Cyclic mechanical stimulation favors myosin heavy chain accumulation in engineered skeletal muscle constructs

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ABSTRACT

Purpose: **Since stretching plays a key role in skeletal muscle tissue development in vivo, by making use of an innovative bioreactor and a biodegradable microfibrous scaffold (DegraPol®) previously developed by our group, we aimed to investigate the effect of mechanical conditioning on the development of skeletal muscle engineered constructs, obtained by seeding and culturing murine skeletal muscle cells on electrospun membranes.**

Methods: **Following 5 days of static culture, skeletal muscle constructs were transferred into the bioreactor and further cultured for 13 days, while experiencing a stretching pattern adapted from the literature to resemble mouse development and growth. Sample withdrawal occurred at the onset of cyclic stretching and after 7 and 10 days. Myosin heavy chain (MHC) accumulation in stretched constructs (D) was evaluated by Western blot analysis and immunofluorescence staining, using statically cultured samples (S) as controls.**

Results: **Western blot analysis of MHC on dynamically (D) and statically (S) cultured constructs at different time points showed that, at day 10, the applied stretching pattern led to an eight-fold increase in myosin accumulation in cyclically stretched constructs (D) with respect to the corresponding static controls (S). These results were confirmed by immunofluorescence staining of total sarcomeric MHC.**

Conclusions: **Since previous attempts to reproduce skeletal myogenesis in vitro mainly suffered from the difficulty of driving myoblast development into an architecturally organized array of myosin expressing myotubes, the chance of inducing MHC accumulation via mechanical conditioning represents a significant step towards the generation of a functional muscle construct for skeletal muscle tissue engineering applications.**

Key words: Tissue engineering, Muscle, Myosin heavy chain, Bioreactor, Electrospinning

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INTRODUCTION

Both passive tension (i.e. unrelated to innervation) and active mechanical forces (i.e. induced by neuronal recruitment) play an important role in the transition of skeletal muscle from the embryonic to the mature state. For example, the continuous passive tension applied to skeletal muscle by bone growth during both embryogenesis and neonatal development will help to organize the proliferating mononucleated myoblasts into the oriented multinucleated myofibers of a functional muscle, and will also influence muscle weight and length as well as myofilament organization (1). Postnatally, passive stretching and contractile activation cooperate and are responsible for many in vivo muscle adaptations after early development, including the regulation of protein synthesis, accumulation and degradation rate (1-4) and of total RNA and DNA content (1, 3, 5). Increased work load and exercise were demonstrated to change mature muscle morphology by increasing both the number (hyperplasia) and diameter (hypertrophy) of muscle fibers and by regulating longitudinal and cross-sectional myofiber growth (6).

Based on the finding that stretching plays a key role in skeletal muscle tissue development in vivo, in the last 20 yrs several in vitro models (7, 8) have been developed to improve structure and function of engineered constructs by means of the mechanical stimulation of cultured skeletal muscle cells. Stretching patterns resembling in vivo (9-13) developmental phases were mainly shown to induce myosin heavy chain (MHC) accumulation and to im-

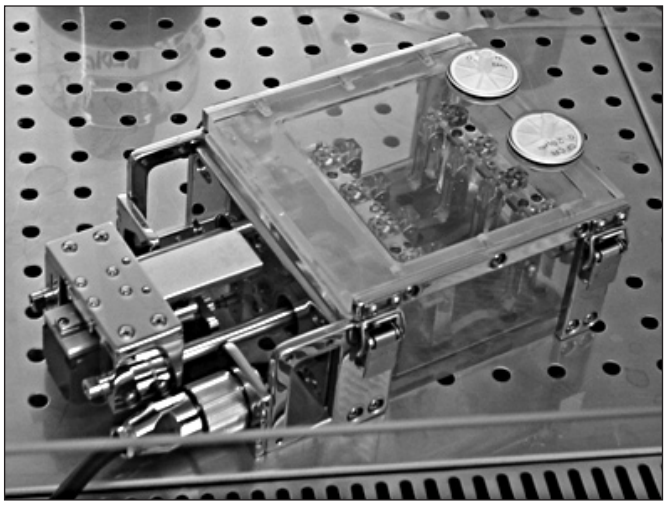

Fig. 1 - *The bioreactor culture chamber with a mechanical stimulation system. The Plexiglas® culture chamber, that houses four biological constructs holding them by means of a system of symmetrical grips, and the stepping motor are housed in the incubator, while the PLC is positioned outside the incubator. For a detailed description see (16).*

prove construct architectural organization. In this context, we recently developed (i) a biodegradable microfibrous scaffold for skeletal muscle tissue engineering (made of DegraPol®, a block polyesterurethane processed by electrospinning) (14, 15) and (ii) an innovative bioreactor, able to provide mechanical stimulation to engineered skeletal muscle constructs (16).

