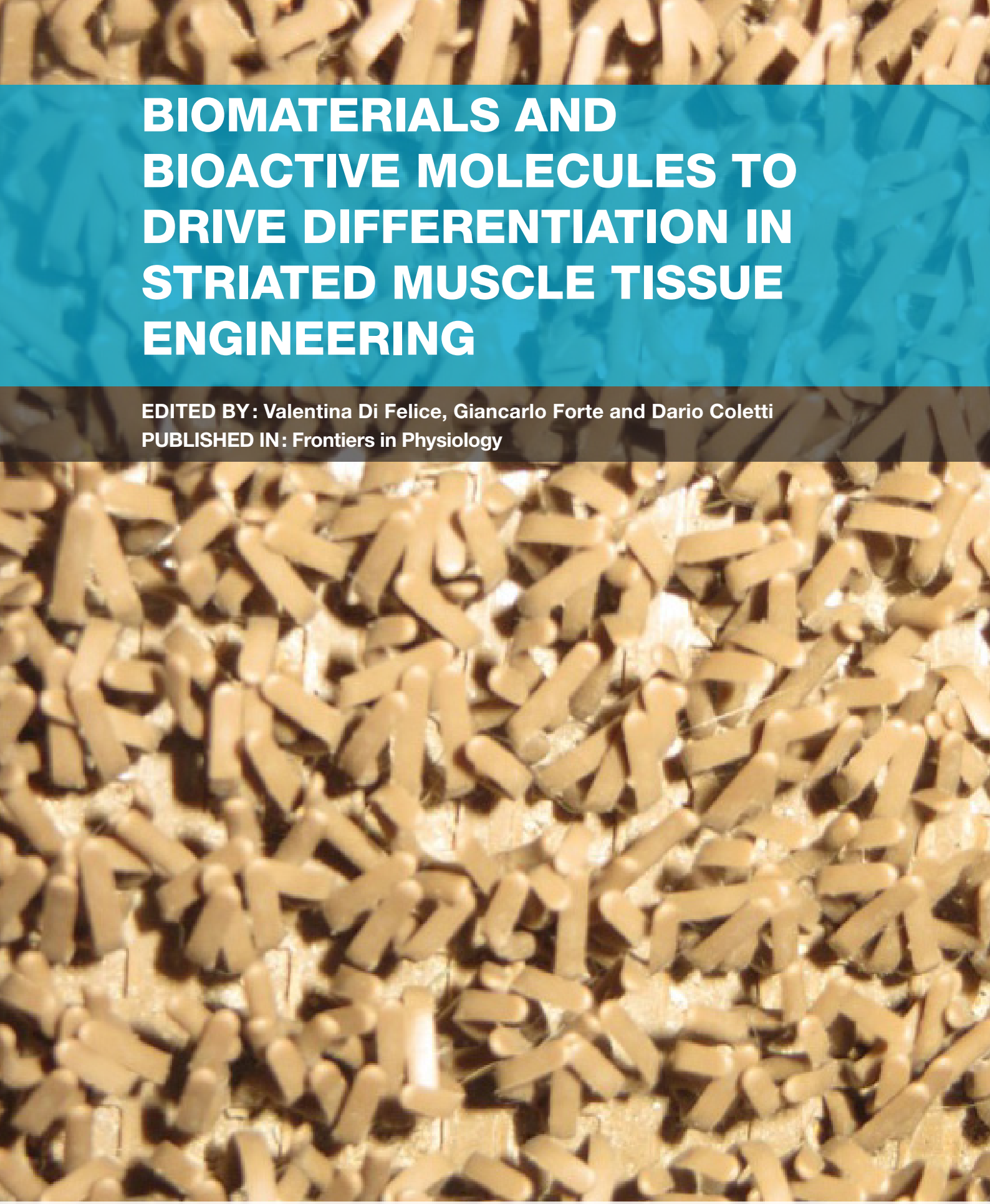

BIOMATERIALS AND BIOACTIVE MOLECULES TO DRIVE DIFFERENTIATION IN STRIATED MUSCLE TISSUE ENGINEERING

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BIOMATERIALS AND BIOACTIVE MOLECULES TO DRIVE DIFFERENTIATION IN STRIATED MUSCLE TISSUE ENGINEERING

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Tissue engineering is an innovative, multidisciplinary approach which combines (bio)materials, cells and growth factors with the aim to obtain neo-organogenesis to repair or replenish damaged tissues and organs. The generation of engineered tissues and organs (e. g. skin and bladder) has entered into the clinical practice in response to the chronic lack of organ donors. In particular, for the skeletal and cardiac muscles the translational potential of tissue engineering approaches has clearly been shown, even though the construction of this tissue lags behind others given the hierarchical, highly organized architecture of striated muscles.

Cardiovascular disease is the leading cause of death in the developed world, where the yearly incidence of Acute MI (AMI) is approx 2 million cases in Europe. Recovery from AMI and reperfusion is still less than ideal. Stem cell therapy may represent a valid treatment. However, delivery of stem cells alone to infarcted myocardium provides no structural support while the myocardium heals, and the injected stem cells do not properly integrate into the myocardium because they are not subjected to the mechanical forces that are known to drive myocardial cellular physiology.

On the other hand, there are many clinical cases where the loss of skeletal muscle due to a traumatic injury, an aggressive tumour or prolonged denervation may be cured by the regeneration of this tissue.

In vivo, stem or progenitor cells are sheltered in a specialized microenvironment (niche), which regulates their survival, proliferation and differentiation. The goal of this research topic is to highlight the available knowledge on biomaterials and bioactive molecules or a combination of them, which can be used successfully to differentiate stem or progenitor cells into beating cardiomyocytes or organized skeletal muscle in vivo. Innovations compared to the on-going trials may be: 1) the successful delivery of stem cells using sutural scaffolds instead of intracoronary or intramuscular injections; 2) protocols to use a limited number of autologous or allogeneic stem

cells; 3) methods to drive their differentiation by modifying the chemical-physical properties of scaffolds or biomaterials, incorporating small molecules (i.e. miRNA) or growth factors; 4) methods to tailor the scaffolds to the elastic properties of the muscle; 5) studies which suggest how to realize scaffolds that optimize tissue functional integration, through the combination of the most up-to-date manufacturing technologies and use of bio-polymers with customized degradation properties.

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Biomaterials and bioactive molecules to drive differentiation in striated muscle tissue engineering

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Keywords: cardiac tissue engineering, regenerative medicine, scaffolds, vasculature niche, stem cell transplantation, skeletal muscle

The generation of engineered tissues and organs has entered into the clinical practice in response to the chronic lack of organ donors. In particular, for the skeletal and cardiac muscles the translational potential of tissue engineering approaches has clearly been shown, even though the construction of these tissues lags behind others given the hierarchical, highly organized architecture of striated muscles. Failure of the cardiac tissue leads to cardiovascular diseases, which are the leading cause of death in the developed world (Di Felice et al., 2014). On the other hand, there are many clinical cases where the loss of skeletal muscle due to a traumatic injury, an aggressive tumor, or prolonged denervation may be cured by the regeneration of the muscle tissue (Perniconi and Coletti, 2014).

In this volume, we have included articles from renowned researchers in the fields of skeletal and cardiac muscle engineering who have contributed with methods, original research, and review articles covering various aspects of native and synthetic biomaterials or three-dimensional (3D) structures able to induce stem cell differentiation and which may be used in pre-clinical and clinical studies.

Of the two bio-artificial systems described, one is a silicon chamber system for the generation of skeletal muscle constructs described by Snyman et al. (2013). This inexpensive and readily adaptable system (distance between pins, cell number, and matrix-cell volume can be readily changed) may be used with different hydrogels and applied to any existing culture chambers. The other is a PEG-fibrinogen (PF)-based hydrogel scaffold used to rejuvenate aged adult skeletal muscle-derived pericytes (MP) from pig skeletal muscle. In this 3D environment pericytes were able to recover their differentiation potential toward myogenic differentiation and vessel formation. Fuoco and colleagues demonstrated that the 3D PF environment was beneficial for swine-derived MP by mimicking the stiffness and mechanical properties of young muscle extracellular matrix (ECM), thus, "rejuvinating" aged MP may be an alternative to the use of myogenic progenitors; indeed, swine derived MP represent a valid alternative to build human size comparable artificial muscle units from the swine muscle (Fuoco et al., 2014).

The method of choice to seed stem cells in 3D cultures is important, in order to form a densely packaged proto-tissue, independently from the scaffold used. This step is fundamental for the cells of the proto-tissue to complete their maturation process and interconnect with the native tissue upon *in vivo* implantation. Pagliari et al. (2014a) showed a method for sequential cell seeding, where first gelatin scaffolds were colonized with human mesenchymal stem cells in a static condition to favor endothelialization; then the scaffolds were loaded with pre-committed cardiac progenitors and cultured in perfusion bioreactor in cardiogenic conditions. The authors obtained a well-packed cardiac proto-tissue, rich in vessels, but without functional contractile or vascular structures. They concluded that even the more complex 3D dynamic culture system needs to be improved with stretching or electric stimulation to obtain mature, functional cardiac tissue.

A valid alternative to biosynthetic scaffolds are de-cellularized organs, for example the muscle acellular scaffold (MAS) from skeletal muscle. Perniconi et al. (2014) demonstrated that MAS may be used to induce the differentiation of cultured myoblasts and this is an excellent support for 3D myogenic cultures also *in vivo* in orthotopical engraftments. MAS is histocompatible, porous, degradable and non-toxic. This structure is stable in anatomical sites other than the skeletal musculature, but does not provide enough signals to trigger myogenesis by the colonizing cells. The possibility to use MAS for the reconstruction of tissue different from the skeletal muscle should be investigated. For example, considering similarities between the skeletal and the cardiac tissue, MAS might be used as an autologous native scaffold for cardiac tissue transplantation.

The same authors also contributed a review article on the usefulness of the MAS as a scaffolding platform able to re-create the natural structure of the muscle. MAS is naturally embedded with active native molecules which remain active after the decellularization process and help the implanted proto-tissue to integrate

in the host organ. Tissue-derived ECM helps in structuring niches rich in adhesive and signaling molecules supporting stem cells self-renewal and differentiation (Teodori et al., 2014).

When the muscle lesion is so extensive that the function of the muscle is impaired, the definition of volumetric muscle losses (VMLs) applies. In this case the muscle should be replaced by devices able to re-establish the function of the musculature and which preserve force transmission and continuity of the architecture within the host tissues. Hence, Cittadella Vigodarzere and Mantero (2014) described the architecture of the native skeletal tissue, identifying all the single elements which should be taken into consideration in a skeletal muscle tissue engineering construct. They focused the attention on the vasculature structures of constructs and, since decellularized ECM promotes vascularization of the implanted construct, they concluded that decellularized scaffolds are ready for a clinical application on human.

A clinical example of the latter is described in an interesting review discussing the importance of angiogenesis and the pro-angiogenic and pro-myogenic effects of the vascular endothelial growth factor (VEGF)/VEGF receptor pathway as a therapeutic strategy to cure muscle weakness and cardiomyopathy in Duchenne muscular dystrophy (DMD) patients (Shimizu-Motohashi and Asakura, 2014).

Apart from the structures of ECM and vasculature, also growth factors, cytokines, pleiotropic signaling pathways, and cell-specific regulators play an important role in the differentiation and self-renewal of cardiac as well as skeletal muscle stem cells. Pagliari et al. (2014b) described the most important signaling pathways which influence the *in vivo* differentiation of cardiac progenitor cells. Many of these pathways and factors have different and distant effects on the differentiation process (JAK/STAT, Hippo pathway, Wnt and Notch signaling, etc.).

This book provides a comprehensive up-to-date review on cardiac and skeletal muscle tissue engineering and highlights the several elements which have to be taken into consideration when engineering a functional proto-tissue.

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Simple silicone chamber system for *in vitro* three-dimensional skeletal muscle tissue formation

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Bioengineering skeletal muscle often requires customized equipment and intricate casting techniques. One of the major hurdles when initially trying to establish *in vitro* tissue engineered muscle constructs is the lack of consistency across published methodology. Although this diversity allows for specialization according to specific research goals, lack of standardization hampers comparative efforts. Differences in cell type, number and density, variability in matrix and scaffold usage as well as inconsistency in the distance between and type of adhesion posts complicates initial establishment of the technique with confidence. We describe an inexpensive, but readily adaptable silicone chamber system for the generation of skeletal muscle constructs that can readily be standardized and used to elucidate myoblast behavior in a three-dimensional space. Muscle generation, regeneration and adaptation can also be investigated in this model, which is more advanced than differentiated myotubes.

Keywords: three-dimensional assays, tissue engineering, hydrogel constructs, *in vitro* skeletal muscle tissue

INTRODUCTION

Three-dimensional (3D) skeletal muscle constructs can be bioengineered *in vitro*. 3D models have advantages over 2D cell cultures in mimicking *in vivo* conditions as they allow for the study of dimensionality, cellular architecture, cell polarity and function. Constructs can be adapted for the generation of *in vitro* drug screening assays as well as *in vivo* tissue repair following transplantation of constructs (Vandenburgh et al., 2008; Corona et al., 2012). If genetically modified to express recombinant protein, these constructs can be used for therapeutic protein delivery (Vandenburgh et al., 1996).

An assortment of methods for the generation of bio-artificial skeletal muscle have been previously described (**Table 1**), with variations on aspects including chamber construction, matrix composition and ultimate tissue size generated. The chambers employed for 3D culture of skeletal muscle may be divided into two main categories: the uncomplicated silicone tubing model and the more intricate models constructed in chamber slides and multi-well plates (**Table 1A**) and chambers and micro-patterned wells that are precast via photolithographic moulds (**Table 1B**). Confluent myoblast monolayers cultured in a matrix-coated petri dish under differentiating conditions may also form scaffold-free 3D muscle tissue due to contractility of the differentiating fibers (**Table 1C**). These methods naturally reflect the thrust of the particular research group. While each model has specific advantages, key methodological aspects differ considerably between the various models which may potentially hamper efficient comparison. A critical overview of the various models is required before describing our simple chamber system.

In general culture vessels for 3D skeletal muscle constructs consist of tubes, standard pre-fabricated laboratory-based culture plates or dishes that contain two tissue adhesion points that mimic tendons and consist of either cast silicone posts, metal pins and mesh, sutures or Velcro pads (Vandenburgh et al., 1996, 2008; Dennis and Kosnik, 2000; Powell et al., 2002). Some models require post-modification with custom-made inserts that replace pins or Velcro adhesion points. In the models consisting of a silicone tube or a precast chamber slide, the adhesion points are on average 18–30 mm apart, which may dictate factors including the volume and concentration of cells initially seeded (**Table 1A**) (Powell et al., 2002; Vandenburgh et al., 2008). The cell seeding number ranged from 1×10^6 to 6×10^6 cells per tube, and the hydrogel-cell suspension volume varied between 400 µl required for the tube models and 3.2 ml for the adapted single well-chamber slide (**Table 1A**) (Vandenburgh et al., 1996; Hinds et al., 2011; Smith et al., 2012). The matrix mixture and culture periods are similarly diverse. This is, however, also an indication of the ease with which tissue-engineering models that use silicone tubes with adhesion points may be constructed and adapted for a range of purposes (Vandenburgh et al., 2008; Hinds et al., 2011; Smith et al., 2012).

While Vandenburgh initially generated *in vitro* 3D muscle tissue constructs from C2C12 myoblasts in silicone tubing containing Velcro pads ~25 mm apart (**Table 1A**), this group subsequently employed a more complex custom-built silicone mould cast around a Teflon template with two flexible silicone posts inserted into a standard 96-well plate 4 mm apart (Vandenburgh et al., 1996, 2008). In other more recently-developed models these anchorage points and the distance between them in custom-built models varied from 4 to 50 mm, according to the size of the wells and aspects required for the experiment. The flexibility of the cantilever posts in the more advanced models

Table 1 | A comparison of published methods for the generation of bio-artificial skeletal muscle.

Model description	Purpose	Anchor points	Cell type and seeding conditions	Matrix (final concentrations)	References
(A) CAST CHAMBERS CONTAINING TWO ADHESION POINTS					
Silicone rubber tubes cut lengthwise in 35 mm dishes	Reversible gene therapy	Velcro pads or stainless steel mesh	C2C12 mouse myoblasts (1–4 × 10^6 cells/well)	Collagen (1.6 mg/ml) and Matrigel (Ratio of 6:1 v/v)	Vandenburgh et al., 1996
Sylgard moulds produced in a vacuum-moulding process around Teflon spacers; 96-well plates	*In vitro* drug screening	Flexible silicone posts	Primary mouse myoblasts (0.2 × 10^6 cells/well)	Collagen (1 mg/ml) and Matrigel (Ratio of 6:1 v/v) Fibrinogen–thrombin (0.5 mg/ml) and thrombin (1 U/ml)	Vandenburgh et al., 2008
Moulds cast from 2% agarose in PBS around Teflon spacers; 24-well plates	Heart muscle kinetics	Flexible Sylgard posts	Neonatal rat heart cells (0.62 × 10^6 cells/well)	Fibrinogen (5 mg/ml) and Matrigel (100 μl/ml) polymerized with thrombin (32:1 v/v)	Hansen et al., 2010
Rectangular casting moulds (see Hansen et al., 2010)	Interaction between cells and surrounding matrix	Silicone pins	Primary human myoblasts (0.66 × 10^6cells/well)	Fibrin-based matrix	Chiron et al., 2012
Mechanical Cell Stimulator, version 4.0 (MCS4) Silicone rubber tissue moulds; 6-well plates	Mechanical stimulation to improve tissue-engineered human skeletal muscle	Stainless steel pins	Primary human skeletal muscle cells (1 × 10^6 cells/well)	Collagen 1 (0.8 mg/ml) and Matrigel (Ratio of 6:1 v/v)	Powell et al., 2002
Silicone tube cut lengthwise (ends sealed with PDMS); 6-well plates	Hydrogel matrix combinations; influence on contractile function of engineered muscle tissue	Velcro adhesion pads	Primary rat skeletal myoblasts (6 × 10^6 cells/well)	Collagen 1 (1.4 mg/ml) and Matrigel Fibrinogen (2, 4 or 6 mg/ml) and Matrigel (10%, 20% or 40% v/v)	Hinds et al., 2011
Commercially available single-well chamber slides	Optimized culture parameters improved reproducibility and the cellular architecture	Polyethylene mesh	Primary rat muscle derived cells (Cell count not stated; 3.2 ml/well)	Collagen 1	Smith et al., 2012
(B) PHOTOLITHOGRAPHIC MOULDS AND MICRO-PATTERNED WELLS WITH POSTS					
Sylgard tissue moulds cast from patterned master templates of coated photo-resistant silicone wafers	Muscle cell alignment	Array of silicone posts	C2C12 mouse myoblasts (1 × 10^6 cells/well) Primary rat skeletal myoblasts (2 × 10^6 cells/well)	Collagen I (1 mg/ml) and fibrinogen (2 mg/ml) (ratio of 1:0, 3:1, 1:1, 1:3, 0:1) Thrombin (0.4 U/mg fibrinogen) Matrigel added to all combinations	Bian and Bursac, 2009
Precast micro-patterned wells	Formation of muscle for use in bioactuators	PDMS cantilevers	C2C12 mouse myoblasts (400 cells/micro-patterned well)	Collagen 1 (2 mg/ml) and Matrigel	Sakar et al., 2012

(Continued)

Table 1 | Continued

Model description	Purpose	Anchor points	Cell type and seeding conditions	Matrix (final concentrations)	References
(C) SCAFFOLD-FREE CONFLUENT MONOLAYER CONTRACTION INTO CYLINDER BETWEEN PINNED SUTURES					
Sylgard-based 35 mm culture dish coated with laminin	Excitability and contractile properties of muscle engineered from co-cultured primary cells	Silk sutures coated with 50 μg/ml laminin	Co-culture of primary myogenic precursors, fibroblasts and all related cell types	Laminin base layer (1 μg/cm^2)	Dennis and Kosnik, 2000
Sylgard-coated 35 mm culture dish coated with laminin	Skeletal muscle construct from C2C12 myoblasts; AIM-V media	Silk sutures	C2C12 myoblasts AIM-V serum-free medium; 0.02 × 10^6 cells / dish	Laminin base layer (2 μg/ml) Laminin top layer (10 μg/ml)	Fujita et al., 2009b

The chambers employed for three-dimensional culture of skeletal muscle may be divided into three main categories: Cast chambers such as the uncomplicated silicon tubing model (A), Photolithographic moulds and micro-patterned wells (B), and Scaffold-free confluent monolayers (C).

These are fitted into the wells of standard culture plates.

The combination of hydrogel components as well as cell type, number and volume seeded per well vary considerably between published methods, and are summarized. The concentration of Matrigel is 10% (v/v) unless otherwise indicated.

is an improvement from the originally employed metal pins or large, fixed Velcro pads or metal mesh (Vandenburgh et al., 2008). The advantage of these custom-built systems with standardized dimensions and with flexible cantilever posts is that the cultured constructs could be used to investigate muscle kinetics (force generation, internal strain or contractile forces) on mechanical stimulation, or in response to compounds such as insulin-like growth factor 1 and cholesterol-lowering statins (Eastwood et al., 1998; Nirmalanandhan et al., 2007; Vandenburgh et al., 2008; Smith et al., 2012).

The actual vessels used for myooid culture may also be custom-built via micro-pattern technology or photolithography (**Table 1B**) (Dennis and Kosnik, 2000; Vandenburgh et al., 2008; Fujita et al., 2009a; Hansen et al., 2010; Smith et al., 2012). Using this technology a negative template is generated and a mould is subsequently cast from biological grade silicone (Bian and Bursac, 2009; Sakar et al., 2012). The use of an array of posts in a wafer pattern rather than simply two adhesion points permits the formation of a sheet-like culture that allows for the investigation of muscle cell alignment. This is in contrast to the 3D construct generated between two cantilevers.

Even though reproducibility is improved, these casting methods and inserts demand specialized equipment, which commonly translates into an increase in cost. In addition the system may not easily be adaptable to different well-types or tissue sizes.

Currently, various hydrogel components are routinely used, both individually and in combination, to successfully engineer muscle tissue containing striated and aligned myotubes. These include collagen 1, Matrigel from Engelbrecht-Holm-Swarm (EHS) sarcoma cells, laminin I, and fibrin-based gels (**Table 1**) (Vandenburgh et al., 2008; Bian and Bursac, 2009; Hansen et al., 2010). The hydrogels differ in their molecular composition, macromolecular orientation and the degree of cross-linking (Dennis and Kosnik, 2000; Hinds et al., 2011). This has practical implications; for instance, Matrigel polymerization is temperature-sensitive, while laminin I and collagen 1 are able to spontaneously form 3D gels at room temperature (Yurchenco et al., 1992). Furthermore, myogenesis itself is differentially affected by these matrix factors; collagen 1 has been shown to suppress differentiation, whereas Matrigel promotes myotube formation in both 2D and 3D models (Langen et al., 2003; Grefte et al., 2012). Laminin I promotes myoblast adherence, proliferation and myotube fusion (Schuler and Sorokin, 1995; Vachon et al., 1996). Finally, the matrix combinations as reported for the different models vary considerably (**Table 1**). The most conventional combination consists of collagen 1 in combination with Matrigel in a ratio of 6:1 (v/v) or a 10–20% Matrigel component (**Table 1**). The concentration of fibrinogen on its own or used in combination with other matrix components also varies considerably (**Table 1**) (Vandenburgh et al., 2008; Hinds et al., 2011). Such diversity may hamper direct comparison of results.

Successful formation of scaffold-free cylindrical 3D myooids in 35 mm culture dishes coated first with Sylgard and then laminin has also been described (Dennis and Kosnik, 2000; Fujita et al., 2009b) (**Table 1C**). After an initial low seeding density (2–10 × 10^3 cells) in a coated 35 mm dish, cells are cultured in a 2D monolayer to confluence. This is followed by replacement of the growth media (GM) with differentiation media (DM) to stimulate differentiation into muscle fibers. Subsequent formation of cylindrical myooids is due to contraction of the fibers and release from the underlying Sylgard coating (Huang et al., 2005; Fujita et al., 2009a). Silk sutures pinned into the Sylgard base act as handling points and mimic flexible tendons for the cylindrical myooid. The laminin matrix employed merely forms a separating layer between the Sylgard coating and the cultured cells, while

the final histology and molecular characteristics reflect that of skeletal muscle. It is believed that the interaction between co-cultured primary fibroblasts and myoblasts in this model allows for the investigation of functional and molecular development, as reflected by the contractile properties and expression of transcription factors MyoD and muscle-related myosin heavy chain of such fabricated skeletal muscle (Dennis and Kosnik, 2000; Huang et al., 2005; Fujita et al., 2009b). The culture period to establish this model is, however, considerably longer than the previously-mentioned models.

Although numerous methods for bioengineering skeletal muscle exist, currently no standardized culture vessel and protocol has been proposed. Also, many are prohibitive due to the required customized equipment and intricate casting techniques. Below we describe an inexpensive, accessible hydrogel-based system that may be readily standardized, yet is easily customized to reflect desired matrix combinations and tissue size. This model is ideal for laboratories expanding into three-dimensional assays.

