity. Error bars, s.d.; n = 3 (E) and n = 4 (G) independent biological replicates. *: p-value < 0.05; **: p-value < 0.01 (two-sided t-test).

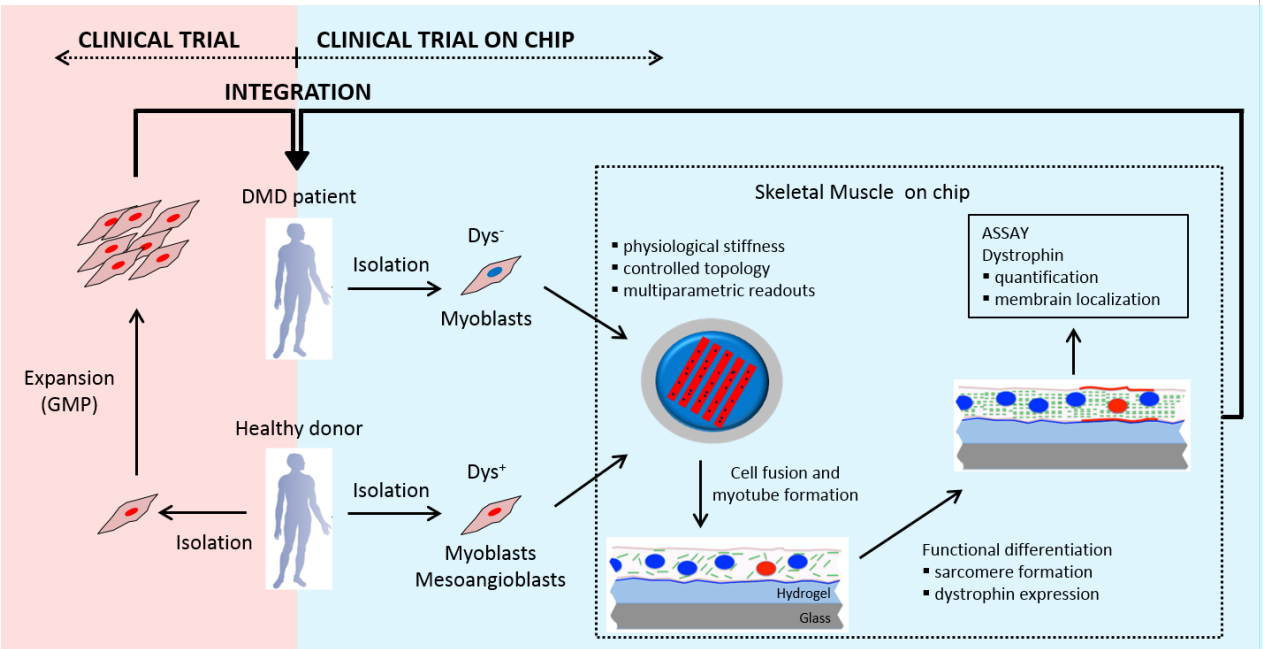
Figure 3: Analysis of the accumulation of dystrophin within co-culture experiments. A: representative western blot of the co-culture experiments of Dys^+ and Dys^- myoblasts (Mbs) and Dys^+ mesoangioblasts (Mabs) and Dys^- myoblasts. H = sample with only Dys^+ cells (Mbs or Mabs); DYS = dystrophin, MyHC = myosin heavy chain; β-ACT = β-actinin B: quantification of the percentage of dystrophin restoration as function of MyHC expression. Error bars, s.d.; n = 3 independent biological replicates.

Figure 4: Analysis of the length of dystrophin domain restored in the co-colture experiments. A-D: immunofluorescence against dystrophin in the 1:9 co-culture of Dys^+ and Dys^- myoblasts (A, B) and 1:9 co-culture of Dys^+ mesoangioblasts and Dys^- myoblasts (C, D). E: quantification of dystrophin domain length. Error bars, s.d.; n = 3 (MYB) and n = 2 (PE) independent biological replicates.

Figure 1S: cell population (Dys^- and Dys^+) staining. Representative images of several strategies to stain the two cell populations. A: hoechst staining of living myoblasts, we observed reduced cell viability after 5 days of culture. B: infection with a GFP adenovirus, which efficiency was low (around 50%). C: infection with a LacZ lentivirus, which stained the cells with a high efficiency (90%), however, the β-galactosidase was translocated to all the nuclei inside the myotube and after 10-12 days, we observed myotubes with all positive nuclei or with all negative nuclei and only in few cases gradient of staining. D-F: staining with lipophilic tracers DiI (red, Dys^-) and DiO (green, Dys^+), which allowed us to visualize and quantify the myotubes formed by Dys^- and Dys^+ cells.