In this preliminary study we aimed to investigate the effect of mechanical conditioning on the development of skeletal muscle engineered constructs, obtained by culturing C2C12 (murine myoblast cell line) on electrospun DegraPol® membranes. The overall goal of this work was to assess a dynamic culture procedure to develop structurally optimized muscle constructs in vitro, evaluating the influence of a stretching pattern adapted from the literature (17) on the production of the typical skeletal muscle marker of terminal differentiation, total sarcomeric MHC in dynamically cultured myofibers.

MATERIALS AND METHODS

DegraPol® microfibrous membranes. DegraPol® block co-polymer was synthesized and purified as previously described (14). DegraPol® microfibrous membranes were manufactured by electrospinning as previously described (15). Briefly, meshes with highly orientated fibers (about 10 µm thick, investigated by scanning electron microscopy (SEM) analysis) on their surface were fabricated from a 25% (w/w) polymer-chloroform solution by ending the electrospinning sessions with a 15 min cycle at high speed (linear rate of the rotating cylindrical collector 7.6 m/sec). After drying the membranes in a high vacuum at room temperature (rt), DegraPol® strips were obtained using a rectangular 50 x 5 mm punch, washed in absolute ethanol (30 min, at -20 °C) and hexane (30 min, rt) and subsequently sterilized with ethylene oxide at 37 °C, as previously reported (15). The scaffold thickness, measured using a digital micrometer (Mitutoyo America Corporation, USA), was 212 ± 14 µm.

The bioreactor. Dynamic culture experiments were carried out with a bioreactor specifically designed and previously realized (16). Briefly, the device is composed of a Plexiglas® culture chamber, able to house four strip samples lying parallel during culture, and a mechanical stimulation subsystem (a stepping motor, two drive shafts and four pairs of grips holding the specimens at the extremities). The device components can be sterilized in ethylene oxide, allowed to de-gas for 5 days and easily assembled under a laminar flow hood due to support tools specifically designed for the purpose. Figure 1 shows the bioreactor chamber. During culture, a PLC controller drives the stepping motor, enabling reliable and reproducible stretching of four engineered constructs in parallel. A couple of dynamic diaphragms (Bellofram®, Bellofram Corporation, USA), positioned in the chamber wall and wrapping the drive shafts, ensure the maintenance of sterility together with the transmission of motion, while sterile gas exchange with the incubator was possible through 0.22 µm filters positioned on the lid.

Cell seeding and static culture on DegraPol® membranes. All chemicals were of reagent grade or higher quality and were purchased from Sigma-Aldrich Srl (Italy) unless specified otherwise. C2C12 (murine myoblast cell line, CRL-1772, ATCC, USA) were plated in 75 cm^2 tissue-culture flasks and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Laboratories Inc, USA) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Flasks were incubated at 37 °C in a humidified 5% CO_2 atmosphere. For the prevention of premature differentiation, cells were sub-cultured at semi-confluence (70-80% area). DegraPol® strips for each experiment were seeded as follows: after centrifuging harvested cells at 1,200 rpm for 5 min, supernatant was discarded and the pellet re-suspended in fresh DMEM to a final concentration of 4 x 10^5 cells/ml suspension. Seven 25 µl drops of this suspension were dispensed at regular intervals on each microfibrous sterile DegraPol® strip, resulting in a cell seeding density of 2.8 x 10^4 cells/cm^2. Samples were subsequently incubated for 30 min at 37 °C, before being transferred to different Petri dishes and immersed in complete DMEM (8 ml/strip). In order to provide differentiating myoblasts with a new source of myogenic cells, in an attempt to increase the efficiency of the incorporation of myoblasts into myotubes, an additional amount of cells was seeded on the cultured samples at day 4 after initial seeding (1 day before dynamic culture initiation). Seven 10 µl drops