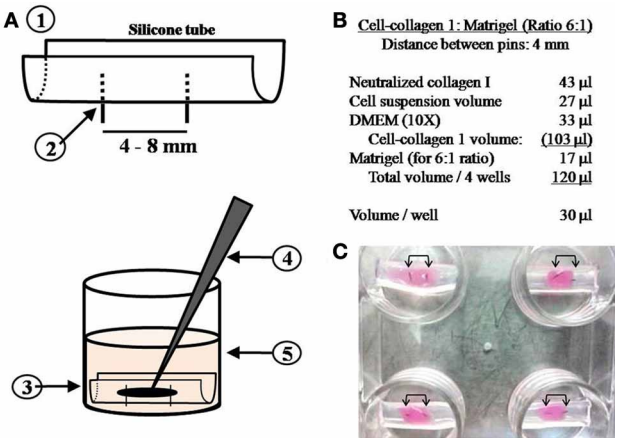

FIGURE 1 | Details on simple silicone tube chamber construction and hydrogel-cell preparation. (A) Biological grade silicone tubing is cut to fit the diameter of the well; the tube is then cut lengthwise in half (1). Surgical grade stainless steel pins are inserted through the silicone wall at predefined distances from each other (2). The silicone tube is secured in place within the well with Sylgard 182 to form a chamber (3). The hydrogel-cell suspension is pipetted into the silicone chamber around the pins (4) and the well is flooded with growth media once the gel construct has set (5). **(B)** Calculations for preparation of the hydrogel-cell mixture (Ratio of 6:1). **(C)** A multi-well plate containing a hydrogel-C2C12 mix seeded in silicone tube chambers (arrows indicate pins positioned 4 mm apart).

MATERIALS AND METHODS

SELECTION AND CONSTRUCTION OF CHAMBER

An adaptable chamber system was generated by using 18 mm sections of biological grade silicone tubing (outer diameter: 5 mm) which was cut in half (lengthwise) (**Figure 1A**). Surgical grade stainless steel pins (3 mm long, outer diameter: 0.2 mm) were inserted into the tubes to act as adhesion points; distances between the pins ranged from 4 to 8 mm, depending on the size of the muscle tissue required. The tubes were fitted into each well of a 4- or 24-well-culture plate and secured within the wells using Sylgard 182 (Dow Corning Corporation, cat. 3097358-1004) (**Figure 1A**). Sylgard was allowed to cure for 24 h and the plates sterilized overnight under an ultraviolet light.

SELECTION AND PREPARATION OF CELLS

Murine C2C12 myoblasts (ATCC, cat. CRL-1772) were maintained in GM containing Dulbecco's Modified Eagle Serum (DMEM, Highveld, cat.CN3193-9), L-glutamine (2% v/v, Cambrex, cat.17-605E), PenStrep (2% v/v, Cambrex, cat.17-602E), and Fetal Calf Serum (FCS; 10% v/v, Invitrogen, cat.10108165). Primary cultured human skeletal myoblasts (HSKM, Lonza, cat. CC-2561) were cultured in Ham's-F10 (Gibco, cat.15140), FCS (20% v/v), Penstrep (2% v/v), L-glutamine (2% v/v), fibroblast growth factor (FGF; 2.5 ng/ml, Promega, cat.G507A).

PREPARATION OF HYDROGEL/MATRIX

Rat tail collagen 1 (3.6 mg/ml; Sigma, cat. C9791) was neutralized with 10% NaOH (~30 µl per ml of collagen 1) until a color change was observed (yellow to pink due to pH indicator in DMEM). All solutions were kept on ice to restrict matrix polymerization prior to seeding.

CELL-HYDROGEL SUSPENSION

Initially a cell suspension (27 µl; in GM) containing 3.2×10^6 HSKM cells or 6.4×10^6 C2C12 cells and 33 µl 10X DMEM was added to the neutralized collagen 1 solution (43 µl) (total volume: 103 µl). To achieve a cell-collagen 1:Matrigel ratio of 6:1 (v/v) as is often used in the generation of skeletal muscle (Vandenburgh et al., 1996, 2008; Powell et al., 2002), 17 µl Matrigel (10.1 mg/ml stock concentration, BD Biosciences, cat.356231) was added to the cell-collagen 1 suspension to achieve a final volume of 120 µl (**Figure 1B**).

HSKM cells are larger than C2C12 myoblasts; this accounts for the lower number of human myoblasts in the hydrogel mix when compared to mouse myoblasts. The cell/hydrogel suspension containing C2C12 or HSKM cells was pipetted into each silicone tube chamber around the pins. Constructs were subsequently incubated at 37°C overnight and the wells then flooded with 350 µl GM (**Figure 1A**). Twenty-four hours later, culture GM was replaced with DM, which contained DMEM, L-glutamine (Lonza; 2% v/v), PenStrep (Lonza; 2% v/v) and horse serum (HS; 1% v/v, Invitrogen, cat.16050-130).

PREPARATION FOR IMAGE COLLECTION

Brightfield images were captured at various stages of muscle development using a Motic 3.0 MP camera and an Olympus stereo microscope (VMZ, Japan). For histochemical investigation of desmin expression and the actin cytoskeleton, muscle constructs were fixed for 2 h in the wells in paraformaldehyde (4% prepared in PBS). Subsequent removal of the pins allowed constructs to be transferred from the chambers to culture wells where they were incubated with either a polyclonal rabbit anti-desmin antibody (1/600, Abcam cat.AB15200) for 2 h at room temperature followed by a Dylight488 donkey anti-rabbit antibody (1/1000, Jackson, cat.711-485-152) for 1 h at room temperature,

or TRITC-conjugated Phalloidin (0.5 ng/ml, Sigma; cat. P1951) for 2 h at room temperature. Nuclei were stained with Hoechst (2.5 μg/ml, Sigma, cat C8890) for 10 min at room temperature and constructs mounted on glass slides with Moviol and viewed with the Zeiss 710 confocal microscope. Constructs cultured for 15 days in DM were also fixed with glutaraldehyde (2%, 2 h at room temperature), dehydrated with a graded series of alcohols and embedded in Spurr's resin. Thin sections were cut and DIC images were obtained with the 710 Zeiss confocal microscope.

This adapted technique proved to be advantageous for skeletal muscle formation with constructs from both C2C12 and HSKM cells successfully spanning the pins after a 3–7 day culture period (**Figures 1C**, **2A–C**). After 12–15 days in culture, differentiated C2C12 myotubes showed clear formation of actin fibers (**Figure 2D**). In addition, aligned myotubes expressed desmin (**Figure 2E**) and longitudinal sections showed evidence of organization into multinucleated myotubes (**Figure 2F**), which is an initial requirement for functionality.

DISCUSSION

Muscle tissue engineering is no longer in its infancy, nor is it the prerogative of only a few laboratories. Nonetheless, only a few research groups have made major advances in this field (Vandenburgh et al., 2008; Hansen et al., 2010; Vandenburgh, 2010; Chiron et al., 2012; Corona et al., 2012; Sakar et al., 2012; Smith et al., 2012). One of the major hurdles when initially trying to establish *in vitro* tissue engineered muscle constructs is the lack of consistency across published methodology. Such lack of uniformity was highlighted by a summary of the range of moulds already employed in muscle tissue engineering (**Table 1**). A careful view of **Table 1** indicates that particular moulds have been designed with specific purposes in mind (Powell et al., 2002; Bian and Bursac, 2009; Sakar et al., 2012). However, differences in cell type, seeding density as well as variability in hydrogel/scaffolds used, and also variations in the distance between and type of adhesion posts (i.e., stainless steel, sutures, Velcro, etc.) hampers initial establishment of the technique with confidence. In the current study we describe an inexpensive, readily adaptable silicone chamber system for the generation of skeletal muscle constructs.

We highlight the basic steps and requirements needed to form differentiated muscle tissue from either C2C12 or HSKM myoblasts in a collagen 1/Matrigel hydrogel. This model is adaptable to fit into any existing culture dish or chamber used in most laboratories. Despite this simplicity, variations in the distance between pins, as well as cell number and matrix-cell volume can be readily achieved. In addition, the use of appropriate pins as anchor points allows for future mechanical stimulation to investigate contractile forces and allows for the study of internal stresses during muscle differentiation in 3D cultures. It is also useful for investigations into genetic manipulation, drug therapy or co-culture of complimentary cell phenotypes.

This model has several practical advantages. Biological grade silicone is inexpensive, readily available and allows for ease of pin insertion and subsequent tissue manipulation. In addition, after initial use, the various components of the chamber system may also be reused following de-cellularization with ammonium hydroxide and cleaning with alcohol and sonication. This model, with standardized parameters, may be used as an optimized system for initial evaluation of factors involved in skeletal muscle generation from both primary cultured myoblasts and established cell lines.

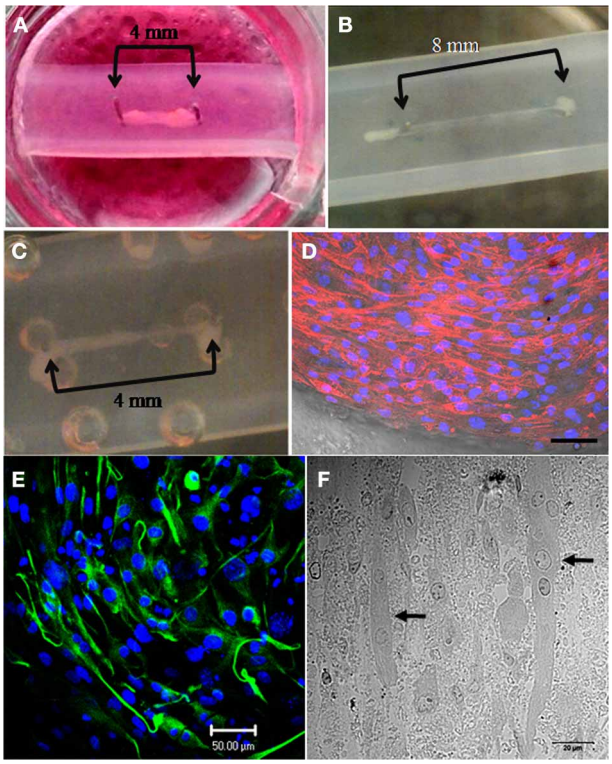

FIGURE 2 | Successful generation of mouse and human skeletal muscle constructs using the simple silicone chamber system. (A) When seeded in a matrix of collagen 1 and Matrigel (14%), mouse C2C12 cells formed tissue (day 7 in differentiation media) between pins placed 4 mm apart. **(B)** When seeded in a matrix of collagen 1 and Matrigel (14%), mouse C2C12 cells formed tissue (day 3 in differentiation media) between pins placed 8 mm apart. **(C)** When seeded in a matrix of collagen 1 and Matrigel (14%), human skeletal muscle (HSKM) cells formed tissue (day 3 in differentiation media) between pins placed 4 mm apart. **(D)** After 12 days in differentiation media, actin fibers stained with TRITC-phalloidin and were clearly visible in the differentiated mouse C2C12 myotubes. Nuclei were stained with Hoechst (scale bar = 50 μm). **(E)** After 15 days in differentiation media, elongated myotubes were aligned and contained desmin, an intermediate filament required for myotube contractile function (scale bar = 50 μm). **(F)** Thin sections of resin-embedded C2C12 myoblasts culture for 15 days in differentiation media showed the formation of multi-nucleated (arrows) myotubes (scale bar = 20 μm).

Imaging of myoblasts functioning in a three-dimensional space is also more closely aligned to *in vivo* behavior. With this model we have described the expression of desmin, an intermediate filament that plays a key role in the integration of striated muscle morphology and function (Capetanaki et al., 2007). We also showed the transition of myoblasts into elongated, multi-nucleated myotubes, one of the relevant steps during myoblast differentiation. It must be noted, however, that the use of C2C12 myoblasts to generate functional striated tissue may require electrical stimulation during the generation of the

bioengineered tissue; this is not the case for primary muscle progenitor cells (Langelaan et al., 2011). Engineered tissue may further be processed for histological purposes or immunocytochemical investigation of transcription factors and expressed proteins. We propose that the method we describe may allow skeletal muscle research groups utilizing 2D cell culture models to move into 3D tissue models with relative ease. This will be important to enable more rapid enhancement of our understanding of muscle synthesis, repair and adaptation *in vitro*, in a model more advanced than differentiated myotubes.

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Angiogenesis as a novel therapeutic strategy for Duchenne muscular dystrophy through decreased ischemia and increased satellite cells

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Duchenne muscular dystrophy (DMD) is the most common hereditary muscular dystrophy caused by mutation in *dystrophin*, and there is no curative therapy. Dystrophin is a protein which forms the dystrophin-associated glycoprotein complex (DGC) at the sarcolemma linking the muscle cytoskeleton to the extracellular matrix. When dystrophin is absent, muscle fibers become vulnerable to mechanical stretch. In addition to this, accumulating evidence indicates DMD muscle having vascular abnormalities and that the muscles are under an ischemic condition. More recent studies demonstrate decreased vascular densities and impaired angiogenesis in the muscles of murine model of DMD. Therefore, generation of new vasculature can be considered a potentially effective strategy for DMD therapy. The pro-angiogenic approaches also seem to be pro-myogenic and could induce muscle regeneration capacity through expansion of the satellite cell juxtavascular niche in the mouse model. Here, we will focus on angiogenesis, reviewing the background, vascular endothelial growth factor (VEGF)/VEGF receptor-pathway, effect, and concerns of this strategy in DMD.

Keywords: muscular dystrophy, regeneration, angiogenesis, VEGF, Flt-1, satellite cell, *mdx* mice, skeletal muscle

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common hereditary muscular dystrophy affecting approximately 1 in 5000 live male births (Mendell et al., 2012). It is caused by mutations in *dystrophin* gene located on Xp21 (Monaco et al., 1986), leading to progressive muscle weakness in which respiratory and cardiac failures are the main reasons of their early mortalities. Dystrophin is a protein which forms the dystrophin-associated glycoprotein complex (DGC) at the sarcolemma which links the muscle sarcomeric structure to the extracellular matrix (Davies and Nowak, 2006). When dystrophin is absent due to the gene mutation, muscle fibers become vulnerable to mechanical stretching (Pasternak et al., 1995). Currently there is no curative therapy for this disease and glucocorticoid is the only medication available that slows the decline in muscle strength and function in DMD (Bushby et al., 2010).

Since the identification of *dystrophin* in the mid 1980's (Monaco et al., 1986), several therapeutic approaches have been investigated. Gene replacement with virus vector, induction of protein expression by exon skipping or read through, compensation with dystrophin surrogates, and delivery of muscle stem cells or pluripotent stem cells have been investigated so far (Leung and Wagner, 2013; Rodino-Klapac et al., 2013). Recently, it was announced that phase 3 clinical trial for Drisapersen, an antisense oligonucleotide for exon skipping, could not meet the endpoint of statistically significant improvement (http://www.gsk.com/media.html). Although exon skipping could still be considered as one of the most promising therapeutic approaches available, there is a necessity for developing further therapeutic strategies. We have recently reviewed vasculature-related strategies for DMD, with a major focus on therapeutic methods to increase blood flow in existing blood vessels (Ennen et al., 2013). In the current review, we examine the evidence for reduced formation of blood vessels in DMD muscle and the therapeutic approach to augment angiogenesis by using vascular endothelial growth factor (VEGF)-based strategies. We provide an update of current evidence for changes in vasculature in DMD, approaches available to increase vasculature, and further discuss the pros and cons of the underlying rationale.

EVIDENCE FOR VASCULATURE CHANGES IN DMD

The necrotic fibers in DMD are often seen in groups, a simultaneous necrosis of contiguous muscle fibers, and it had been thought that this phenomenon was due to local reduction of blood supply by common capillaries in that group of necrotic fibers (Rando, 2001). The dystrophin deficiency in vascular smooth muscle (Miyatake et al., 1989) and absence of nitric oxide synthase (NOS) from the sarcolemma have indicated that DMD muscle is subjected to impaired blood flow (Brenman et al., 1995; Rando, 2001; Ennen et al., 2013).

More recent studies demonstrate that *mdx* mice muscle has decreased vascular density. Immunostaining of arterioles has revealed decreased vascular density in heart and gracilis muscles of *mdx* mice (Loufrani et al., 2004). Matsakas et al., have

visually shown that vasculature of the tibialis anterior (TA) in *mdx* mice is reduced compared to wild type by microfil-perfused whole mount imaging (Matsakas et al., 2013).

Another report shows that angiogenesis is impaired in *mdx* mice, not only in muscle, but systemically, which was proven by laser Doppler perfusion imaging in the hind limb ischemic model, VEGF induced neovascularization quantification in the corneal model, Matrigel subcutaneous angiogenic assay, and quantification of tumor growth and vascularization in the tumor implant model (Palladino et al., 2013).

Rhoads et al., have indicated satellite cells isolated from aging and *mdx* mice exhibit decreased expression of hypoxia-inducible factor 1α (HIF-1α) and VEGF and reduced capacity to promote angiogenesis *in vitro*, using a co-culture model of conditioned media from *mdx* mice or wild type satellite cells co-cultured with microvascular fragments (MVFs) (Rhoads et al., 2009, 2013). They also demonstrated that VEGF mRNA expression was decreased in proliferating satellite cells in dystrophic muscle. Furthermore, hypoxic conditions increase VEGF mRNA expression in satellite cells (Flann et al., 2013).

Taken together, there is a rationale to believe that DMD has significant defect in vasculature in terms of its quality, quantity, and angiogenesis, and that the muscles are under an ischemic condition.

It should be critically discussed whether the vascular change seen in DMD is a primary effect of the disease or not. Studies of blood perfusion in *mdx* mice indicate age or disease progression may have a major effect in vascular changes. A study with 2-month-old *mdx* mice showed increased blood flow compared to the wild type control in the hindlimb ischemia model, whereas older mice of 6 months showed decreased blood flow (Straino et al., 2004; Palladino et al., 2013). In wild type mice, it has been indicated that aged mice have a reduced response to angiogenesis after ischemia (Palladino et al., 2011, 2012). These data imply that the vascular changes seen in *mdx* mice could be the physiological response to aging and disease progression, rather than being the primary disease effect.

In one study of gene expression profiling of DMD patients' muscles, VEGF appears to be lower in DMD than the controls (Bakay et al., 2002), whereas in another study, a significant difference was not shown (Haslett et al., 2002). This inconsistency may be due to the data analysis and differences in experimental design (Haslett et al., 2002). It also has been reported that blood VEGF levels are significantly lower in DMD with mean age of 8.1 ± 1.9 years (Abdel-Salam et al., 2009). Others reported that VEGF levels in serum samples from DMD patients with mean age of 14.6 ± 0.8 years was elevated (Saito et al., 2009). Although the latter study was not compared to age-matched controls, these data imply that VEGF secretion fluctuates according to age or disease progression.

Further studies are required to elucidate the mechanism of vascular change in DMD, however, the studies designed to improve vasorelaxation capacity (Ennen et al., 2013) or to increase vasculature in order to improve tissue perfusion in DMD animal models have demonstrated the amelioration of dystrophic phenotypes (Asai et al., 2007; Verma et al., 2010; Kawahara et al., 2011). As far as we are aware, the approaches to increase vascular density have not been applied to humans yet, with animal model studies showing promising results for DMD therapy (**Table 1**).

DIFFERENT APPROACHES TO INCREASE VASCULAR DENSITY IN DMD

VEGF OVEREXPRESSION

VEGF-A (also known as VEGF) is crucial for blood vessel formation during early embryogenesis (Shibuya, 2013). It binds to VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) which are the membrane-spanning tyrosine kinase receptors and both have pro-angiogenic effects. The major pro-angiogenic effect is generated through Flk-1, on the other hand, Flt-1 can act negatively in angiogenesis. Although there is higher affinity to VEGF in Flt-1 compared to Flk-1 (Sawano et al., 1996), its kinase activity is lower than Flk-1. Besides full length Flt-1, there is a truncated soluble type (Kendall and Thomas, 1993) which lacks transmembrane and tyrosine kinase domains, and is considered to act as decoy receptor of VEGF.

Overexpression of VEGF via AAV gene transfer in muscles of *mdx* mice showed an increased number of capillaries/fibers (Messina et al., 2007). The treated *mdx* mice had increased forelimb strength, reduced necrotic fiber areas, and increased regenerative fiber areas. Increased capillary density was only seen in regenerating areas. Although this study also highlights the role of direct pro-regenerative effect of VEGF to skeletal muscle, they discuss the possible beneficial effects of VEGF-induced muscle neovascularization on dystrophic muscle as (1) promoting macrophage recruitment and removal of cellular debris; (2) increasing release and circulation of factors secreted by mononuclear cells and activating myogenic cells, and (3) increasing the recruitment of bone marrow derived mononuclear cells, which in turn release factors that activate the myogenic process.

VEGF RECEPTOR MODULATION

We recently reported that *mdx* mice crossed with heterozygous *Flt-1* gene knockout mice (*Flt-1*$^{+/-}$) showed increased vascular density and ameliorated phenotype compared to control *mdx*:*Flt-1*$^{+/+}$ mice (Verma et al., 2010; Ennen et al., 2013). Our data showed that when *Flt-1*$^{+/-}$ mice were compared with their wild-type (*Flt-1*$^{+/+}$) littermates, *Flt-1*$^{+/-}$ mice had a significantly increased number of endothelial cells (ECs) and increased tissue perfusion in TA muscle. When *Flt-1*$^{+/-}$ mice were crossed with *mdx* mice to create double mutant *mdx* mice with the heterozygous allele for *Flt-1*$^{+/-}$ (*mdx:Flt-1*$^{+/-}$) and compared with their littermates *mdx* mice with control Flt-1 (*mdx:Flt-1*$^{+/+}$) mice, *mdx:Flt-1*$^{+/-}$ mice exhibited a higher number of ECs, increased blood flow and improved muscle function. In these double mutant mice (*mdx:Flt-1*$^{+/-}$), muscle histology suggested decreased fiber turnover and increased fiber stability. Importantly, *mdx:Flt-1*$^{+/-}$ mice display increased number of satellite cells in the muscle compared to *mdx:Flt-1*$^{+/+}$ mice. Satellite cells are a muscle stem cell population in adult skeletal muscle and are essential for postnatal muscle growth and regeneration. As muscle ages or is afflicted by disease, muscle regeneration is impaired due to the decreased number and decreased differentiation capacity of satellite cells (Mounier et al., 2011). Therefore, it is possible that an increase in the vascular niche might promote muscle

Table 1 | Different approaches that could increase vascular density in DMD model animals.

Approach	Age of mice at treatment onset	Outcome	References
VEGF overexpression via AAV gene transfer in *mdx* mice	4 weeks	Increased capillary density in regenerating areas Reduced necrotic fiber areas Increased regenerative fiber areas Increased forelimb strength	Messina et al., 2007
VEGF overexpression via muscle-derived stem cell (MDSC) transplantation into *mdx/scid* mice	8–10 weeks	Increase in angiogenesis Increase in muscle regeneration Reduction in fibrosis	Deasy et al., 2009
Genetic modulation of VEGF receptor (Flt-1) level in *mdx* and *mdx/utrn*$^{-/-}$ mice	2–3 months	Increased vascular density Decreased muscle membrane permeability Less area of fibrosis and calcification Decreased centrally located nuclei Increased tissue perfusion Improved maximum isometric force and whole-body tension analysis	Verma et al., 2010
Overexpression of estrogen-related receptor-γ (ERRγ)	6–8 weeks	Enhanced vasculature and blood flow Increased number of oxidative myofibers Improved exercise tolerance	Matsakas et al., 2013
Mesoangioblast transplantation into the heart of *mdx/utrn*$^{-/-}$ mice	4–6 weeks	Prevented onset of cardiomyopathy Increased capillary in the heart	Chun et al., 2013
Treatment with aspirin	4 weeks (treatment continued for 7 months)	Increased vascular density Decreased muscle membrane permeability Less area of fibrosis Increased numbers of regenerating fibers Increased tissue perfusion Improved resistance to physical exercise	Palladino et al., 2013

regeneration via stimulation of satellite cell proliferation or survival. These data strongly suggest that *Flt-1* haploinsufficiency ameliorates muscular dystrophy phenotype by developmentally increased vasculature in *mdx* mice, further implying the possibility of VEGF receptor modulation as a therapeutic strategy (**Figure 1**).

OTHER APPROACHES THAT CAN INCREASE VASCULAR DENSITY IN MUSCLE

Estrogen-related receptor-γ (ERRγ) is known to be highly expressed in skeletal muscles, and it has been demonstrated that ERRγ can induce angiogenic factors including VEGF to increase angiogenesis in muscle (Narkar et al., 2011). Matsakas et al., reported that ERRγ expression and downstream metabolic and angiogenic target genes are down-regulated in the skeletal muscles of *mdx* mice (Matsakas et al., 2013). In this study, overexpression of ERRγ selectively in the *mdx* mice skeletal muscle could enhance vasculature, blood flow, and oxidative myofibers, and improve exercise tolerance.

Palladino et al., hypothesized that aspirin has beneficial effects on the angiogenic properties of ECs in dystrophic mice, due to its ability to enhance NO release from vascular ECs and protective effect on ECs via the NO cGMP pathway (Palladino et al., 2013). Treatment with aspirin could enhance production of NO and cGMP, and long-term low dose aspirin could increase capillary density, improve resistance to physical exercise, and muscle fiber permeability.

It is known that exercise training promotes many adaptations in skeletal muscle, including enhanced angiogenesis (Andersen and Henriksson, 1977; Gavin et al., 2004). Although excessive exercise may exacerbate the DMD phenotype, these studies imply that an adequate amount of exercise may be beneficial to DMD.

