An Engineered Multiphase Three-Dimensional Microenvironment to Ensure the Controlled Delivery of Cyclic Strain and Human Growth Differentiation Factor 5 for the Tenogenic Commitment of Human Bone Marrow Mesenchymal Stem Cells

An engineered multiphase 3D microenvironment to ensure the controlled delivery of cyclic strain and *h*GDF-5 for the tenogenic commitment of *h*BMSCs

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ABSTRACT

At present, injuries or breakages of tendons are treated by surgical repair and/or conservative approaches with poor clinical outcome. Alternative strategies able to repair tendon defects without the undesirable side effects associated with the current reconstructive options are needed. In this view, a tissue engineering approach has gained considerable attention as a promising strategy.

Here, we investigated a synthetic 3D microenvironment able to interacting with onboard stem cells and inducing, via coupled biochemical and physical signals, their early commitment towards the tenogenic lineage. This multiphase 3D construct was organized with a braided hyaluronate elastic band merged with *human* bone marrow mesenchymal stem cells (*h*BMSCs) and poly-lactic-co-glycolic acid microcarriers loaded with human growth differentiation factor 5 (*h*GDF-5) by means of fibrin hydrogel. The multiphase structure allowed *h*BMSCs culture under cyclic strain within a microenvironment with a controlled amount of *h*GDF-5 regularly delivered. The cooperative biochemical and physical stimuli induced significantly increased expression of tenogenic markers, such as collagen type I and III, decorin, scleraxis and tenascin-C, within only three days of dynamic *h*BMSCs culture. This approach opens a perspective for future development of engineered tendon tissue substitutes.

INTRODUCTION

Tendons allow force developed by muscle contraction to be transmitted to bone for the stabilized movement of a joint. Tendon lesions obviously impair joint motion and can produce disability[1]. Their spontaneous healing produces reparative, scar-like tissue, lacking the mechanical properties of native tissue, with limited functionality[2-3]. Therapeutic options presently available are direct suture repair, reconstruction with an auto/allograft, or use of prosthetic devices[4]. However, even under the best circumstances, the mechanical and biological properties of the joint remain permanently impaired[5].

Providing tissue-engineered substitutes grown *in vitro* and able to integrate with the host tissues is a promising alternative[6]. Scaffolds composed of various biomaterials manufactured via several fabrication techniques have been used for tendon tissue engineering (TE), but there is still the need for substitutes with adequate biological and mechanical properties able to provide support for cell attachment, proliferation and differentiation[7-8]. A combination of appropriate cells, biomaterials/scaffolds, biochemical cues/growth factors and physical stimulation seems a successful approach to this aim. Tenocytes, fibroblasts and stem cells (SCs) have all been tested as potential candidates for the purpose[9]. Adult mesenchymal SCs (MSCs) are multi-potent cells able to differentiate into several lineages both *in vitro* and *in vivo*. Given their proliferation potential, bio-molecular production, cell-to-cell signaling and formation of appropriate extracellular matrix (ECM), MSCs appear as the most promising cell source to improve structural and biomechanical features of an injured tendon upon autologous administration[10]. In particular, bone marrow MSCs (BMSCs) have been studied for tendon repair and regeneration[11] and two clinical trials are in progress investigating the efficacy of BMSCs in the treatment of refractory Achilles tendinopathy (source: Australian New Zealand Clinical Trials Registry, Trial Number: ACTRN12610000985088) and rotator cuff tear repair (source: ClinicalTrials.gov, Trial Number: NCT01687777). Several growth factors (GFs), including insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), basic fibroblastic growth factor (bFGF), bone morphogenetic proteins (BMP), transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) are involved in the activation and regulation of the

cellular responses during tendon repair[12]. The inclusion of one or more of them into a TE protocol should improve/accelerate the tissue substitute's fabrication by activating signaling cascades leading to the transcription of tendon tissue specific genes[13-14]. In particular, several authors reported the ability of growth differentiation factor 5 to induce genes consistent with tenogenic differentiation and significantly stimulate neo-tendon phenotype[15-18]. On the other hand its deficiency is reported to delay Achilles tendon healing in mice[19-20]. Thus, it has been recently suggested that a sustained release of GFs inside a 3D scaffold may provide an effective level of control when addressing cell phenotype, with respect to the simple dilution of GFs into the culture medium**[21]**. Poly-lactic-co-glycolic acid (PLGA) microcarriers (MCs) were proposed as transient scaffold components to ensure GFs sustained and controlled delivery[22-23].

Mechanostimulation may also act as a significant input to maintain[24] or acquire[25] the tendon phenotype. In particular, mRNA expression of type I and III collagen, and tenascin-C – all of them tenogenic markers – significantly increased in MSCs subjected to 10% stretching for 48 hrs; this effect persisted 48 hrs later[26]. Also, evidence is published about the promotion of tenogenic differentiation *in vitro*, when a mechanical stimulus was applied to braided nanofibrous scaffold[27]. Several bioreactors have been used to transfer a mechanical stimulus to cells in culture[28-29]. Some of them can also act as a stand-alone cell culture incubator, easy to operate and effective in transferring a controlled, recordable and adjustable (cyclic) deformation to a 3D scaffold[30].

The choice of a suitable biomaterial to fabricate a scaffold to support cell growth also is a relevant issue[31]. Scaffolds should have appropriate mechanical properties to provide support, which is critical to the early phase of repair. In addition, biocompatibility of the substrate for cell attachment and proliferation, along with its biological signals, are especially important for SC-based approaches to tendon regeneration[8]. Biomaterials used for scaffold fabrication include both biomimetic synthetic polymers and biological molecules[32], but an ideal biodegradable scaffold has not been identified yet. Scaffolds from hyaluronan polymer with braided fibers have been used; some weeks after surgery, these scaffolds produced improved biomechanical properties of the regenerated tendon tissue in the

rotator cuff, and bolstered production of type I collagen in rabbits[33]. Also, PLGA scaffolds have been reported to improve tendon healing, both histologically and mechanically, and to facilitate the production of type I and III collagen fibrils improving both *in vitro* and *in vivo* tendon mechanical properties[34-35].

Although each relevant signal, useful to induce tenogenic lineage commitment, is individually explored in the literature resumed above, very few protocols have proposed to exploit their synergic action within a cooperative approach[36]. Barber et al.[37] presented a protocol where *h*MSCs were cultured in presence of mixed tenogenic growth factors (BMP-2, FGF-2 and GDF-5) and stimulated with cyclic tensile strain, reporting their commitment into the tenogenic lineage, as evidenced by the significant up-regulation of mainly Scleraxis gene expression after 17 days in culture. Although this evidence corroborates the synergistic effect of supplying cooperative biochemical and mechanical signals to the cells, neither information about the specific contribution of single GFs and their active concentration within the engineered tissue construct, nor the minimum time needed for obtaining significant commitment were presented and discussed.

Aiming at providing a protocol where a 3D microenvironment ensures the controlled delivery of cooperative biochemical and mechanical signals to differentiating cells, GDF-5 was chosen, as the relevant biochemical signal. The goal was to develop a protocol where cells within a 3D microenvironment are stimulated with controlled mechanical deformation and a locally-released, kinetically-controlled biochemical signal, to induce their fast tenogenic lineage commitment. To this aim a multiphase 3D microenvironment was designed, comprising: (i) a braided hyaluronate elastic band, (ii) poly-lactic-co-glycolic acid microcarriers loaded with human GDF-5 (*h*GDF-5), (iii) human BMSCs (*h*BMSCs) merged together within a fibrin hydrogel and cultivated under cyclic strain culture. The early induction of cell tenogenic markers was evidenced after only three days of this dynamic culture.