Figure 2S: nuclei distribution. Quantification of nuclei distribution as function of dystrophin domain length. We divided the myotubes based on the domain length of dystrophin expression and counted the number of nuclei in each myotube within the same dystrophin domain. Error bars, s.d.; n = 2 independent biological replicates

Figure 1


CLINICAL TRIAL
CLINICAL TRIAL ON CHIP
INTEGRATION
DMD patient
Isolation
Dys-
Myoblasts
Expansion
(GMP)
Healthy donor
Isolation
Isolation
Dys+
Myoblasts
Mesoangioblasts
Skeletal Muscle on chip
▪ physiological stiffness
▪ controlled topology
▪ multiparametric readouts
ASSAY
Dystrophin
▪ quantification
▪ membrain localization
Cell fusion and
myotube formation
Hydrogel
Glass
Functional differentiation
▪ sarcomere formation
▪ dystrophin expression

+

Figure 2

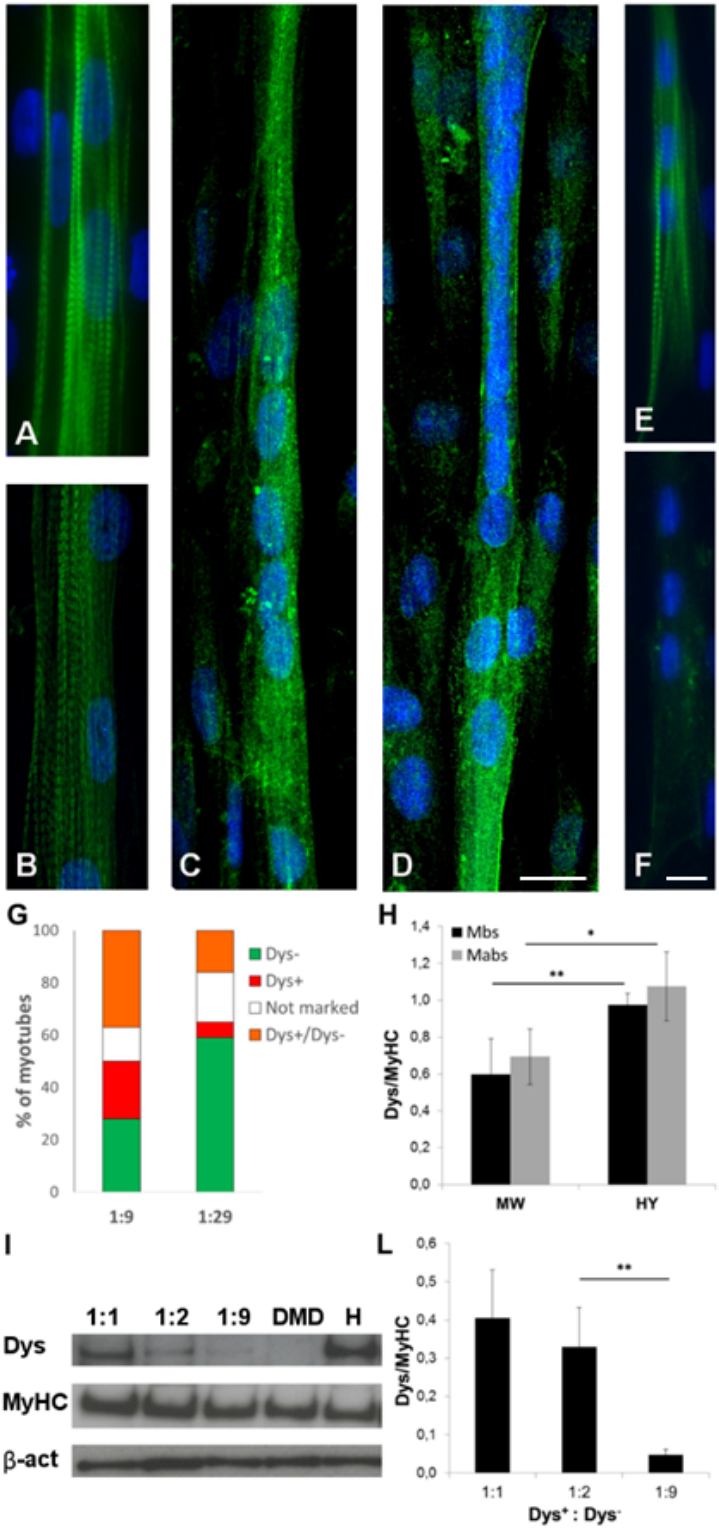

A
B
C
D
E
F
G
% of myotubes
100
80
60
40
20
0
1:9
1:29
Dys-
Dys+
Not marked
Dys+/Dys-
H
Mbs
Mabs
*
**
Dys/MyHC
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
MW
HY
I
1:1
1:2
1:9
DMD
H
Dys
MyHC
β-act
L
**
Dys/MyHC
0,6
0,5
0,4
0,3
0,2
0,1
0,0
1:1
1:2
1:9
Dys⁺ : Dys⁻