CONCERNS REGARDING VEGF ADMINISTRATION AND ITS RECEPTOR MODULATION

Upon targeting the VEGF/VEGF receptor pathway for therapy, the greatest concern would be whether the newly generated vasculature was morphologically and functionally sound *in vivo*. Although VEGF is a well-known factor for angiogenesis, it was first described as vascular permeability factor, and these vascular permeability-producing effects of VEGF

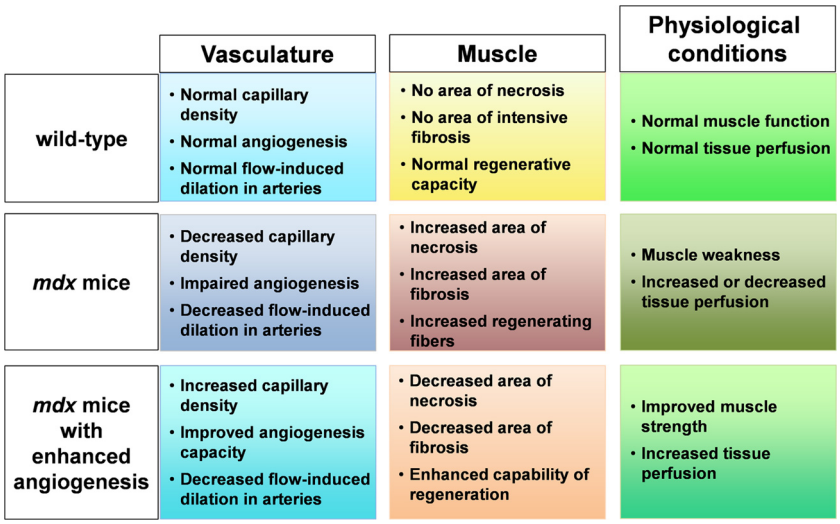

FIGURE 1 | Schematic figure of relationship between muscle, capillary, and physiological condition. In *mdx* mice, capillary density is decreased, angiogenesis is impaired with decreased flow induced dilation in arteries. When angiogenesis is enhanced in *mdx* mice, increased capillary density and improved angiogenesis capacity leads to decreased area of necrosis and fibrosis and increased regenerative capacity in muscle accompanied with increased tissue perfusion and improved muscle weakness.

may have profound negative consequences in ischemic disease by augmenting the level of infarcted tissue (Senger et al., 1983; Weis and Cheresh, 2005). An *in vivo* imaging study performed in rat skeletal muscle with prolonged expression of VEGF via AAV vector revealed impaired perfusion of the tissue (Zacchigna et al., 2007). Despite the histological evidence of neoangiogenesis, resting muscle blood flow measured by positron emission tomography did not improve, and moreover, post-exercise muscle blood flow measurements showed decreased perfusion. This phenomenon was explained by the formation of leaky vascular lacunae which accounted for the occurrence of arteriovenous shunts that excluded the downstream microcirculation.

Although the precise role of *Flt-1* in postnatal and adult tissue angiogenesis still remains ambiguous, a recent study demonstrated Cre-loxP-mediated conditional knockout mice with ablated *Flt-1* expression in neonatal and adult periods displaying increased angiogenesis in various tissues including cornea, lung, heart, brain, kidney, and liver (Ho et al., 2012). Moreover, the vasculature seen in the conditional *Flt-1* knockout mice matured and perfused properly. These findings are especially encouraging for developing therapeutic strategies targeting VEGF/Flt-1 interaction.

COMBINATION THERAPY OF DELIVERING AN ANGIOGENESIS AND MYOGENESIS FACTOR

Borselli et al. reported that a combination of VEGF to promote angiogenesis and insulin-like growth factor-1 to directly promote muscle regeneration could induce functional recovery in an ischemic injured skeletal muscle in a more prominent manner than VEGF alone (Borselli et al., 2010). It is also noteworthy that in their study, sustained delivery of the factors via injectable gel was effective, whereas bolus delivery did not show any benefit in terms of angiogenesis, regeneration, and muscle perfusion. These data imply alternative strategies may be used to obtain more benefits of angiogenesis therapy in DMD.

THE SIGNIFICANCE OF VEGF/VEGFR PATHWAY IN DMD

DIRECT EFFECT OF VEGF ON SKELETAL MUSCLE

Other than the pro-angiogenic effect, a pro-myogenic effect of VEGF has been reported in skeletal muscle; however, the effect of VEGF on muscle cell function is still unclear. The presence of VEGF receptors has been investigated by several groups. *In vitro* experiments using C2C12 cells and primary mouse myoblasts showed that both Flt-1 and Flk-1 were detected in western blotting analysis and RT-PCR (Germani et al., 2003; Arsic et al., 2004), and their expression levels were modulated during the course of differentiation. With normal muscle tissue, immunohistochemistry data indicated Flt-1 and Flk-1 expression levels in muscle fibers are either very low or null (Arsic et al., 2004; Wagatsuma et al., 2006; Messina et al., 2007), but once the muscle was subjected to experimental injury, both receptors became detectable in satellite cells and regenerating muscle fibers (Arsic et al., 2004; Wagatsuma et al., 2006).

When VEGF was administered to C2C12 cells, cell migration and survival were enhanced (Germani et al., 2003). Others have shown an increased number of myotubes expressing myosin heavy chain (MHC), a myogenic differentiation marker, in differentiated C2C12 cell cultures supplemented with VEGF (Arsic et al., 2004). These MHC-positive fibers contained more multinucleated myofibers while non-VEGF-treated cells consisted mainly of mononuclear myocytes. In addition to these findings, VEGF-treated cells had significantly longer mono- or multinucleated MHC-positive cells. Also, when C2C12 cells were treated with an apoptosis-triggering agent, VEGF could decrease

the fraction of cells expressing markers of necrosis. It also has been reported that when human myogenic precursor cells were incubated with VEGF, there was an increase in cell density (Christov et al., 2007).

Taken together, it is indicated that VEGF may have a direct proliferative effect on muscle in addition to promotion of myogenic fiber growth and an anti-apoptotic effect.

THE PRO-ANGIOGENIC AND PRO-MYOGENIC EFFECTS OF THE VEGF/VEGFR PATHWAY PLAY AN IMPORTANT ROLE IN THE REGENERATIVE PROCESS IN MUSCLE

Recent studies highlight the importance of angiogenic and regenerative effects of VEGF in muscle regeneration (Beckman et al., 2013; Bobadilla et al., 2013). Mechanical stimulation (MS) can increase VEGF secretion (Payne et al., 2007; Cassino et al., 2012), and MS increases the effectiveness of tissue repair in muscle-derived stem cell (MDSC) transplantation experiments in *mdx/scid* mice (Beckman et al., 2013). Inhibition of VEGF with soluble Flt-1 (sFlt-1) or short hairpin RNA (shRNA) in mechanically stimulated MDSCs (MS-MDSCs) resulted in reduction of cells' differentiation and angiogenic capacity within the transplantation area, both of which are increased in MS-MDSC transplantation cases (Beckman et al., 2013).

Bobadilla et al., indicated metalloproteinase-10 (MMP-10) has a role in muscle regeneration in injured or dystrophic muscle through VEGF/Akt signaling (Bobadilla et al., 2013). MMP-10 protein levels were upregulated in *mdx* mice, and ablation of MMP-10 in *mdx* mice deteriorated the dystrophic phenotype. Although it was not significant, MMP-10 knockout mice (MMP-10 KO) crossed with *mdx* mice had fewer arterioles in the muscle, and MMP-10 KO had significantly lower number of arterioles than the wild type control. Various MMPs induce VEGF secretion, and in their study, MMP-10 mRNA silencing in injured wild type muscle, a decrease in VEGF protein level was observed, while treatment with recombinant human MMP-10 showed elevated VEGF (Mott and Werb, 2004; Bobadilla et al., 2013). Collectively, these data suggest that pro-myogenic and pro-angiogenic effects of VEGF/VEGFR play an important role in the muscle regeneration process in DMD.

ENDOTHELIAL CELL AND SATELLITE CELL RELATIONSHIP

ECs and factors secreted by them are able to induce satellite cell proliferation and survival. Christov et al., reported that in adult normal muscle, satellite cells are located preferentially close to capillaries, and reciprocally interact with ECs to support the angio-myogenesis relationship (Christov et al., 2007). Their study further indicated that there is a correlation between capillary and satellite cell numbers. In muscle specimens from patients with amyopathic dermatomyositis (aDM), an inflammatory disease, capillary density in the muscle is decreased but is spared from both myofiber damage and inflammation. Interestingly, a proportionate decrease in the mean satellite cell number per myofiber was also observed. Conversely, athletes' muscles, which have workload-induced increased capillary density, showed increased numbers of both capillaries and satellite cells per myofiber. Indirect coculture using chambers with human umbilical vascular ECs (HUVECS) or human microvascular ECs (HMECS) cultured in the upper chamber, and separated with porous filter, human myogenic precursor cells cultured in the lower chamber, revealed that an endothelial cell monolayer could increase myogenic cell growth through soluble factors. EC derived IGF-1, HGF, bFGF, PDGF-BB, and VEGF were indicated as effectors for myogenic cell growth promotion. These data suggest that satellite cell and muscle regeneration are under influence of vasculature and that they receive supportive cues from ECs to expand and further commit and differentiate (Mounier et al., 2011).

VASCULAR NETWORK IN DMD CARDIAC MUSCLE AND POSSIBLE EFFECT OF ANGIOGENESIS

In *mdx* mice, it has been reported that there is reduced vasculature in cardiac tissue compared to the wild-type (Loufrani et al., 2004). A recent study of postnatal *Flt-1* gene ablation, in combination with left anterior descending artery ligation to create ischemic cardiomyopathy, showed reduced infarction size and increased capillary density after ligation (Ho et al., 2012). Chun et al., could prevent the onset of cardiomyopathy by transplanting mesoangioblast stem cells into the heart of *mdx/utrn*$^{-/-}$ mice. They reported increased number of capillaries in the treated hearts, indicating a possible positive effect on angiogenesis, a well-known indirect effect of stem cell therapy (Chun et al., 2013). These data imply that angiogenesis may be beneficial also for DMD cardiomyopathy.

CONCLUSIONS

Mounting evidence for DMD vascular abnormality indicates that vascular therapy is a logical approach. Currently, the most frequently used method to increase vasculature is by modulating VEGF/VEGFR pathways. DMD muscle may benefit from angiogenesis in multidimensional aspects. Increasing capillary density simply resolves ischemia. Direct regenerative and anti-apoptotic effects of VEGF can be expected. Increased vascular niches that house satellite cells imply enhanced proliferation of satellite cells under the influence of supportive cues from vasculature.

The concern would be whether therapeutically generated vasculature is morphologically and functionally beneficial. Although this issue must be investigated before treating human DMD, increasing data currently provide strong support that angiogenesis is a promising therapeutic strategy for DMD.

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3D hydrogel environment rejuvenates aged pericytes for skeletal muscle tissue engineering

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Skeletal muscle tissue engineering is a promising approach for the treatment of muscular disorders. However, the complex organization of muscle, combined with the difficulty in finding an appropriate source of regenerative cells and in providing an adequate blood supply to the engineered tissue, makes this a hard task to face. In the present work, we describe an innovative approach to rejuvenate adult skeletal muscle-derived pericytes (MP) based on the use of a PEG-based hydrogel scaffold. MP were isolated from young (piglet) and adult (boar) pigs to assess whether aging affects tissue regeneration efficiency. *In vitro*, MP from boars had similar morphology and colony forming capacity to piglet MP, but an impaired ability to form myotubes and capillary-like structures. However, the use of a PEG-based hydrogel to support adult MP significantly improved their myogenic differentiation and angiogenic potentials *in vitro* and *in vivo*. Thus, PEG-based hydrogel scaffolds may provide a progenitor cell "niche" that promotes skeletal muscle regeneration and blood vessel growth, and together with pericytes may be developed for use in regenerative applications.

Keywords: stem cells, perycite, skeletal muscle, myogenic differentiation, tissue engineering, PEG-firbinogen, biomaterials

INTRODUCTION

Adult skeletal muscle tissue has a remarkable capability to regenerate after injury, although this property is related to damage entity. Muscle harm evokes the activation of different mononucleated cell populations that, in response to myofiber degeneration, proliferate and differentiate into myocytes, enabling muscle regeneration (Carlson, 1973). The main actors of this process are muscle satellite cells, quiescent resident stem cells located between the sarcolemma and the basal lamina. This specific microenvironment, often referred to as a "cellular niche," is instrumental in keeping the satellite cell in a quiescent state, but promotes activation of the muscle progenitor cells after injury. In addition, many other cell types, either residing in different muscle compartments or attracted from the circulation following injury, contribute to the regeneration process (Bentzinger et al., 2013).

Similarly to the niches of specialized cells that have been described in skeletal muscle and bone marrow, the wall of skeletal muscle vessels is believed to possess a niche of regenerative mesenchymal cells, namely pericytes (Birbrair et al., 2013). Pericytes play a crucial role in the control of angiogenesis, both in the early stages and in the stabilization of the nascent structure. This effect is exerted via direct and paracrine factor-mediated interaction with the endothelium. In particular, crucial biological functions of endothelial cells (EC) such as migration, proliferation, permeability, and contractility, are affected by pericytes (Armulik et al., 2005; Ribatti and Crivellato, 2011; Dulmovits and Herman, 2012). Pericytes reside in anatomical proximity to ECs, sharing the basement membrane, making specialized junctions, and surrounding them at the tip of capillaries, ready to guide the vasculogenic process. The EC:pericyte ratio varies throughout the body, ranging from 1:1 in neural tissues to 10:1 in skeletal muscles (Armulik et al., 2011). In response to pro-angiogenic conditions, pericytes contribute to the mobilization of ECs, promoting the formation and stabilization of the nascent vessel until the accomplishment of the process preventing microvascular regression. Their ability to interact with ECs and to regulate angiogenesis as well as their documented capacity of multilineage mesenchymal differentiation render pericytes suitable for investigations in the field of tissue regeneration and promotion of angiogenesis in ischemic diseases and in wound healing

(Abou-Khalil et al., 2010). In this respect, pericytes resident in the adventitia of vessels, such as the saphenous vein, have been shown by our group to represent a potent class of pro-angiogenic cells *in vitro* and *in vivo* in experimental models of ischemia (Campagnolo et al., 2010; Katare et al., 2011).

We address here the potential of pericytes resident in adult skeletal muscle to support muscle differentiation and angiogenesis *in vitro* and *in vivo*. The underlying hypothesis is that the bi-directional differentiation potential of skeletal muscle-derived pericytes (MP)—i.e., for vasculogenesis and myogenesis—may represent a plus in the development of new regenerative approaches for ischemic or wounded tissues. Although their contribution to the *in vivo* muscle repair process seems to be marginal when compared with that of satellite cells, their noteworthy myogenic capability makes them attractive as alternative source of myogenic progenitors for stem cell-mediated muscle regeneration approaches (Sirabella et al., 2013). Indeed, skeletal muscle tissue is unable to duly repair in response to a severe injury, so reconstructive strategies, such as autologous muscle transplantation or intra-muscular injection of progenitor cells, are needed to recover the damaged area. Moreover, ageing negatively affects muscle regeneration and myogenic stem cell potential, resulting in muscular tissue pauperization or sarcopenia (Carosio et al., 2011; García-Prat et al., 2013). In particular, several studies have demonstrated that aged muscle satellite cells present functional alterations, such as a decreased capacity to form colonies, when cultured *in vitro*, and an impaired capability to activate and proliferate in response to damage (Conboy et al., 2003; Baj et al., 2005; Shefer et al., 2006; Day et al., 2010; Shadrach and Wagers, 2011). Aging also significantly affects the capacity of muscle satellite cells to differentiate, to fuse into myotubes and to replenish reserve pools in culture (Carlson and Faulkner, 1996; Grounds, 1998; Shavlakadze et al., 2010).

Tissue engineering is a novel discipline aiming to mimic organogenesis. In principle, one could consider generating new skeletal muscle tissue *in vitro* and implanting it *in vivo* to replace severely injured muscle. The skeletal muscle engineering approach contemplates two main components: stem cells and a biomaterial, ensuring a regenerative capacity and tissue scaffolding, respectively. In order to guarantee a suitable *in vivo* support for new tissue generation, the biomaterial must have specific characteristics: it must be inert, with minimal cytotoxicity, resorbable by cell-mediated biodegradation processes and modifiable, its stiffness included (Rossi et al., 2010; Fuoco et al., 2012; Rizzi et al., 2012; Seliktar, 2012). Extracellular matrix (ECM) constituents have been used as a model and as components for scaffold materials, guaranteeing skeletal muscle microenvironment reconstitution and muscular regeneration amelioration. Several natural or synthetic polymers have also been studied as biomaterials in rats and dogs for striated muscle regeneration, mainly supporting cardiac repair; nevertheless, just a few have been tested in human clinical experimentation (Zammaretti and Jaconi, 2004; Habib et al., 2011).

Here, we report on the effects of polyethylene glycol (PEG)–fibrinogen (PF) on the skeletal muscle regeneration capacity of pericytes. We found that seeding onto a PF scaffold recuperated the reduced myogenic/vasculogenic ability of aged pericytes *in vitro*, allowing these cells to generate a vascularized muscle *in vivo* that was indistinguishable from that formed with the use of younger cells.

METHODS

MUSCLE PERICYTE ISOLATION

Muscle biopsies from piglet and boar (Large White X Landrace) were kindly provided by Dr. La Torre of the animal house facility of Tecnopolo Castel Romano, Italy. Skeletal muscle biopsies (approximately 2 cm^3) were sterilely isolated from porcine quadriceps, soaked in PBS and processed within 24 h. Muscular tissue was finely minced with tweezers until no intact muscle pieces could be distinguished. The minced tissue was digested for 45 min with collagenase II (0.1 mg/ml, GIBCO) at 37°C. After filtration through a 70 μm strainer, cells were washed in PBS and plated in DMEM GlutaMAX (GIBCO) supplemented with heat-inactivated 20% fetal bovine serum (FBS), 100 iu/ml penicillin and 100 mg/ml streptomycin on plastic dishes. We selected pericytes by their capacity to grow in this relatively poor condition at low cell confluence (0.1–1 × 10^4 cell/cm^2), as opposed to mesoangioblasts (Tonlorenzi et al., 2007). The first fibroblast colony-forming units (CFU-F) formed 7–10 days after seeding (Sacchetti et al., 2007), after which pericyte colonies were expanded and tested for their myogenic and angiogenic potential. For myogenic differentiation analysis, swine muscle-derived pericytes (MP) were cultured on plastic dishes as above cited for 24–48 h, until 80% confluent, then medium was replaced with DMEM supplemented with 5% horse serum (differentiation medium) to promote muscle differentiation.

IMMUNOFLUORESCENCE

Cells and tissue were fixed in 2% PFA and processed for histology and immunocytochemistry as previously described (Gargioli et al., 2008). Briefly, MP were cultured in fibronectin-coated 8-well chamber slides (Nunc) as described above. After fixation with 2% PFA for 10 min, cells were incubated overnight at 4°C with the following primary antibodies: anti-α-smooth muscle actin (SMA; Dako), platelet-derived growth factor receptor beta (PDGFR-β; Cell Signaling Technology), chondroitin sulfate proteoglycan (NG2; Merk Millipore) and myosin heavy chain (MyHC; DHSB), diluted in accordance with the manufacturers' instructions. Cells were then incubated with the appropriate secondary fluorophore-conjugated antibody. Nuclei were stained with DAPI. Fluorescence photomicrographs were taken with an Axio Observer A1 microscope equipped with a fluorescence detection system at 20 × magnification. Imaging analysis was performed with AxioVision Imaging System software (Zeiss).

IMMUNOBLOTTING

Tissue samples were pulverized in liquid nitrogen and then immediately homogenized in RIPA buffer (20 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.1% SDS, 1% NP40, 1% NaDOC, and Roche protease inhibitor cocktail). Homogenates were centrifuged at 12,000 g for 10 min at 4°C to discard nuclei and cellular debris. Protein concentration was determined with bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin (BSA) as the standard. Total homogenates were separated by sodium

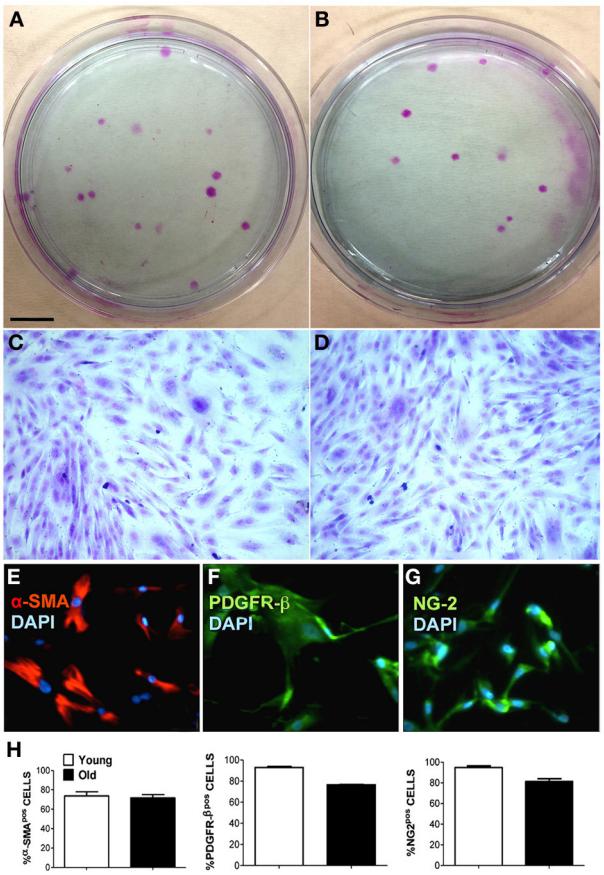

FIGURE 1 | Characterization of muscle-derived pericytes (MP) from swine. MP were analyzed for their behavior and their expression of characteristic pericyte markers to define their cellular identity. **(A,B)** Young **(A)** and old **(B)** swine-derived MP showing fibroblast colony-forming units (CFU-F) when seeded at low confluence. Giemsa staining on plastic 9 cm Petri dishes. **(C,D)** Single CFU-F morphology of young **(C)** and adult **(D)** MP, presenting identical spindle-like morphology. **(E–G)** Representative immunofluorescence staining of young MP showing positivity (pos) for alpha-smooth muscle actin (α-SMA) **(E)**, chondroitin sulfate proteoglycan (NG-2) **(G)**, and platelet-derived growth factor receptor beta (PDGFR-β) **(F)**. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI). **(H)** Average data scored from immunofluorescence in **(E–G)**. Scale bar: **(A,B)** 1 cm; **(C–G)** 15 μm.

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a concentration opportunely chosen on the base of molecular weight of the proteins analyzed. For Western blot analysis, proteins were transferred onto Immobilon membranes (Amersham), saturated with 5% non-fat dry milk (Biorad) in 0.1% Tween-20 (Sigma) PBS (blocking solution) and hybridized with 1:5 MF20 mouse monoclonal antibody against MyHC (DHSB) or with 1:5000 anti-GAPDH (clone 71.1, Sigma) for 1 h at RT. The filters were washed three times (15 min each at RT) with wash solution (0.1% Tween-20 in PBS) and then reacted with anti-mouse, anti-rat, or anti-rabbit secondary antibody conjugated with 1:3000 horseradish peroxidase IgG (Biorad) for 1 h at RT, washed three times and finally visualized with ECL (Amersham). Optical density (OD) was calculated with ImageJ software.

TUBE FORMING ASSAY

Human umbilical vein endothelial cells (HUVEC) and MP were seeded in 8-well Permanox chamberslides (Nunc) coated with 150 μl Matrigel (3D; BD Biosciences), either alone (3.75×10^4 cells/well) or as a 1:4 MP-to-HUVEC ratio co-culture, in medium supplemented with 0.1% BSA. Cells were incubated for at least 5 h post-seeding to allow tube formation. Images of the networks were taken with a 5× objective (Axio Observer A1, Zeiss, Germany). Images of branches at the nodes of a ramification of formed tubes were taken with a 20× objective. All experiments were conducted in duplicates.

CREATION OF PEG-FIBRINOGEN CONSTRUCTS

The PEG-fibrinogen precursor solution was prepared and photopolymerized as described elsewhere (Fuoco et al., 2012). We prepared PEG hydrogels containing MP by mixing a PBS cell suspension with PEGylated fibrinogen precursor solution containing 0.1% Igracure 2959 photoinitiator (Ciba Specialty Chemicals) in order to have a final concentration of 8 mg/ml. We added 100 μl of the suspension into cylindrical silicon molds and placed them under a long-wave UV lamp (365 nm, 4–5 mW/cm^2) for 5 min in a laminar flow hood. DMEM culture medium (containing 20% FBS) was added immediately to the polymerized hydrogels to ensure cell growth. The plugs were cultured for 24 h in serum-supplemented growth medium and then transferred into serum-depleted differentiation medium for 5 days in order to promote muscle fiber formation. For *in vivo* experiments, molds were immediately implanted subcutaneously in the backs of mice.

IMPLANTATION OF CONSTRUCTS

Two-month-old male RAG2/γChain immunocompromised mice were anesthetized with an intramuscular injection of physiologic saline (10 ml/kg) containing ketamine (5 mg/ml) and xylazine (1 mg/ml) and then PF constructs containing 1.5×10^6 swine-derived MP implanted subcutaneously in their backs (one side received a construct seeded with piglet MP, and the other side a construct seeded with boar MP). In order to ensure good placement of the constructs, we performed a limited incision on the medial side of the back, separated the dorsal muscle from the skin, placed the plug as desired and then sutured the wound. Mice were sacrificed 30 days after implantation for molecular and morphological analysis. Experiments on mice were conducted according to the rules of good animal experimentation I.A.C.U.C. n° 432, March 12 2006.

STATISTICAL ANALYSIS

Continuous variables are expressed as means ± standard error (SEM). Statistical significance was tested using GraphPad Prism 5 software and applying Student's *t*-test or One-Way ANOVA followed by Bonferroni multiple comparison post-test or Kruskal–Wallis test if data had a skewed distribution.