MATERIAL & METHODS

1.1. SEE technology

PLGA MCs were obtained using Supercritical Emulsion Extraction (SEE). This innovative technology allows the fast production of polymer MCs from multiple emulsions by supercritical extraction of the oily phase in a countercurrent packed tower operating in continuous mode[38]. In this experiment, unloaded and *h*GDF-5 loaded MCs were fabricated utilizing a fixed *water-oil-water* emulsion ratio of 1:19:80. Briefly, recombinant *h*GDF-5 (PeproTech, Rocky Hill, NJ, USA) was dissolved into a 0.04% w/v human serum albumin (*h*SA, Sigma-Aldrich, Milan, Italy) containing poly-vinyl alcohol (PVA) used as surfactant. This solution was added to the oily phase composed of ethyl acetate (EA, purity 99.9%) and poly-lactic-co-glycolic acid (PLGA 50:50, RESOMER® RG 504H, Mol wt: 38000-54000 kDa from Boehringer Ingelheim, Germany) at 10% w/w. The primary water-oil (w_1/o) emulsion was obtained after 90 seconds sonication with a digital ultrasonic probe at 50% of amplitude (Branson Ultrasonics Corporation, Danbury, CT, USA). The emulsion was immediately poured into a 0.6% w/w EA-saturated aqueous Tween 80 solution, which is used as outer water phase to form the secondary emulsion by a high-speed homogenizer (mod. L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, UK) for 6 min at 10°C in an ice bath with a stirring rate of 2800 rpm. The emulsions were processed by SEE immediately after preparation. Operative pressure and temperature conditions in the high-pressure column were 8 MPa and 38°C, respectively, with an SC-CO_2 flow of 1.4 kg/h and an L/G ratio of 0.1 on mass based[39]. The suspension was continuously collected at the bottom of the extraction column, washed to eliminate the surfactant and lyophilized. To obtain a cell culture-grade preparation the washing steps were performed in presence of a Pen/Strep (1% w/v) solution to reduce contamination risks. Each run allowed the recovery of 98% of the loaded biopolymer, and assured an excellent batch-to-batch reproducibility. Empty and loaded MCs were produced using the same process conditions.

1.2.Microcarrier morphology and size distribution

The droplets formed in the emulsion were observed using an optical phase contrast microscope (Olympus, Tokyo, Japan). The shape and morphology of the MCs were investigated by Field Emission-Scanning Electron Microscopy (FE-SEM) (mod. LEO 1525, Carl Zeiss, Oberkochen, Germany). Samples of powder were placed on a double-sided adhesive carbon tape previously stuck to an aluminum stub and coated with a 250Å thick gold film using a sputter coater (mod.108 A, Agar Scientific, Stansted, UK). Scaffold structure was also fixed it in 4% paraformaldehyde (PFA; Sigma-Aldrich) for 3 hrs, followed by an overnight incubation in 0.1 M sodium cacodylate/4% PFA (Sigma-Aldrich). The scaffolds were then dehydrated in a series of ethanol dilutions (75, 90, and 100%), embedded in paraffin (Fisher Scientific, Milan, Italy), and sectioned. The structure was then coated with gold as described above. Droplet size distributions (DSD) of the emulsion and particle size distributions (PSD) of the suspension were measured using a laser granulometer (mod. Mastersizer S, Malvern Instruments Ltd., Worcherstershire, UK), based on dynamic light scattering (DLS). Sizes are expressed as volume mean size (MS, μm) with standard deviation (SD, ± μm).

1.3. hGDF-5 loading, release study and diffusion coefficient calculation

*h*SA was used in the MCs production process as a *h*GDF-5 stabilizer in the internal water phase of the double emulsion. The specific amount of *h*SA loaded into PLGA MCs was determined by dissolving 10 mg of dried powder in 600 μL of acetonitrile and sonicating it in 1400 μL of water. The remaining undissolved PLGA was separated by centrifugation at 2000 rpm for 2 min. The resulting clear supernatant solution was directly analyzed at room temperature (RT) by high-performance liquid chromatography (HPLC) (Agilent Technologies Inc., Santa Clara, CA, USA). The amount of *h*SA in solution was calculated by HPLC-UV analysis using a calibration curve and then converted into the effective loading in terms of amount (mg) of protein loaded into PLGA (g). Similarly, *h*GDF-5 loaded into PLGA MCs was extracted with the same procedure and monitored by ELISA-based assay (PeproTech), then calculated as μg/g (protein loaded/PLGA).

*h*GDF-5 release profiles were monitored *in vitro*, using the same ELISA-based assay. Microparticles of 20 mg were suspended in 2 mL of Dulbecco's modified Eagle medium (DMEM) and placed in an incubator at 35°C and stirred continuously at 50 rpm. At fixed time intervals, the samples were centrifuged at 4000 rpm for 15 min and the supernatant was completely removed and replaced with fresh media to maintain sink conditions. Released *h*GDF-5 concentrations from collected samples were then measured with an ELISA (PeproTech). Release experiments were performed in duplicate (n=2), and the proposed curve is the mean profile obtained.

*h*GDF-5 diffusion coefficient from the release data was calculated assuming a spherical geometry of the system, a constant diffusion coefficient, and a fixed initial molecule concentration. In this way, the diffusion controlled mass transfer equation can be written as the *equation 1*:

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \cdot \sum_{n=1}^{\infty} \frac{1}{n^2} \cdot \exp\left(- \frac{D \cdot n^2 \cdot \pi^2 \cdot t}{r^2} \right) \quad (1)$$

where, **Mt** represents the amount of the released molecule at given time (t), **M**∞ the maximum amount released, **D** is the diffusion coefficient of the molecule, and **r** the radius of the considered system. PLGA degradation follows pseudo-first order kinetics, and is influenced by polymer molecular weight and MCs size. PLGA degradation is not affected by the presence/absence of the encapsulated molecule[40]. After exposure to the release medium, water imbibes into the MCs leading to an autocatalytic reaction that produces a reduction of the macromolecular weight of the PLGA[41]; therefore, in addition to diffusional processes, the degradation of the polymer can be taken into account by considering that drug diffusivity increases as the molecular weight of the polymer decrease. This dependence of **D** on $\mathbf{M_w}$ is described by *equation 2*.

$$D = D_0 + \frac{k}{M_W} \quad (2)$$

where $\mathbf{D_0}$ is the diffusion coefficient of the molecules in the undegraded polymer (t=0) and **k** is a constant that characterizes the dependence of the diffusivity on the molecular weight of the polymer.

This parameter is not a universal constant, but depends on the physicochemical properties of the system and a value of 5.9 10^{-10} cm^2/kDa/sec was used for the data fitting[41]. The fitting procedure for D calculation was based on the minimization of the resulting differences between experimental and theoretical values (least squares method; R^2 value = 0.98).

1.4. hBMSC isolation and harvesting.