Figure 3

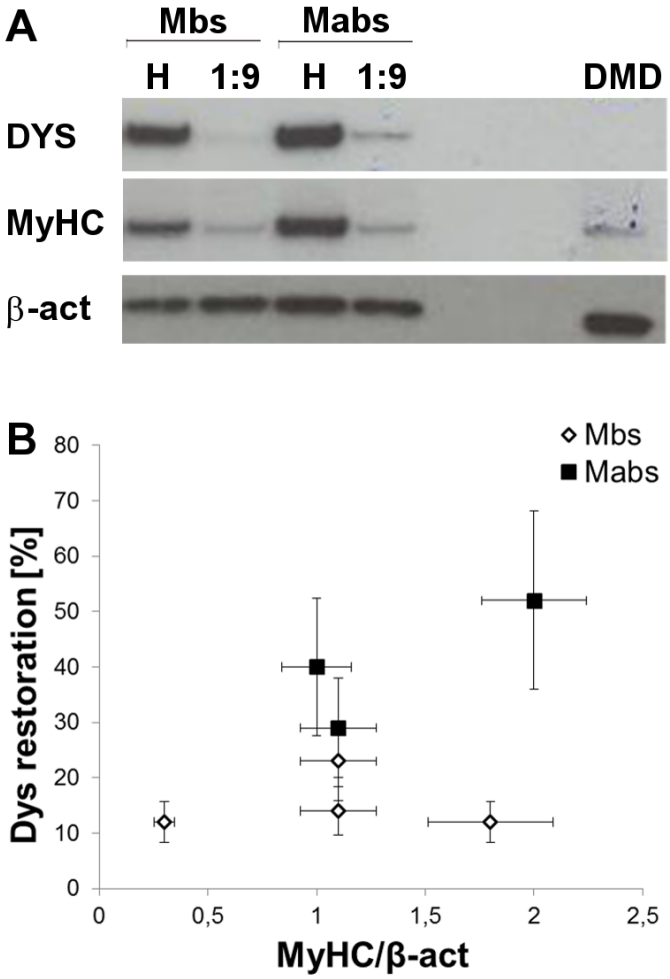
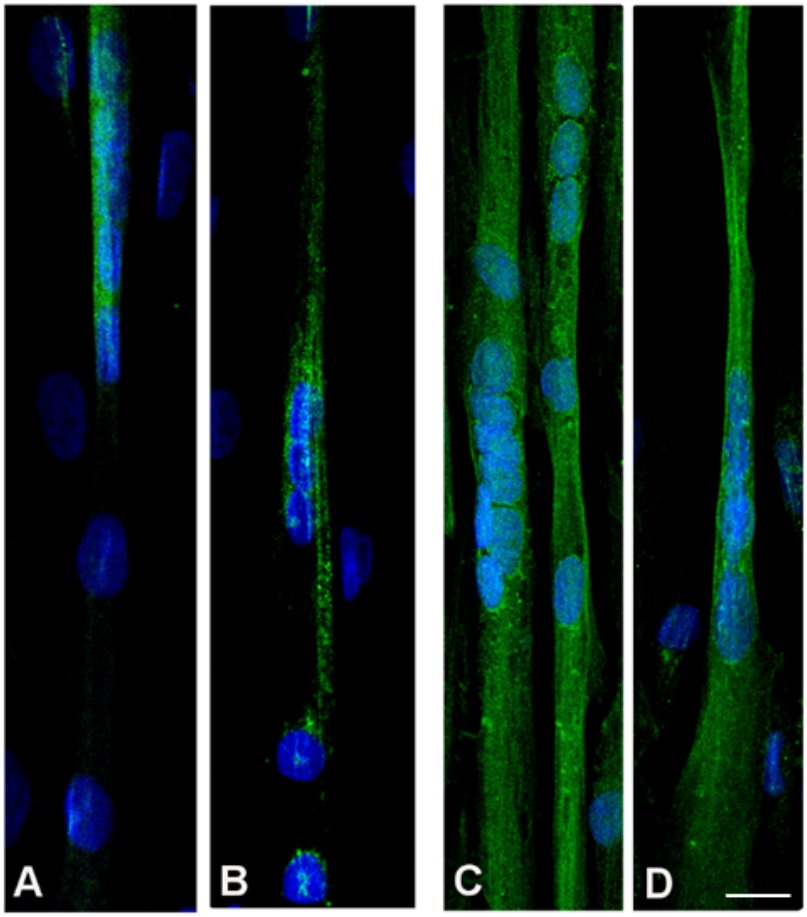
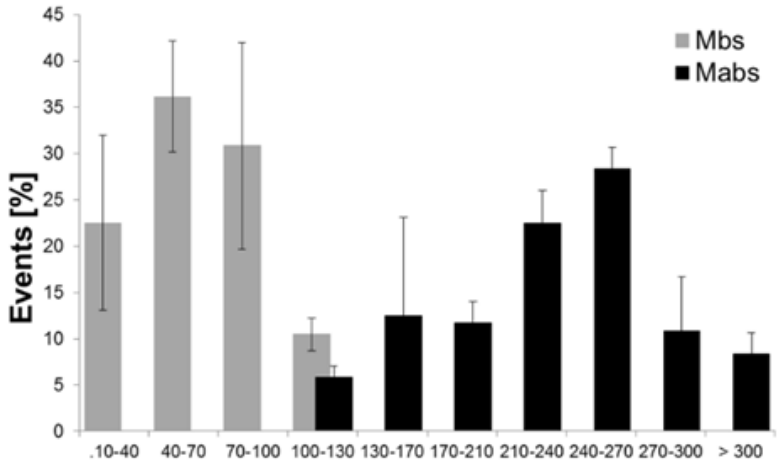