RESULTS

AGING DOES NOT IMPACT ON MORPHOLOGY, CFU CAPACITY OR MARKER EXPRESSION OF SWINE MUSCLE-DERIVED PERICYTES

MP were isolated and cultured as described in the Methods section. Freshly isolated MP were cultured in plastic Petri dishes at

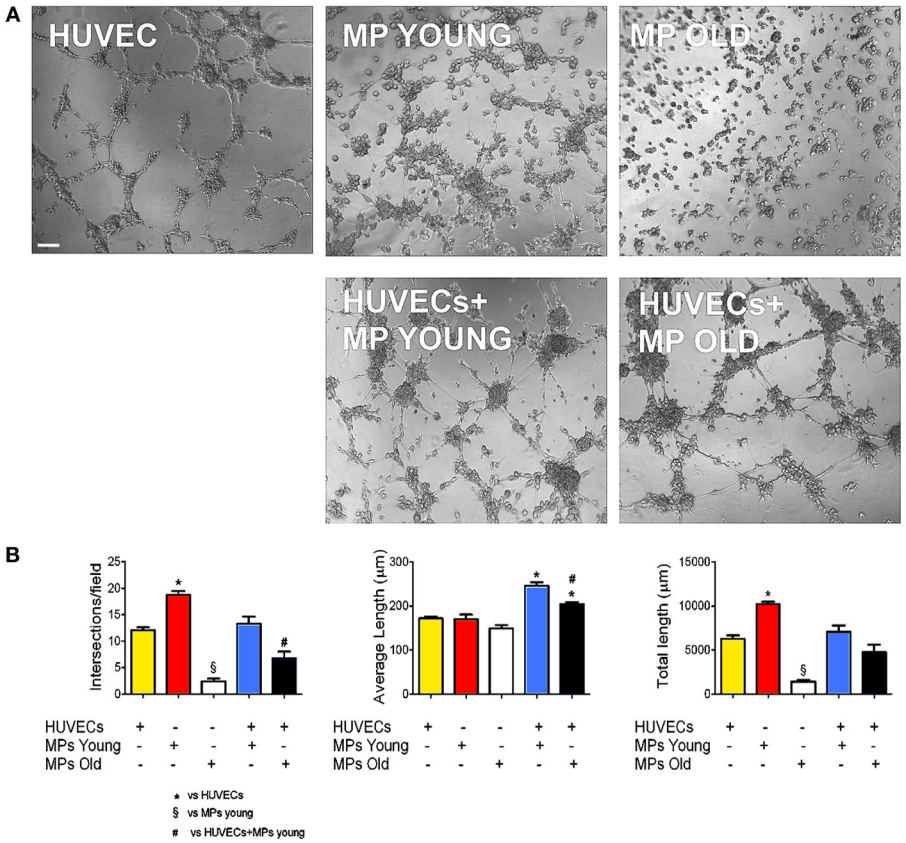

FIGURE 2 | Ability of MP to form capillary-like structures *in vitro*. Endothelial cell networking *in vitro* on Matrigel. **(A)** Phase-contrast microphotographs showing human umbilical vascular endothelial cell (HUVEC) and muscle-derived pericyte (MP) capacity to form networks on Matrigel, alone and in co-culture (1:4 MP:HUVEC ratio). **(B)** The efficiency of tube formation was quantified as number of intersections, average, and total tube length. Values are expressed as mean ±s.e.m. of $n = 3$ donor/group assayed in duplicate. $^{*}p < 0.05$ vs. HUVECs alone; $^{\S}p < 0.05$ vs. young MP; $^{\#}p < 0.05$ vs. HUVECs + young MP. Scale bar: 100 μm.

a low density ($0.1–1 \times 10^4$ cell/cm^2) in order to evaluate CFU-F capacity and for immunofluorescence staining using specific pericyte antigenic markers. We found that piglet- and boar-derived MP had a similar CFU-F capacity and cell shape (**Figures 1A–D**). Isolated swine MP were positive for typical pericyte markers, such as SMA, PDGFR-β and NG2 (**Figures 1E–G**), and there was no difference in the percentage of positive cells from piglets and boars (**Figure 1H**). These findings suggested that age does not affect MP morphology or CFU-F capability.

ADULT MUSCLE-DERIVED PERICYTES HAVE IMPAIRED ANGIOGENIC AND MYOGENIC DIFFERENTIATION CAPACITIES *IN VITRO*

Adequate blood supply is indispensable to guarantee cell survival and engraftment in skeletal muscle tissue engineering applications. Because pericytes are active players in capillary sprouting and vessel stabilization, we assessed the *in vitro* capacity of MP to form networking structures and to cooperate with endothelial cells (HUVEC) to form capillary-like structures (**Figure 2A**). We found that piglet MP had an enhanced ability to form capillary-like structures on Matrigel compared with HUVEC (**Figure 2B**). In boar MP, this ability was severely hampered and was not recuperated by co-culture with HUVEC. The estimates of the average number of intersections and length of capillary-like structures consistently pointed to a negative impact of age on the ability of porcine MP to support vessel formation. MP can differentiate into myoblasts that fuse into multinucleated fibers (Cappellari and Cossu, 2013). Together with the formation of capillary like-structures, this property is crucial for the reparative function of MP. We therefore tested whether aging had an effect on this ability too. High confluency and serum depletion were used to promote myogenic differentiation in MP (Péault et al., 2007). Piglet-derived MP differentiated spontaneously into multinucleated myotubes when they became confluent (**Figures 3A,B**), an ability that was markedly greater than in adult MP (**Figures 3C,D**). In fact, we found that the number of MyHC-positive fibers formed by boar MP was significantly lower (**Figure 3E**). However, this was not accompanied by a decreased fusion index (i.e., nuclei/fiber), suggesting that a lower percentage of adult MP retained an efficient myogenic ability.

PEG-FIBRINOGEN IMPROVES THE MYOGENIC CAPABILITY OF ADULT MP

PF is a relatively novel material that transitions into a gel under a UV trigger (Almany and Seliktar, 2005). It was recently

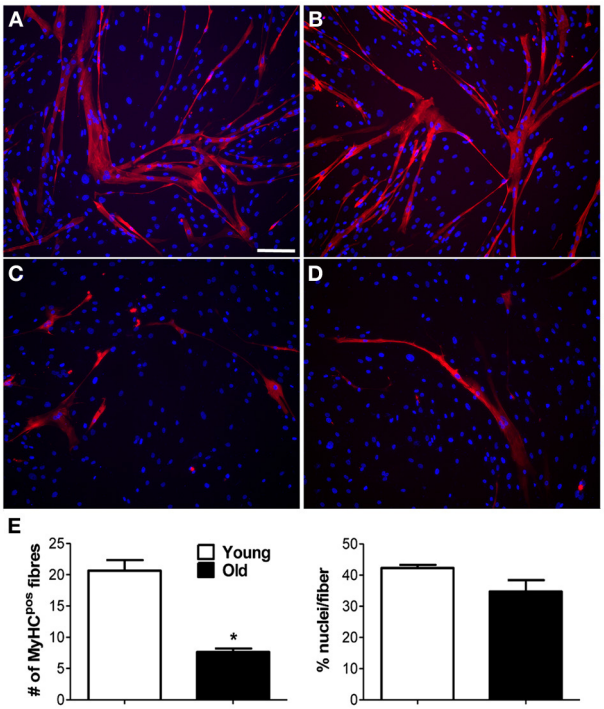

FIGURE 3 | Myogenic capability of MP *in vitro*. Myogenic ability was assessed for muscle-derived pericytes (MP). **(A–D)** Immunofluorescence staining with anti-myosin heavy chain (MyHC) antibody. Representative images showing MyHC-positive (pos) myotubes (red) formed from young **(A,B)** and old **(C,D)** MP, demonstrating that age affects MP muscle differentiation capacity. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). **(E)** Average data extracted from **(A–D)** immunolabeling. The number of positive fibers was quantified with respect to the total number of nuclei. The efficiency of cell fusion into syncytial myofibers was measured as percentage of nuclei per fiber. The analysis was done on five pictures from three donor/group. Values are expressed as mean ±s.e.m. $*p < 0.05$ vs. young MP. Scale bar: **(A–D)** 50 μm.

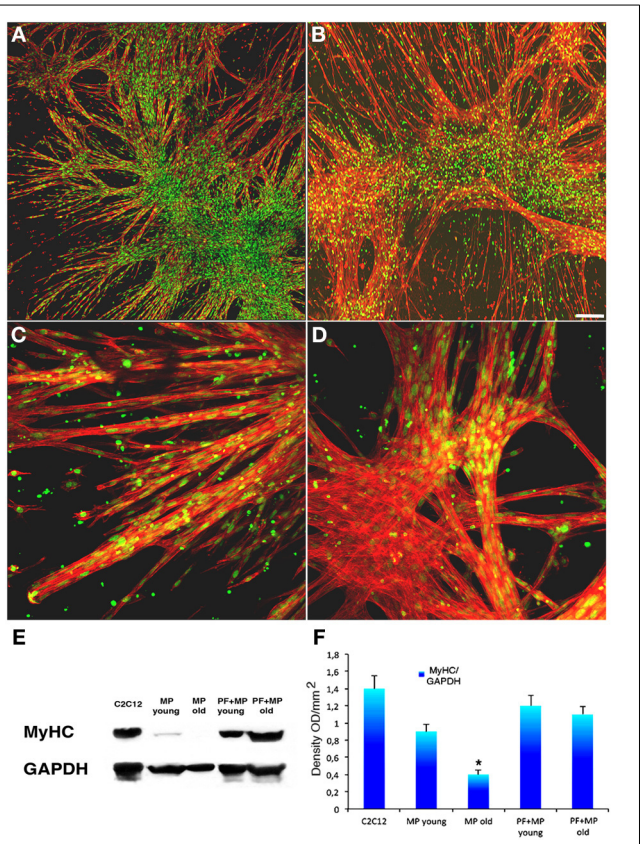

FIGURE 4 | PEG-fibrinogen re-establishes the myogenic capability of adult MP. (A,B) Confocal images of immunofluorescence labeling with an antibody against MyHC (red) and SYTOX Green for nuclei (green) on myotubes developed from young **(A)** and adult **(B)** pericytes cultured on a PEG-fibrinogen scaffold (5 days). **(C,D)** Enlarged view respectively of young **(C)** and adult **(D)** pericytes cultured on a PEG-fibrinogen showing multinucleated muscle fibers. **(E)** Representative Western blot of protein extracts from differentiated C2C12 cells as control and young and adult muscle-derived pericytes (MP) cultured on plastic or encapsulated in a PEG-fibrinogen scaffold (PF+), showing myosin heavy chain (MyHC) expression. **(F)** Densitometric analysis of three different Western blots quantifying the level of expression of MyHC. mean ±s.e.m., normalized to GADPH. $*p < 0.05$ vs. Young MP. Scale bar: **(A,B)** 50 μm; **(C,D)** 10 μm.

demonstrated that PF has a remarkable influence on myogenic differentiation of progenitor muscle cells by providing a three-dimensional microenvironment suitable for myofiber development (Fuoco et al., 2012; Rizzi et al., 2012). We therefore assessed whether PF could improve the poor myogenic capability of adult MP. To this end, cylindrically shaped silicon molds were filled with 100 μl PF seeded with 5×10^5 MP from piglets or boars, and then exposed to a UV source, as described in the Methods section. The scaffolded MP were cultured for 24 h in serum-supplemented growth medium and then transferred into serum-depleted differentiation medium for 5 days to promote muscle differentiation and myotube formation. Immunofluorescence for MyHC indicated that piglet-derived MP encapsulated in PF had an enhanced myogenic activity, whereas adult MP recovered their myogenic capability almost completely, becoming comparable to that of piglet MP (**Figures 4A–D**). Western blotting analysis supported the immunofluorescence findings (**Figures 4E,F**). To assess whether porcine MP could be used as a source of myogenic precursor cells for skeletal muscle tissue engineering, MP–PF constructs were implanted subcutaneously into the backs of immunocompromised mice immediately after the polymerization step, and explanted 30 days later. We found that the PF cylinders seeded with either young or adult MP formed ectopic tissue (**Figure 5A**) that contained vessel-like structures (**Figure 5B**). Histological analysis revealed the presence of a tissue with a muscle-like organization (**Figures 5C–H**) containing MyHC-positive fibers (**Figures 5E,F**) and blood vessels, probably generated by the recruitment of host EC and/or by host vessels branching into the ectopic tissue (**Figures 5G,H**).

DISCUSSION

Over the past few years, we have witnessed a remarkable progress in our understanding of ageing on tissue deterioration and stem cell functionality (Carosio et al., 2011; García-Prat et al., 2013). It is now evident that the stem cell microenvironment—the cellular niche—plays a fundamental role in muscle progenitor cell

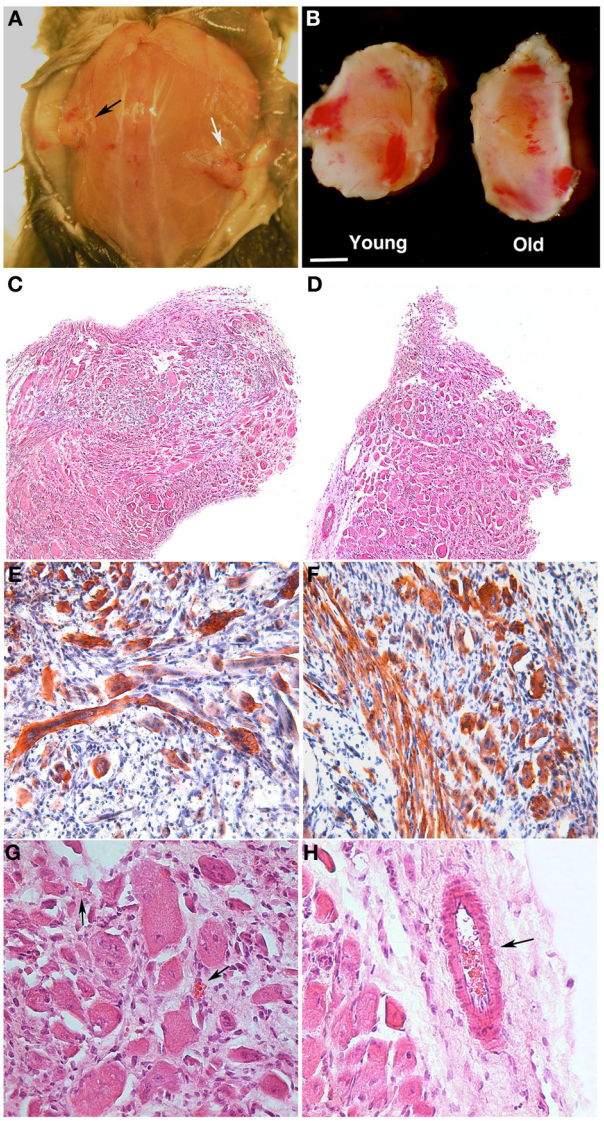

FIGURE 5 | PEG-fibrinogen-pericyte constructs form ectopic muscle *in vivo*. Muscle-derived pericytes (MP) seeded onto PEG-fibrinogen scaffolds (MP + PF) were implanted subcutaneously in immunocompromised mice and extracted 30 days after grafting ($n = 6$). **(A,B)** Macroscopic comparison of subcutaneous MP + PF implants loaded with 5×10^5 young MP (black arrow) and 5×10^5 adult MP (white arrow) **(A)**, revealing an ectopic, vascularized tissue-like formation **(B)**. **(C,D)** Hematoxylin/eosin stained sections of ectopic tissue revealing similar muscular-like structures generated from young **(C)** and adult **(D)** MP. **(E,F)** Immunohistochemistry on young- **(E)** and old- **(F)** MP-derived ectopic tissue showing muscle fiber positive for myosin heavy chain (red/brown). Nuclei stained with hematoxylin (blue). **(G,H)** Enlarged view of, respectively **(C)** and **(D)**, revealing the presence of vessels (arrows) in a muscle-like tissue. Scale bar: **(A)** 10 mm; **(B)** 2.5 mm; **(C,D)** 300 μm; **(E,F)** 100 μm; **(G,H)** 50 μm.

rejuvenation, as elegantly demonstrated by hetero-parabiosis and hetero-grafting skeletal muscle experiments (Conboy et al., 2005). Thus, regenerative medicine approaches based on the use of adult stem cells could benefit from methods that rejuvenate the cells used. On this point, the present study reports on an innovative strategy to rejuvenate adult muscle progenitor cells. We studied pericytes because they offer a noteworthy advantage over other cell types on account of their bi-directional—vasculogenic and myogenic—commitment capacity, which can be exploited for skeletal muscle tissue engineering. With respect to pericytes from piglets, we found that aged pericytes had an impaired functionality in regards to their myogenic differentiation capacity and ability to support vessel formation. This reduced potential was recuperated by culture on a PEG-fibrinogen scaffold (PF). The PF has been chosen as rejuvenescence inducing matrix on the basis of our foregoing researches revealing the PF to be a very suitable material supporting muscle differentiation (Fuoco et al., 2012). Moreover we strongly believe that the hybrid nature of PF, represented by tunable synthetic PEG and natural fibrinogen molecules as integrant parts of the scaffold, favors cell adhesion, survival and differentiation. Thus PF guarantees a protective and inductive environment promoting swine-derived stem cells rejuvenation by recovering muscle differentiation and angiogenic capabilities of aged porcine perycites. We can speculate that vascularization of ectopic muscle like tissue comes from influence generated by swine derived perycite on host blood vessel recruitment for their intrinsic property to form the suitable "angiogenic niche" and than to retrieve EC for vessel branching and sprouting (Sacchetti et al., 2007; Ribatti et al., 2014). Furthermore recent publications on skeletal muscle tissue engineering demonstrate that exogenous engineered muscle like tissue can be vascularized by host vessel colonization or in case of co-culture with a source of EC (Levenberg et al., 2005; Juhas et al., 2014). These obtained results reflect the effect of a healthy three-dimensional ECM on muscle stem cells. In fact, aging modifies the ECM by increasing endo- and perimysial connective tissue, which alters the mechanical properties of muscle (Seene et al., 2012). Young, healthy ECM has a profound effect on muscle stem cell capabilities compared with an aged, stiffer environment (Antia et al., 2008; Sicari et al., 2012). Therefore, we can speculate that the three-dimensional environment of the PF plugs has a positive action on swine-derived MP by mimicking the stiffness and mechanical cues of young muscle ECM, since evoking into aged MP young-old hetero-parabiosis and /or hetero-grafting promoting swine derived MP rejuvenation. This opens a new scenario for future developments exploiting adult pericytes as myogenic progenitors and an angiogenic trigger to generate engineered muscle for use in humans, overall for the possibility to build with swine derived MP porcine artificial muscle like human size comparable.

AUTHOR CONTRIBUTIONS

Cesare Gargioli and Gaia Spinetti designed the research and wrote the paper. Claudia Fuoco and Elena Sangalli isolated the cells and carried out most of the experimental work; Roberto Rizzi and Claudia Bearzi helped with data interpretation; Rosa Vono performed cell characterization; Benedetto Sacchetti helped with histological technique and analysis; Dror Seliktar produced and implemented PF for muscle experiments; Sergio Bernardini and Stefano Testa did the histology and immunostaining; Michael V. G. Latronico, Paolo Madeddu, Gianni Cesareni and Stefano Cannata helped

with study design, data analysis interpretation, and paper writing.

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A multistep procedure to prepare pre-vascularized cardiac tissue constructs using adult stem sells, dynamic cell cultures, and porous scaffolds

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The vascularization of tissue engineered products represents a key issue in regenerative medicine which needs to be addressed before the translation of these protocols to the bedside can be foreseen. Here we propose a multistep procedure to prepare pre-vascularized three-dimensional (3D) cardiac bio-substitutes using dynamic cell cultures and highly porous biocompatible gelatin scaffolds. The strategy adopted exploits the peculiar differentiation potential of two distinct subsets of adult stem cells to obtain human vascularized 3D cardiac tissues. In the first step of the procedure, human mesenchymal stem cells (hMSCs) are seeded onto gelatin scaffolds to provide interconnected vessel-like structures, while human cardiomyocyte progenitor cells (hCMPCs) are stimulated *in vitro* to obtain their commitment toward the cardiac phenotype. The use of a modular bioreactor allows the perfusion of the whole scaffold, providing superior performance in terms of cardiac tissue maturation and cell survival. Both the cell culture on natural-derived polymers and the continuous medium perfusion of the scaffold led to the formation of a densely packaged proto-tissue composed of vascular-like and cardiac-like cells, which might complete maturation process and interconnect with native tissue upon *in vivo* implantation. In conclusion, the data obtained through the approach here proposed highlight the importance to provide stem cells with complementary signals *in vitro* able to resemble the complexity of cardiac microenvironment.

Keywords: cardiac tissue engineering, adult stem cells, vascularized three-dimensional (3D) scaffolds, dynamic culture, patient-derived stem cells

INTRODUCTION

The successful regeneration of injured areas of the myocardium by tissue-engineered constructs relies on the long time viability and persistence of the bio-substitute *in vivo*, given the harsh conditions cells in the infarcted milieu are exposed to. Previous investigations revealed the sudden disappearance of cells administered by injection—systemically or locally—independent of cell type. This negative outcome has been ascribed to the low retention and high mortality of cells in the hypoxic environment characterized by an inflammatory response and the lack of local blood supply (Gnecchi et al., 2008; Menasche, 2011). The issue of promoting ischemic area vascularization has been lately addressed by cardiac tissue engineers through different approaches: (i) the administration of pro-angiogenic factors supplied by direct injection or through drug-releasing carriers (Sato et al., 2001; Chiu and Radisic, 2010; Singh et al., 2012); (ii) the infusion of endothelial progenitors (EPCs) or mature endothelial cells (ECs; Lian et al., 2008); and (iii) the pre-vascularization of the tissue constructs produced *in vitro* before implantation (Caspi et al., 2007; Dvir et al., 2009). Although the first two strategies are potentially interesting in a therapeutic perspective, they rely on the *in situ* generation and organization of vascular structures which depend either on the bioavailability of beneficial molecules or on the growth and differentiation capacity of vascular cells or their progenitors (Lovett et al., 2009). The early clinical trials in which growth factors or cells were delivered to the injured heart yielded disappointing results in terms of improvement of cardiac function (Urbich et al., 2005; Dubois et al., 2010; Simón-Yarza et al., 2012). The pre-vascularization of cardiac patches is also appropriate for providing a capillary network to support cells in the inner core of the implant, while biocompatible substrates are deemed to contribute to the improvement of retention and engraftment of the transplanted cardiac tissue (Terrovitis et al., 2010; Segers and Lee, 2011). The advantage of the pre-vascularization of thick muscle constructs was underlined by the demonstration that co-cultures including skeletal myoblasts, endothelial cells (or their progenitors) and embryonic fibroblasts on biocompatible porous scaffolds can enhance the overall survival and functionality of the constructs *in vivo* (Levenberg et al., 2005). Moreover, the adoption of scaffolds displaying an interconnected porosity itself

could foster host vascular cell recruitment, with the possibility of vessels branching throughout the core of the construct. Alternatively, scaffoldless thick cardiac constructs were provided with a vascular bed (Sekine et al., 2013), or with microchannels (Sakaguchi et al., 2013) to favor vessel ingrowth, although biocompatible supports improve the handling of the grafts and can provide cells with appropriate bio-mechanical signals to better induce tissue regeneration and repair. In this context, the use of porous gelatin scaffolds represents a suitable tool for cardiac tissue engineering application (Sakai et al., 2001; Akhyari et al., 2002). In fact, gelatin is a cheap polymer derived from collagen denaturation and hydrolysis, and, due to its natural origin, it displays excellent cell adhesion property (Wu et al., 2011). It also features high biocompatibility, low immunogenicity, and biodegradability (Xing et al., 2014). In addition, gelatin sponges have been proven effective in inducing angiogenesis (Dreesmann et al., 2007) and their porous structure can favor the vascularization of the construct by supporting the diffusion of cells and nutrients within its core area. Its mechanical properties can be easily adjusted to match those encountered in living tissues.

The use of autologous stem cells has been proposed for various cell therapy applications as a mean to avoid the immune rejection issues raised by allogeneic or xenogeneic derivatives and the ethical concerns due to the use of embryonic material. Human bone marrow-derived mesenchymal stem cells (hMSCs) are an excellent candidate for regenerative medicine applications due to their autologous origin, their immunomodulatory properties and relative safety in clinical practice (Lalu et al., 2012). The *in vitro* multilineage differentiation potential of mesodermal progenitors has been proven in a number of studies (Pittenger et al., 1999; Muraglia et al., 2000) and their ability to express endothelial markers upon growth factor stimulation (Oswald et al., 2004; Jazayeri et al., 2008; Portalska et al., 2012) and response to bio-mechanical stimulation (stretching, shear stress, substrate mechanical properties tuning; Lozito et al., 2009; Bai et al., 2010) has been shown. More importantly, the benefits of MSC-based therapy have mainly been ascribed to their ability to generate endothelial cells and exert pro-angiogenic and cardioprotective effects by paracrine mechanisms rather than to direct the generation of new contractile cells (Gnecchi et al., 2008; Meyer et al., 2009; Wöhrle et al., 2010; Loffredo et al., 2011).

Among the adult stem cell subsets so far proposed for cardiac muscle repair, resident cardiac stem/progenitor cells (CSCs or CPCs) were shown to retain the ability to differentiate into all the cardiac tissue cell types (Beltrami et al., 2003; Forte et al., 2011) and favor cardiac healing by direct production of contractile cells *in vivo* (Smits et al., 2009a,b).

By taking advantage of the peculiar differentiation potential of hMSCs and human cardiomyocyte progenitor cells (hCMPCs), in the present investigation we propose a multistep procedure to obtain human pre-vascularized three-dimensional (3D) cardiac bio-substitutes based on highly porous gelatin scaffolds displaying the stiffness of cardiac tissue. Given the thickness and the dimensions of the bio-construct, a modular dynamic culture system has been used to guarantee scaffold perfusion and promote cell colonization of the inner layers.

Although being here tested exclusively *in vitro*, the present method allows for the formation of a 3D cardiac construct based on a physiological environment given by the gelatin scaffold and featuring the use of autologous stem cells with unique potential.