The data presented in this manuscript were obtained using *h*MSCs out of bone marrow from 3 male donors (age between 33 and 39), each of them for a single experiment (n = 3) including the static and the dynamic cultures with either empty or *h*GDF5-loaded microcarriers. Cells were used at passage number two. Written informed consent was obtained (Immune-Haematology U.O.C. Ethical Committee prot. Nr. 071/2015). *h*BMSCs were isolated and cultured as previously described with some modifications[42]. Briefly, mononuclear cells were enriched using a Ficoll-Paque PLUS gradient (GE Healthcare Life Sciences, Uppsala, Sweden), then seeded in T75 plastic flasks (10^6 MNCs/cm^2) with MesenCult™-SF Culture Kit (StemCell Technologies INC, Vancouver, BC, Canada) and incubated at 37°C, in a 5% CO_2 atmosphere and 95% relative humidity. The medium was changed every 3-4 days. Once the cell cultures reached 70–80% confluence (12-17 days for the first passage), the cells were dissociated with StemPRO accutase (Invitrogen, CA, USA), and replaced every 6-8 days at approximately 80% confluence. The first passage (P1) of the cells was used if not otherwise stated. To validate the purity of the *hB*MSCs at the end of the *in vitro* culture, FACS-analyses were performed. Isolated cells were detached using StemPRO accutase, stained for MSC markers (CD105, CD90 and CD73) and hematopoietic SC markers (CD14, CD20, CD34, CD45) (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed by flow cytometry (FACSCalibur, Becton Dickinson Headquarters). *h*MSCs were characterized using the following directly conjugated monoclonal antibody: Mouse Anti-Human CD73 PE (phycoerythrin), clone AD2; Mouse Anti-Human CD90 FITC (Fluorescein isothiocyanate), clone 5E10; Mouse Anti-Human CD105APC (Allophycocyanin), clone 266; Mouse Anti-Human CD14 APC, clone MφP9; Mouse Anti-Human CD20 FITC, clone L27; Mouse Anti-Human CD34 PE, clone 8G12; Mouse Anti-Human CD45 FITC, clone 2D1

1.5.Multiphase 3D microenvironment preparation

The SCs-based scaffold was prepared by hydro-gelling a fibrin mesh on hyaluronate braided fibers (Hyalonect®, courtesy of Anika Therapeutics, Abano Terme, PD, Italy) to embed *h*BMSCs and PLGA MCs (w/wo *h*GDF-5) into a multiphase deformable tissue construct able of biochemical signal delivery. To prepare each sample, a mixture of fibrinogen from human plasma (Sigma-Aldrich), aprotinin (Sigma-Aldrich) and DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (referred to as growing medium, GM), was added at 1:1:1 ratio to 100 mg of PLGA microcarries (56 µg/g w/wo *h*GDF-5) and then to an average of 5 x 10^5 cells. The amount of loaded MCs was chosen in order to achieve a physiologically relevant level of *h*GDF-5 inside the scaffold during the first 3 culture days[18]. The cells/MCs suspension was homogeneously pipetted into a mold (30x20x0.45 mm) to fill all the available volume in which the band was previously placed. After a quick addition of 100 U/mL thrombin (Merck-Millipore, Vimodrone (MI), Italy), the mold was placed in a 37°C humidified incubator for 30 min to allow fibrin polymerization. As soon as the hydrogel was formed, the band was entrapped inside the hydrogel that uniformly distributed on both sides of it. Then, engineered constructs were transferred from the molds to standard polystyrene culture plates, each containing 15 mL of the GM, and cultured in an incubator at 37°C in a 5% CO_2 atmosphere and 95% relative humidity. After 24 hrs, the medium was changed, and the engineered constructs were subjected to static (referred to as static group, SG) or dynamic culture (referred to as dynamic group, DG).

1.6. Static and dynamic culture

The SG-engineered constructs (w/wo *h*GDF-5, referred to as SG + /SG - respectively) were cultured statically within culture plates at 37°C in a humidified atmosphere containing 5% CO_2 for 72 hrs. The mechanical stretch was applied to DG-engineered constructs in a custom-made bioreactor device[30]. Briefly, the Hyalonect® scaffold was clamped at both free ends, held by one motionless and one sliding arm (operated by a linear motor actuator), and assembled into the stand-alone culture chamber. After selection of stretching parameters using a custom-written software developed with LabVIEW

v. 8.2 (http://www.ni.com/labview), the sliding arm of the apparatus moved to a pre-tensioning position, representing the maximal load. This load was then relaxed to a minimum value cycling between these two positions at the imposed frequency. In addition, continuous feedback signals provided by a load cell positioned on the fixed arm allowed to maintain the defined load on the scaffold in response to physical modifications in the growing pseudotissue in culture, by automatic adjustment of the pre-tensioning position. For the mechanical stimulation of DG, the engineered constructs (w/wo *h*GDF-5, referred to as DG + /DG - respectively) were placed into the stand-alone culture chamber of the bioreactor, and stretched at 1 Hz of frequency and 0.1 N of strain force (which is about 10% elongation in length). These operating parameters were set after mechanical testing of the mechanical behavior of the simple braided hyaluronate band and of the tridimensional multiphase construct. These measurements were performed according to the ASTM 1708 by a CMT 6000 dynamometer (SANS, Shenzen, China) equipped with a 1 kN load cell. Samples conditioned in DMEM medium during 1 hr were shaped following manufacturer instructions to obtain specimens having a gauge length (Lo) of 22 mm and a width (W) of 5 mm. For each sample, thickness (S) was measured with a thickness gauge brand at three different averaged points. A monoaxial deformation was applied to the sample with a speed 22 mm/min, and force (F) and elongation (L) during traction was registered. The value of force (F) provided by the instrument was divided by the sample area (A = WxS) to obtain the strength values (σ = F/A). The deformation values L during the run were compared to the initial length to obtain values of strain (ε = (L- Lo)/Lo); the ultimate tensile strength (σ max, expressed in MPa) was calculated as load to failure/cross sectional area of the sample.

1.7. RNA Isolation and Gene Expression Profile

At the end of each experiment for SG or DG, cells were collected, and the mRNA expression of type I and III collagen (*Col1A1*, *Col3A1*), decorin (*DCN*), scleraxis (*SCX*), and tenascin-C (*TNC*) was analyzed by RT-qPCR. Total RNA was extracted from *h*BMSCs seeded into the 3D construct of each experimental group using TRIzol® reagent (Life Technologies, Monza, Italy) and chloroform (Sigma-Aldrich) as previous described[43]. For each sample, 400 ng of total RNA was reverse-transcribed using iScript® cDNA synthesis kit (Bio-Rad, Milan, Italy). Relative gene expression analysis was performed in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad), using the SoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) with the validated primers for *Col1A1*, *Col3A1*, *DCN*, *SCX* and *TNC* (Bio-Rad), and following MIQE guidelines[44]. Amplification was performed in a 20 µl final volume including 5 µl of cDNA as template. Specificity of the formed products was addressed performing a melting curve analysis. Duplicate experiments (two replicates each) were performed for each condition explored and data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) expression (reference genes), applying the geneNorm method[45] to calculate reference gene stability between the different conditions (calculated with CFX Manager software; $M < 0.5$). Fold-changes in gene expression were determined by the $2^{-\Delta\Delta Cq}$ method, and are presented as relative levels vs. *h*BMSCs cultured in static condition in the presence of unloaded MCs (SG –). Comparison between groups was performed using the unpaired Student's *t-test*. Differences were considered statistically significant when $P < 0.05$[46].

2. RESULTS

A SC-based tridimensional multiphase construct was fabricated with the aim to test a reliable strategy to rapidly commit *h*BMSCs toward a tenogenic phenotype (**Fig. 1**). The cells used were 95% positive for CD73, CD90, CD105, and negative for CD14, CD20, CD34, CD45 prior to their use; their early induction of tenogenic markers was evaluated after a 72 hrs-long of synergic stimulation using cyclic strain and locally released *h*GDF-5. Cyclic deformation of 3D multiphase construct was applied via a proprietary device, extensively described in Govoni et al.[30], set to transfer a mean load of 0.1 N over 1 Hz cycles to the scaffold throughout the entire experiment. The load parameters were applied after evaluating the tensile strength and the elastic modulus of the 3D multiphase construct.