A
Mbs
Mabs
H
1:9
H
1:9
DMD
DYS
MyHC
β-act
B
Mbs
Mabs
Dys restoration [%]
MyHC/β-act
0
10
20
30
40
50
60
70
80
0
0,5
1
1,5
2
2,5

Figure 4


A
B
C
D


Mbs
Mabs
Events [%]
45
40
35
30
25
20
15
10
5
0
.10-40
40-70
70-100
100-130
130-170
170-210
210-240
240-270
270-300
> 300
Dystrophyn domain [µm]

E

Figure 1S

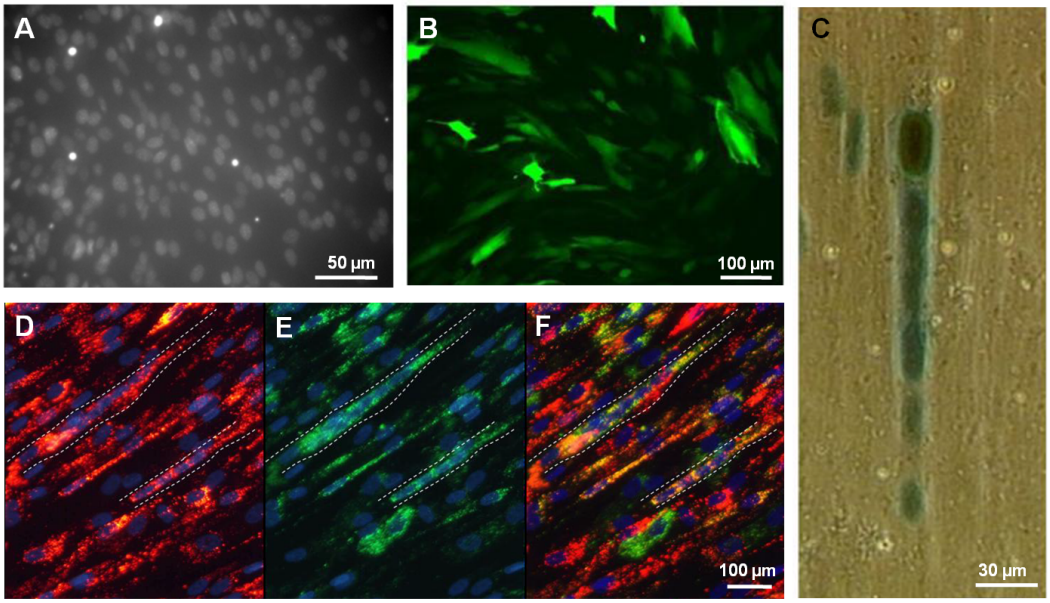


Figure 2S