MATERIALS AND METHODS

PREPARATION OF POROUS GELATIN SCAFFOLDS

All materials used (unless specified) were purchased from Sigma-Aldrich (Italy). A 5% w/v gelatin solution was prepared by dissolving gelatin (Type A, G1890, 300 bloom strength) in deionized water. Porous gelatin scaffolds were prepared with a multi-step procedure. The solution was stirred for 1 h at 50°C, allowing complete dissolution, then casted in cylindrical shaped mold and physically gelled at room temperature. Samples were kept at 4°C for 1 h, and then at −20°C overnight. Gelatin samples were then freeze-dried (−50°C, 150 mBar) to obtain a porous structure as described elsewhere (Lien et al., 2009). Samples were swollen in deionized water and then cross-linked by immersion in a 10 mM glutaraldehyde (GTA) solution in 40% v/v ethanol/deionized water. The crosslinking reaction was controlled by keeping the glutaraldehyde/gelatin ratio (defined as molar concentration of GTA versus gelatin weight) constant. The scaffolds were immersed in GTA solution at 4°C for 48 h, until the cross-linking reaction occurred. Therefore, the samples were immersed in 0.1 M glycine solution in deionized water for 2 h at room temperature in order to stop any further cross-linking reaction (and remove any excess of GTA). Phosphate buffered solution (PBS) was sequentially used to rinse samples. Samples were kept at −20°C overnight, and finally freeze-dried (−50°C, 150 mBar) until all water content was removed. Samples were then stored at room temperature and sterilized with gas plasma before using.

SCAFFOLD MECHANO-ARCHITECTURAL PROPERTIES: SWELLING, POROSITY, AND STIFFNESS

Water absorption capability, porosity and stiffness of the porous gelatin scaffolds were evaluated using the procedure described elsewhere (Spinelli et al., 2012). Swelling ratio (Q) was calculated from the ratio of the weight of a dry (W_0) and a completely swollen (W_{eq}) sample (Brannon-Peppas and Peppas, 1990) returning the amount of adsorbed water. For the measurements, cryogel were swollen in deionized water at room temperature and weighted (W_i) at different time points until a swelling equilibrium was reached. A precision microbalance (AE240, Mettler, Italy) was used: in case of wet samples, blotting paper was used to remove the water in excess. Porosity was indirectly evaluated by the imbibition method (Mwangi and Ofner, 2004; Martucci et al., 2006), while pore size was directly measured by processing both Scanning Electron Microscopy (SEM) and optical microscopy acquisition of sample sections with ImageJ (Abramoff et al., 2004). Sample stiffness was measured by compressive mechanical tests. Prior to the tests, samples were completely swollen in deionized water, compressive tests were then performed using a Zwick-Roell Z005 Instron twin column-testing machine (Zwick Testing Machines Ltd., UK). Samples were compressed up to 5% of their initial length using a 0.01 $mm \cdot s^{-1}$ strain rate; tests were performed with the samples partially immersed in water to preserve their hydration. Data were then post-processed

and stress-strain curves were obtained. Samples stiffness was evaluated within 1% strain (first linear zone) of the stress-strain curve.

BIOREACTOR WORKING CONDITIONS

A computational fluid-dynamic (CFD) analysis of the modular chamber bioreactor with a porous scaffold was performed, assessing the perfusion and oxygenation of the millimeter sized scaffold. The analysis was performed using Brinkman and Incompressible Navier-Stokes equations were combined with reaction and diffusion equations using Comsol Multiphysics (COMSOL AB, Stockholm, Sweden). The system was then modeled with a porous domain representing the scaffold (porous section of 2×12 mm in size, with permeability of 1.68×10^{-10} m^2 and 90% porosity) placed on the bottom of the bioreactor perfusion chamber, which is represented by a fluid domain. A preliminary analysis was performed in order to verify scaffold oxygenation as function of bioreactor flow rate (Supplementary Figure 1A). Chosen an inner flow rate to a value of 200 μL/min, fluid flow inside the perfusion chamber and scaffold's perfusion was analyzed (Supplementary Figure 1B).

CELL CULTURE

hMSCs were purchased from Lonza and cultured in MSCGM™ BulletKit™ (hMSC basal medium, Lonza Japan Ltd, Tokyo, Japan). The cells were expanded to the desired number and used between passages 3 and 7. For the endothelial differentiation in 2D conditions, hMSCs were seeded onto tissue culture polystyrene plates (TCPS) and cultured for 7 days in endothelial differentiation medium [(EDM: hMSC basal medium supplemented with 50 ng/ml VEGF165 (R&D systems)]. hCMPCs were isolated from healthy donor atrial biopsies, cultured in basal medium (M199/EGM-2 (Lonza) (3:1) 10% FBS (Equitech-Bio Inc., Kerrville, Texas, USA), 1% MEM nonessential amino acids (from 50× stock; Life Technologies Gaithersburg, Maryland, USA) and 2% penicillin/streptomycin (from 50× stock; Invitrogen, Carlsbad, California, USA) and differentiated in cardiac differentiation medium (CDM: IMDM/Hams F12 (1:1, Life Technologies) with L-glutamine (Life Technologies), 2% horse serum (Life Tchnologies), nonessential amino acids, insulin–transferrin–selenium (Invitrogen), penicillin/streptomycin) as previously described (Smits et al., 2009a,b). Briefly, minced auricle were digested in collagenase A (1 mg/ml) for 2 h at 37°C while stirring. Afterwards, the solution was filtered through 40-μm cell strainer and centrifuged at 300 g for 5 min at RT. Then, the pellet was resuspended in cold buffer containing EDTA (2 mM) and 2% FBS and filtered through 40-μm cell strainer. The filtered cell population was subjected to magnetic cell sorting (Miltenyi Biotec, Sunnyvale, CA, USA) using Sca-1-coupled beads, following the manufacturer's protocol, in order to isolate Sca-1-like + progenitor cells. The cells were cultured on 0.1% gelatin- (Sigma-Aldrich, St Louis, Missouri, USA) coated plates in basal medium. For cardiac differentiation, cells were grown in differentiation medium supplemented with 5 μM 5-azacytidine (Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 72 h. After induction, the medium was replaced with differentiation medium supplemented with 10^{-4} M ascorbic acid (Wako) and TGF-β1 (1 ng/ml, PeproTech, Rocky Hill, NJ, USA) and changed every 3 days for up to 3 weeks. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, grown in EGM-2 bullet kit media and used between passage 2 and 4. The media were replenished every other day.

GENERATION OF TNT-GFP hCMPCs

The pGreenZeo lentiviral expression vector carrying the full-length copGFP gene under the control of cardiac Troponin T type 2 (TNNT2) promoter was purchased from SBI (System Biosciences Inc. Mountain View, CA, USA) and delivered into mammalian cells according to the manufacturer's instructions. Briefly, hCMPCs were transduced at an approximate MOI of 20 and incubated at 37°C overnight. The following day, half of the culture medium was replaced with fresh medium and 48 h after the transduction, the medium was discarded and fresh medium added. The cells (hereafter referred as cTNT-GFP hCMPCs) were cultured on TCPS or gelatin scaffolds in the presence of basal medium or CDM.

CELL SEEDING AND 3D DISTRIBUTION IN GELATIN SCAFFOLDS

Prior to cell seeding, gelatin scaffolds were swollen in cell culture medium in Ultra-low Attachment 24-well plates (Corning® Incorporated) for 24 h. The cells (2.0×10^5) were directly seeded on top of each scaffold in a small volume (100 μl) of appropriate medium and allowed to adhere for 60 min before adding the rest of medium. Then, the scaffolds were placed in the incubator for 12 h and transferred in another low-adhesion well. Cardiac TNT-GFP hCMPCs were cultured into scaffolds for 7 days in complete hCMPCs basal medium or CDM, while hMSCs-seeded scaffolds were cultured for 4 days in a mixture of Matrigel™/hMSCs basal medium (1:10) with or without VEGF (50 ng/mL) before being processed or co-cultured with pre-conditioned cardiac progenitors. The formation of vascular-like structures on gelatin scaffolds was followed by labeling hMSCs with the membrane-permeable live-cell labeling dye Calcein AM (Invitrogen) after 4 days of culture in EDM. Briefly, Calcein AM was added to hMSC-seeded scaffolds at a final concentration of 2 μM for 30 min at 37°C and 5% CO_2 in the dark. Five scaffolds have been used for any single experiment. The experiments have been performed independently three times.

DYNAMIC CULTURE

A commercial modular chamber bioreactor (Quasi-Vivo®, QV500, Kirkstall, United Kingdom) was used to perfuse the scaffolds. The main feature of this system is the ability to apply high flow rates and provide high nutrient turnover to cells without imposing high shear stress or turbulent flow (Mazzei et al., 2010). Pre-seeded scaffolds were gently transferred into the bioreactor chamber, and then the chamber was filled with cell culture media prior to perfusion. Two Quasi-Vivo® chambers and one mixing chamber (growth medium reservoir) were then connected in series to a peristaltic pump (High Performance Perista BIO-MINI PUMP, ATTO) giving a final volume of cell culture media of about 21 mL. The use of the mixing chamber guarantees medium oxygenation (**Figure 1**). To establish the optimal flow rate, the gelatin porous scaffold was included in the fluid-dynamic and oxygen

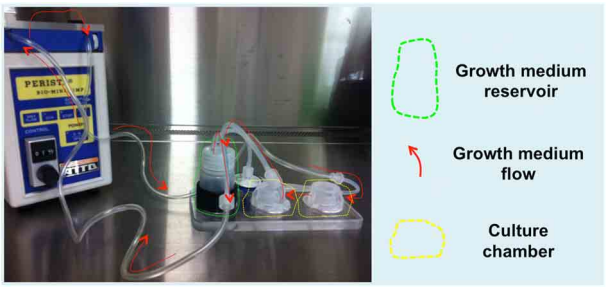

FIGURE 1 | QuasiVivo Bioreactor (QV500). The bioreactor is composed of a peristaltic pump keeping a constant medium flow rate (200 μl/min) from the growth medium reservoir to two culture chambers connected in series, thus allowing scaffold inner layer perfusion.

transport model previously described (Mazzei et al., 2010), giving consistent results (Supplementary Figure 1). Cardiac TNT-GFP hCMPCs grown for 7 days onto gelatin scaffolds in the bioreactor were used to study the effect of dynamic culture conditions on cell growth, differentiation potential and gene expression profile. The medium was replenished every third day. For the generation of vascularized cardiac grafts, scaffolds were soaked for 12 h in diluted Matrigel™ (1:10 in hMSC basal medium) and switched to a new low-adhesion plate in EDM. The pre-conditioned scaffold was loaded with 2.0×10^5 hMSCs and kept for 4 days in the incubator at 37°C, 5% CO_2. After a 2 week pre-conditioning passage in CDM on TCPS, 2.0×10^5 cTNT-GFP hCMPCs were seeded onto the vascularized scaffold and allowed to attach for 24 h. Thence, the cell-loaded scaffolds were carefully moved to Quasi-vivo bioreactor chambers and perfused with CDM at 200 μl/min (the rate suggested by the computational model) for 7 days. Cells grown on TCPS and 3D scaffolds under static conditions were used as controls. Five scaffolds have been used for any single experiment. The experiments have been performed independently three times.

REAL-TIME QUANTITATIVE PCR ANALYSIS

Total RNA was extracted by TRIZOL® Reagent according to the manufacturer's instructions (Invitrogen). One microgram of RNA for each sample, measured by NanoDrop 2000 (Thermo Scientific) and assessed on ReadyAgarose Precast Gel (Bio-Rad), was retro-transcribed using RT^2 First Strand Kit including DNAse treatment to remove genomic DNA (SA Biosciences Corp., USA). The resulting cDNA was diluted 1:10 in DNase/RNase-free water and the expression profile of genes involved in different pathways analyzed by the following RT^2 Profiler™ PCR Arrays (SA Biosciences Corp.): (i) RT^2 Profiler™ PCR Array Human Angiogenesis (PAHS-024Z); (ii) RT^2 Profiler™ PCR Array Human Extracellular Matrix & Adhesion Molecules (PAHS-013); (iii) qBiomarker iPSC PCR Array Cardiomyocytes differentiation (iPHS102); (for a comprehensive list of genes included in these arrays please refer to Supplementary Table 1). Real-time PCR was performed on ABI 7500 Real-Time PCR System (AB Applied Biosystems, Foster City, CA) using RT^2 SYBR Green/ROX qPCR Master Mix (SA Biosciences Corp.) and the following cycling parameters: 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 s, and 60°C for 1 min. Data was analyzed by the ΔΔCt method with the PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). The graphs show the mean values of obtained fold changes by analyzing independently two samples per each experimental condition.

STATISTICAL ANALYSIS

Data are represented as mean ± SD. Student's *T*-test was used to analyze the data. Comparisons between different conditions were considered statistically significant for $P < 0.05$.

For a more extensive description of the Materials and Methods used, please refer to Supplementary Information.

RESULTS

MODELING THE MULTI-DIMENSIONAL *IN VITRO* SYSTEM THROUGH GELATIN SCAFFOLDS DISPLAYING CARDIAC-LIKE MECHANICAL PROPERTIES

Three dimensional gelatin scaffolds were characterized for their structure, water affinity, and stiffness. The analysis of the sections showed a distinctive bimodal pore size distribution. Although the average pore diameter ranged between 50–200 μm (with a few having a maximum size of 500 μm), the smaller pores were approximately double the size of the bigger pores (diameters of 61.62 ± 24.01 and 140.38 ± 19.51 μm, respectively; **Figure 2A**). Swelling tests were performed to assess the scaffold capacity to retain water. The scaffolds were monitored over 24 h of immersion in cell culture media, showing a complete rehydration after 8 h with a swelling ratio of about 10.92 ± 0.32 (**Figure 2B**). These data were also used to measure scaffold porosity, which was found to be approximately 90% (also confirmed in Spinelli et al., 2012). Finally, compressive mechanical tests were also performed on swollen samples (after 24 h in cell culture media), and on samples kept immersed in cell culture media and in the incubator for 1 week. Scaffold stiffness (evaluated within the first linear region of stress-strain plot, **Figure 2C**) was 10.5 ± 1.4 kPa, which was stable in time. After scaffold characterization and assessment of mechano-architectural properties, attention was dedicated to define the most suitable working conditions of the bioreactors to guarantee a homogeneous scaffold oxygenation in the bioreactor. Specifically, literature values of hypoxic limits (1%) (Cipolleschi et al., 1993) and oxygen consumption of stem cells ($K_m = 0.001$ mM, $V_{max} = 1$ pm/min/10^4 cells) (Varum et al., 2011) were used to estimate the minimum oxygen concentration at the base of the scaffold as a function of flow rate and cell density, while the Wang and Tarbell approximation was used to calculate the shear stress at the pore walls. Results obtained from the computational models (reported in Supplementary Information) evidenced that—by using a flow rate of 200 μL/min—an oxygen concentration of 0.15 mM at the base of the scaffold (14%) and a shear stress of 1.10^{-6} Pa in the pores are obtained (Wang and Tarbell, 1995; Boschetti et al., 2006). The use of lower flow rates in the bioreactor may compromise oxygen supply rates to cells, especially as they proliferate, while higher flow rates may cause shear-related damage (Mazzei et al., 2010). Bioreactor parameters were set according to these results for all the following experiments.

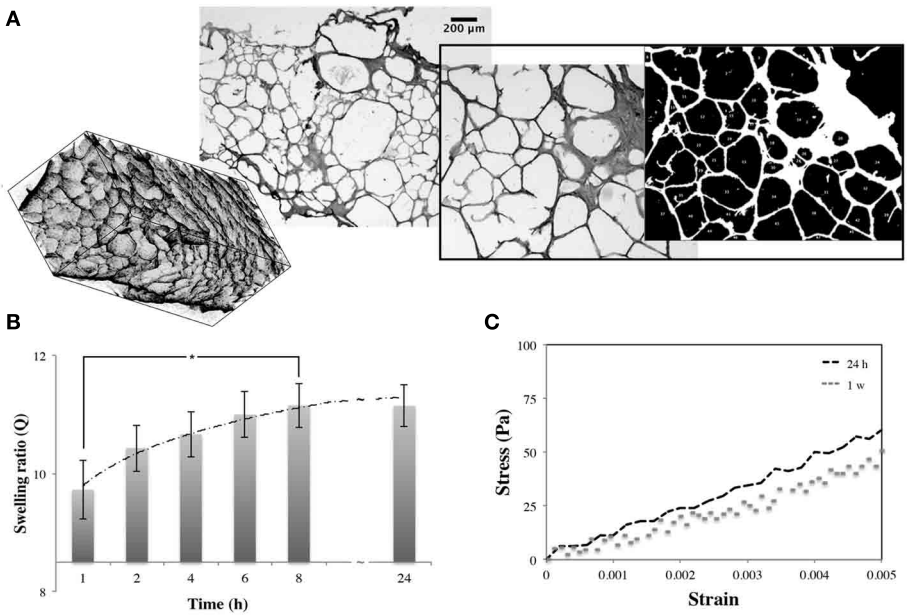

FIGURE 2 | The porous gelatin scaffold. Three-dimensional rendering of scaffold, brightfield images of scaffold section and pore dimension analysis **(A)**. Modifications of swelling ratio of scaffold over 24 h in cell culture media **(B)**. Stress-strain plot of gelatin samples **(C)** obtained after 24 h and 1 week in culture medium (37°C, 5% CO_2). $^*P < 0.05$.

3D CULTURE INDUCES A GENERAL REMODELING IN THE EXPRESSION OF GENES INVOLVED IN CELL-MATRIX INTERACTION

Establishing a physiologically relevant system requires the development of 3D culture conditions resembling the *in vivo* microenvironment (Pampaloni et al., 2007). As reported above, the scaffolds here used were designed to have mechanical properties similar to those of cardiac tissue (Engler et al., 2007). Preliminary experiments demonstrated that culturing human hCMPCs on 3D gelatin scaffolds for 7 days induced a clear upregulation in the expression of genes (fold change $\geq$2, as compared to cells grown on TCPS) coding for proteins involved in cell-matrix remodeling (collagens (COL), laminins (LAMA), metalloproteinases (MMP) and their inhibitors (TIMP) and cell-cell adhesion (integrins) (**Figure 3**, for a complete list of the genes analyzed see Supplementary Table 1) and testified the actual activity of the cells in remodeling the scaffold.

3D POROUS GELATIN SCAFFOLDS REPRESENT A PERMISSIVE ENVIRONMENT FOR STEM CELL CARDIAC AND ENDOTHELIAL COMMITMENT

The hCMPC reporter cell line (cTNT-GFP hCMPCs) was driven toward a cardiac phenotype in both 2D (Supplementary Figure 2) and 3D conditions, when stimulated with CDM (**Figure 4A**). Cell commitment was confirmed by the up-regulation of a number of cardiac-specific genes like actinin alpha 2 (ACTN2), adrenoceptor beta 1 (ADRB1), desmin (DES), cardiac troponin T type 2 (TNNT2), cardiac troponin I type 1 (TNNI1), myosin light chain (MYL) 2, 3, 7, myosin heavy chain b (MYH7) in cardiac progenitors stimulated on the scaffold as compared to their respective control (3D culture in basal medium). Moreover, when compared to cells cultured in CDM on TCPS, hCMPCs grown onto gelatin scaffolds encountered the up-regulation of few cardiac genes, confirming a superior performance of 3D vs. 2D culture conditions (**Figure 4B**, for a complete list of the genes analyzed see Supplementary Table 1). On the other hand, the ability of hMSCs to acquire an endothelial phenotype (Supplementary Figure 3) was exploited to obtain endothelial cells from hMSCs under 3D culture conditions. The scaffolds were treated with a mixture of EDM and Matrigel™ for 24 h. SEM analysis showed that the interconnected porosity of the scaffold was not modified by Matrigel™ treatment (highlighted by yellow dotted line in **Figure 4C**) and that the successful adhesion of human cells was achieved (**Figure 4C**, right). Consequently, hMSCs grown on 3D gelatin scaffolds in EDM displayed the up-regulation of a wide range of angiogenic markers as compared to cells cultured on scaffolds in basal medium (**Figure 4D**, for a complete list of the genes analyzed see Supplementary Table 1). Confocal analysis of 3D scaffolds after 4 day hMSC culture demonstrated the establishment that cells loaded with Calcein AM or expressing endothelial marker VCAM-1 colonized the whole scaffold, while aligning around the pores (**Figure 4E**).

DYNAMIC CULTURE CONDITIONS PROVIDE SUPERIOR PERFORMANCE IN TERMS OF CARDIAC COMMITTMENT OF hCMPCs

The use of dynamic culture conditions using a bioreactor is thought to increase cell survival within a 3D structure by favoring inner core perfusion and catabolite removal (Cimetta et al., 2007; Burdick and Vunjak-Novakovic, 2009; Vozzi et al., 2011). Indeed, a slight although not significant increase in DNA content, indicative of cell proliferation, could be noticed 7 days after 3D cell culture in the bioreactor, as compared to static conditions (**Figure 5A**). Given the ability of human cardiac progenitor

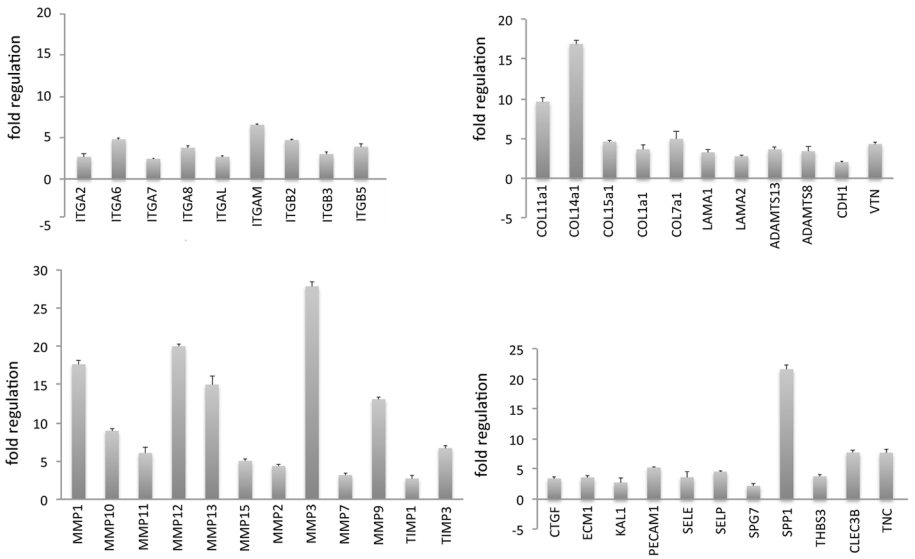

FIGURE 3 | Three-dimensional (3D) stem cell culture induces a significant remodeling in the expression of genes involved in cell adhesion and ECM deposition. Human cardiac progenitor cells (hCMPCs) were grown for 1 week on 3D gelatin scaffolds and the expression of 73 genes involved in cell-matrix interaction resulted upregulated, as evidenced by real time PCR (for a complete list of genes analyzed please refer to Supplementary Table1).

cells to proceed to cardiac maturation on 3D gelatin scaffolds, their capacity to activate the cardiac program when exposed to dynamic conditions in a modular bioreactor was assessed. For this experiment, cTNT-GFP hCMPCs were pre-committed in CDM for 2 weeks on TCPS. Afterwards, an aliquot of this cell population was seeded onto scaffolds and further stimulated for 1 week under static or dynamic conditions. The data confirmed that the commitment of cTNT-GFP hCMPCs could be achieved by CDM in dynamic conditions (**Figure 5B**) and that a minor although significant spontaneous commitment of hCMPCs was induced by 3D culture conditions *per se*, as also described in static culture (white columns). Although the dynamic culture conditions did not significantly modify the percentage of GFP-positive cells with respect to the static protocol, real time PCR array analysis demonstrated that dynamic stimulation—when combined with differentiation medium—could lead to the up-regulation of few cardiac-specific genes (**Figure 5C**, for a complete list of the genes analyzed see Supplementary Table 1).

GENERATION OF 3D PRE-VASCULARIZED CARDIAC CONSTRUCTS BY DYNAMIC CULTURE AND POROUS SCAFFOLDS

Given the positive effect of dynamic culture conditions on cardiac gene activation, we sought to obtain vascularized cardiac 3D tissues by co-culturing hMSCs-derived vascular cells and pre-differentiated hCMPCs in the bioreactor system for 7 days, as described in **Figure 6A**. Gelatin sponges were first seeded with hMSCs under endothelial differentiation conditions. In the meantime, cTNT-GFP hCMPCs seeded on TCPS were challenged with CDM for 2 weeks, seeded onto hMSCs-loaded scaffolds and allowed to adhere for 24 h before being exposed to continuous medium flow. The analysis of the co-cultured constructs after 1 week testified massive scaffold colonization by the cells (**Figure 6B**). The scaffold thickness was not significantly modified by medium perfusion (data not shown) that affected, instead, cell migration inside the scaffold. In fact, the side of the colonized scaffolds directly exposed to flow showed a more uniform and extensive distribution of vascular-like cells (in red) and hCMPC-derived cardiomyocytes (green) inside the scaffold, while the cells were mostly distributed on the scaffold surface in the static control (**Figure 6C**). The expression of VCAM-1 and CD144 endothelial markers confirmed the presence of endothelial-like cells, while GFP expression testified the persistence of a number of cardiac-like cells within the bio-construct (**Figure 6D**). Sections from the perfusion group showed VCAM-1-positive cells aligned forming tube-like structures around the pores and contacting GFP-positive cells (**Figure 6E**). The extensive cell distribution within the scaffold accounted for the formation of a densely packed multicellular tissue derived from the two different stem cell types used (**Figure 6F**).

