

A double chamber rotating bioreactor for enhanced tubular tissue generation from human mesenchymal stem cells: a promising tool for vascular tissue regeneration

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Abstract

Cardiovascular diseases represent a major global health burden, with high rates of mortality and morbidity. Autologous grafts are commonly used to replace damaged or failing blood vessels; however, such approaches are hampered by the scarcity of suitable graft tissue, donor site morbidity and poor long-term stability. Tissue engineering has been investigated as a means by which exogenous vessel grafts can be produced, with varying levels of success to date, a result of mismatched mechanical properties of these vessel substitutes and inadequate *ex vivo* vessel tissue genesis. In this work, we describe the development of a novel multifunctional dual-phase (air/aqueous) bioreactor, designed to both rotate and perfuse small-diameter tubular scaffolds and encourage enhanced tissue genesis throughout such scaffolds. Within this novel dynamic culture system, an elastomeric nanofibrous, microporous composite tubular scaffold, composed of poly(caprolactone) and acrylated poly(lactide-co-trimethylene-carbonate) and with mechanical properties approaching those of native vessels, was seeded with human mesenchymal stem cells (hMSCs) and cultured for up to 14 days in inductive (smooth muscle) media. This scaffold/bioreactor combination provided a dynamic culture environment that enhanced (compared with static controls) scaffold colonization, cell growth, extracellular matrix deposition and *in situ* differentiation of the hMSCs into mature smooth muscle cells, representing a concrete step towards our goal of creating a mature *ex vivo* vascular tissue for implantation. Copyright © 2016 John Wiley & Sons, Ltd.

Received 29 February 2016; Revised 18 August 2016; Accepted 20 October 2016

Keywords bioreactor; dynamic culture; mesenchymal stem cell; composite scaffold; vascular grafts

1. Introduction

Over the last 60 years, transplantation of a wide variety of tissues and organs, reconstructive surgical techniques and replacement with artificial devices has significantly enhanced patient life expectancy and quality. Unfortunately, these solutions still suffer from many limitations, including donor shortage and lifelong immunosuppression, increased risk of infections, unwanted side-effects and, in some cases, finite durability (Fuchs *et al.*, 2001; Nasser *et al.*, 2001). This situation has led to an increasing interest in tissue engineering (TE) as a possible solution to develop *in vitro* cellularized functional substitutes able to restore or improve tissue and organ function (Martin *et al.*, 2014; Peloso *et al.*, 2015; Vacanti, 2010).

Significant progress has already been made in the field, and examples of successful clinical implementation of *in vitro* TE products include skin substitutes (Catalano *et al.*, 2013; Gauvin *et al.*, 2013), bone grafts, cartilage, operational bladders and trachea (Asnaghi *et al.*, 2009; Atala *et al.*, 2006; Fulco *et al.*, 2014; Jungebluth *et al.*, 2011).

Whilst these progresses offer significant promise, besides appropriate preclinical models to investigate the host response (e.g. neovascularization, re-modelling; Piola *et al.*, 2013a) and the behaviour of the produced substitute once grafted, more effort is still required in terms of elucidating basic mechanisms regulating cell responses to these scaffolds under dynamic vs. static culture conditions and, further, the behaviour of the engineered constructs during *ex vivo* maturation (Couet and Mantovani *et al.*, 2012; Martin *et al.*, 2014). Moreover, it is acknowledged that each tissue target and each pathological condition will likely require a specific approach for optimal results, and hence having a modular and flexible approach to *ex vivo* TE will be important.

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Tissue-engineered tubular tissues are seen as an alternative source of tissues for the replacement of small-diameter damaged hollow organ structures (< 6 mm \varnothing), such as blood vessels and urethra (Atala *et al.*, 2012). There is a critical demand for the production of TE vascular substitutes capable of meeting the functional requirements of the vessel without inducing immune or inflammatory responses, or losing function over time (Martin *et al.*, 2014).

The TE approach is based on the well-established triad comprising cells, scaffolds (synthetic or natural) and stimuli (Martin *et al.*, 2014; Asnaghi *et al.*, 2014). Several studies have highlighted the important roles of mechanical and biochemical stimulation in driving the development of various cell phenotypes from various cellular starting points in order to create a mature and functional tissue substitute (Moore *et al.*, 2012; Prandi *et al.*, 2015; Seifu *et al.*, 2013; Tresoldi *et al.*, 2015). Bioreactors thus have the potential to play a critical role in TE, having the ability to provide such stimulation in a dynamic but controlled manner to create the appropriate environment for tissue growth, remodelling and maturation, prior to implantation (Asnaghi *et al.*, 2009; Flanagan *et al.*, 2007; Piola *et al.*, 2013b, 2015; Uzarski *et al.*, 2014).

To address this potential, numerous groups of researchers have developed dynamic culture systems suitable for applying, in a controlled manner, single or combined biomechanical stimuli to seeded scaffolds, such as shear stress, pressure, axial stretching or torsion loads (Nieponice *et al.*, 2008; Sivaratna *et al.*, 2015; Syedain *et al.*, 2011; Uzarski *et al.*, 2015). However, these devices still have their limitations, mainly associated with cumbersome handling and assembly procedures, and an inability to simultaneously produce different stimuli during culture that are required for the successful production of suitable TE grafts (Fishman *et al.*, 2014; Mantero *et al.*, 2007; Seifu *et al.*, 2013).

The present study aimed to redesign and validate an innovative multifunctional bioreactor, previously used for large hollow organs engineering (Asnaghi *et al.*, 2009), for the generation of small-diameter tubular tissues. The whole dynamic culture system was redesigned to suit small-diameter tubular constructs and two new important features were added, namely pre-tensioning and luminal perfusion. Furthermore, as adaptability is considered nowadays a key element for TE (Morgan and Black, 2014), great importance was given to modularity and scalability during the development process, in order to allow regeneration of patient-specific graft, always considering as mandatory sterility maintenance during long-term culture (Mollet *et al.*, 2015).