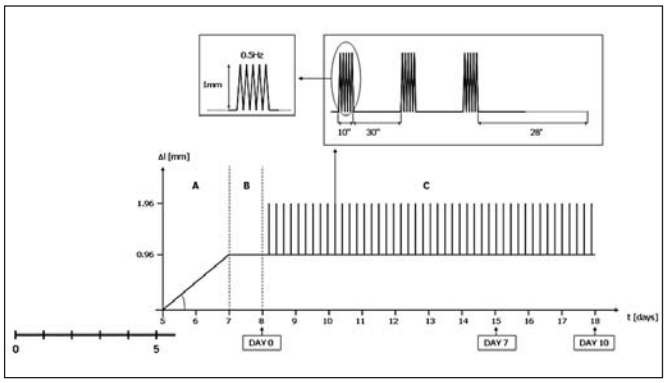

Fig. 2 - *Stretching pattern applied during mechanical conditioning, adapted from the literature to myoblasts of murine origin. The unidirectional stretching phase is meant to mimic bone growth during embryonic development, while the phase of cyclic stretch resembles increasing functional demand of the developing fetus.*

of a C2C12 suspension at 4 x 10^5 cells/ml were dispensed at regular intervals along the strip length and, after 30 min of incubation aimed at favoring cell adhesion, complete DMEM was added to the Petri dishes (8 ml/strip).

Mechanical conditioning. Five out of nine statically cultured DegraPol® strips were sterilely transferred into the bioreactor: while four of them were grasped in order to be subjected to dynamic mechanical stimulation (named D - dynamic - samples in the following), the fifth (non-stretched) remained floating in the same culture chamber for the rest of the experiment (13 days), serving as an internal control. The remaining four control samples (named S - static - in the following) were positioned in the same incubator and contemporaneously cultured in static conditions in Petri dishes. The medium was exchanged every third day. Figure 2 describes the stretching pattern that the D samples experienced during conditioning and was adapted from the mechanical activity pattern of embryonic avian skeletal muscle cells (17, 18) to myoblasts of murine origin: the unidirectional stretching phase (phase A) is meant to mimic bone growth-associated muscle lengthening during embryonic development (24 hr of stretching at 0.02 mm/hr, up to 960 μm displacement), while the phase of cyclic stretch (phase C) resembles cyclic functional demand of the developing fetus. In this phase, the constructs were stimulated with three consecutive displacement-controlled 5-pulse bursts (pulse frequency 0.5 Hz, amplitude 1 mm), separated by 30 sec rest and followed by 28 min rest. This phase involved stretching the samples for less than 2% of the time and inducing a 3.4% substrate deformation (calculated considering a uniform deformation of the free length of the specimen, 29 mm). The overall deformation of the samples, including unidirectional stretching of phase A, was 6.7%. Figure 2 also shows the time points at which sample withdrawal occurred. Taking the day of onset of phase C as a reference point (DAY 0 = day 8 after seeding), dynamic samples and static controls of four independent sets of experiments were collected at DAY 0 (D0 and S0, n=1 both), at DAY 7 (D7 and S7, n=1 both), and at DAY 10 (D10, n=5; S10 n=6; NS10, n=2), following timings previously proposed by Khodabukus and Baar (19) (S), immediately frozen in cold isopentane, air-dried, cut into pieces and stored at -80 °C until use.

Western blot analysis of MHC. Total protein extraction from the samples was obtained as previously described (20). After thawing, a few pieces of each specimen were minced and chopped in 200 μl of 50 mM Hepes, pH 7.4, containing protease inhibitors (1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin) and 1% Triton X-100 and subsequently incubated for 45 min at 4 °C. Following 5 min centrifugation (5,000 rpm, 4 °C) to remove scaffold debris, the supernatant, containing the solubilized proteins, was collected and protein content determined using a BCA Protein Assay Kit (Pierce Chemical Company, USA). For Western blot analysis of total myosin, 9.2 μg of protein homogenates were resolved in 8% SDS-polyacrylamide gels (Bio-Rad Laboratories, Italy). Proteins were transferred to polyvinylidene fluoride (PVDF, Invitrogen, Italy) membranes (0.22 μm) by electro-blotting. Membranes were incubated with mouse anti-MHC antibodies (MF20, 1:1,000 dilution, Developmental Studies Hybridoma Bank, USA). Loading and protein transfers were verified with a mouse anti β-actin antibodies (A 2228, 1:5,000 dilution). Following incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (115-035-062, 1:10,000 dilution, Jackson ImmunoResearch Laboratories Inc, USA), immunoreactive signals were visualized with LiteAblot enhanced chemiluminescent detection kit (EuroClone Life Sciences, UK). MHC bands were quantified by scanning densitometry using a Kodak 440 CF Image Station equipped with Kodak Molecular Imaging Software (Kodak, Italy). Data were reported as Net band intensity.