DISCUSSION

Rebuilding functional portions of the myocardium requires the generation of bio-substitutes that best recapitulate the structure and function of the healthy myocardium, thus providing new cardiomyocytes with a functional vascular network, which may prevent or reduce pathological decline and improve cardiac function after injury (Simons and Ware, 2003; Kang et al., 2013). Exploiting cell sheet technology, our group and others successfully achieved the preparation of 3D cardiac tissues in the absence of scaffolds by different cell types having a cardiac significance (Haraguchi et al., 2006; Forte et al., 2011; Matsuura et al., 2012; Sekine et al., 2013). Nonetheless, the scaling up of solid engineered tissues to obtain a critical size substitute with therapeutical relevance is limited by the diffusion of oxygen, nutrients and waste products to and from the inner portion of the construct, impairing the survival of the newly formed tissue. In this

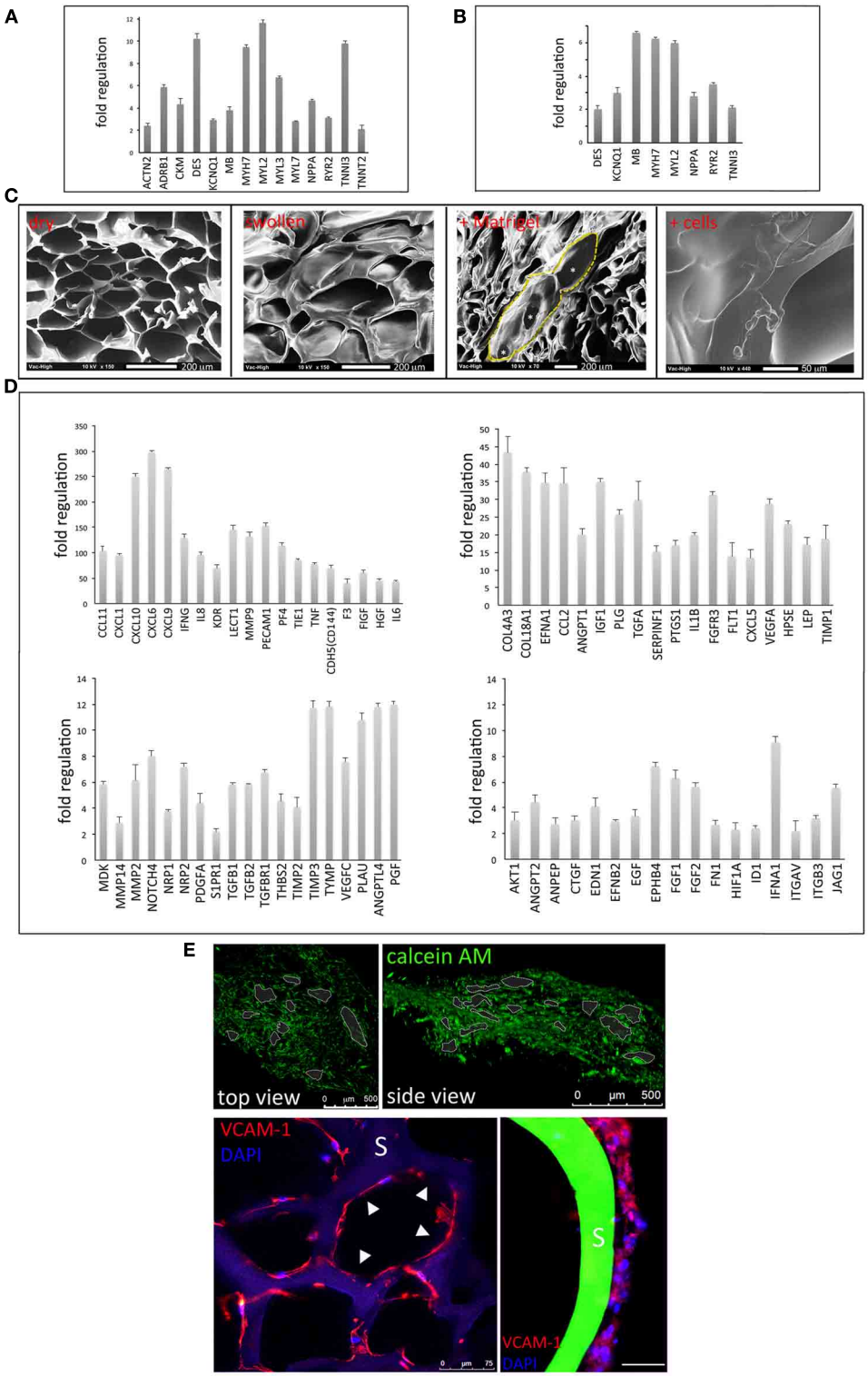

FIGURE 4 | Three-dimensional (3D) gelatin scaffolds support multilineage stem cell commitment. After 2 weeks of cardiac pre-commitment on TCPS, human cardiac progenitors (hCMPCs), seeded on 3D gelatin scaffolds and stimulated with cardiogenic differentiation medium (CDM) for further 1 week, up-regulate some cardiac-specific genes, as compared to 3D culture in basal medium **(A)**. 3D differentiation conditions show a higher performance **(B)** as compared to tissue culture polystyrene (TCPS). The interconnected porosity of the scaffold (as outlined by yellow dashed line and asterisks) is not modified by Matrigel™ treatment and can

(Continued)

FIGURE 4 | Continued
accommodate human cells **(C)**. Human mesenchymal stem cells (hMSCs) are differentiated to endothelial cells on 3D gelatin scaffolds when stimulated with endothelial differentiation medium (EDM) and low concentration of Matrigel™, as demonstrated by the upregulation of several vascular genes as analyzed by real time PCR **(D)**. Calcein AM staining shows the network of living cells spreading throughout the scaffold (**E**, upper panels). Scaffold (S) pores are covered by lining cells expressing VCAM-1 as highlighted by white arrowheads (**E**, lower panel). A magnification of a cord-like structure aligned along a pore is visible on the right; scale bar: 100 μm.

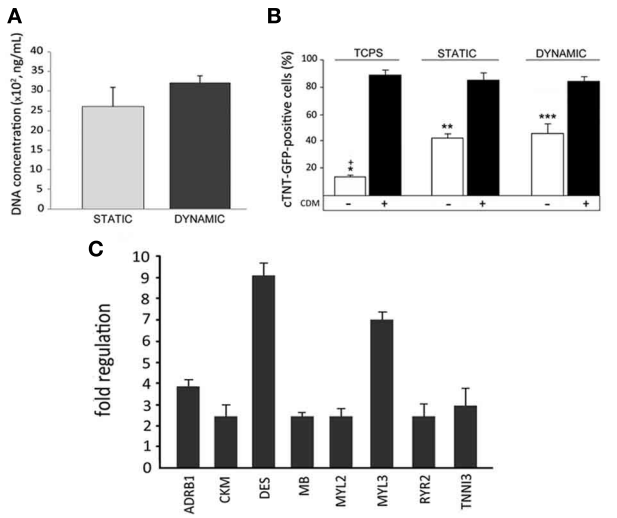

FIGURE 5 | Dynamic culture conditions induce a superior cardiac commitment in human cardiac progenitors (hCMPCs). Dynamic culture conditions provide a higher, although not significant, increase in scaffold cell colonization as compared to static conditions **(A)**. Percentage of differentiated cTNT-GFP CMPCs stimulated with cardiogenic differentiation medium (CDM) and cultured on TCPS and scaffolds, under dynamic, or static conditions, as evidenced by FACS analysis **(B)** (*, **, ***$P < 0.05$ between hCMPC basal medium and CDM). Importantly, a significant commitment can be induced, equivalently, by 3D culture itself in both static and dynamic conditions compared to TCPS ($^{+}P < 0.05$ between TCPS and 3D scaffolds). Nonetheless, quantitative PCR analysis demonstrates the up-regulation of few cardiac-specific genes in hCMPCs **(C)** cultured in dynamic conditions, as compared to its static control (for a complete list of genes analyzed please refer to Supplementary Table 1; among the genes analyzed, those not showing a consistent and significant difference in the expression as compared to their respective controls, are not shown).

context, the use of porous scaffolds to generate *in vitro* artificial bio-substitutes gives the opportunity to choose the shape and the size of the graft, while second-generation scaffolds are currently proposed as active templates which can be removed in a timely fashion compatible with tissue growth and exert an instructive role in the formation of the new tissue (Forte et al., 2013; Pagliari et al., 2013). In the present study, we propose a multistep procedure to prepare adult stem cell-derived pre-vascularized thick cardiac patches by using 3D porous gelatin scaffolds and dynamic culture conditions. The procedure here adopted is based on the peculiar differentiation potential of two different autologous adult cell subsets, mesenchymal, and cardiac resident stem cells. Human MSCs can be routinely obtained from patient bone marrow and display a minimal, if any, ability to give raise to cardiomyocytes (Gnecchi et al., 2005; Loffredo et al., 2011), while they are supposed to retain an intrinsic vascular potential (Oswald et al., 2004). In the present investigation, hMSCs were efficiently driven to acquire an endothelial phenotype by means of EDM in 3D culture conditions. In particular, when grown on 3D porous gelatin scaffold, hMSCs showed a robust vascular commitment as confirmed by the up-regulation of 73 genes involved in angiogenesis. Among these, angiopoietin 1 (ANGPT1), which has been shown to promote blood vessel maturation by regulating endothelial cell survival and the recruitment of mural cells (Thomas and Augustin, 2009), encountered a 20-fold increase, thus suggesting stability of the newly formed vascular network. At the same time, CPCs can be extracted from patient atrial biopsies and are known to possess the potential to generate new cardiomyocytes *in vitro* and *in vivo* (Beltrami et al., 2003; Goumans et al., 2007) making them an effective and safe cardiogenic cell type (Bolli et al., 2011; Makkar et al., 2012). In the present investigation, we explored the possibility to generate a 3D cardiac tissue *in vitro* by pre-committed hCMPCs. Although most of the attempts to regenerate damaged myocardium have used undifferentiated cells, concerns have been raised due to the poor differentiation capability of adult progenitors *in vivo*, together with the potential of pluripotent stem cells to give birth to undesired phenotypes. Recently, it has been suggested that the transplantation of pre-committed cells could improve the clinical outcome in cardiac patients (Heng et al., 2004; Mehta and Shim, 2013). For this reason, stem/progenitor cells were appropriately stimulated to undergo tissue specific pre-commitment before being used for all co-culture experiments. However, given that the mechanical properties of the gelatin scaffolds used here are similar to those of cardiac tissue, a spontaneous commitment of cardiac progenitor cells could be obtained through 3D culture *per se*, as demonstrated by the increased percentage of GFP-positive cells and similarly to what we and others previously demonstrated (Engler et al., 2006; Pagliari et al., 2011; Gaetani et al., 2012; Mosqueira et al., 2014). However, despite the fact that the gene expression profile analysis confirmed a moderate commitment of hCMPCs induced by the scaffold itself, biological factors were needed to improve this effect. In particular, when cells seeded on the scaffold were stimulated with CDM, we observed an increased expression of cardiac troponin genes (TNNT2, 3) along with the up-regulation of ryanodine receptor 2 (RYR2, involved in muscle excitation-contraction coupling, Bround et al., 2012) and natriuretic peptide precursor A (NPPA) genes. These data suggest that the combination of 3D porous gelatin and CDM represents a suitable combination to provide cells with biological and mechanical signals synergistically supporting the commitment of hCMCPs at a later stage. Nonetheless, neither complete sarcomeric structures nor any beating activity could be noticed in prompted cells. This evidence suggested that the system implemented represents a good tool to induce an efficient cardiac commitment and study the behavior of late cardiac progenitors, but this also implicates that more complex devices providing stretching and/or electrical

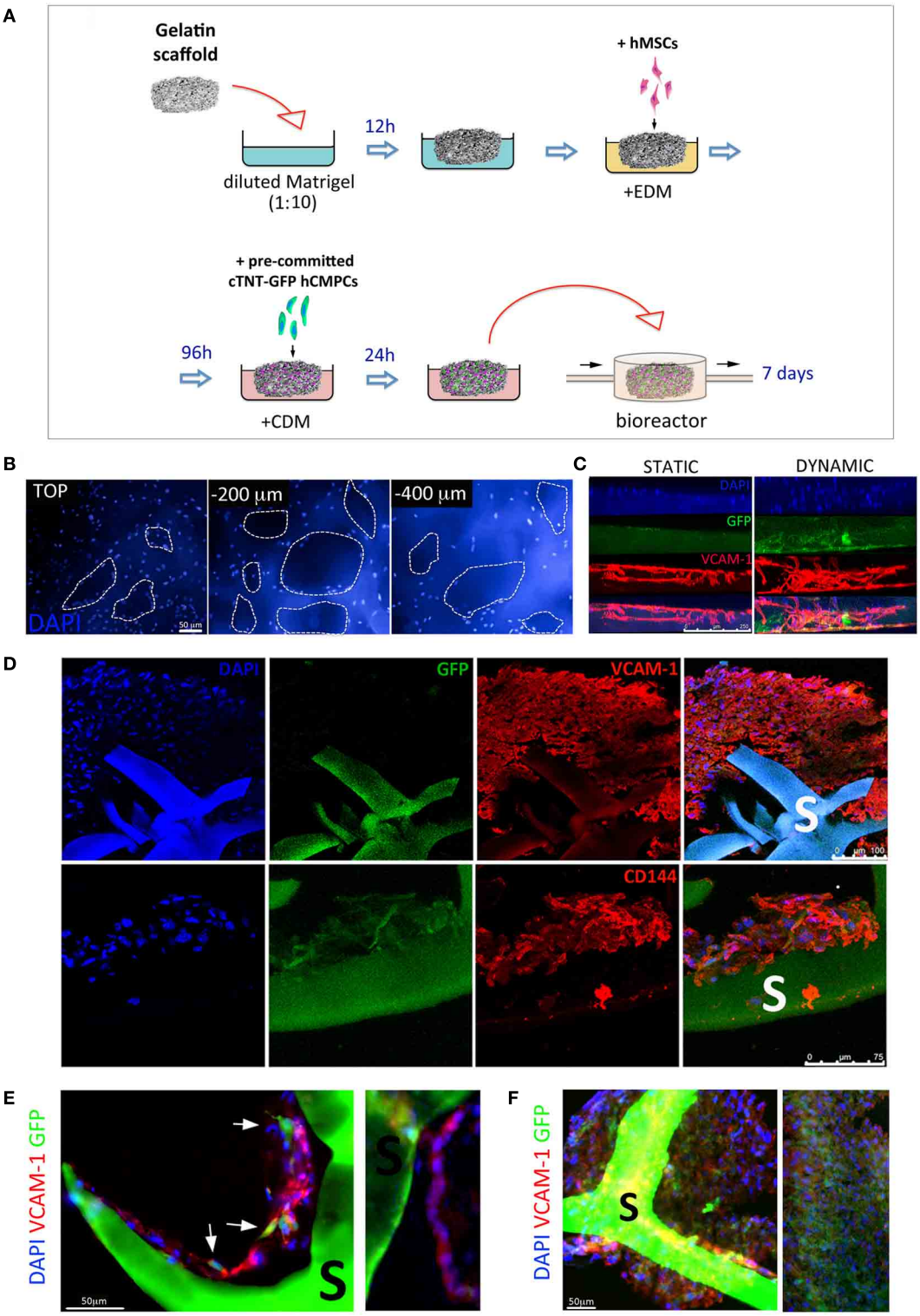

FIGURE 6 | Generation of a vascularized 3D cardiac construct by adult stem cells grown in dynamic conditions onto gelatin porous scaffolds. Schematic illustration of the protocol used to produce vascularized 3D cardiac construct **(A)**. Gelatin porous sponges were dipped in diluted Matrigel™ and then switched to endothelial differentiation medium (EDM). Therefore, hMSCs were allowed to colonize the scaffold and differentiate toward the endothelial phenotype for 4 days. Cardiac TNT-GFP progenitor cells were pre-committed for 2 weeks with cardiac differentiation medium (CDM) on TCPS, and then cultured on vascularized scaffold for further 7 days in CDM

(Continued)

FIGURE 6 | Continued
and in a perfusive modular bioreactor. Human cells colonize the scaffold inner layers as shown by nuclei staining at different depths **(B)**. Side view of cellularized scaffolds cultured under static or dynamic conditions **(C)**; infiltration of GFP- (cardiomyocyte-like cells, green) and VCAM-1-positive cells (endothelial-like cells, red) into scaffold is improved by dynamic culture. Immunohistochemistry analysis of the colonized scaffolds shows the massive infiltration of VCAM-1, CD144 cells in the core of the construct. Higher magnification images **(D)** shows the VCAM-1-positive cells aligned in tube-like structures around the pores and contacting GFP-positive cells **(E,F)**. S, scaffold.

stimulation are probably necessary to deliver more specific stimuli and improve the outcome in terms of cardiac differentiation. The activation of genes involved in matrix remodeling observed in 3D testified the ability of cells to actively interact with the natural support they grow on. In fact, while the up-regulation of Matrix metalloproteinase (MMP) genes and their regulators, tissue inhibitors of metalloproteinase (TIMP), is predictive of gelatin remodeling and breakdown, the activation of genes encoding for different collagens and laminins suggest the concomitant substitution of the artificial matrix with neo-formed tissue.

Bioreactors have been widely proposed to intensify oxygen and nutrient transport and increase the viability and function of thick cardiovascular tissue-engineered substitutes (Zimmermann et al., 2006; Cimetta et al., 2007; Burdick and Vunjak-Novakovic, 2009). In our experimental setting, dynamic culture conditions positively affected cardiac commitment, leading to a significant increase in GFP-expressing cells as compared to TCPS and independently of biological stimulation. Since the increase in GFP was not significantly different from that observed under 3D static culture conditions, it might be concluded that providing cells with continuous medium flow does not positively affect on cardiac progenitor differentiation potential on the scaffold. Nevertheless, the expression of genes recognized to be essential for the formation or regulation of the contractile apparatus was positively influenced when dynamic flow was applied to cells grown in a 3D environment. Hence, the use of a modular bioreactor providing continuous media turnover to the scaffold resulted in a superior performance in terms of cardiac gene activation. In the light of these promising results, we also expected that direct perfusion of cells could promote the formation of a vascularized cardiac patch. In line with previous descriptions (Maidhof et al., 2012), we observed more uniform and dense spatial cell distribution in the scaffold, with cells migrating toward the substrate inner core under dynamic conditions. Thus we established a method for sequential cell seeding: gelatin scaffolds were colonized with hMSCs and maintained in static conditions for 4 days in order to favor the endothelialization of the substrate, then scaffolds were loaded with pre-committed GFP-positive cardiac progenitors and cultured in perfusion bioreactor for 1 week in the presence of cardiogenic medium. As a result, a 3D cardiac proto-tissue composed of densely packed cardiomyocyte-like cells intertwined with vessel-like structures was obtained. Although being promising in terms of cell colonization and survival, these co-culture conditions did not yield the formation of functional contractile and vascular structures. Thus additional experiments will be necessary to provide cells with other cues *in vitro* to complete the organization of a proper vascularized cardiac tissue.

Nonetheless, irrespective of the stem cell subsets used, the procedure here described provides a novel platform for the preparation of complex 3D vascularized bio-constructs to be used for *in vitro* studies aiming at the understanding of stem cell behavior in a more physiological context. Pre-clinical animal studies addressing the long-term *in vivo* survival and the relevance of the constructs will be necessary before this technique may represent a suitable platform for future clinical applications.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2014.00210/abstract

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Native extracellular matrix: a new scaffolding platform for repair of damaged muscle

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Effective clinical treatments for volumetric muscle loss resulting from traumatic injury or resection of a large amount of muscle mass are not available to date. Tissue engineering may represent an alternative treatment approach. Decellularization of tissues and whole organs is a recently introduced platform technology for creating scaffolding materials for tissue engineering and regenerative medicine. The muscle stem cell niche is composed of a three-dimensional architecture of fibrous proteins, proteoglycans, and glycosaminoglycans, synthesized by the resident cells that form an intricate extracellular matrix (ECM) network in equilibrium with the surrounding cells and growth factors. A consistent body of evidence indicates that ECM proteins regulate stem cell differentiation and renewal and are highly relevant to tissue engineering applications. The ECM also provides a supportive medium for blood or lymphatic vessels and for nerves. Thus, the ECM is the nature's ideal biological scaffold material. ECM-based bioscaffolds can be recellularized to create potentially functional constructs as a regenerative medicine strategy for organ replacement or tissue repopulation. This article reviews current strategies for the repair of damaged muscle using bioscaffolds obtained from animal ECM by decellularization of small intestinal submucosa (SIS), urinary bladder mucosa (UB), and skeletal muscle, and proposes some innovative approaches for the application of such strategies in the clinical setting.

Keywords: biomaterials, extracellular matrix, tissue engineering, regenerative medicine, skeletal muscle, scaffold, decellularization

INTRODUCTION

Tissue engineering aims to mimic neo-organogenesis to produce *ex-vivo* living tissue (Carosio et al., 2013). Initial clinical experiences with bioengineered tissues have been reported in skin, cartilage, vascular grafts, bones, and several other specialized internal tissues, such as liver and kidney (Olson et al., 2011). However, owing to its intrinsic complexity, skeletal muscle remains a challenge for *in vitro* tissue engineering. Most engineered muscle structures have been obtained by employing an artificial scaffold, such as matrigel (Lü et al., 2009, 2012), or native or modified collagen (van Wachem et al., 1996; Okano and Matsuda, 1997, 1998). Decellularization of tissues and whole organs is a recently introduced platform technology for creating scaffolding materials composed of an extracellular matrix (ECM) for skeletal muscle tissue engineering. The resulting bioscaffolds (i.e., scaffold of biological origin) can then be recellularized to create potentially functional constructs as a regenerative medicine strategy for organ replacement or tissue repopulation. Indeed, the ECM represents the secreted product of the resident cells of each tissue or organ. It includes both functional and structural molecules arranged in a unique three-dimensional ultrastructure that supports the phenotype and the function of the resident cells (Reing et al., 2009, 2010). Appropriate tissue decellularization preserves not only the ECM integrity, bioactivity and spatial structure, but also the vascular, lymphatic and nervous network (Badylak et al., 2012). Moreover, a native ECM scaffold obtained by means of decellularization is also biodegradable, thereby responding to another important requirement of an ideal biomaterial for tissue engineering. Thus, a tissue-derived ECM is the ideal bioscaffold, and all the components that are retained during its preparation are likely to contribute to the success of the ECM upon implantation. Indeed, the ECM is not merely a static entity that supports the tissues, but plays a critical role in cell signaling and tissue homeostasis, provides molecules for cell-matrix interactions (such as laminin and fibronectin), maintains the appropriate physico-chemical properties, and represents a fundamental structure for mechano-transduction signals (Chiquet, 1999; Badylak et al., 2012). The ECM helps to structure niches spatially and modulate the concentration of adhesive and signaling molecules locally (Kim et al., 2011). A niche is

considered as a subset of tissue, cells and extracellular substrates (matrix and soluble factors) that support stem cells and control their self-renewal *in vivo* (Escobedo-Lucea et al., 2012). In this regard, recent studies provide strong evidence that the niche is composed of both soluble factors and ECM macromolecules that direct cell fate (Brown and Badylak, 2014). Thus, the niche represents a specialized local microenvironment that contributes to the establishment and maintenance of the stem cell phenotype and stem cell differentiation (Jones and Wagers, 2008). Indeed, the use of ECM-derived scaffolds in tissue engineering is strictly dependent on its niche properties in stem cell recruitment and differentiation. When implanted *in vivo*, an ECM-derived scaffold elicits an immune response from the host (Keane and Badylak, 2014), which in turn is responsible for one of the key events of tissue regeneration/remodeling: ECM-derived scaffold degradation. Some authors have demonstrated that ECM degradation releases both growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor, hepatocyte growth factor (Crapo et al., 2012; Choi et al., 2013; Hoganson et al., 2010), and cryptic peptides (Agrawal et al., 2011a,b; Ricard-Blum and Ballut, 2011) which activate cell surface receptors (Davis, 2010) and are required for cell viability, motility, and differentiation (Voytik-Harbin et al., 1997; Hodde et al., 2001, 2005; Badylak et al., 2009). They all may subsequently induce the events that lead to tissue regeneration. In particular, these factors are hypothesized to polarize the macrophage phenotype toward an M2 anti-inflammatory phenotype (Turner and Badylak, 2013), rather than toward an M1 pro-inflammatory phenotype, and to recruit different stem or progenitors cells that may give rise to new tissue formation (Agrawal et al., 2011a,b), vasculature and innervation (Agrawal et al., 2009; Sicari et al., 2012; Turner et al., 2012). Indeed, it has recently been demonstrated that native ECM scaffolds from skeletal muscle elicit M2 macrophage polarization during the host inflammatory response (Valentin et al., 2009; Turner and Badylak, 2013). M2 macrophages play a key role in the resolution of inflammation as well as in the activation of satellite cells during skeletal muscle regeneration (Kharraz et al., 2013). In skeletal muscle tissue engineering, the use of an ECM-derived scaffold *in vivo* has been shown to recruit $CD133^+$ cells (Turner et al., 2012), recently identified as progenitors of a myogenic cell population, as well as $Sca1^+/PW1^+$ cells identified as muscle interstitial stem cells, named PICs (Perniconi et al., 2011), Sox2+, and Sca1+,Lin- cells (Agrawal et al., 2011a,b). The suggestion that ECM degradation products directly affect macrophage polarization is also supported by evidence indicating that chemically cross-linked ECM scaffolds, which are not biodegradable. The fact that the ECM of each specific tissue has a specific biochemical composition and 3D structure that influence the host response through the release of suitable GFs (Hoganson et al., 2010; Agrawal et al., 2011a,b; Crapo et al., 2012; Choi et al., 2013) and of specific cryptic peptide (which are retained after decellularization and are released during ECM degradation), implies that the tissue specific ECM may elicit the growth and differentiation of those tissue specific cells and has some advantages over ECM from non-homologous tissues.

The maintenance of 3D architecture has a significant relevance to the regeneration of complex organs and tissues. In particular, skeletal muscle functionality is strictly dependent on the correct alignment of myofibers (Boontheekul et al., 2007; Klumpp et al., 2010). A native ECM scaffold from skeletal muscle tissue presumably preserves the correct architecture of the native ECM surrounding each myofiber. On the basis of these considerations, a novel approach to tissue engineering of skeletal muscle, which involves the use of three-dimensional bioscaffolds made of an allogeneic or xenogeneic ECM derived from skeletal muscle tissue, has been proposed. The large scaffolds required for this approach may be of cadaveric origin and may to some extent be stored. However, the harvesting of muscle tissue from the same patient may be considered for minor defects or for plastic surgery. Owing to the scarcity of studies on skeletal muscle-derived ECM, the present review investigates the reconstruction of skeletal muscle tissue based on both skeletal muscle and non-skeletal muscle (SIS and UB) ECM decellularized tissue. The state of the art regarding studies on ECM derived from decellularized tissues for skeletal muscle tissue regeneration/remodeling worldwide, their current applications in clinics and future perspective are also discussed. A review of cartilage- and bone-derived ECM bioscaffold production and use, as opposed to skeletal muscle-derived ECM, has been recently published (Cheng et al., 2014).