The functionality and the benefit of the bioreactor system were assessed in the context of small-calibre vessel engineering, using human mesenchymal stem cells (hMSCs) and a promising elastomeric composite poly(caprolactone) and poly(lactide-co-trimethylene-carbonate) (PCL/aPLA-co-TMC)-based electrospun

(Stefani and Cooper-White, 2016), having first determined an optimized medium composition to induce hMSCs differentiation into mature smooth muscle cells (SMCs). Under dynamic culture within the bioreactor system for 14 days, the small-diameter tubular scaffold and the novel optimized differentiation media resulted in substantial improvements in scaffold colonization, cell growth, SMC extracellular matrix (ECM) secretion and differentiation of hMSCs into mature SMCs throughout the tubular scaffold as compared with static controls.

2. Materials and methods

2.1. hMSC two-dimensional culture and elucidation of optimal SMC differentiation media

Human MSCs were purchased from Lonza (donors: 8006, 7219, 0956). hMSCs were seeded at 4000 cells/cm², expanded in growth media (GM) and subsequently, when needed, exposed to differentiation media for 7 or 14 days before collecting samples for analysis. GM consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μ g/ml; Gibco, Australia). Four different myogenic media were tested and consisted of GM supplemented with: 1 ng/ml transforming growth factor (TGF)- β 1, 30 μ M ascorbic acid; 10 ng/ml TGF- β 1, 30 μ M ascorbic acid; 1 ng/ml TGF- β 1, 30 μ M ascorbic acid, 10% FBS; 10 ng/ml TGF- β 1, 30 μ M ascorbic acid, 10% FBS. The different media are coded as (X Y), where X is the % of FBS and Y is TGF- β 1 ng/ml added to low-glucose DMEM. Media were changed every 2 days.

2.2. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy Mini Kit with on-column DNase treatment (QIAGEN VWR, Stockholm, Sweden) according to the manufacturer's protocol. The concentration and purity of RNA were determined by using a Nano-Drop spectrophotometer (NanoDrop Technologies, Rockland, DE, USA; data not shown). cDNA was synthesized from 100 ng of RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies) in a total volume of 21 μ l, as per manufacturer's instructions. An equivalent volume of DNase and RNase-free water (Sigma) was used in place of RT Enzyme Mix for no-RT controls.

Quantitative (q)PCR reactions were set up in triplicate, with each reaction having a total volume of 10 μ l containing 1 \times Platinum SYBR Green qPCR SuperMix-UGD (Invitrogen), 0.2 μ M forward and reverse primers, and 1 μ l cDNA. A CFX Connect™ Real-Time PCR Detection System (Bio-rad, USA) was used at standard cycling parameters: 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 2 min, and then 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 30 s for a total of 40 cycles.

Results of qPCR were analysed using the Pfaffl model to normalize gene expression to the reference gene GAPDH (Pfaffl, 2001). Analysed genes (Table 1) were chosen due to their previous use within literature as SMC differentiation markers (Ball *et al.*, 2004; Cameron *et al.*, 2011, 2014; Murrell *et al.*, 2011; Narita *et al.*, 2008; Tang *et al.*, 2012).

2.3. Cell morphology and differentiation analysis

Cells seeded on 48 multi-well plates (triplicate samples, 1, 7 and 14 days post-seeding) were fixed in 4% paraformaldehyde [PFA; in phosphate-buffered saline (PBS)] for 20 min and rinsed with PBS before being permeabilized with a 0.1% (*w/v*) Triton 100-X solution for 5 min. After PBS rinsing, samples were blocked in 3% bovine serum albumin (BSA) in PBS solution for 1 h.

The following staining solutions (in 3% BSA in PBS) were used (150 μ l/well): 1/200 anti-alpha smooth muscle actin [1A4] antibody and 1/100 anti-calponin antibody [CALP] (Abcam, UK) overnight (4 °C) followed by 1/500 AlexaFluor 568 (Life Technologies) and 1/400 AlexaFluor 633 (Life Technologies) 60 min (room temperature, RT); finally 1/200 Phalloidin 488 (actin staining; Life Technologies, Australia) 30 min (RT) and 1/1000 Hoechst 33342 (nucleus staining; Life Technologies, Australia) 5 min (RT).

Wells were then rinsed in PBS three times and left immersed at 4 °C until imaging. High-resolution images were obtained using an LSM Zeiss 710 confocal microscope.

Pictures of cell populations for chosen treatment conditions (more effective myogenic medium and control) were processed with a self-made method implemented with cell-image analysis open source software CellProfiler (Broad Institute, USA; Carpenter *et al.*, 2006) to identify, count and measure individual cells. The method recognized nuclei first, in order to generate a cytological profile, containing a collection of measurements of features of each cell, like fluorescence intensity for all the different channels.

CellProfiler Analyst system then presented individual cells for classification, sampled randomly from the screen-wide population (Jones *et al.*, 2009). After 50 cells were classified by the operator as α -SMA and calponin-positive/negative, the iterative machine-learning phase, in which the computer generates a tentative rule based on the classified cells and presents the researcher with

cells classified according to that rule, began. After correcting classification errors and retraining for several rounds, the automatic classification became more accurate. When the accuracy was considered sufficient, the trained method was used to classify all cells in the experiment in order to calculate the number of double-positive cells in each sample.

2.4. PCL/aPLA-co-TMC scaffold

A previously developed PCL/aPLA-co-TMC tubular electrospun porous scaffold (SF-1) was chosen due to the promising chemical and mechanical feature for vascular TE. The three-dimensional support is composed of fibres with diameter $\phi = 2.0 \pm 0.6$ micrometers, presenting a spontaneous in-process phase separation: the inner core is formed from a stiffer material (PCL), and the outer layer is crosslinked aPLA-co-TMC. The structure of the scaffold is porous, but tightly packed (pore size = 12.5 ± 0.8 μ m). The outer surface presents axially aligned structures (SF-1D; Stefani and Cooper-White, 2016). Five-centimetre-long scaffolds with internal diameter of 4 mm and a wall thickness of 0.75 mm were sterilized with 70% ethanol wash and rinsed in 1% antibiotic-antimycotic (Gibco, Thermo Fisher Scientific, USA) solution in PBS. Before cell seeding, scaffolds were kept in GM without FBS overnight.

2.5. Bioreactor design

The bioreactor design and development focused on achieving the following criteria for the system: (1) simple cell seeding procedures on both sides of a tubular matrix, to permit a homogeneous cell distribution; (2) the ability to culture different cell types on either side of the tubular support, with the possibility to supply different media; (3) the ability to enhance oxygenation of the culture medium and mass transport (oxygen, nutrients and catabolites) between media and adhering cells; (4) the ability to perfuse the internal lumen, and connect the bioreactor to different perfusion systems; (5) the ability to introduce scaffold tensioning in order to assure a pervious lumen; (6) the achievement and maintenance of sterility and other criteria of good laboratory practice.