MHC immunostaining. After thawing cryopreserved fragments and washing isopentane residuals in PBS, a piece of approximately 10 x 5 x 0.2 mm of each of the six samples harvested at different time points was fixed in 1% paraformaldehyde (PFA) in PBS and subsequently stained for MHC and nuclei as follows. Following incubation in PBS supplemented with 1% bovine serum albumin (BSA) and 0.5% Triton X-100, samples were incubated at 4 °C overnight with monoclonal antibodies for total sarcomeric MHC (MF20, 1:100 dilution) in PBS containing 1% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$. Finally, samples were incubated at rt with FITC-conjugated secondary antibodies (F 6257, 1:100 dilution) in PBS containing 0.5% BSA and 0.25% Triton X-100. Following rinsing with PBS, nuclei were stained with Hoechst (1:1,000 dilution, rt, Invitrogen, Italy) and fixed at 4 °C in 1% PFA. Immunostained samples were subsequently mounted on glass slides and viewed under an Olympus

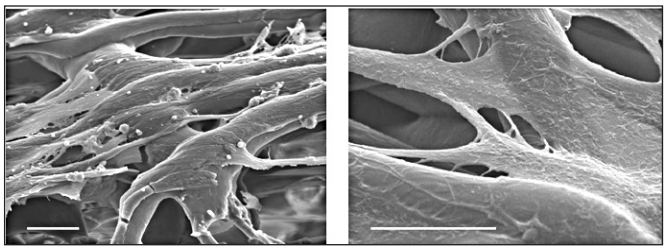

Fig. 3 - *SEM micrographs at different magnifications of C2C12 cells cultured for 5 days in static conditions (DAY 0) on DegraPol® membranes. Scale bars = 10 μm.*

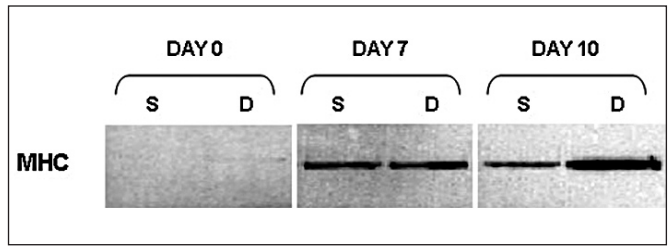

Fig. 4 - *Typical Western blot picture representing the accumulation of total myosin heavy chain (MHC) at days 0, 7 and 10 from the onset of cyclic stretching (phase C in Fig. 2) on stretched and control engineered constructs.*

BX51WI epifluorescence microscope, equipped with an Olympus U-CMAD3 camera and the analiSYS software (Olympus, Italy).

Scanning electron microscopy (SEM). In order to visualize myoblast spreading and alignment along the scaffold fibers before mechanical stimulation, a piece of two samples cultured for 5 days in static conditions (before the onset of phase A) was fixed with osmium tetroxide and dehydrated in a graded series of alcohols. Samples were air dried, glued onto SEM stubs, lightly coated with gold and observed at 9 kV using a Stereoscan S260 microscope (Leica Microsystems SpA, Italy).

Statistical analysis. Statistical analysis was carried out by GraphPad analysis (version 5; GraphPad Software Inc, USA).The mean and standard error of the mean (SEM) were calculated for the measured data of each group. The groups were compared with paired t-tests, with differences considered to be significant if $p<0.05$.

RESULTS

Figure 3 shows the morphology of cells proliferating on microfibrous DegraPol® scaffolds, investigated by SEM analysis. Micrographs represent cells cultured for 5 days in 10% FBS supplemented medium, showing the tendency of the myoblasts to spread along the scaffold fibers by developing filopodia. As previously described, once attached to the fiber surface, C2C12 myoblasts spread out and multiplied, forming layers of cells aligning parallel to the fiber axis (14-16).