The curves of stress (MPa) vs. deformation (dL/Lo, mm/mm) are plotted in **Fig. 2a**. The load -bearing Hyalonect® band thickness is 0.22 mm, whereas the multiphase stem cell-based construct showed a thickness of 0.45 mm. Both structures had a uniform behavior until failure. The values of maximum stress and tensile strength at failure are reported for each sample in **Table 1,** together with their elastic modulus. The braided band showed an ultimate tensile strength of 3.40 (± 0.03) MPa and an average elastic modulus (passive stiffness) of 6.84 (± 0.02) MPa with a percentage elongation at yield of 57%. The complete multiphase scaffold band showed an ultimate tensile strength of 2.15 (± 0.5) MPa and an average elastic modulus of 4.56 (± 0.7) MPa with a percentage elongation at yield of 70%. The load (expressed in Newton) vs. strain plot is also given in **Fig. 2b**. These data indicate that our 3D multiphase construct maintained the tensile strength and the elastic modulus comparable with the braided hyaluronate core and is suitable for the applied cyclic deformation protocol.

PLGA MCs were used for the controlled release of *h*GDF-5; these particles showed a spherical shape (see FE-SEM image in **Fig. 3),** a mean size of 2.0 ± 0.4 μm and a growth factor encapsulation efficiency of 93%. MCs were produced by a proprietary and patented apparatus described in Della Porta et al.[38] and were embedded, together with the *h*BMSCs, within a fibrin mesh around hyaluronate fibers (see SEM images of the 3D multiphase scaffold in **Fig. 4**).

Several test were performed in order to optimize the *h*GDF-5 amount delivered each day from the MCs to be inserted into the scaffold, after fixing the number of cells within the 3D structure. Preliminary, the MCs mean size and loading were engineered in order to obtain a rapid release of about 20% of the total protein loaded (56 µg/g *h*GDF-5/PLGA) along the first 3 days, followed by a lower (3% of the total load per day) and constant rate of *h*GDF-5 released **(Fig. 5**) for the following 15 days, when roughly half of the initial load was released**.** The *h*GDF-5 diffusion coefficient was calculated as 7.5 x 10^{-10} cm^2/sec by fitting the release profiles of free MCs monitored at 37°C in DMEM. Then, following the suggested physiological *h*GDF-5 values proposed in the litterature[18], each scaffold (surface 600 mm^2, thickness 0.45 mm) was loaded with 100 mg of PLGA MCs (with an overall content of 5.6 µg of *h*GDF-5) and 500.000 cells to achieve a physiologically relevant level of GF delivered within the scaffold during the first 3 days of cultivation. Using this protocol, an overall amount of 1 µg of *h*GDF-5 is expected to be available within the scaffold for the cells throughout the 72 hrs of experiment. In detail, the expected amount released is illustrated in the insert within **Fig. 5,** at day 1 (600 ng) and at day 3 (1000 ng). The measured amount of *h*GDF-5 in dynamic (120±20 ng at day 1and 240±50 ng at day 3) and static (60±23 ng at day 1 and 320±48 ng at day 3) media are also illustrated for comparison.

The 3-day lag was the minimal time chosen to monitor the synergistic effect of biochemical (*h*GDF-5) and physical (cyclic strain) stimuli over *ColA1* and *SCX* gene expression (**Fig. 6**), which we are referring to as the "*early commitment*" toward the tenogenic phenotype. The choice of 72 hours was done after preliminary experiments, in where we monitored the gene expression during culture time; a fragment of our constructs after 24 and 72 hrs, was collected and cells mRNA expression for Col1A1, Col3A1, DCN and TCN was analyzed (in this preliminary experiment mRNA expression of SCX was not analyzed). Although 24 hrs of stimulation still increased the mRNA expression of tenogenic markers analyzed in DG +, a time range of 72 hrs was selected to assure a statistically significant data for all genes tested. Only when both cyclic strain and locally released *h*GDF-5 were supplied to the cells, a significant up-regulation of the mRNA levels of the relevant tenogenic markers

Col1A1 ($P < 0.05$), *Col3A1* ($P < 0.05$), *DCN* ($P < 0.05$) *SCX* ($P< 0.01$) and *TNC* ($P < 0.05$) was evidenced in *h*BMSCs within three days of culture (**Fig. 6**), pointing at a priming of a tenogenic differentiation program within the tissue engineered construct. *h*GDF-5 alone induced an not significative up-regulation for *DCN* mRNA level ($P < 0.18$). An up-regulation is evidenced only for *SCX* mRNA level ($P < 0.01$) when, on the other hand, the mechanical stimulus was applied alone.

3. DISCUSSION

Even under the best circumstances, the surgical treatment of tendon injuries or breakages with auto/allograft or prosthetic devices does not grant the recovering of the mechanical and biological properties of the original tissue[5]. Since these traumas are frequent and relevant at any age, more performant reconstructive options are thus expected. Providing tissue-engineered substitutes with adequate biological and mechanical properties, grown *in vitro* and intended for subsequent integration with the host tissues is a promising alternative. A combination of appropriate cells, biomaterials/scaffolds, biochemical cues/growth factors and physical stimulation seems a successful approach to this aim.

We thus assembled *h*BMSCs together with *h*GDF-5 releasing MCs within a hydro-gelling fibrin mesh onboard of an elastic and braided hyaluronate fiber that underwent a cyclic mechanical stimulation. In fact, although each among this relevant signal useful to induce tenogenic lineage commitment has already been individually explored, very few protocols have been proposed to exploit their synergic action within a cooperative approach. The proposed engineered microenvironment resulted able to combine physical and biochemical stimulation and deliver both of them to the cells onboard with the aim to promote their fast commitment into the desired tissue phenotype.

A main issue in the proposed approach concerns avoiding the administration of free growth factors into the culture medium. *h*GDF-5 was rather locally administered with a controlled delivery to the cells within the 3D microenvironment where they are cultured. As a consequence, *h*GDF-5

quantities required to stimulate the cells, i.e. the inherent culture costs, were significantly reduced and a concurrent higher stability in the biochemical signal presented to the *h*BMSCs was obtained. This was allowed by the use of a novel SEE approach to fabricate PLGA MCs loaded with *h*GDF-5 with a very accurate size tailoring coupled with high encapsulation efficiency (drug encapsulated/drug loaded*100) thanks to the very fast emulsion processing that prevents any droplets coalescence and allows a rapid polymer hardening. These performances are due to the reduced density and the enhanced diffusivity typical of the supercritical fluid extraction performed by SEE technology with respect to a liquid/liquid one[23,38]. Moreover, the MC size and composition is tunable by changing the emulsion formulation and droplet size and composition; these factors can affect the sustained delivery of significant molecules[23].