In order to ensure applicability of the device for different purposes [i.e. different scaffold sizes (diameter up to 1 cm and length up to 10 cm) and cell types], the

Table 1. Primer sequences used in qPCR analysis

Gene	Forward	Reverse	Reference or NCBI sequence
GAPDH	ATGGGGAAGGTGAAGGTCTG	TAAAAGCAGCCTGGTGACC	(Murrell <i>et al.</i> , 2011)
α -SMA	ACGTGGGTGACGAAGCACAG	GGGCAACACGAAGCTCATTGTA	(Ball <i>et al.</i> , 2004; Cameron <i>et al.</i> , 2011; Narita <i>et al.</i> , 2008)
CNN1	TCGGCAACAACCTCATGG	GCCTGGCTGCAGCTTATT	(Ball <i>et al.</i> , 2004)
DES	GAGACCATCGCGGCTAAGAAC	GTGTAGGACTGGATCTGGTGT	NM_001927.3
SM1	ATTGCCAAGCCAGAGAC	GAAGCCCTCCAAAAGCAA	(Ball <i>et al.</i> , 2004)
Col1A1	CCTGCGTGTACCCCACTCA	ACCAGACATGCCTCTTGCCTT	(Cameron <i>et al.</i> , 2014; Tang <i>et al.</i> , 2012)
Col4A1	GCCGTGGGACCTGCAATTACT	CGTAGGCTTCTGAACATCT	NM_001845

major design principles were high versatility and modularity of the whole system. Furthermore, two perfusion systems were developed to bench-test the device under laminar and pulsatile ($P = 120\text{--}180$ mmHg) flows.

2.6. Bioreactor dynamic culture

After sterilization (autoclave), the culture chamber was assembled in a biosafety cabinet. The sterilized scaffold was positioned on the developed shafts (Figure 1A). Approximately 2–3 mm linear motion of the pre-tensioning system was required, depending on the experiment, to maintain the used scaffold straight and pervious. The electrospun scaffold was not visibly modified: no dimensional change was visible; no modification in porosity or fibre alignment is expected. hMSCs (Donor 9056, Lonza – Passage 5) were harvested from culture flasks; a cell density of 2×10^6 cells/cm was calculated, considering 4 cm of scaffold as ‘seedable’ (0.5 cm each side was used to block the scaffold with the developed pinches). The entire cell pellet was re-suspended in 320 μl of GM, and 10 μl drops were pipetted on the external surface of the tube positioned in the

bioreactor, slowly rotating the scaffolds during the seeding phase (Figure 2).

The bioreactors were then placed in the incubator (37°C , 5% CO_2) and left for 3.5 h, setting a rotation speed of 2 rpm (Arrigoni *et al.*, 2008), to encourage more even cell distributions. The static controls (1 cm each), positioned in an ultra-low attachment 24-well plate, were seeded, under sterile conditions, rotating the matrix 90° every 30 min. After 3.5 h, the bioreactors were stopped and filled with myogenic media (scaffold submerged), and left in the controlled environment of the incubator overnight. At the same time myogenic media was also applied to the static controls. The following day, 15 ml of media was removed from the main chambers and the bioreactors were turned on with a speed of 3 rpm exposing the scaffold to double-phase (Zeisberg *et al.*, 2005; Figure 2). Optimal myogenic media was changed every 2 days. After 14 days of culture, scaffolds were harvested and samples were collected for scanning electron microscopy (SEM) analysis and histology imaging.

2.7. Histological and SEM analysis

After 14 days, samples cultured in the bioreactor and the static controls were processed for histological analysis and SEM observation. Briefly, they were fixed with 4% PFA for 6 h at 4°C , left overnight in a 30% sucrose (w/v) solution, then immersed for 12 h in OCT embedding media and finally frozen in fresh OCT. Samples were stored at -80°C prior to cutting. Sections (10 μm thick) of cultured tubes were collected on SuperFrost Plus slides (Thermo Scientific), washed with PBS, rinsed with MilliQ water, and left to dry overnight. Lillie’s allochrome stain was performed and the slides scanned with an Aperio AT slide-scanner (Leica Biosystems, Australia) to analyse the cell distribution

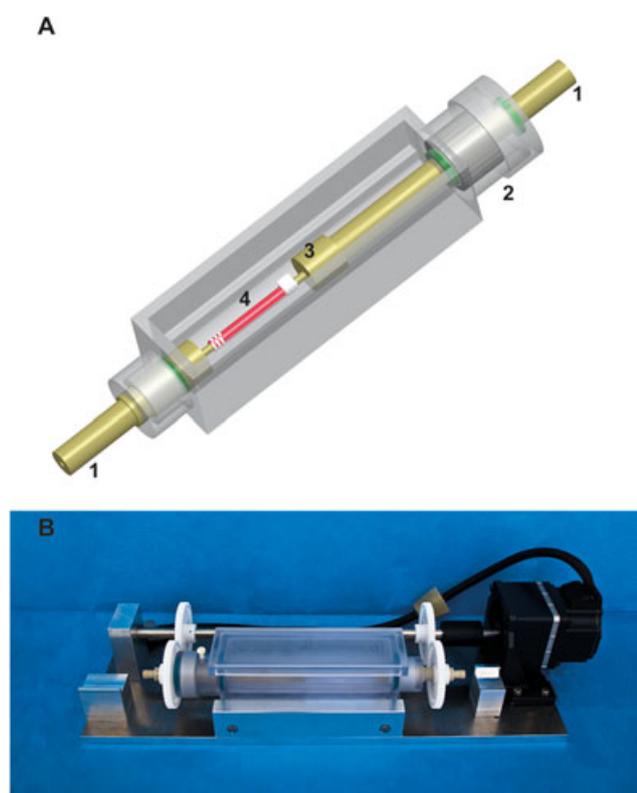


Figure 1. (A) Three-dimensional rendering of the developed bioreactor. (1) Shafts for rotation and pre-tensioning system, produced in different sizes in order to house scaffolds of different lengths. (2) The pre-tensioning system (in green Viton® dynamic-seals, in dark grey the ball bearing holder). (3) Scaffold supports. They are interchangeable and can be inserted in the chamber after the scaffold housing outside of the chamber. (4) Scaffold and custom-made clips, specifically designed to stably hold the scaffold in place. (B) Picture of the developed bioreactor coupled with the motion unit: the whole base can be easily positioned in a standard incubator and left in it for the whole time of the culture, while the culture chamber can be independently moved for medium change procedures or samples collection