The effect of the applied stretching pattern (Fig. 2) on the development of skeletal muscle engineered constructs was evaluated by investigating MHC accumulation in stretched constructs, using statically cultured samples (S) as controls. Western blot analysis on protein homogenates and immunostaining of total sarcomeric MHC, known as a marker of terminal differentiation, were performed. Figure 4 shows the result of Western blot for MHC on dynamically (D) and statically (S) cultured samples, at DAY 0 (D0, S0), DAY 7 (D7, S7) and DAY 10 (D10, S10) after the onset of cyclic stretching in the bioreactor (phase C in Fig. 2). Due to the preliminary nature of this work, at DAY 0 and DAY 7 we compared only one sample from a set of experiments in order to show the trend of MHC accumulation fully quantified at DAY 10. While S and D bands are comparable at time points DAY 0 and DAY 7, indicating the poor short-term effect of mechanical conditioning on MHC accumulation, MHC demonstrates a strong positive response to the applied stretching pattern between DAY 7 and DAY 10, with an eight-fold increase in myosin content in D samples with respect to the corresponding S controls at the time point DAY 10 (10377 ± 2047 Nbi in D10 vs. 1254 ± 205 Nbi in S10, $p<0.05$). Figure 5 represents densitometric analysis of Western blot assays. Statistical significance among groups and small standard errors (intragroup variability) demonstrated a fairly homogeneous production of MHC among D10 and S10 samples obtained from different sets of experiments. Moreover, while MHC accumulation is shown to decrease about 36% in S samples from DAY 7 to DAY 10, dynamically cultured D samples demonstrated accumulation of MHC as culture progressed, showing a 67% increase in MHC accumulation at DAY 10 with respect to DAY 7. MHC accumulation at the onset of cyclic stretching (DAY 0) was close to zero for both the S and D samples, suggesting that phases A and B of the stretching pattern have a negligible effect on MHC accumulation.

The effect of mechanical conditioning on skeletal myogenesis, inferred from Western blot analysis shown in Figure 4, was confirmed by immunofluorescent staining of MHC. Fluorescent micrographs of stretched (D) and static constructs (S) cultured for 0, 7 and 10 days from the onset of cyclic stretching (phase C in Fig. 2) are shown in Figure 6. While at DAY 0 no sign of myotube formation and myosin expression is detectable either in static or in stretched samples, at DAY 7 regular arrays of parallel, unbranched, multinucleated myotubes are visible in both dynamic and control constructs, suggesting that the applied stretching pattern does not interfere with the contact-induced myotube arrangement along the scaffold orientated fibers previously observed (15). When compared to statically

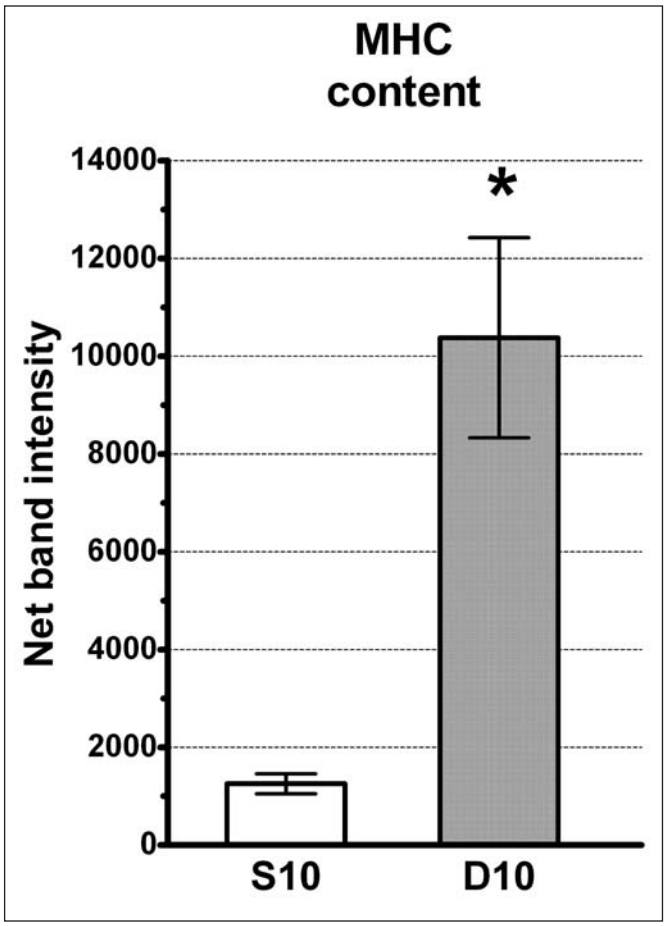

Fig. 5 - *Western blot quantification of total myosin heavy chain (MHC) in dynamically (D10, n=5) and statically cultured constructs at DAY 10 (S10, n=6) after the onset of cyclic stretching. Values are expressed as Net band intensity (sum of the background-subtracted pixel values in each band rectangle). Data, expressed as mean ± standard error of the mean (SEM) of at least five samples for each group, were analyzed by paired t-tests (*p<0.05).*

cultured constructs at DAY 10, stretched samples show marked myotube packing into more dense fiber arrays, demonstrating that mechanical stimulation has a positive effect on myotube development and arrangement.