PLGA was selected as a biopolymer to fabricate the MCs, because it offers an accurate drug release over prolonged periods thanks to: (1) a diffusion-controlled mass transfer of the drug accessible at the solid/media interface (2) an enhanced release caused by polymer bulk erosion via hydrolysis of the ester bonds[41, 47]. Moreover, no polymer swelling is reported, and therefore no tridimensional multiphase structure deformation is expected during cell cultivation. Molecular release profiles from PLGA MCs have also been extensively studied, and several authors calculated the diffusion coefficient values of different encapsulated drugs/proteins. In our settings, the Diffusion Coefficient calculated value is of two order of magnitude higher than the ones reported by Faisant et al.[41] (10^{-10} cm^2/sec vs 10^{-11}/10^{-12} cm^2/sec), but it seems consistent with the SEE-fabricated MCs, considering that we used PLGA with lower molecular weight (averaged 46 kDa against 104 kDa reported[41]), and we fabricated MCs with mean size ten times smaller than the ones reported by the same authors (1 vs. 10 mm, as mean size). It is also worth noting that the calculated diffusion coefficient is of several orders of magnitude lower than the ones reported in the literature for the free peptides diffusion through hydrogel scaffolds of thickness similar to the one used in the present work[22]. Hence, i) the release obtained from PLGA MCs resulted in a more delayed profile when compared to free GFs molecules diffusing through hydrogels scaffolds, ii) it can be considered the

predominant step, and iii) the diffusion of free protein through the fibrin mesh of the hydrogel can be neglected. Consequently, we suppose that the cells are directly exposed to, and are affected by, released GFs inside the fibrin hydrogel structure, both in static and dynamic experiments.

Looking at the *h*GDF-5 release profile of SEE fabricated MCs, it seemed well tailored for a strong *h*GDF-5 signaling during the first 3 days of stimulation (20% of the load released in the first 3 days) and for a sustained release (3%/day) during a hypothetical subsequent *in-vivo* implant of the bioengineered structure. Moreover, the amount of *h*GDF-5 measured in the culture media after a 3-day culture (see insert in **Fig. 5**), in both static and dynamic runs**,** was a small fraction of the total *h*GDF-5 released from the loaded microspheres (1000 ng in each scaffold). This suggests that most of the *h*GDF-5 was retained within the scaffold and available to bind to the cells.

Cyclic tensile strain was also applied using a proprietary bioreactor for the controlled deformation of the overall 3D multiphase scaffold. As the braided hyaluronate band, holding the overall 3D structure and transmitting the applied load, tolerates a maximum load of 5 N and shows an elastic modulus of 6.8 MPa (see **Fig 2a-b**); so that, when a load of 0.1 N was transferred over 1 Hz cycles, the bioreactor operated within the elastic range of the construct and with an effective strain delivery to the overall components of the 3D structure. The structure also showed a uniform behavior until its breakage indicating that all the cells dispersed within were uniformly stretched via the applied cyclic strain.

To check for the induction of tenogenesis in *h*BMSCs, several marker genes, such as *Col1A1*, *Col3A1*, *DCN*, *SCX* and *TNC*, were investigated in this work (see **Fig 6**). Col1A1 is the major substance in tendon tissue (75-85% of the dry mass of tendon) and is considered to be responsible for its mechanical strength, while elevate amounts of Col3A1, whose fibers are thinner and more extensile, have been reported at the rupture site of the human tendon[48]. A significant ($P < 0.05$ vs. SG –) enhancement, up to fourfold, of the mRNA level of *Col1A1* compared with control (SG –) was shown when *h*BMSCs were co-cultured for 72 hrs within the 3D scaffold with *h*GDF-5–releasing MCs under mechanical stimulation. On the other hand, when mechanical or chemical stimuli were

applied alone no differences from SG – control were observed. Interestingly, when *Col3A1* mRNA level reached statistical significance ($P < 0.05$ vs. SG –) upon DG + protocol application, a favorable ratio of *Col3A1* to *Col1A1* was maintained, whereas only *h*GDF-5 stimulation (SG +) resulted in an atypical collagen ratio[49].

In addition to Col1A1, also a small leucine-rich proteoglycan implicated in the regulation of fibrillogenesis, i.e. DCN, is a fundamental protein in the ECM matrix of tendon[50]. Our results show a twofold increase of mRNA level ($P < 0.05$ vs. SG –) upon DG + treatment. Not significant *DCN* up-regulation ($P < 0.18$ vs. SG –) was observed upon SG +; the behavior may be in agreement with *Youngstrom et al*., who showed a peak in *DCN* upregulation of only 3% in presence of GDF-5, rather than a 5% after cyclic strain application. This suggests that the 10% elongation level used in our study might exceed a physiological stimulus for this marker, although it appears as the proper one to induce *Col1A1* and *TNC* expression[49]. Indeed, *TNC* transcript – translated into a six-armed hexabrachion-shaped ECM glycoprotein considered an early marker of tendineous differentiation abundantly expressed in the musculoskeletal tissues during embryogenesis[51] – also reached statistical significance vs. SG –, upon DG + treatment in our experiments ($P < 0.05$).

SCX is a tendon-specific basic helix-loop-helix transcription factor responsible for the transition of MSCs into tendon progenitors, as recently demonstrated by the work of Alberton et al.[52]. In addition, more recently, Chen et al.[53] showed how the down-regulation of *SCX*, caused by dexamethasone treatment, inhibited the differentiation of rat tendon MSCs into tenocytes, confirming the key role of this gene in the promotion of a tenocyte-like phenotype in MSCs. Our results showed a fivefold increase in *SCX* mRNA upon DG – treatment ($P < 0.01$ vs. SG –), in accordance with Mendias et al.[54], that showed how *SCX* gene was particularly responsive to exercise. This level raised up to eightfold ($P < 0.01$ vs. SG –) when combined physical and biochemical stimulation (DG +) were applied to the construct.

Taken together, these data suggest that *h*BMSCs display within 72 hrs an early commitment toward tenogenesis guided by a mechanical stimulation and a synergetic biochemical signaling, as

anticipated by Aspenberg[55] and in agreement with very recent evidence in the literature for other tissue phenotypes[56]. Indeed, as a member of the Transforming Growth Factor-β (TGF-β) superfamily of proteins, *h*GDF-5 (also known as Bone Morphogenetic Protein 14) binds to a specific receptor inducing phosphorylation of the downstream substrate proteins Smads. Phosphorylated Smad1/5/8 associates with the co-Smad (Smad4), and the complex translocates to the nucleus where it further associates with coactivators or corepressors to regulate gene expression[57]**.** On the other hand RhoA/ROCK, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of *h*MSCs[58-59]. The convergence of these different signaling pathways onto common downstream gene promoters appears as a reasonable explanation for the observed synergism of the biochemical and mechanical stimuli.

The proof of concept proposed in this work opens a perspective for developing 3D bioengineered model to understand specific molecular and cellular compositions of damaged systems, as well as, for fabricating implantable tissue substitutes. The hydrogel/hyaluronate multiphase structure displays higher elasticity and less tensile strength and passive stiffness (reduced elastic modulus value) when compared to the Hyalonect® band alone, without losing resistance (**Fig. 2b).** The apparently less tensile strength of multiphase structure (**Fig. 2a)** might only result from its larger cross-section, which is almost double (0.45 mm) for the hydrogel when compared with the simple braided band (0.22 mm). The initial tensile strength of the proposed structure seems within the range of forces reported for passive or unresisted motion in human flexor tendons[12], because it shows a good mechanical behavior under a load of 5 N. This maximum load is still inadequate to replace most human tendons[60]; however, a strong improvement of the hyaluronate band by weaving fibers differently and/or in a cylindrical shape to enlarge the support cross-sectional area can increase the strength of the construct, and longer stimulation times might induce a more substantial mechanical improvement of the construct. Moreover, the presence of *h*GDF-5-loaded within the 3D multiphase structure, which allows the biochemical signal delivery within the scaffold, this action has to be expected to last also after the scaffold implant by surgery.