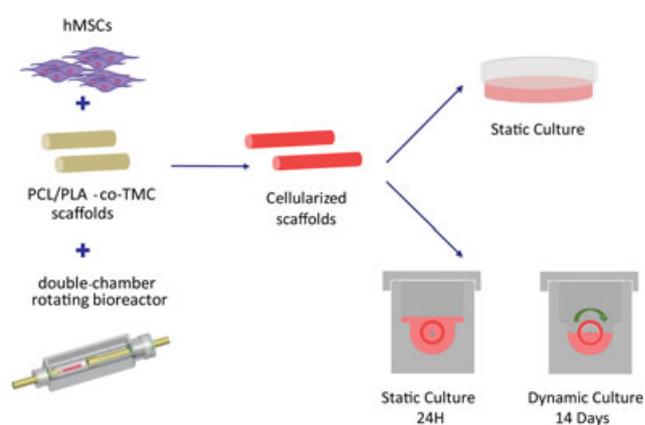


Figure 2. Experimental scheme for tissue-engineered (TE) vascular grafts study. A summary of the approach we followed to cellularize a synthetic scaffold: seeding and culture conditions are reported to simplify the understanding of the set-up of the study. Human mesenchymal stem cells (hMSCs) were seeded dropwise on the developed synthetic scaffold and cultured under dynamic condition for 14 days. Static culture was carried out as control. PCL/aPLA-co-TMC, poly(caprolactone)/poly(lactide-co-trimethylene-carbonate)

and ECM deposition in the tube cross-sections. Lillie's allochrome method was adapted from Culling (1974).

As for immunofluorescence staining, a 0.05% trypsin solution in 0.1% calcium chloride was used to unmask the antigens and epitopes for protein staining (20 min, RT). After PBS rinsing, sections were blocked in 3% BSA in PBS solution for 1 h. The following staining solutions (in 3% BSA in PBS) were used: 1/400 anti-collagen IV antibody, 1/300 anti-fibronectin antibody, 1/300 anti-laminin antibody, 1/400 anti-collagen I antibody (Abcam, UK) 60 min (RT) followed by 1/500 AlexaFluor 488 (Life Technologies) 60 min (RT); finally fluorescence mounting media was used to preserve the sections. Sides were analysed using a LSM Zeiss 710 confocal microscope.

Furthermore, samples were analysed using a SEM Stereoscan 360 (Cambridge Instruments, UK). Before analysis, the samples were dehydrated, with an increasing ethanol scale, and coated with a 20 nm layer of gold (SC7620, Quorum Technologies, UK).

3. Results

3.1. Differentiated MSCs gene expression (qPCR) and CellProfiler analysis

The cells cultured with TGF- β 1 increased the gene expression level of early SMC-specific markers in a dose-dependent manner (9056 Donor; Figure 3; other donors SF- 2), consistently with previous publications (Wang *et al.*, 2004; Au *et al.*, 2008). A significant increase in gene expression from an increase in TGF- β 1 from 1 ng/ml to 10 ng/ml was only seen across all the three donors for calponin F (CNN1) and collagen IV. Throughout the time course, desmin (DES) and SM1 expression levels remained similar to day 1; DES is an early marker of a SMC phenotype, whilst SM1 is expressed later in more mature SMCs (Narita *et al.*, 2008). It is therefore probable that at days 7 and 14 the cells that were analysed were in an intermediate stage, past the high DES expression, but still not in a final differentiation stage, expressing SM1. The upregulation trend is still visible at day 14 of exposure.

Whilst there was an alteration in gene expression profiles between the conditions of 10% vs. 20% FBS, this was not considered significant enough to justify the increase of concentration of FBS for further experimental studies, with 10% FBS selected due to an improved cost to performance ratio, whilst reducing potential confounding elements from such an undefined serum. The following media was thus chosen as our optimal media for all work using the bioreactor: low-glucose DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μ g/ml), plus 10 ng/ml TGF- β 1 and 30 μ M ascorbic acid.

Further investigations were carried out to quantify the efficiency of differentiation along the time course with the chosen medium. CellProfiler was utilized to analyse 20 images (examples reported in Figure 4), coming from

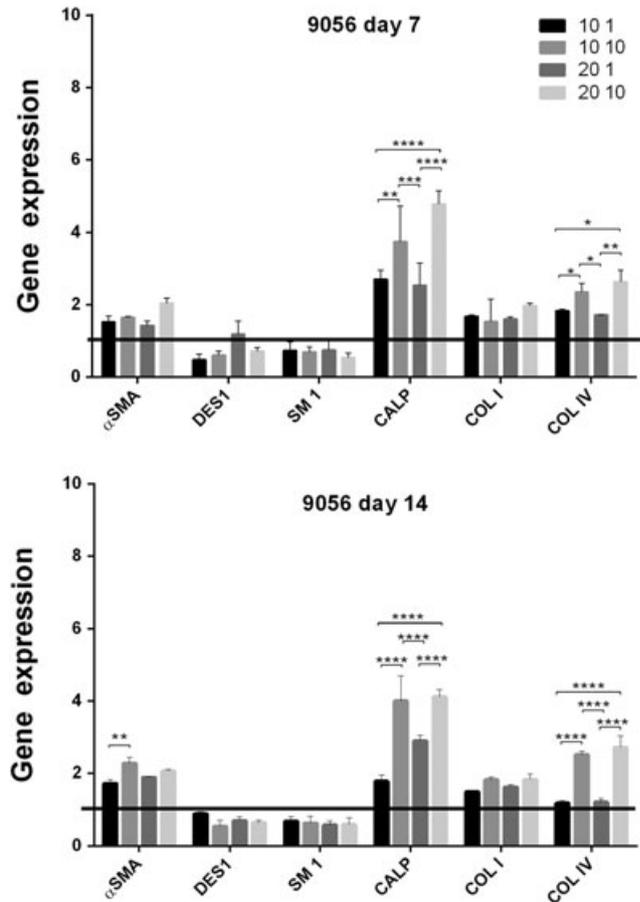


Figure 3. Gene expression in human mesenchymal stem cells (hMSCs; donor 9056 used for dynamic experiment later in this paper) exposed to four different myogenic media for 7 (LHS) and 14 (RHS) days. Samples number represents the % of fetal bovine serum (FBS; 10 or 20) and the concentration of transforming growth factor (TGF)- β 1 (1 or 10 ng/ml). Data are normalized on GAPDH and growth media (GM) exposed to hMSCs gene expression data (black line). Data were not combined due to similar trends, but different expression values due to donor variability (** = $p \leq 0.01$; **** = $p \leq 0.0001$)