DISCUSSION

A number of studies in the last 30 yrs have been carried out in order to investigate the effects of passive tension and electrical activation on the development of differentiated myofibers in vitro (21-24). Together the results of these studies, in the vast majority of the cases conducted on bi-dimensional culture substrates or employing collagen gels as matrices, demonstrated that electromechanical conditioning plays a key role in inducing in vitro skeletal myogenesis (25, 26). In particular, stretching patterns

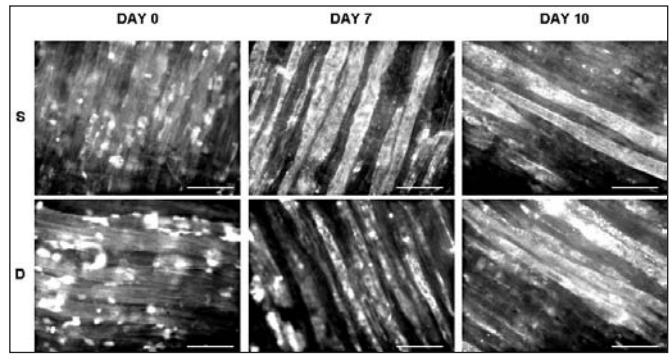

Fig. 6 - *Micrograph of C2C12 differentiating on DegraPol® microfibrous membranes after 7 and 10 days of static (S) or dynamic (D) culture. Following 5 days of culture in static conditions (DAY 0), the constructs were transferred in the bioreactor and subjected to a stimulation pattern adapted from literature. Staining represents myosin heavy chain (MHC), a marker of myogenic terminal differentiation. Scale bars = 100 μm.*

meant to resemble the mechanical cues acting during muscle embryogenic development and postnatal growth were demonstrated to induce cell hyperplasia and hypertrophy, besides favoring the structural optimization of cultured skeletal muscle constructs induced by the highly ordered architecture of scaffolds (i.e. inducing myotube alignment into parallel arrays of packed fibers along the direction of the applied stretch) (26, 27).

In this context, aiming to realize a comprehensive culture model system for in vitro skeletal muscle tissue engineering, in our previous works we designed and developed a degradable microfibrous scaffold for culturing skeletal muscle cells (14, 15) and a mechanical stimulator (bioreactor) suitable for dynamic conditioning of skeletal muscle cultured constructs (16). As to the scaffold, DegraPol®, a degradable biocompatible block polyesterurethane, it was processed by electrospinning in the form of microfibrous membranes with highly orientated fibers, which proved able to drive myoblast fusion and differentiation into myotubes along the preferential direction of fibers (15). The bioreactor, instead, was revealed to be a reliable and safe device that enables the delivery of stretching patterns (tuneable in frequency and intensity) to four engineered muscle constructs lying in parallel in a culture chamber. By allowing the precise control of topographical, chemical and mechanical environmental cues to which the cultures are subjected, our culture model system enables an orderly investigation of independent and concurrent effects of the several factors determining skeletal muscle development in vitro.

In this study we aimed to investigate the effects of mechanical stimulation on the development of skeletal muscle engineered constructs obtained by seeding and culturing C2C12 line cells on electrospun DegraPol® strips. First, cell seeding and alignment parallel to the fiber axis of scaffolds was verified by SEM analysis. More-

over, a stretching pattern adapted from the literature (17) to resemble mouse embryonic growth was applied to cell-scaffold constructs, and subsequently evaluated for MHC accumulation by Western blot and immunofluorescence analysis.