4. CONCLUSIONS AND PERSPECTIVES

Tendon development and healing is a complex process whose most important aspects in the context of TE remain to be established. Our approach is a potential strategy to address this problem examining their relative importance during a tightly controlled *in vitro* protocol. To the best of our knowledge, we did not find many studies assessing the use of growth factor delivery devices within the scaffold coupled with mechanical training in cell addressing versus tenogenic phenotype. The very fact is that mechanical and chemical stimuli are traditionally treated separately. In order to tackle this issue, we hypothesize that the mechanical stimulus coordinated by the chemical stimulus can provide an integrated outline to deal with systems where a combination of both stimuli plays an important role. The encouraging results of the proof of concept proposed in this work will be improved with further experiments exploring on a longer time scale the consistent histological evolution of the engineered tissue construct. Into the future this will pave the way towards the *in vivo* test of the functional potential of the substitute, whose macroscopic folding is reasonably expected to engender geometries bearing a suitable joint load. We expect that addressing these issues will facilitate effective TE procedures, and drive the synthesis of functional engineered tissues for the successful treatment of tendon injuries.

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AUTHOR DISCLOSURE STATEMENT

Emanuele Giordano, Marco Govoni, and Claudio Muscari are listed as inventors in the application with International Publication No. WO2011013067, filed to the World Intellectual Property Organization (WIPO) and entitled 'Bioreactor for stem cell stimulation'. Della Porta Giovanna and Ernesto Reverchon are listed as inventors of the US Patent n. US Patent US/8628802 B2 Jan 2014 entitled "Continuous process for microspheres production by using expanded fluids".

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TABLES

Table 1. Mechanical tests values of hyaluronate band and multiphase scaffold.

	Hyaluronate band	Multiphase Scaffold
Gauge length, mm	22	22
Length of sample, mm	22	22
Width of sample, mm	5	5
Thickness of sample, mm	0.28	0.45
Humidity (%)	100	100
Modulus of elasticity (MPa)	6.84 (± 0.3)	4.56 (± 0.5)
Elongation at break (%)	57.42	70.69
Tensile strength at break (MPa)	3.40 (± 0.2)	2.15 (± 0.7)

Values are mean ± SD; n = 2.

FIGURE LEGENDS

Figure 1. Image and schematic illustration of the 3D multiphase stem cell-based construct. The scaffold was formed by fibrin hydrogel coating hyaluronate braided fibers to embed *h*BMSCs and PLGA microcarriers (w/wo *h*GDF-5) into a multiphase deformable tissue construct able of both biochemical signaling and mechanical strain delivery. The free ends of the hyaluronate band allowed the docking into the holders of the mechanical actuation system for cyclic load transfer.

Figure 2a-b. Stress–Strain plot and elastic modulus values **(a)**, and maximum load **(b)**, of hyaluronate braided band (continuous) and of multiphase stem cell-based scaffold (dashed).

Figure 3. Scanning Electron image of PLGA microcarriers loaded with *h*GDF-5 fabricated by SEE processing at 8 MPa and 38°C a *water-oil-water* emulsion (1:19:80); **(insert)**Size distribution data are expressed as volume percentage.

Figure 4. Scanning Electron images of the 3D multiphase scaffold formed by: hyaruronate fibers (diameter of about 10 μm) assembled with PLGA microcarriers and *h*BMSCs by fibrin hydrogel.

Figure 5. *In-vitro h*GDF-5 release data (blue square) obtained in Dulbecco's modified Eagle medium (DMEM) at 37°C with a continuous stirring at 50 rpm; fitting profiles are reported as dashed line. The percentage indicates the released amount of the total load contained in the MCs to be inserted in the scaffold. Values are mean with n = 2. **(Insert)** Total amount of *h*GDF-5 released each day from the 3D scaffold (MCs: 100 mg) in DMEM; *h*GDF-5 measured in the day 1 and 3 during static and dynamic cultivations is also reported, for comparison purpose.

Figure 6. Mean (± SD) gene expression data for *Col1A1*, *Col3A1*, *DCN*, SCX and *TNC* (*GAPDH*and *HPRT1* used as reference genes). *hB*MSCs under static conditions in presence of unloaded microcarriers were used as control. SG – : Static Group with empty microcarriers; DG – : Dynamic Group with empty microcarriers; SG + : Static Group with *h*GDF-5–loaded microcarriers; DG +: Dynamic Group with *h*GDF-5–loaded microcarriers. * = $P < 0.05$ vs. SG –; ** = $P < 0.01$ vs. SG –.

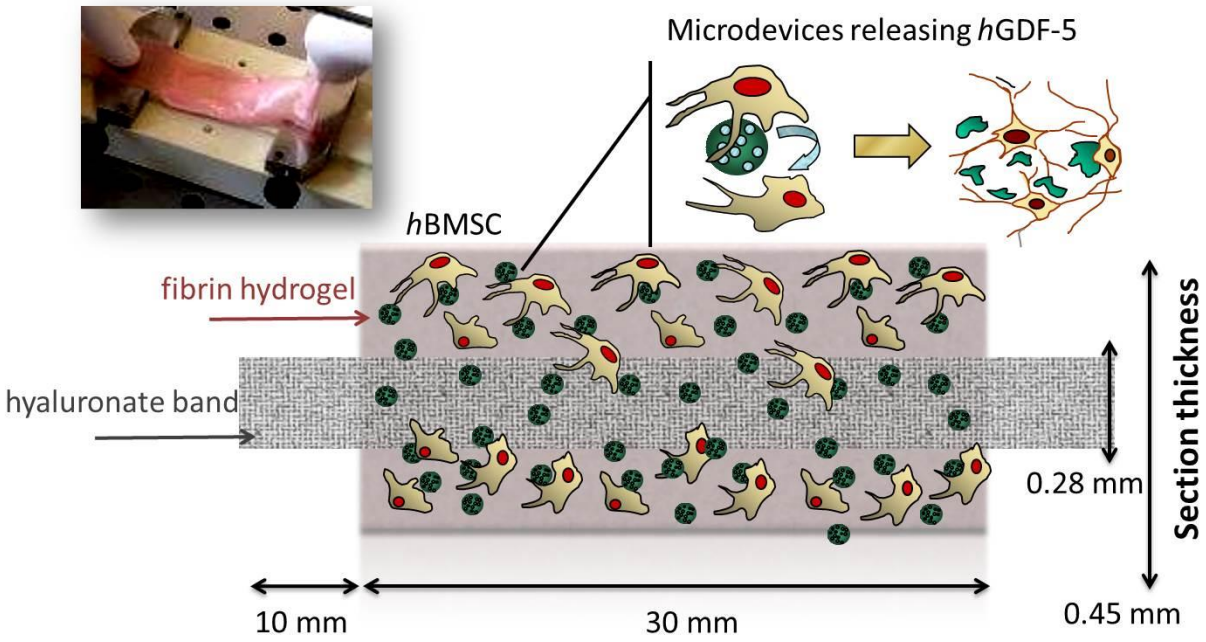
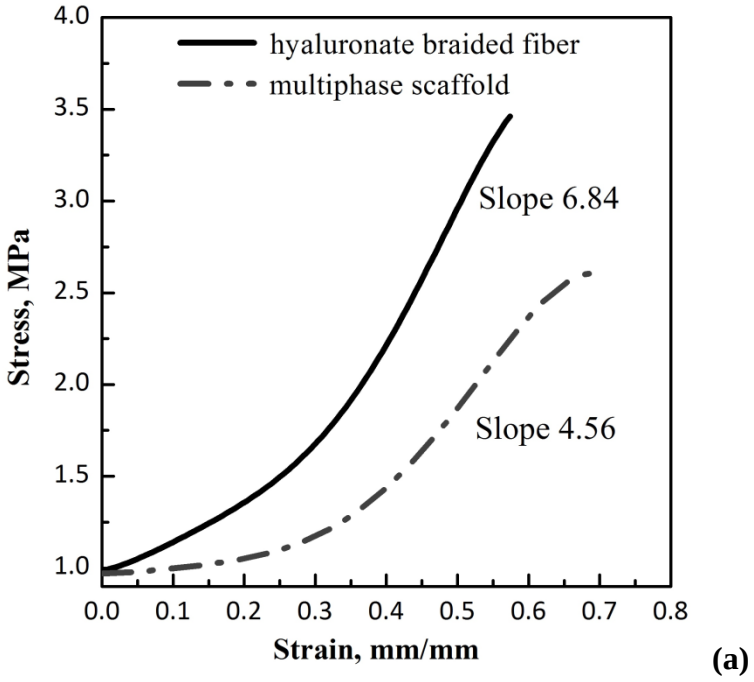
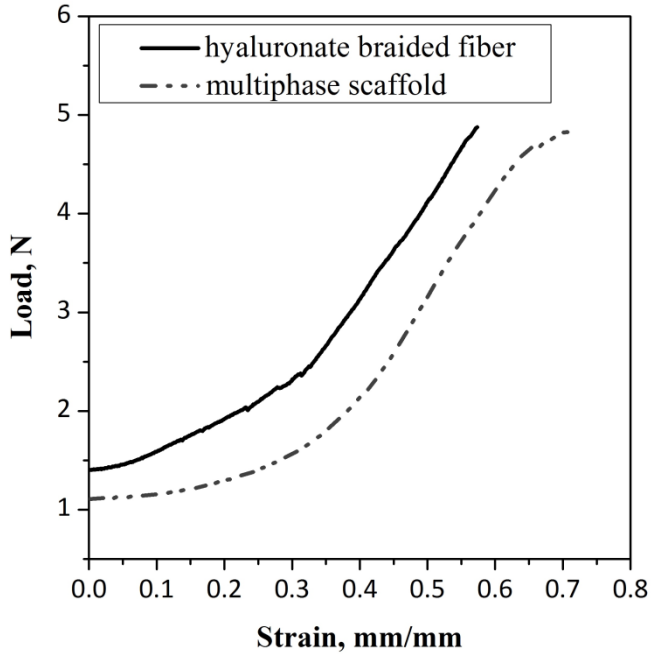