each tested donor, for each time point. At day 7, $48 \pm 10\%$ positive cells were detected, while at day 14 the number increased to $90 \pm 4\%$ positive cells; the control did not show any double-positive staining. Confocal imaging also allowed the study of cell morphology: MSCs gradually acquired an elongated shape, reaching a smooth muscle-like morphology, after 14 days of myogenic media culture in the optimal media.

3.2. Bioreactor final design and bench validation

A rectangular-based bioreactor structure ($40 \times 190 \times 45$ mm) was manufactured, out of polycarbonate, with access from the top, to allow easy insertion and removal of the scaffold construct and facilitating cell seeding of the external surface. The lid design mimicked a Petri dish configuration to permit gas exchange, while preserving sterility of the culture environment. Two orifices for the insertion of the shafts were created on two of the chamber walls, in order to define the structure that allows rotational motion. Viton® dynamic seals (highlighted in green in Figure 1A), positioned into slits properly

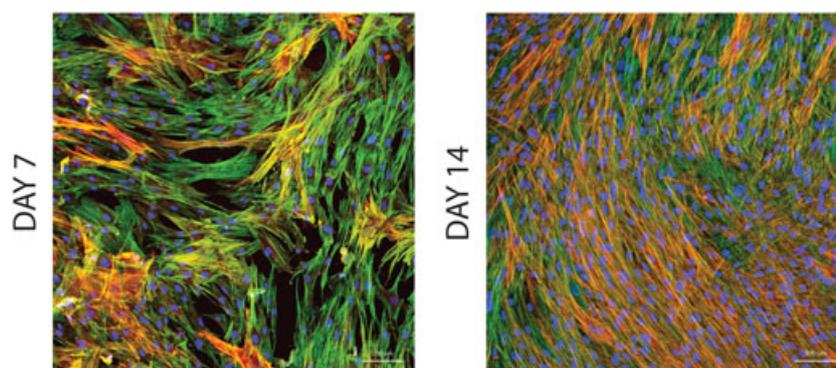


Figure 4. Confocal pictures of mesenchymal stem cells (MSCs) after 7 and 14 days of exposure to 10-10 differentiation medium. Nuclei (blue), actin (green), smooth muscle actin (α -SMA; red) and calponin (yellow) were stained. Images were loaded into CellProfiler method as separate channels, as required from the software

manufactured on chamber orifices, guaranteed isolation and sterility of the culture milieu in correspondence of critical interfaces.

The tubular scaffold is positioned on two polyether ether ketone (PEEK) supports, developed to securely connect with the rotating shafts, in an easy and reliable way; inside the supports, two O-rings prevent medium infiltration. Along the axis of the shafts, a 2-mm-wide conduit was made, to allow perfusion of the inner lumen by connecting it to a hydraulic circuit; the choice of two rotating shafts instead of a single chuck maximizes the exposure of the scaffold-lumen of small-calibre constructs to medium flow, during perfusion. Connections between chamber and actuator were designed to allow easy positioning and extraction of the device from the incubator. The bioreactor rendering and prototype are shown in Figure 1. Thanks to the modularity of shafts and scaffold-supports, together with their scalability, it is possible to host scaffolds of different sizes (both length and diameter) by simply changing the scaffold supports. The pre-tensioning system (Figure 1A-2) allows extension of the scaffold, after securing in the culture chamber, to avoid any geometrical change (due to bending) of the scaffold, maintaining its lumen pervious. The pre-tensioning mechanism allows for a maximum increase in length of 10 mm. All the described components can be autoclaved in order to reduce contamination risks.

Rotation of the scaffold is provided by a DC brushless motor AXHM230KC-GFH (Oriental Motor, UK), with a fine speed regulation (AXHD30K, Brushless DC Speed Controller, Oriental Motor) even at low revolutions per minute, and ensuring reliability inside the incubator.

All prototypes were manufactured by Zerotre Meccanica Srl (Costa di Mezzate, Italy) using in-house technologies, a parallel lathe (OPTI D 240 \times 500 G-Vario, Optimum, Bamberg, Germany) and a numerical control machine (Roland MDX-40, Roland DG Mid Europe s.r.l, Peschiera Borromeo, Italy).

A custom-made hydraulic circuit was developed to supply pulsatile pressure stimuli in the physiological range of 80–130 mmHg, as we wanted to test critical points like the connection between shafts and supports and the luer-lock joints for high-pressure resistance. The

system was assembled and left in working condition (rotation and pulsatile perfusion) for 7 days, and connection stability assessed for absence of leakages within the perfusion circuit and main chamber, and within the bioreactor and external environment. The validity of the pulsatile system was ensured using two pressure transducers PX600F (Edwards Lifescience LLC, USA) connected in line with the bioreactor shafts.

3.3. Bioreactor culture outcomes

Scaffolds from static controls (24-well plate) and dynamic experiments (bioreactor) were first analysed with SEM imaging (Figure 5). The external surfaces of each scaffold were scanned to visualize differences in ECM production and distribution. Differences at 14 days of culture are clear. Bioreactor samples showed complete coverage of the scaffold outer surface with significant production of ECM. It was possible to observe the underlying morphology of the fibres, but the surface was homogeneously covered. The static cultured scaffolds, on the other hand, presented covered zones as well as matrix-free areas, typical of the cell-suspension drop seeding technique. The comparison allowed us to prove that the rotation conditioning, starting from the seeding time point, in the bioreactor, during the adhesion timeframe for the MSCs, permitted improved cell seeding and distribution and hence a more favourable starting point for more even matrix production and distribution throughout the scaffold (Wendt *et al.*, 2003), whilst in the controls cells were not exposed to the whole scaffold outer surface and settled to a large extent where they were initially seeded. The production of ECM was also lower in the static condition, confirming previous reports suggesting dynamic culture promote protein production and deposition, and cell proliferation (Au *et al.*, 2008; Scotti *et al.*, 2012; Wendt *et al.*, 2006).