In this study we evaluated MHC accumulation in C2C12 skeletal myoblasts seeded and cultured on electrospun DegraPol® strips, in response to a stretching pattern (Fig. 2) adapted from the literature (17) to cells of murine origin. Phase A, 48 hr of displacement-controlled unidirectional stretching up to 960 μm, was meant to resemble bone-associated skeletal muscle growth during embryonic development; such a stretching phase, when applied during differentiation of cultured myoblasts, was previously demonstrated to increase the incorporation of myoblasts into myotubes and, above all, to orient the developing myofibers along the direction of the applied load (28, 29). Phase B represents a quiescence period, in which extracellular matrix components should strengthen the cultured myotubes, while phase C, a series of repetitive stretch-relaxation cycles 1 mm in amplitude, was thought to resemble the start of active embryonic movement and the functional load applied to the developing muscles. Periodic stretching was previously shown to induce both hyperplasic and hypertrophic effect in cultured skeletal muscle cells (6). Despite the preliminary nature of this study, interesting trends were noticed in MHC accumulation when mechanical stimulation was applied to skeletal myoblasts cultured on DegraPol® strips. Phases A and B were shown not to induce a significant effect on MHC accumulation in cultured skeletal muscle cells. In fact, observing Figure 4, only a negligible difference in MHC accumulation can be noticed at time point DAY 0 (corresponding to the onset of phase C) when the stretched sample (D) is compared to the corresponding statically cultured control (S). Fluorescent micrographs shown in Figure 6 confirm the absence of myosin expressing differentiated myotubes at this time point, both in the stretched and in the static sample. This finding is not surprising and it correlates coherently with our previous observations (14, 16) concerning C2C12 differentiation on DegraPol® electrospun membranes, as determined by RT-PCR and immunostaining. The most significant finding of this work, however, is that the applied pattern of stretch-relaxation cycles induced a greater accumulation of MHC in stretched samples with respect to their static controls. Comparing time points DAY 7 and DAY 10 in Figures 4 and 5, it is evident that, while MHC accumulation dramatically dropped when the constructs were cultured in static conditions, stretched samples accumulated MHC, resulting in an eight-fold increase in total MHC content after 10 days of stretching (D) compared to static conditions (S). These results are in agreement with previous works showing the influence of contractile activity on the expression and maintenance of MHC in C2C12 cells (19) and the importance of in vitro mechanical stimulation of human muscle precursor cells (MPCs) to accelerate maturation and function of engineered skeletal muscle in vivo (30). Several observations can be derived from the trends described. Although the mechanisms underlying stretch-induced cell growth are not known in detail, different hypothesis can be formulated in order to interpret the above reported results. Two are the major phenomena that could lie behind MHC accumulation: cell hyperplasia due to the increase in cell proliferation prior to differentiation processes and cell hypertrophy with an increase in incorporation of nuclei into myotubes or stretch-mediated gene regulation leading to an increase in MHC synthesis and/or a decrease in MHC degradation. Since we did not perform any quantification of cell proliferation (e.g. via DNA quantification or direct cell counting), we are not able currently to determine whether the leading phenomenon inducing MHC accumulation in stretched samples is cell hyperplasia, cell hypertrophy, or a combination of both. Nonetheless, based on the literature (5, 10), we believe that cell proliferation may be augmented in stretched samples during phase A by a release of contact inhibition associated with an increase of the available surface area (not quantified) induced by substrate stretching. Some other likely mechanisms that could justify MHC accumulation in stretched constructs are a mechanically-induced sustained release of growth factors, positively influencing protein synthesis and preventing myotubes from atrophy (11, 31), and stretch-associated enhancement of diffusion among the substrate fibers, allowing catabolite/anabolite exchange. In particular, nutrients that have penetrated the construct may be pushed toward its center with mechanical perturbation, thus contributing to a general improvement of cell metabolic capabilities.

CONCLUSIONS

In conclusion, since previous attempts to reproduce skeletal myogenesis in vitro mainly suffered from the difficulty of sustaining myosin-expressing myotube cultures for longer than 3-10 days, our preliminary findings herein showing that cyclic stretching induces MHC accumulation and contributes to myotube maintenance in a three-dimensional environment (Fig. 6) will pave the way for effective engineered skeletal muscle construct development.

Many issues are currently under investigation and will be the object of future studies, such as the identification of the leading phenomenon inducing MHC accumulation in stretched samples, the effect of increasing the time span of mechanical stimulation on MHC accumulation, the possibility to combine mechanical conditioning with gene therapy approaches (32-35) for effective guidance strategy (36) for skeletal muscle cell differentiation and tissue development.

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Conflict of interest: The authors have no financial interest in any of the products or devices described in this article.

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