Figure 1. Image and schematic illustration of the 3D multiphase stem cell-based construct. The scaffold was formed by fibrin hydrogel coating hyaluronate braided fibers to embed *h*BMSCs and PLGA microcarriers (w/wo *h*GDF-5) into a multiphase deformable tissue construct able of both biochemical signaling and mechanical strain delivery. The free ends of the hyaluronate band allowed the docking into the holders of the mechanical actuation system for cyclic load transfer.



(a)



(b)

Figure 2a-b. Stress–Strain plot and elastic modulus values **(a)**, and maximum load **(b)**, of hyaluronate braided band (continuous) and of multiphase stem cell-based scaffold (dashed).

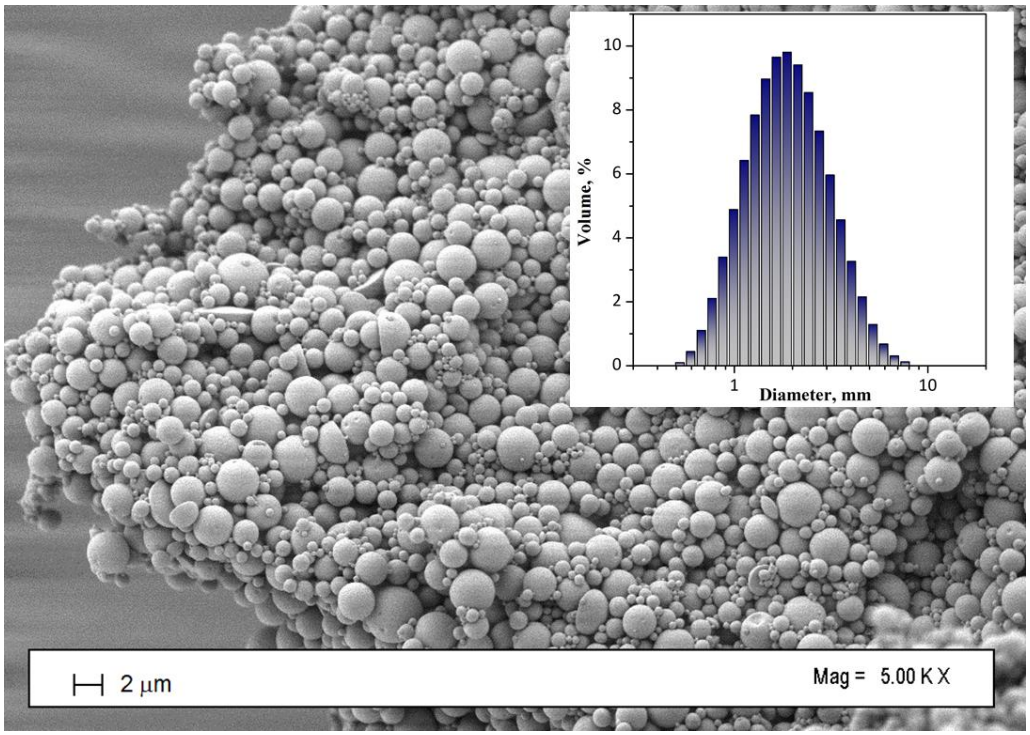


Figure 3. Scanning Electron image of PLGA microcarriers loaded with *h*GDF-5 fabricated by SEE processing at 8 MPa and 38°C a *water-oil-water* emulsion (1:19:80); **(insert)**Size distribution data are expressed as volume percentage.