Histological analysis using Lillie's allochrome (Figure 6), a connective tissue stain, permitted further analysis of the developed tissue structure, as it clearly distinguishes between nuclei (black), collagen (blue), muscle reticulin (reddish purple) and fibrin (red to pink),

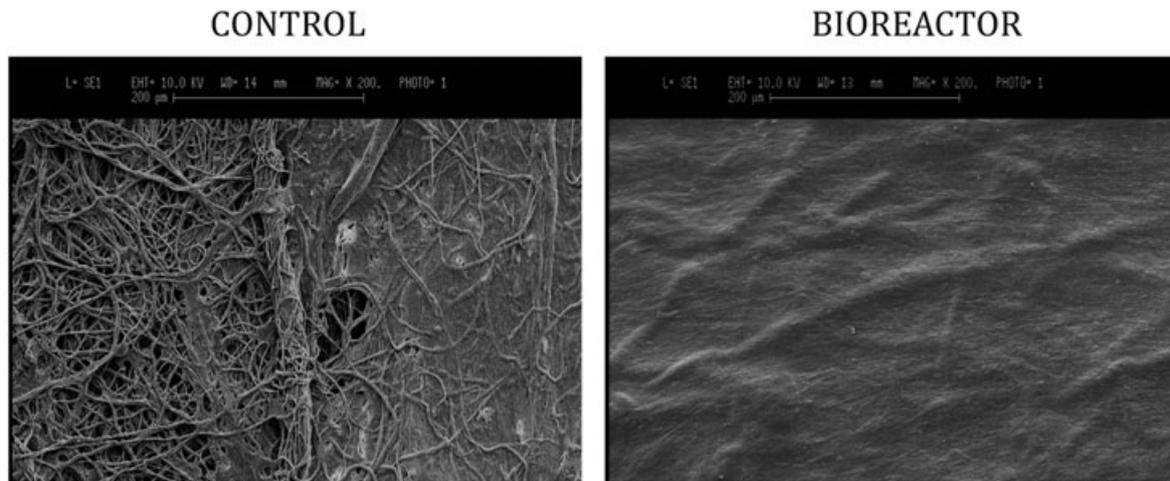


Figure 5. Scanning electron microscopy (SEM) of poly(caprolactone)/poly(lactide-co-trimethylene-carbonate (PCL/PLA-co-TMC) scaffold seeded with human mesenchymal stem cells (hMSCs) and cultured in myogenic media for 14 days. Outer surfaces of static (CONTROL) and dynamic (BIOREACTOR) cultured samples were analysed to investigate cells morphology and extracellular matrix (ECM) deposition. Scale bar: 200 µm

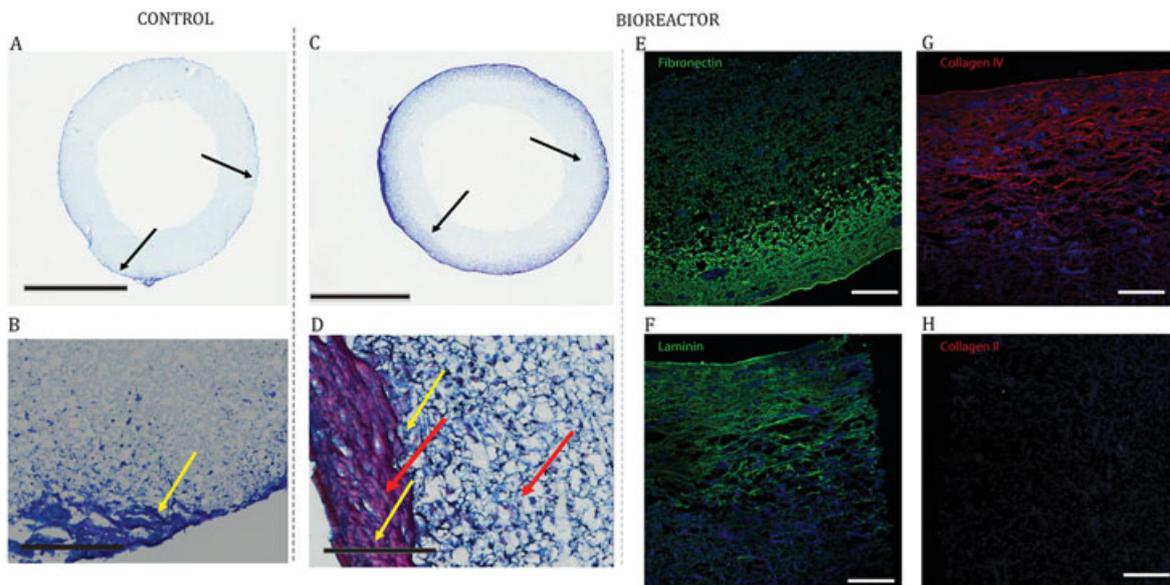


Figure 6. (A–D) Lillie's allochrome staining. Control and bioreactor scaffolds were analysed circumferentially. Black arrows in (A) and (B) present the extent of cell penetration. The deposition of different matrix proteins was investigated throughout the scaffolds. Nuclei are stained in black, collagen in blue (yellow arrows), muscle reticulin reddish purple (red arrows), fibrin red to pink. Scale bars: 3 mm (top); 200 µm (bottom). (E–H) Immunohistochemistry was performed on histology sections from the bioreactor samples in order to highlight the main components of the produced extracellular matrix (ECM; E: fibronectin; F: laminin; G: collagen IV; H: collagen II). The scaffold is visible in blue. Scale bar: 200 µm

highlighting differences in cell and protein distributions across the two conditions tested. Circumferential and axial sections of the bioreactor cultured samples gave important information showing how cells were colonizing more than half of the scaffold thickness. Matrix deposition was relevant at the outer surface and it was possible to see, at the highest magnification (20 \times), tissue developing around fibres. The contrast between the bioreactor samples (Figure 6C–H) with the static control (Figure 6A and B) was clear: only an outer and non-continuous layer of cells was present in the static control, probably due to the highly packed structure (pore size ≤ 30 µm) of the matrix, which does not facilitate spontaneous cell

migration in the absence of intra-mural flow – further encouraged by the air-aqueous rotating interface in the bioreactor. Static cultured scaffolds contained considerably fewer cells, mainly producing collagen, whereas a complete network of proteins, with collagen, reticulin and fibrin, was present in the samples cultured in the bioreactor. A clear homogenous distribution of cells and ECM could be seen on the outer half of the tubular scaffold. It was possible to recognize differently stained structures also in the inner part of the scaffold thickness, confirming that nutrient and growth factors could reach the inner zone of the structure easily with the rotation stimuli.