DECELLULARIZATION OF TISSUES FOR ECM-BASED SCAFFOLD PRODUCTION

Decellularization is the first step of a strategy that attempts to obtain a biologically engineered construct that resembles the native tissue or organ as closely as possible (Badylak et al., 2012). This strategy is particularly suitable for complex tissues or organs that require the maintenance of spatial architecture for translational applications.

Processing methods play a critical role in determining the type of host response (Valentin et al., 2009; Faulk et al., 2014a,b). The challenge faced by each decellularization method is to completely remove the cellular component and DNA content (less than 50ng of dsDNA per 1 mg dry weight of ECM scaffold) while preserving the ECM biochemical features, architecture, ultrastructure, and porosity (Crapo et al., 2011). Decellularization protocols are based on physico-chemical agents, enzymes, detergent solutions, or a combination of these (Crapo et al., 2011). Physical reactions, such as freeze-thaw cycles, and mechanical forces, such as hydrostatic pressure, are sufficient to induce cell lysis. Chemical agents include: per-acetic and acetic acid, which remove nucleic acids but affect collagen; bases (such as NaOH), which destroy growth factors; hypertonic or hypotonic solutions, which lyse cells through osmotic shock (Crapo et al., 2011). Commonly used detergents include Triton X-100 and CHAPS for thick tissues, or low percentages of SDS (sodium dodecyl sulfate) for whole organ decellularization. These detergents solubilize the cell membrane and disrupt DNA (Meyer et al., 2006; Jones and Wagers, 2008; Giusti et al., 2009; Petersen et al., 2010; Reing et al., 2010; Crapo et al., 2011), though they have negative effects on protein and ultrastructure. Chelating agents, such as EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylene glycol tetraacetic

acid), help to dissociate cells from the ECM, while serine protease inhibitors, such as PMSF (phenylmethylsulfonyl fluoride), aprotinin, and leupeptin, prevent ECM damage (Crapo et al., 2011). Several types of enzymes, such as nuclease, trypsin, and collagenase, can be used to specifically and quantitatively perform some of the afore-mentioned tasks. A balance must be achieved between chemical and physical treatments during the decellularization process (Macchiarini et al., 2008; Ott et al., 2008; Escobedo-Lucea et al., 2012). The most effective agent for decellularization of a specific tissue or organ depends on many factors, including the tissue size, cellularity, density, lipid content, and thickness. For thin tissue laminae, such as the urinary bladder, intestine, pericardium, and amnion, decellularization techniques include freezing and thawing, mechanical removal of muscle or submucosa, and brief treatment with detergents or acid (Crapo et al., 2011). For thick tissue laminae, such as dermis, a more extensive biochemical exposure and a longer incubation time are required. Adipose tissue, brain, and pancreas typically require the addition of lipid solvents (Crapo et al., 2011). For whole organ decellularization, the action of detergent and biological agents is enhanced by antegrade or retrograde perfusion, as demonstrated for the heart (Ott et al., 2008; Wainwright et al., 2010), lung, liver, and other organs (Petersen et al., 2010; Price et al., 2010; Shupe et al., 2010; Bonvillain et al., 2013; Tsuchiya et al., 2014), or even by agitation in the decellularization solution, as is done for the majority of tissues (Crapo et al., 2011; Perniconi et al., 2011). As far as skeletal muscle tissue decellularization is concerned two of the most recent methods are very different. Some authors described a method requiring several days for tibialis anterior murine muscle (TA), based on treatment with latrunculin B that disrupts actin, osmotic shock for cell lysis, and ionic salt solution for depolymerizing myosin. The resulting bioscaffold is completely decellularized, but preserves the amount of collagen and glycosaminoglycans (GAGs) required, the overall architecture of the native ECM, and the mechanical integrity (Gillies et al., 2011). By applying the same protocol, Fishman et al. (2013) also achieved the decellularization of rabbit cricoarytenoid dorsalis muscle (CAD). The second method (Perniconi et al., 2011) is based on a 1% SDS detergent solution and requires only 48 h to obtain a murine TA scaffold. This method resulted in the decellularization of skeletal muscle, while preserving the biochemical features (such as collagen, laminin, and fibronectin) and 3D-architecture of the native ECM. Moreover, when implanted to replace the whole host homologous muscle *in vivo*, this bioscaffold represented a pro-myogenic environment (Perniconi et al., 2011). Previous studies that have addressed skeletal muscle tissue engineering using a native-ECM approach were based on long and complex decellularization processes. One such example is the decellularization of rat abdominal muscles achieved by means of a protocol based on osmotic shock, 4% sodium deoxycholate and DNase-I, which required more than 3 days (Conconi et al., 2005). The resulting scaffold supported myoblast growth and differentiation *in vitro*. When the decellularized muscle seeded with myoblasts was implanted between the *obliquus externus abdominis* and the *obliquus internus abdominis*, neovascularization and the formation of new myofibers occurred within 2 months (Conconi et al., 2005). Merritt et al. decellularized a rat gastrocnemius (GAS) by means of a protocol based on osmotic shock and detergent solutions that required several days (Merritt et al., 2010b). The resulting decellularized GAS was used as a patch to repair a muscle defect: the graft did not elicit an immune response and was capable of supporting the growth of myofibers (between 28 and 42 days after transplantation) and blood vessels (between 7 and 28 days after transplantation). More recently, Fishman et al. (2013) decellularized rat TA by cycles of freeze-thaw and distilled water for 72 h, followed by enzymatic treatment (trypsin) and Triton X-100 for a further 4 days. The acellular native ECM was transplanted to repair a volumetric muscle injury. Two weeks after transplantation, the graft displayed extensive infiltration of CD68 positive cells, few regenerating myofibers in the areas surrounding the injured muscle and vascularization (Wu et al., 2012). To sum up, the afore-mentioned studies demonstrate (i) that native ECM-derived scaffold from various tissues can be obtained by means of different protocols that may require several days (Gilbert et al., 2006), and (ii) that this scaffold supports growth and survival of myogenic cells both *in vitro* and *in vivo*, and that it represents a pro-myogenic environment.

The importance of decellularization methods and their effect upon the resulting ECM structure and composition is currently highly relevant, as is demonstrated by the fact that at least two other reviews, besides ours, are being published in 2014. Since the advantages and drawbacks of several decellularization methods have been systematically reviewed (Badylak, 2014; Brown and Badylak, 2014; Faulk et al., 2014a,b), we recommend that they be referred to for details on these issues. We believe that each of these studies unveils important information on the native-ECM derived scaffold used to reconstruct skeletal muscle tissue: on its ability to support cell viability and growth *in vitro*, to provide a pro-myogenic environment *in vivo*, to elicit an immune response polarized toward an M2 macrophage phenotype and to preserve its molecular and structural features, as well as on the time and mode of decellularization and possible storage conditions. However, none provides details leading to the full reconstruction of a whole muscle. An overview of the literature on the topic is shown in **Table 1**.

CHARACTERIZATION OF ECM BIOSCAFFOLDS

FROM XENOGENIC MATRIXES TO SKELETAL MUSCLE

The biomechanical and biochemical properties of ECM scaffolds derived from porcine small intestinal submucosa (SIS) and porcine urinary bladder mucosa (UB) have been well characterized (Derwin et al., 2006; Freytes et al., 2008a,b). Porcine SIS ranges in thickness from 0.05 to 0.22 mm and has a variable porous microstructure, though it is always sufficient for oxygen diffusion to sustain cell proliferation and viability and is biodegradable in 3 months. It is primarily composed of type I collagen fibers, but also contains a minor amount of elastin and collagen types III, IV, and VI. Multi-domain glycoproteins such as fibronectin and laminin, which mediate cell adhesion to the extracellular matrix, have also been identified in SIS. Moreover, SIS contains glycosaminoglycans and proteoglycans that provide cell attachment and growth factor binding sites, sequesters matrix-degrading enzymes and enhances cellular infiltration into injured tissue. It has also been documented that SIS releases growth

Table 1 | Overview of the literature on ECM-based bioscaffolds.

Who, When	Where	Source	Target
Agrawal et al., 2009	University of Pittsburgh, Pennsylvania, USA	Porcine urinary bladder matrix	Canine oesophagus; rat abdominal muscular wall
Alberti and Xu, 2013	Tufts University, Medford, USA	Bovine achilles tendon	Potential role for nerve guidance conduits and blood vessel tissue engineering
Borschel et al., 2004	University of Michigan, Ann Arbor, USA	Murine skeletal muscles	C2C12 culture *in vitro*
Chen and Walters, 2013	United States Army Institute of Surgical Research, Extremity Trauma and Regenerative Medicine Research Program, San Antonio, TX, USA, and Wake Forest Institute for Regenerative Medicine, Winston-Salem, NC, USA	Rat muscle-derived extracellular matrix	Reconstruction of VML in rat latissimus dorsi
Carmignac and Durbeej, 2012	Lund University, Lund, Sweden	ECM-cell membrane-cytoskeleton interactions	Treatment of muscle disorders
Conconi et al., 2005	University of Padova, Italy	Rat skeletal muscle seeded with myoblasts	Muscle fibers in syngeneic host
Corona et al., 2013	US Army Institute of Surgical research, Fort Sam, Houston, USA	Rat skeletal muscle	Enhanced mechanical stability in VML
Dai and Xu, 2011	Tufts University, Medford, USA	Adult cow tendons	Future application in other natural materials, e.g., muscles
Gillies et al., 2011	University of California San Diego, La Jolla, USA	Murine skeletal muscles	C2C12 culture *in vitro*
Mase et al., 2010	Institute of Surgical Research, Houston, USA	Porcine intestinal submucosa	Human right thigh medialis muscle
Merritt et al., 2010a	University of Texas, Austin, USA	Rat muscle-derived ECM seeded with bone-marrow-derived mesenchymal stem cells	Rat lateral gastrocnemius skeletal myofibers
Milner and Cameron, 2013	University of Illinois, Urbana, USA	Skeletal muscle	Amphibian limbs
Perniconi et al., 2011	Sapienza University of Rome, Rome, Italy	Murine skeletal muscles	Muscle fibers in syngeneic host
Sicari et al., 2012	University of Pittsburgh, Pennsylvania, USA	Porcine intestinal submucosa	VML in murine quadriceps
Stern et al., 2009	Wake Forest University School of Medicine, Winston-Salem, USA	Hamster skeletal muscles	Coating for C2C12 colture *in vitro*
Turner et al., 2010	University of Pittsburgh, Pennsylvania, USA	Xenogeneic ECM	Dog distal gastrocnemius musculotendinous junction
Turner et al., 2012	University of Pittsburgh, Pennsylvania, USA	Dog small intestinal submucosa	Dog quadriceps skeletal muscle
Valentin et al., 2010	University of Pittsburgh, Pennsylvania, USA	Carbodiimide-crosslinked porcine SIS; autologous tissue; polypropylene mesh	Rodent abdominal wall
Vindigni et al., 2004	University of Padua, Italy	Rat rectus abdominis seeded with satellite cells	Reconstruction of homologous muscle
Wolf et al., 2012	University of Pittsburgh, Pennsylvania, USA	Dog skeletal muscle; porcine small intestine submucosa	Rat abdominal muscular wall
Wu et al., 2012	University of Texas, San Antonio, Texas, USA	Rat tibialis anterior muscle	Rat tibialis anterior muscle

List of works dealing with the production, characterization, and use of ECM-derived bioscaffolds obtained by means of tissue/organ decellularization and proposed for tissue engineering applications.

factors, including FGF-2, TGF-β1, and VEGF (Voytik-Harbin et al., 1997; Badylak et al., 1998; Mase et al., 2010). In a body wall repair, a canine model SIS has been shown to elicit the formation of new ECM associated with skeletal muscle and adipose tissue regeneration (Badylak et al., 2002). Thus, SIS is an ideal remodelable biomaterial for muscle tissue engineering, thanks to its size, thin membranous configuration, relative uniformity, and availability. SIS has so far been used to repair inguinal hernia and large

abdominal wall defects, the urinary tract, achilles tendon, musculotendinous tissues and dura mater, and as vascular graft material and for dermal wounds (Prevel et al., 1995; Cobb et al., 1996, 1999; Dejardin et al., 1999, 2001; Kropp, 1999; Shalhav et al., 1999; Portis et al., 2000; Badylak et al., 2002).

ECM scaffold materials derived from porcine UB have been extensively studied recently. Two ECM bioscaffolds can be derived from adjacent layers of porcine UB: urinary bladder matrix and urinary bladder sub mucosa, derived from tunica mucosa including the basement membrane and from tunica sub mucosa, respectively. The advances made in ECM scaffold technology have led to the development, manufacturing, and commercialization of a naturally-occurring porcine urinary bladder matrix (Lecheminant and Field, 2012; Kruper et al., 2013; Rommer et al., 2013). It has been demonstrated that it allows the retention of multiple growth factors including VEGF, transforming growth factor beta (TGFβ), platelet derived growth factor (PDGF), bone morphogenetic protein 4 (BMP4), and basic fibroblast growth factor (BFGF), which contribute to regeneration and healing. Recent studies have demonstrated the antimicrobial properties of UB-ECM to both *S. aureus* and *E. coli* (Brennan et al., 2006) as well as a pre-dominance of macrophage phenotype 2 in the early post-operative period, which has been shown to reduce scar formation and enhance healthy functional tissue regeneration with increased chemotactic activity (Turner and Badylak, 2013).

FROM SKELETAL MUSCLE TO SKELETAL MUSCLE

In spite of the extensive characterization of SIS and UB, as yet very little information has been provided on ECM derived from skeletal muscle tissue. The need for a bioscaffold that preserves the native 3D-architecture of the ECM is particularly evident for skeletal muscle tissue engineering applications, in which the correct parallel alignment of myofibers and the maintenance of mechanical properties are essential for tissue functionality. Indeed, the only extensive report demonstrating that decellularized skeletal muscle preserved its biochemical features, such as collagen, laminin, and fibronectin, as well as the 3D-architecture of the native tissue, is the one by Perniconi et al. (2011). Their study provides evidence indicating that such a muscle ECM-derived scaffold provides the correct support for myofiber development and the appropriate architecture for muscle fiber formation. In particular, when this bioscaffold was orthotopically grafted in syngeneic animals, the activation of myogenic cells and the formation of new myofibers in areas within the bioscaffold was observed. However, 4 weeks after transplantation, the grafts had almost completely degraded, probably owing to digestion by neutrophils and macrophages. In this regard, immunosuppressors such as Cyclosporin A have been reported to enhance the myogenic process and delay graft digestion (Perniconi et al., 2011). The decellularized bioscaffold, together with the removal of the host muscle, is likely to have elicited an inflammatory response dominated by neutrophils and macrophages. The inflammatory process peaked 2 weeks after transplantation and then declined. This process was, however, accompanied by the presence of stem cells expressing PW1, a global stemness marker described by Sassoon and co-workers (Besson et al., 2011) and by the formation of new myofibers within the bioscaffold from 1 to 3 weeks after transplantation. In the 4th week, the graft started undergoing biodegradation (Perniconi et al., 2011). This study also demonstrated that the decellularized scaffold can be stored for several weeks in sterile conditions at either +4°C in PBS or +36°C in DMEM in culture conditions, though laminin preservation in the latter case was worse. The possibility of storing the acellular scaffold is highly relevant to the use of such devices in future clinical applications. Other recent skeletal muscle acellular scaffold preparations (described in the decellularization section) from TA and CAD have been shown to retain the overall architecture of native ECM. In particular, Gillies et al. (2011) demonstrated that TA-derived acellular scaffolds not only maintained their collagen and glycosaminoglycan (GAG) levels, the overall architecture of the native ECM, their mechanical integrity, but also supported murine myogenic cells C2C12 survival *in vitro*. By contrast, decellularized CAD implanted in the rat abdominal wall modulated the immune response, polarizing macrophages toward the M2 phenotype and inducing T-cell hypo-responsiveness (Fishman et al., 2013).

STATE OF THE ART OF ECM BIOSCAFFOLD GRAFTING

Although the use of the decellularized ECM for skeletal muscle tissue engineering is a recent innovation, the results of studies that have been published to date on this topic are promising. Indeed, the majority of data have demonstrated that the ECM can support muscle and blood vessel regeneration, though recovery of function is not complete (Merritt et al., 2010b).

Animal models, consisting mainly of rats, mice, rabbits, and dogs subjected to experimentally induced skeletal muscle injury, have been used as recipients of xenogeneic ECM scaffold grafts to test surgical treatment for volumetric muscle loss. To our knowledge, such treatment has been applied to humans a very limited number of times. One such case was described in a case report by Mase et al., who used porcine SIS to restore multiple injuries in the quadriceps muscle in a young soldier (Mase et al., 2010). Implantation of the SIS supported by intensive physical therapy improved the pick torque, total work, and average power of the grafted limb 16 weeks post-operatively (Mase et al., 2010). A very recent study demonstrated the remodeling characteristics of xenogeneic porcine SIS ECM bioscaffolds when used to surgically treat volumetric muscle loss in five male patients (Badylak et al., 2012; Sicari et al., 2014). That study showed that ECM bioscaffold implantation was associated with perivascular stem cell mobilization and accumulation within the site of injury and *de novo* formation of skeletal muscle cells. Overall, the studies in the literature demonstrate that ECM bioscaffold implantation recruits stem cells within the bioscaffold and promotes differentiation and *de novo* formation of skeletal muscle cells. Although the mechanism of action underlying stem and progenitor cell recruitment is not yet fully understood, it might be ascribed to extracellular matrix chemotactic cryptic peptide (Agrawal et al., 2011a,b; Ribeiro et al., 2012) and the host's immune response, which induces the macrophage 2 phenotype (Brown et al., 2012; Turner and Badylak, 2013). This is in agreement with our observation that macrophages largely account for the inflammatory invasion of muscle ECM-derived bioscaffolds, and are consequently likely to play a major role in muscle graft integration (Perniconi et al.,

2011). The ability of bioscaffolds to alter the macrophage phenotype response, coupled with the release of latent growth factors and chemotactic degradation products, means these materials lend themselves to being used as scaffolds to promote skeletal muscle reconstruction following trauma and volumetric loss (Mase et al., 2010; Turner and Badylak, 2013). The clinical results of these applications may vary for a wide range of reasons that include not only the characteristics of the source tissue, methods, and efficacy of tissue decellularization, and methods of processing/manufacturing, but also the response of the host to these implanted biological scaffold materials, which may hamper the success of the implant. Although several studies on the remodeling characteristics of ECM scaffolds are currently in progress, to the best of our knowledge a systematic temporal evaluation of the structural and functional muscle remodeling following the implantation of acellular scaffolds is still lacking in the literature.

PROBLEMS AND CHALLENGES

The use of ECM biologic scaffolds for skeletal muscle tissue reconstruction is attracting a high degree of interest in regenerative medicine. Recent advances have opened new perspectives for the replacement of skeletal muscle tissue in clinical applications, such as traumatic injury and pathological conditions. However, these highly promising applications in the field of regenerative medicine require a greater understanding of the biochemical, cellular, and mechanical mechanisms that stimulate the constructive remodeling response. Although ECM bioscaffolds implanted in an injury site have been shown to promote the migration and proliferation of progenitor cells, the triggers that cause these cells to differentiate into site appropriate tissue are still unknown. Moreover, a better characterization of ECM bioscaffolds derived from different tissues may help to select ECM bioscaffolds that are most suited to constructive remodeling in different sites. Improved methods of decellularization and ECM bioscaffold preparation may improve the retention and release of growth factors, and consequently increase the success of implantation. Last but not least, improved quality control should be aimed at the optimization of ECM scaffold decellularization and implantation so as to enhance the likelihood of survival and reduce that of rejection (Koch et al., 2012). For example, image cytometry technologies of tissues (also referred to as tissue cytometry Heindl et al., 2013) could easily be adapted to quantify the remaining cells or cell fragments on scaffolds after staining with appropriate fluorescent markers for DNA, RNA, organelles, specific cells, etc. By adopting suitable approaches, image cytometry may also be adopted for acellular or hypocellurar tissues (Fueldner et al., 2012). Moreover, like all cytometry techniques, tissue cytometry may be standardized to optimize reproducibility (Mittag and Tarnok, 2009), and since tissue cytometry can be multiplexed by using different colors for different targets (Gerner et al., 2012), minimal specimen amounts are needed. Non-invasive label free detection methods may also be applied (Nallala et al., 2013), implying that this technique may prove useful for pre-implantation quality assessment. Last, despite progresses made in the research and clinical application of skeletal muscle tissue engineering in recent decades, further efforts aimed at whole muscle engineering are required. Indeed, the use of generic ECM scaffolds for a wide range of applications are unlikely to be sufficient for complex organs, and ECM derived from homologous organs may be required to support site specific differentiation of appropriate cells. Investigations on whole organ scaffolds are needed to shed further light on this issue in the future.

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Targeting pleiotropic signaling pathways to control adult cardiac stem cell fate and function

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The identification of different pools of cardiac progenitor cells resident in the adult mammalian heart opened a new era in heart regeneration as a means to restore the loss of functional cardiac tissue and overcome the limited availability of donor organs. Indeed, resident stem cells are believed to participate to tissue homeostasis and renewal in healthy and damaged myocardium although their actual contribution to these processes remain unclear. The poor outcome in terms of cardiac regeneration following tissue damage point out at the need for a deeper understanding of the molecular mechanisms controlling CPC behavior and fate determination before new therapeutic strategies can be developed. The regulation of cardiac resident stem cell fate and function is likely to result from the interplay between pleiotropic signaling pathways as well as tissue- and cell-specific regulators. Such a modular interaction—which has already been described in the nucleus of a number of different cells where transcriptional complexes form to activate specific gene programs—would account for the unique responses of cardiac progenitors to general and tissue-specific stimuli. The study of the molecular determinants involved in cardiac stem/progenitor cell regulatory mechanisms may shed light on the processes of cardiac homeostasis in health and disease and thus provide clues on the actual feasibility of cardiac cell therapy through tissue-specific progenitors.

Keywords: cardiac stem cell, heart regeneration, cell signaling, differentiation, stem cell homeostasis

INTRODUCTION

Early pre-clinical and clinical trials involving skeletal myoblasts (Menasché et al., 2008), mesenchymal (Dixon et al., 2009) and hematopoietic stem cells (Balsam et al., 2004; Murry et al., 2004) proved mild, non-reproducible effects on cardiac diseases. In fact, the intracoronary delivery of bone marrow mesenchymal stem cells (MSCs) in patients affected by acute myocardial infarction (AMI) only proved their worth improving left ventricular ejection fraction by 3%, while failing to modify the negative impact of ventricular remodeling. In the case of chronic heart failure (CHF), skeletal myoblast or bone marrow mononuclear cell implantation failed to provide any benefit to the patient, other than a small improvement ascribed to the secretion of beneficial factors. A different scenario appears when the outcome of the pathology can be improved by neoangiogenesis, like in the case of refractory angina (RA). In this case, bone marrow cells (namely CD34+ hematopoietic progenitors) can provide some degree of endothelial differentiation together with the positive paracrine effect to help the cause (Menasché, 2011).

Anyway, the rate of transdifferentiation obtained by bone marrow or skeletal muscle-derived progenitors is limited. Thus, cardiac resident progenitor cells (CPCs) are considered the elective source of autologous contractile cells for transplantation (Chugh et al., 2012). Among adult cardiac resident stem cells, the few subsets identified on the basis of the expression of c-kit (CD117, Quaini et al., 2002) or Stem Cell Antigen 1 (Sca-1, Bailey et al., 2012) are still questioned for their actual role in contractile tissue homeostasis and regeneration.

Despite the incredible amount of information regarding the role of specific signaling pathways acting during cardiogenesis to finely control cell specification within the forming organ, little is known about the molecular mechanisms governing adult tissue-specific progenitor cell function.

The issue of tissue-resident stem cell fate and function control has been so far addressed by using the instruments and knowledge coming from the study of somatic cells.

Nonetheless, compelling evidence supports the notion that tissue-resident stem cell behavior and fate may be, at least in part, modulated through non-canonical stimuli able to activate cell-specific signaling pathways and in turn leading to a given biological response (Guilak et al., 2009; Kaivosja et al., 2012).

The organogenesis and healing programs present a high degree of complexity because of the precise spatial and temporal activation of signaling pathways that are deemed to interact with each other.

In the present review, we would like to draw the attention on the possibility that the regulation of cardiac resident stem cell fate and function is the result of the interplay between pleiotropic signaling pathways and cell-specific regulators. Thus we summarize the main pathways acknowledged to participate in the regulation of adult CPC fate and function so far.

Wnt SIGNALING PATHWAY

Many biological processes occurring in cardiac stem cells, including cardiac specification, survival and differentiation, have been associated to Wnt signaling pathway (Flaherty et al., 2012). Wnt signals consist of secreted glycoproteins acting in a paracrine fashion by binding to Frizzled (FZD) family of trans-membrane receptors and activating the so-called canonical or non-canonical transduction pathways.

Different co-receptors are independently activated in response to canonical (LRP-5/LRP-6) or non-canonical (ROR2 and RYK) signaling (Katoh and Katoh, 2007) and intracellular Wnt signaling regulators are also involved in the transduction of one or the other pathway. β-catenin is the main effector of canonical Wnt signaling whereas non-canonical Wnt signals are transduced through the Rho/Rac/JNK-dependent or the calcium-dependent signaling cascades.