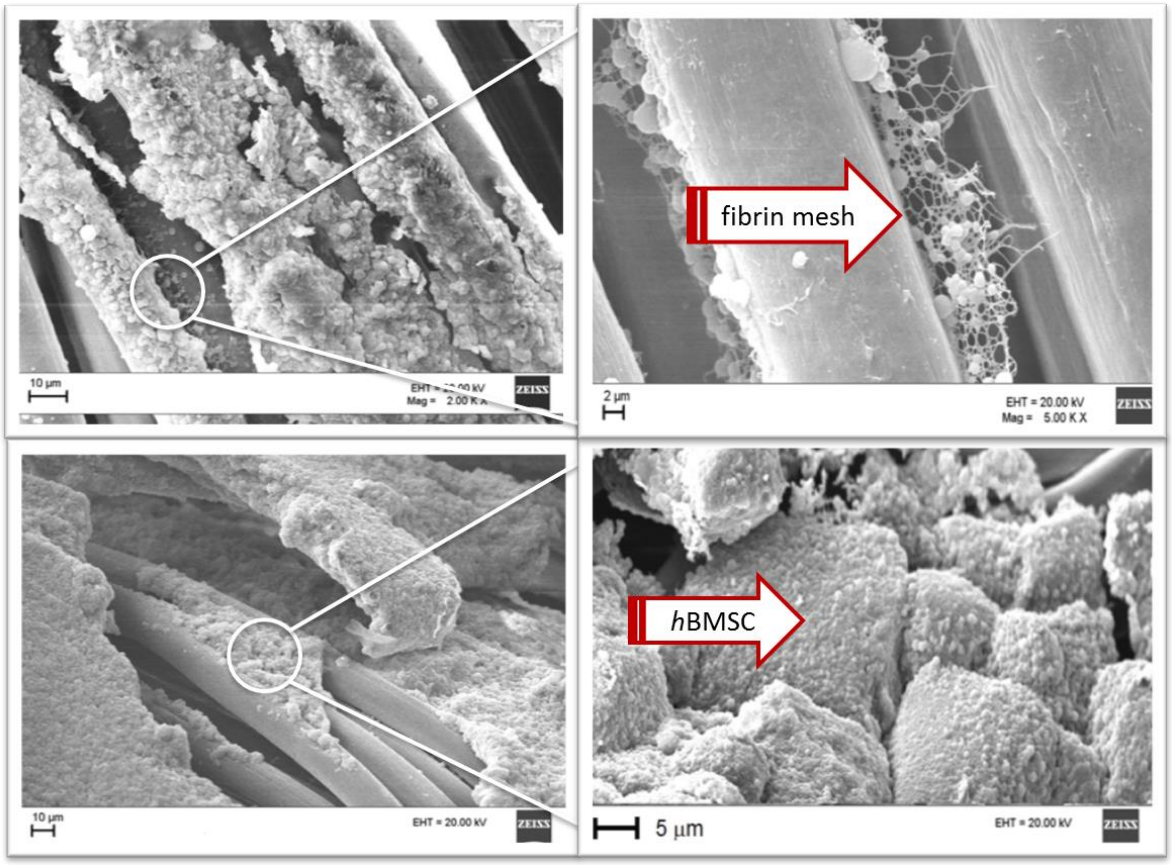


Figure 4. Scanning Electron images of the 3D multiphase scaffold formed by: hyaruronate fibers (diameter of about 10 µm) assembled with PLGA microcarriers and *h*BMSCs by fibrin hydrogel.

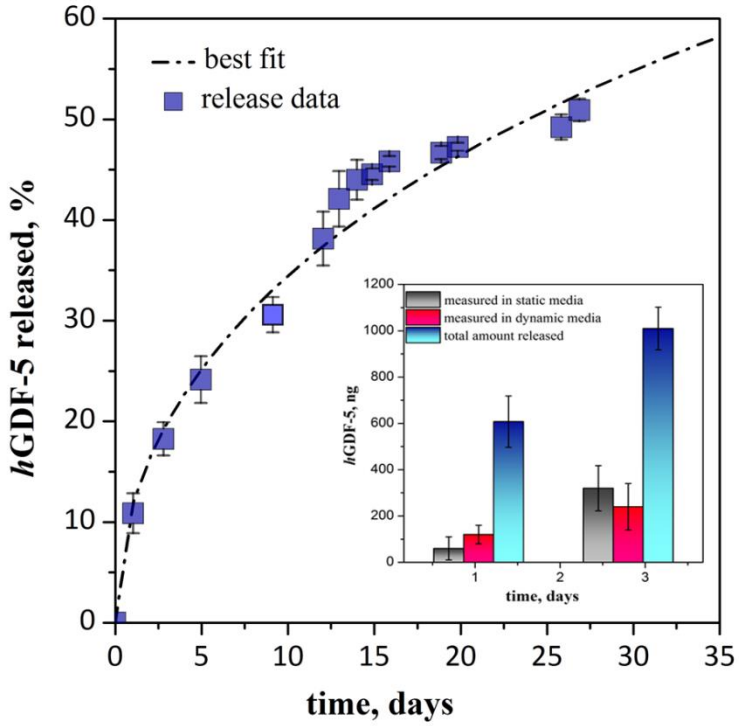


Figure 5. *In-vitro h*GDF-5 release data (blue square) obtained in Dulbecco's modified Eagle medium (DMEM) at 37°C with a continuous stirring at 50 rpm; fitting profiles are reported as dashed line. The percentage indicates the released amount of the total load contained in the MCs to be inserted in the scaffold. Values are mean with n = 2. **(Insert)** Total amount of *h*GDF-5 released each day from the 3D scaffold (MCs: 100 mg) in DMEM; *h*GDF-5 measured in the day 1 and 3 during static and dynamic cultivations is also reported, for comparison purpose.

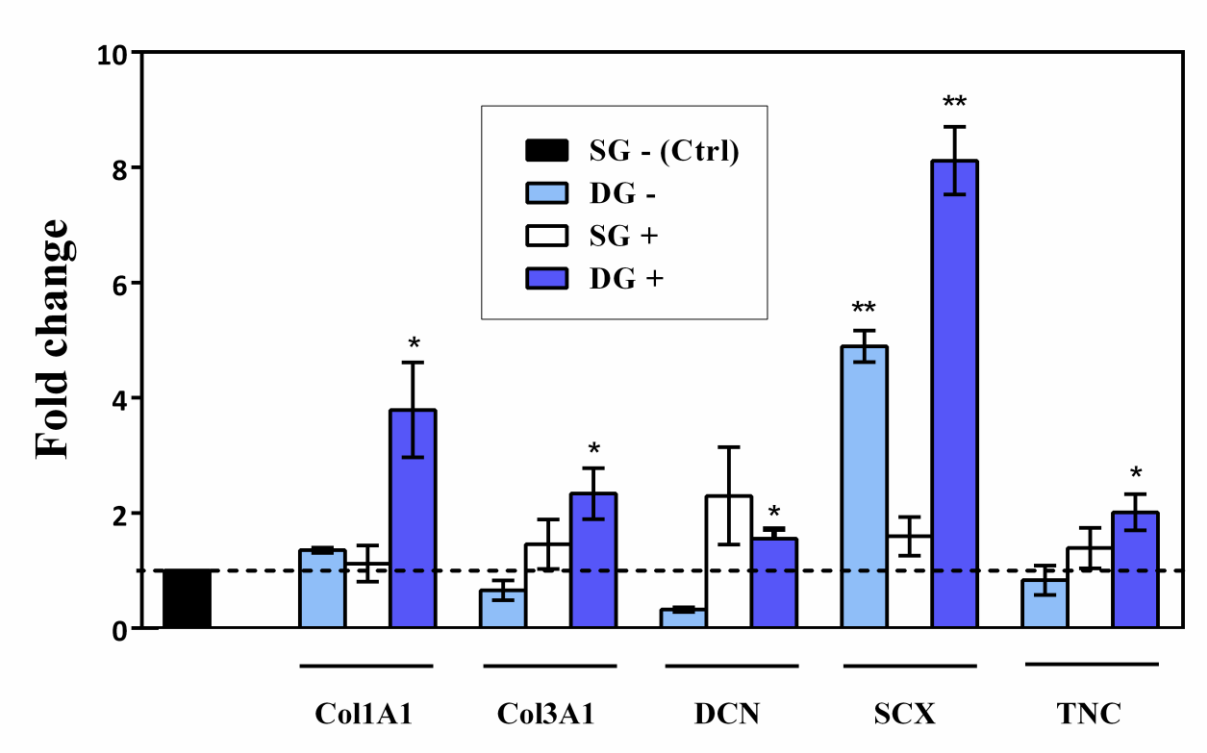


Figure 6. Mean (± SD) gene expression data (n=3) for *Col1A1*, *Col3A1*, *DCN*, SCX and *TNC* (*GAPDH*and *HPRT1* used as reference genes). *h*BMSCs under static conditions in presence of unloaded microcarriers were used as control. SG – : Static Group with empty microcarriers; DG – : Dynamic Group with empty microcarriers; SG + : Static Group with *h*GDF-5–loaded microcarriers; DG +: Dynamic Group with *h*GDF-5–loaded microcarriers. * = $P < 0.05$ vs. SG –; ** = $P < 0.01$ vs. SG –.