4. Discussion

Vascular TE aims to develop a vascular construct that demonstrates biological and mechanical properties as close as possible to those of a native vessel (Couet and Mantovani, 2012), with many different approaches having already been reported in the literature. More than 40 reviews on this topic, plus countless primary research articles, were written in 2014, and given that cardiovascular diseases account for most non-communicable diseases deaths (17.3 million people annually; Lim *et al.*, 2012), the need for a flexible, functional and patient-specific solution is clear.

Bioreactors are a useful tool in regenerative medicine, building a suitable environment for functional tissue growth and maturation. These devices can provide a standardized and reproducible range of stimuli, confined in a safe sterile environment, presenting a possible regulatory-compliant approach to reach the desired clinical substitute (Autors, 2013; Couet and Mantovani, 2012). Many vascular-bioreactors have been reported in the literature, mainly due to the necessity to discover an alternative to small-diameter synthetic prostheses that currently lead to the development of anastomotic intimal hyperplasia, coagulation and thrombogenesis (Graham *et al.*, 1989; Pasic *et al.*, 1995).

Niklason *et al.* (1999) developed and patented a bioreactor applying cyclic pulsatile strain. The device, connected to a closed flow system equipped with a peristaltic pump, provided cyclic pulsatile strain to cell-seeded scaffolds. The main idea was to reproduce the cyclic circumferential stretching imposed on native vessels by the heart (Niklason *et al.*, 1999). The device allowed them to create a decellularized scaffold starting from a polyglycolic acid tubular mesh seeded with porcine SMCs and cultured for 10 weeks in a perfusion bioreactor system. The constructs were then decellularized, leaving behind the mechanically robust ECM of the graft wall. After seeding endothelial progenitor cells on the lumen, the graft was then implanted (Quint *et al.*, 2011), with promising results, but it is important to underline how 10 weeks of culture plus decellularizing and re-seeding significantly enhance the probability of bacterial or fungal contamination.

Mironov *et al.* (2003) thereafter suggested that longitudinal strain is a necessary stimulus to produce functional grafts. Their bioreactor included two media perfusion systems: one for the luminal side of the tubular construct and the other for the watertight main chamber. The device allowed for a manually invoked change of longitudinal strain of the construct during culture, from 0% to 200%. The prototype was also equipped with pressure transducer and a digital TV camera, to analyse culture evolution. The bioreactor was tested using a native bovine carotid artery as a tubular construct, but never exploited for graft culture and tissue regeneration (Mironov *et al.*, 2003).

Bilodeau *et al.* (2005) describe a bioreactor that allows for the possibility of applying an oscillatory/constant strain utilizing a pneumatic cylinder controlled by a

microcontroller, reproducing torsion generally induced by heart beating. The internal and external surfaces of the seeded scaffold are in contact with culture medium to feed seeded cells. Two perfusion systems were then developed to avoid contamination between different cell types (Bilodeau *et al.*, 2005). Although the system was considered of great interest, no biological results were published.

Finally, Bjork and Tranquillo (2009) developed a transmural flow bioreactor allowing for different flow configurations and for measurement of changes in transmural dissolved oxygen concentration. It consisted of a glass external housing with a PEEK internal housing. Culture medium, directed through the internal lumen of a scaffold, was supplied by syringe pumps. The device allowed for the introduction of transmural perfusion, lumen perfusion and a combination of the two. SMCs culture was performed, enhancing production of ECM (Bjork and Tranquillo, 2009). The system allowed seeding of two different cell types, as the structure allowed one to connect two different hydraulic circuits. However, perfusion and transmural flow are the only two stimuli exploited, limiting the potential of this device.

Taking into account these published results, we report in this paper the development of a device that could reproduce the most promising stimuli for vascular tissue regeneration (perfusion, rotation, tensioning) never presented before in the literature in a single bioreactor. Considering modularity and scalability as one of the main goals, the produced prototype can house scaffolds with a variable length from 5 to 10 cm (just by changing one of the shafts) and internal diameter varying from 3 to 10 mm (just by producing a set of supports). This modular design permits the application of the bioreactor to constructs of varied dimensions as required, depending on the targeted tissue structure and function. Furthermore, we focused our design on easy assembly procedures after sterilization and ease of handle.

Taking into consideration the current state of art in vascular TE practices as reported in the literature (Pashneh-Tala *et al.*, 2015), we believe that bone marrow-derived hMSCs show potential as a practical cell source (in particular for SMCs) for a less invasive way of obtaining an appropriate number of cells prior to implantation. Little is known about the mechanisms causing the differentiation of hMSCs into SMCs (Gong *et al.*, 2009; Narita *et al.*, 2008; Wang *et al.*, 2004). TGF- β_1 is recognized as a fundamental signalling protein, playing important roles in many aspects of embryonic development and adult homeostasis (Wu and Hill, 2009). This factor has different effects on cells, mainly depending on the concentration of TGF- β_1 , the environment or cellular phase. TGF- β_1 has been shown to upregulate some smooth muscle markers in hMSCs (Narita *et al.*, 2008; Wang *et al.*, 2004) and embryonic cells (Sinha *et al.*, 2004). We thus decided to investigate four media combinations using low-glucose DMEM and 30 μ M ascorbic acid with two different concentrations of FBS (10/20%) and two concentrations of TGF- β_1 (1/10

ng/ml) across three different MSC donors. Higher FBS was tested as SMC primary cultures are usually performed with 20% of serum (Arrigoni *et al.*, 2008). Real time-PCR analysis was performed and, whilst different magnitudes of gene upregulation across the three hMSC different donors investigated were observed, they all displayed similar trends. Late differentiation markers were still low at day 14, while early differentiation genes were activated. Calponin was significantly upregulated in comparison to other genes only in cultures exposed to 10 ng/ml TGF- β_1 , guiding the decision to choose the 10 ng/ml + 10% FBS media combination. Because no significant differences were noticed with the higher FBS concentration, the FBS concentration was kept at 10%. Our results are in line with previous works demonstrating the important of TGF- β_1 in MSCs differentiation into SMCs lineage (Gong *et al.*, 2009; Narita *et al.*, 2008).

Further analyses were conducted using confocal imaging to quantitate the amount of cells undergoing differentiation at days 7 and 14, and to study changes in cell morphology. After building a method on CellProfiler, images were processed (as described in Materials and methods) with results indicating that at day 14 in the (10 10) media, 90% of cells were positive for a SMC phenotype [(α -SMA + calponin-positive/phalloidin-positive) * 100]. Cells also appeared elongated with less randomly (in terms of direction) attached filopodia, a phenotype characteristic of SMCs (Galvin *et al.*, 2002; Patel *et al.*, 2000).

The experiments that utilized our novel bioreactor produced outstanding results compared with the static culture: SEM analysis showed a non-homogenous distribution of cells on static culture, due to drop seeding, with cells seemingly proliferating where they were positioned without migrating consistently across/throughout the scaffold surface; on the other hand, scaffolds cultured in the multifunctional bioreactor presented a continuous layer of ECM that completely incorporated and covered scaffold fibres. Histological analysis of these two culture methods (bioreactor vs. static) confirmed clear differences between static and dynamic culture: scaffold colonization across a significant fraction of the scaffold thickness was consistent within the bioreactor environment, while cells survived only on the outer surface in the static condition. Moreover, the stimuli given by rotation and exposure to the air-aqueous phases appeared to facilitate cell migration into a scaffold with small pore-size like the one produced.

Matrix deposition was also enhanced in the case of the bioreactor dynamic culture, as shown from Lillie's allochrome staining; different proteins were deposited by differentiating MSCs. Collagen, muscle reticulin and fibrin were all detected. Proteins were arranged in a complex network, incorporating the scaffold structure. Nuclei staining demonstrated cell infiltration exceeding half way through the scaffold thickness. In stark contrast, matrix deposition in the static condition was minimal and uneven, and cell penetration

minimal, underlying the importance of a dynamic culture environment. After only 2 weeks of culture in our bioreactor, the obtained amount of matrix is comparable with most TE vessels reported in the literature, but in these cases, 6–10 weeks of cell culture was needed (L'heureux, 2012; L'heureux *et al.*, 2006; Quint *et al.*, 2011; Syedain *et al.*, 2011).

This work using the described bioreactor focused on vascular TE; however, it is clear that the approach detailed herein could be applied for all tubular organs that have a SMC muscularis and an epithelium or endothelium covering the internal lumen.

Whilst the obtained results are promising, further analysis is now required on the impacts of scaffold rotation and pulsatile perfusion rates, along with simultaneous cell seeding on both the outer surface and in the internal lumen, in order to comprehensively examine the full functionality of the bioreactor in terms of creating a constitutionally correct (in terms of both cell types and ECM) vessel. We believe that the coexistence and right combination of these two stimuli will lead to further increases in matrix deposition, giving rise to a mature muscular layer, facilitating cell differentiation and tissue cohesion.

4.1. Conclusions

In this paper, we have presented an innovative bioreactor specifically designed for the regeneration of small-diameter tubular structures, allowing rotation and perfusion of the entire lumen of cylindrical grafts. With particular focus on small-diameter vascular regeneration, we tested the developed device in combination with a previously developed PCL/a-PLA-co-TMC electrospun tubular matrix, proving promising mechanical properties and excellent biocompatibility. hMSCs were chosen to prove the bioreactor's ability to create a suitable environment for *in vitro* tissue generation, as we strongly believe that hMSCs are a valuable source of cells for our purpose as they can be differentiated in SMCs and, in a future prospective, they are easily harvestable from patients. The combination of our novel polymeric matrix and dynamic culture system created an environment that promoted hMSC growth and differentiation into applicable cell types to generate a functional vessel, result confirmed by histological staining showing a considerable amount of the targeted ECM produced. In conclusion, we have developed an efficient, versatile and valuable tool that in synergy with the produced scaffold is a concrete step towards our goal of creating a mature tunica media for vascular TE constructs.

Conflict of interest

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence this work.

Acknowledgements

This work was performed in part at the Queensland node of the Australian National Fabrication Facility, a company established under the National Collaborative Research Infrastructure Strategy to provide nano- and micro-fabrication facilities for Australia's researchers. Research was supported by 'Progetti di Ricerca Indipendente (Sanità)' Bioart-Decreto

13848 (Regione Lombardia) and the Australian Institute for Bioengineering and Nanotechnology, The University of Queensland. The authors would like to thank ZeroTre Meccanica s.r.l. for the bioreactor fabrication. The authors would like to acknowledge Dr Marco Piola and Dr Virginia Ballotta for technical support, Prof. G. Beniamino Fiore for lending the pressure analysis set-up, and Nick Glass for sharing his confocal microscopy expertise.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1: PCL/PLA-TMC tubular scaffold (A) and section (B: cut post liquid nitrogen treatment). Solution was spun for 2h, with a flow rate of 1ml/h, a collector/tip distance of 18 cm, an applied voltage of 18 kV and collector rotation speed of 400 rpm. UV light was directed on the collector (UV dose (365 nm): 50 mW/cm²) during all the process. C) SEM image of the scaffold wall. The SEM was performed after a vibrotome cut of a spun matrix. Scale Bar = 200 μ m. D) SEM analysis of the scaffold external wall, showing matrix fibers. Scale Bar = 20 μ m.

Figure S2: Gene Expression in hMSCs (donors 8006, 7129) exposed to four different myogenic media over 7 and 14 days. Samples number represents the % of Fetal bovine serum (10 or 20) and the concentration of TGF- β 1 (1 or 10 ng/mL). Data are normalized on GAPDH and growth media exposed hMSCs gene expression data (black-line). Data were not combine due to similar trends, but different expression values due to donor variability.

Figure S3: A-D) Lillie's allochrome staining. Bioreactors' scaffolds were analysed also on axial direction. As in figure 6, matrix deposition was investigated. Nuclei are stained in black, collagen in blue, muscle reticulin reddish purple, fibrin red to pink. (Scale-bar A=3 mm, B=200 μ m).