Wnt pathways play a role in the development and differentiation of both the embryo and adult heart and are highly interconnected. The nuclear translocation of β-catenin was proven to up-regulate the transcription of genes controlling CPC amplification (e.g., c-myc and cyclin D1). On the other hand, the inhibition of β-catenin-dependent transcription via proteasomal degradation of its cytoplasmic form triggers CPC differentiation toward the contractile phenotype (Bergmann, 2010).

Although no evidence has been provided regarding the activation of β-catenin signaling in the healthy adult heart (Cohen et al., 2008), this pathway is being activated after myocardial infarction (Bergmann, 2010). Interestingly, β-catenin depletion is beneficial to infarcted post-natal mouse heart leading to both attenuated mortality and LV remodeling. It also up-regulates the expression of Tbx5 and GATA4 cardiac lineage markers while enhancing Sca-1+ resident cardiac progenitor cell terminal differentiation (Zelarayan et al., 2008).

Consistently Krueppel-like factor 15 (KLF15)—a transcription factor regulating cardiac β-catenin transcriptional activity—was found to be critical in controlling CPC homeostasis. In fact, the *in vivo* proliferative and cardiogenic potential of Sca-1+ CPCs was reduced in hearts derived from Klf15-/- mice in normal and hypertrophic conditions. Additionally, the activation of β-catenin-dependent transcription upon loss of KLF15 prompted the CPCs toward an endothelial phenotype (Noack et al., 2012).

Following this experimental evidence, Wnt/β-catenin signaling inhibition via the local release of effective Wnt scavengers or inhibitors (e.g., soluble frizzle-related proteins sFRPs and the secreted protein Dkk1) has become an attractive therapeutic target to prevent heart failure and preserve cardiac function.

Moreover the reactivation of Wnt pathway could be explored as a suitable strategy to protect adult c-kit$^+$ CPCs from oxidative stress-mediated apoptosis in infarcted heart (Liu et al., 2013) and restore LV function (Bergmann, 2010).

More recently, a biphasic function for Wnt/β-catenin signaling during cardiac development has been also proposed, implying that the effects of Wnt signals (including Wnt1, Wnt2a, Wnt3a, and Wnt8) or their inhibitors can depend on the stage of development (Naito et al., 2006; Tzahor, 2007; Ueno et al., 2007).

Studies on the role of Wnt/β-catenin signaling during cardiac tissue specification demonstrated that the upregulation of Wnt/β-catenin signaling is essential to the formation of mesodermal tissue (Huelsken et al., 2000) and to enhance the cardiac commitment or the expansion of cardiac progenitors during the early phases of cardiogenesis (Qyang et al., 2007; Lindsley et al., 2008).

At a later stage, the inhibition of Wnt/β-catenin signals, mediated by the early cardiac-specific marker Mesp1, is pivotal for the terminal differentiation of precursors into cardiomyocytes (David et al., 2008).

Noteworthy, the Wnt/β-catenin signaling seems also to inhibit early cardiac commitment of progenitors via a non-cell-autonomous mechanism, very likely to be exerted through the release of signals by the associated endoderm in mouse ES cells and embryos (Lickert et al., 2002; Liu et al., 2007). As such, Wnt signals would participate in cardiac specification and differentiation through several phases of activation and inhibition (Gessert and Kuhl, 2010) as well as in the regulation of cell-cell interaction through the involvement of secondary factors acting on cardiac mesoderm formation during heart development. In this respect, the effects of this universal pathway would be the result of its interaction with cell-specific adaptors, thus leading to a context- and cell-specific response.

For instance, a positive effect on the proliferation of neonatal and embryonic Isl-1$^+$ CPCs from both *in vivo* and *ex vivo* explants was exerted by Wnt/β-catenin signaling through the downstream activation of multiple FGF ligands, in particular FGF10 (Cohen et al., 2007). In contrast, an excess of β-catenin signaling as a result of the up-regulation of insulin-like growth factor-binding protein 3 (IGFBP3) was associated to the reduction of the proliferative capacity in adult cultured Sca-1+/Islet-1- cardiac stem cells (Oikonomopoulos et al., 2011).

Recently, an increased β-catenin activity mediated by stromal cell derived factor 1α (SDF1α) has been associated with a reduction in c-kit+ CPCs proliferation. This event might restrain heart endogenous reparative response following conditions (stress or injury) characterized by high levels of the cytokine (Dimova et al., 2014). The reduction of proliferation in response to β-catenin activation might be suggestive of cardiac commitment/differentiation of tissue-specific progenitors. In accordance with this hypothesis, loss-of function experiments established an undisclosed relationship between Sca-1 antigen and canonical Wnt signaling in regulating the balance between c-kit+ CPC proliferation and differentiation. In fact, c-kit+ CPCs from Sca-1-knockout models exhibited poor proliferation and increased cardiogenic commitment, very likely due to the activation of β-catenin signaling (Bailey et al., 2012).

In contrast to canonical Wnt signaling, non-canonical pathway is less well characterized and its role in cardiac stem cell biology remains to be elucidated. Several studies have evidenced a positive effect of non-canonical Wnt signaling in mouse ES cell differentiation (Cohen et al., 2008), with Wnt5a and Wnt11 being requested to promote cardiac differentiation through non-canonical pathway in both the embryo and progenitor cells (Eisenberg and Eisenberg, 1999; Pandur et al., 2002; Brade et al., 2006; Palpant et al., 2007; Flaherty and Dawn, 2008; Cohen et al., 2012).

Current evidence suggests that some degree of overlapping exists between the two pathways, although the actual relevance of this interaction in cardiac tissue formation and repair remains poorly understood.

NOTCH SIGNALING PATHWAY

Notch pathway is involved in the regulation of tissue homeostasis and formation during embryonic and adult life by controlling cell fate decision and the maintenance and differentiation of stem cells (Nemir and Pedrazzini, 2008). Notch activity participates in cardiogenesis at multiple stages, from the commitment of mesoderm to the maturation of myocytes, in a context- and time-dependent fashion (Gude and Sussman, 2012).

In the developing heart, Notch regulates ventricular trabeculation, cardiomyocyte proliferation and differentiation, as well as valve formation (High and Epstein, 2008).

Interestingly, both the over-expression and inhibition of Notch signaling have been associated with the onset of cardiac defects (Kratsios et al., 2010), pointing at its fine regulation as a key determinant in fetal heart development.

In mammals, Notch pathway mediates the interaction between adjacent cells expressing Notch receptors (Notch1–4) and their ligands (Delta-like 1, 3, 4, and Jagged 1, 2) (Chiba, 2006; Bolós et al., 2007).

Consequently, the fate and function of any given cell are strictly regulated by adhesive intercellular interactions and dependent on the fate of the neighboring cells. Upon the binding of the ligand, Notch intra-cytoplasmic domain (NICD) is released by proteolytic cleavage, can translocate to the nucleus, interact with DNA-binding proteins and participate in the control of downstream effectors.

The most well-known downstream effectors of Notch pathway are Hes and Hes-related (Hesr) family of proteins, acting as repressors of the transcription of Notch-dependent genes (Iso et al., 2003).

In the post-natal heart, Notch1 is the predominant isoform, being expressed in immature cardiomyocytes and non-myocyte cells (Croquelois et al., 2008; Gude et al., 2008; Nemir and Pedrazzini, 2008). The activation of this pathway in adult heart evokes pro-survival, proliferative and anti-apoptotic effects in cardiomyocytes, thus contributing to attenuate cardiac hypertrophy, fibrosis and ameliorate cardiac performance (Croquelois et al., 2008; Nemir et al., 2012).

Together with its target gene Hes-1, Notch pathway was found up-regulated in cardiomyocytes at the border zone of the infarcted region (Gude et al., 2008) and also cardiomyocyte treatment with hepatocyte growth factor (HGF), exerting cardioprotective effects through its receptor c-Met, was found to act through the activation of Notch signaling.

NICD over-expression in the infarcted heart caused functional and morphological improvement, although no information on the possible contribution of resident stem cells to this process was given. Nonetheless, since c-kit+ and Sca-1+ cardiac progenitors express c-Met receptor and secrete HGF (Urbanek et al., 2005), the homeostasis of the adult myocardium might, at least in part, be guided by the activation of Notch pathway in undifferentiated cells homing to the damaged site.

Consistently, evidence is being accumulated suggesting that resident CPC recruitment and differentiation is an effect of Notch-mediated induction of reparative mechanisms in the damaged adult myocardium (Campa et al., 2008; Gude et al., 2008; Kratsios et al., 2010; Li et al., 2010).

Additionally, cycling CPCs displayed high Notch activity as a means to restrict their cardiomyocyte differentiation potential, thus predicting for Notch a role in maintaining the self-renewal and stemness state of the cardiac precursor pool residing in mouse and rat normal adult hearts (Collesi et al., 2008; Croquelois et al., 2008).

This result paralleled those obtained in embryonic stem cells in which the activation of Notch signaling hindered the differentiation of mesodermal progenitors into cardiomyocytes (Nemir et al., 2006; Schroeder et al., 2006). Furthermore, in a late stage of the hypertrophic response, Jagged-1 ligand expression was found up-regulated on the membrane of cardiomyocytes: this event was shown to affect the expansion of the neighboring Notch-positive cardiomyocytes and progenitor cells.

However, these results were obtained in a non-purified population of cardiac stem/progenitor cells, exclusively selected for their lack of markers of terminal differentiation.

The *in vivo* analyses of adult murine cardiac tissue highlighted an interesting pattern in Notch distribution: a baseline expression of Notch-1 receptor in c-kit+ CPCs residing in cardiac niche was confirmed. As expected, these cells are surrounded by cells expressing Jagged-1 ligand. A similar correlation between Notch-1 and Sca-1 expression in stem cell niche has been also suggested (Boni et al., 2008).

Although these data seem to imply a linear dependence of stem cell niche regulation by the surrounding tissue, a more sophisticated Notch-based mechanism has been shown to regulate the balance between the proliferation and differentiation in cardiac stem cells, thus questioning the actual role of Notch on the maintenance of stemness phenotype. In fact, upon Jagged-1-mediated stimulation, Notch-1 activation paralleled the up-regulation of Nkx-2.5, an early regulator of cardiac commitment, driving the cell toward a phenotype of highly proliferative myocytes.

These results would provide evidence of the existence of a modular interplay between the ubiquitous Notch pathway and cell-specific actuators (Nkx-2.5) to evoke a unique response of cardiac progenitors to external stimuli.

In the border zone of the infarcted myocardium, the pharmacological inhibition of Notch reduced the number of cycling c-kit+ CPCs and compromised their cardiac commitment. Accordingly, a severe dilated myopathy developed in newborn mice following the inhibition of Notch signaling, as a consequence of the reduced differentiation rate of cardiac progenitors (Urbanek et al., 2010).

The complexity of Notch impact on cardiac progenitor regulation is demonstrated by its role in epicardial cells. Epicardium has been recently suggested to act as a cardiac stem cell niche contributing to the pool of cardiac progenitors in developing and adult heart (Zhou et al., 2008; Popescu et al., 2009; Di Meglio et al., 2010). Indeed MI causes the activation of epicardium-derived progenitors (Limana et al., 2010) to promote cardiac repair by contributing to neovascularization and generating

cardiomyocytes structurally and functionally integrated with the resident myocardium (Smart et al., 2012).

Notch blockade lead to an up-regulation of pluripotency genes in epicardial-derived c-kit+ cells from rat hearts and delayed their differentiation (Zakharova et al., 2012). In particular, Notch signaling was found to favor epithelial-to-mesenchymal transition (EMT) in epicardial precursors in culture.

The transition between an epithelial and mesenchymal cell phenotype is a crucial and reversible process (mesenchymal-to-epithelial transition, MET) operating during embryogenesis and organ development but can also promote the acquisition of stemness properties *in vitro* (Di Meglio et al., 2010; Forte et al., 2012). EMT has been found reactivated in adult cells in pathological conditions (Zeisberg et al., 2007; Thiery et al., 2009) and demonstrated to be crucial in embryonic stem cell differentiation (Martínez-Estrada et al., 2010).

This process is fundamental in the epicardium contributing to coronary vasculature and myocardium formation during heart development (Zhou et al., 2011). At this stage epicardium-derived cells (EPDCs) undergo three successive rounds of EMT and MET to acquire the final differentiated phenotype and spatial organization (Thiery et al., 2009).

Of note, both c-kit+ and c-kit- cardiac progenitor cells migrating out of atrial explants *in vitro* were shown to transiently express epicardial markers and to proceed to EMT in culture. Different signaling pathways belonging to TGFβ superfamily, Wnt/β-catenin, Notch, HGF, FGF, retinoic acid families have been described (von Gise and Pu, 2012; Zakharova et al., 2012) as key players in the fine regulation of EMT process.

JAK/STAT SIGNALING PATHWAY

The hypothesis that JAK/STAT pathway could be involved in cardiogenesis has been initially explored in Drosophila. Drosophila embryos with targeted mutations in the JAK/STAT ligand or in STAT92E transducer developed non-functional hearts with defects in lumen formation very likely as an effect of inappropriate cell-cell interaction and abnormal cell aggregation. This effect has been associated to JAK/STAT pathway regulation of Tinman protein expression prior to heart precursor cell diversification (Johnson et al., 2011). Tinman is the Drosophila homolog of Nkx-2.5 mammalian transcription factor. Thus, the existence of an interaction between JAK/STAT ubiquitous signaling pathway and cardiac-specific regulators during tissue-specific stem cell specification in mammalians can be inferred, although no evidence has been given so far.

JAK-STAT signaling has been demonstrated to be essential for keeping ES self-renewal and pluripotency (Niwa et al., 1998; Matsuda et al., 1999). The inhibition of JAK2/STAT3 signaling in embryonic bodies prevented the formation of beating areas, while its ectopic expression had opposite effects (Foshay et al., 2005).

The same pathway has been found to protect murine cardiac tissue by different means, the most remarkable being the mobilization of endothelial progenitors from the bone marrow (Mohri et al., 2012). Additionally, a direct effect of this signaling pathway on cardiac cells was proposed. For example, the attenuation of cardiac fibrosis after MI through the activation of STAT3 by IL-11 in cardiac myocytes has been demonstrated (Obana et al., 2010). This cytokine, together with CT-1 was found highly expressed in the heart during myocardial infarction.

STAT3 pathway was also activated in Sca-1+ cardiac progenitor cells treated with IL-11 and CT-1. The result of such stimulation on the cells was the up-regulation of endothelial markers (Mohri et al., 2009). This process is thought to be a pro-angiogenic response of resident stem cells to the hypoxic and inflammatory environment of the post-ischemic area.

Surprisingly, a similar effect could not be evoked by the inflammatory cytokine IL-6 in Sca-1+ cells, very likely because of the lack of gp130 receptor on the membrane of this stem cell subset (Mohri et al., 2012).

On the contrary, JAK-STAT pathway can be activated through IL-6 receptor in c-kit cardiac stem cells, thereby inducing their endothelial differentiation. These effects are likely to reflect the tight association between the inflammatory burst occurring after myocardial infarction and the process of neo-vascularization.

HIPPO PATHWAY IN CARDIAC STEM/PROGENITOR CELLS

The Hippo pathway has been recently identified as a fundamental axis in the regulation of organ size and shape during organogenesis and its involvement in the pathogenesis of cancer has been established (Johnson and Halder, 2014).

This pathway negatively controls the nuclear shuttling of the paralog Yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1 (WWTR1 or TAZ) by sequestering them into the cytoplasm and inducing their degradation through proteasome activity (Hong and Guan, 2012).

The downstream effectors of Hippo pathway have also been identified as mammalian proto-oncogenes.

YAP and TAZ have no transcriptional activity *per se* but function as gene co-activators by binding cell-specific transcription factors (Tian et al., 2010).

YAP/TAZ axis has been described as a central regulator of human embryonic stem cell self-renewal through the control of SMAD complex shuttling to the nucleus, with TAZ knockdown resulting in the loss of cell pluripotency (Varelas et al., 2010). The same co-factors control intestinal (Camargo et al., 2007) and neural progenitor cell number and differentiation (Cao et al., 2008) by targeting the Notch signaling. YAP activity has also been proven fundamental in controlling epidermal stem cell homeostasis (Zhang et al., 2011).

The activity of TAZ as a modulator of MSC differentiation has been shown to direct cell differentiation into osteoblasts and adipocytes through its ability to bind lineage-specific master transcription factors: in fact, MSC fate could be controlled by TAZ ability either to activate Runx2 or to inhibit PPARγ, respectively (Hong et al., 2005; Hong and Yaffe, 2006).

Moreover, the same factor has been demonstrated to be crucial in directing myogenic differentiation by physically associating with MyoD in skeletal muscle progenitors (Jeong et al., 2010).

Yap1 is an important regulator of cardiomyocyte proliferation and embryonic heart development, its lack being associated with diminished cardiomyocyte proliferation (von Gise et al., 2012) and embryonic lethality (Heallen et al., 2011) during cardiogenesis.

In the adult heart, Hippo pathway has been connected to cardiomyocyte response and protection against ischemic injury (Del Re et al., 2013).

Our group recently identified the role of YAP/TAZ proteins as regulators of cardiac stem/progenitor cell sensitivity to the stiffness and nanotopography of the microenvironment (Mosqueira et al., 2014).

By using human and murine cardiac resident stem cells isolated on the basis of Stem Cell Antigen 1 (Sca-1) expression, we demonstrated the ability of YAP and TAZ to translocate from the cytoplasm to the nucleus and back in response to dynamic modifications in substrate elasticity and nanostructure.

Similarly to what was shown in mesenchymal stem cells (Dupont et al., 2011), in Sca-1+ cardiac stem cells, YAP/TAZ re-localization in the nucleus seems to be controlled by the availability of adhesion sites within the extracellular matrix (ECM) and the ability of the cells to spread and acquire a given shape.

Along with their key role in mastering CPC migration, YAP/TAZ participate in fate decision in tripotent Sca-1+ cardiac stem/progenitor cells: its inhibition by gene silencing results in a switch between cardiac and endothelial lineage commitment. According to our unpublished results, a direct interaction could occur in the cytoplasm between YAP/TAZ protein and GATA-4, Tbx-5 cardiac-specific transcription factors. This binding would induce the complex to migrate to the nucleus of Sca-1+ CPCs and trigger specific responses (our unpublished results).

PI3K-Akt/Pim-1 KINASE AXIS

Akt is a kinase promoting a number of biological functions including cell survival, proliferation, and differentiation (Sussman, 2007). Akt is involved in a number of cell processes as a result of its interaction with different pathways.

The cardiac-specific overexpression of nuclear Akt was found to increase cell cycling in resident cardiac progenitor cells, an effect which has been associated with cardioprotective significance (Fujio et al., 2000; Shiraishi et al., 2004; Gude et al., 2006; Tsujita et al., 2006).

Through ectopic expression experiments, Akt has been deemed responsible of the paracrine effect implanted mesenchymal stem cells exert on injured cardiac tissue. The molecular basis of this effect has been ascribed to the ability of Akt-transduced MSCs to secrete a massive amount of beneficial molecules, namely VEGF, bFGF, HGF, IGF-1, and thymosin β4 (TB4), thus evoking cardiac tissue remodeling and local neo-vascularization (Gnecchi et al., 2008). In this context, a possible effect of the factors secreted on the resident pool of cardiac stem/progenitor cells has been hypothesized.

Recently, the cooperation between Akt and Pim-1 kinases has been identified in c-kit+ cardiac progenitor cells. Pim-1 is known to exert anti-apoptotic effects on the myocardium as a result of the phosphorylation of Bad and inhibition of caspase cleavage (Muraski et al., 2007).

Also, Pim-1 kinase phosphorylates heterochromatin protein-1 and nuclear mitotic apparatus protein (NuMA), which are crucial in cell division, while promoting cell proliferation in cancer and hematopoietic stem cells (Sun and Schatten, 2006) through the collaboration with c-Myc (Stewart et al., 1999; Zhang et al., 2007, 2008).

In c-kit+ cardiac stem cells, Pim-1 over-expression was found to enhance CPC proliferation through asymmetric division, with Pim-1 transgenic mice displaying a higher proliferative activity in the heart as compared to the controls (Cottage et al., 2010).

A hypothesis on the role of Pim-1 kinase has been formulated in which its overexpression is responsible for CPC increased proliferation during the hyperplastic phases occurring in physiological (during pre- and post-natal development) and in pathological (after myocardial infarction) conditions.

HGF AND IGF1 IN CARDIAC STEM CELLS

The possible role of Hepatocyte Growth Factor (HGF) and Insulin-like Growth Factor-1 (IGF-1) in the homeostasis and differentiation of resident cardiac stem cells has been the subject of a few investigations in which they were studied together.

Hepatocyte growth factor (HGF) is a pleiotropic cytokine of mesenchymal origin, promoting motility, proliferation, invasion, morphogenesis, and survival in a wide spectrum of cells, namely epithelial and endothelial cells (Trusolino and Comoglio, 2002; Birchmeier et al., 2003). HGF controls the tubulogenesis during kidney (Santos et al., 1994) and mammary gland (Yang et al., 1995) development and angiogenesis (Bussolino et al., 1992). The protein, originally identified as scatter factor (SF), acts as a chemoattractant for motor neuron axon (Ebens et al., 1996) and myogenic precursors (Bladt et al., 1995; Maina et al., 1996) and a survival factor for hepatocytes (Schmidt et al., 1995) and placenta (Uehara et al., 1995). Our group identified the presence of HGF putative receptor c-Met on murine mesenchymal stem cells and successfully achieved MSC commitment toward cardiac phenotype through HGF stimulation. The cells failed to acquire a mature cardiomyocyte phenotype by our protocol (Forte et al., 2006). In this study, HGF activity in MSCs was shown to activate the ras-ERK1/2 and p38 MAPKs as well as the PI3K/Akt pathways.

Consistently, HGF has been shown to improve embryonic stem cell differentiation toward the cardiac phenotype (Roggia et al., 2007).

HGF has a cardioprotective role in experimentally induced myocardial infarction, preventing cardiomyocyte apoptosis, inducing angiogenesis, and improving impaired heart function (Aoki et al., 2000; Jayasankar et al., 2003; Sala and Crepaldi, 2011).

c-kit+ cardiac resident stem cells were shown to secrete HGF while expressing its functional receptor on their membrane. The factor acts as a chemoattractant and increases c-kit+ cardiac stem cell invasive ability. This cell subset was shown to express a functional receptor for IGF-1, while its administration had few, if any, motogenic or invasive activity on them. Interestingly, IGF-1 displayed a marked anti-apoptotic activity in CSCs (Urbanek et al., 2005).

Insulin-like growth factor-1 (IGF-1) is a potent mitogen, exerts anti-apoptotic activity, and is necessary for neural stem cell growth (Arsenijevic et al., 2001). IGF-1 also promotes myocyte formation while protecting them from the injury due to myocardial infarction (Li et al., 1997). Moreover, the proliferation and cardiac induction of developing murine mesoderm could be promoted *in vitro* by administering IGF, thus disclosing a novel

role of this cytokine in cardiac mesoderm specification (Engels et al., 2014).

Both IGF-1 and HGF, whose expression was found significantly increased in the infarction border zone after myocardial ischemic insult (Fiaccavento et al., 2005), were shown to enhance cardiac stem cell proliferation. The local delivery of both the factors in the infarcted heart resulted in the migration of c-kit+ cardiac stem cells toward the injured area and the regeneration of dead portions of the myocardium (Urbanek et al., 2005).

Moreover, the regenerative response of endogenous porcine c-kit+/CD45− cardiac stem cells to an ischemic insult could be enhanced *in situ* by administering the two growth factors directly through the coronary circulation (Ellison et al., 2011). This treatment resulted in a persistent dose-dependent and positive effect on cardiac performance, probably due both to a direct effect on the survival of the cardiomyocytes and to an enhancement in cardiac stem cell function.

EXAMPLES OF THE INTERACTION BETWEEN DIFFERENT PATHWAYS IN CARDIAC PROGENITOR CELLS

The interplay among the molecular pathways taking part to cardiogenesis could be based on the sharing of molecular effectors, with WNT/β-catenin, Hippo, TGF β and BMP signaling being considered pivotal in the specification and maturation of cardiac precursors (Attisano and Wrana, 2013).

An example of such an interplay comes from the use of conditional mutagenesis in mouse embryos. By this means, a hierarchical regulation of cardiac progenitor differentiation has been unveiled, in which WNT/β-catenin acts as downstream effector of Notch signaling and upstream of BMP signaling during embryonic heart development (day 9.0). In this context, progenitor cardiac differentiation requires the sequential expression of distinct sets of cardiac transcription factors controlled by WNT/β-catenin and Bmp4 signaling. At an early stage WNT/β-catenin activates *Baf60c*, *Nkx2-5*, and *Isl1* genes but also BMP4 signaling which, in turn, activates Gata4 and SRF (Klaus et al., 2012).

Similarly, the Hippo pathway effector TAZ has been shown to negatively regulate WNT pathway by inhibiting Disheveled, an upstream inhibitor of the β-catenin destruction complex, thus reducing β-catenin stabilization in human embryonic kidney cells (Varelas et al., 2010). Therefore, Hippo pathway appeared to restrict WNT/β-catenin signaling by TAZ cytoplasmic activity (Varelas et al., 2010). The interaction between Hippo and WNT signaling pathways has also been acknowledged as part of the complex mechanisms controlling mammalian heart size, with the first antagonizing the nuclear β-catenin/YAP interaction in differentiating cardiomyocytes (Heallen et al., 2011).

Lately, a study by Azzolin and co-workers proposed a new model in which WNT/β-catenin signaling responses are being mediated, in a context-dependent manner, not only by β-catenin activation, but also by TAZ stabilization and TAZ-dependent transcription, thus suggesting TAZ as downstream co-activator of β-catenin target genes (Azzolin et al., 2012). This study also revealed a Hippo inhibitory activity of β-catenin, inducing TAZ inhibition through its interaction with proteasomal complex. Although these findings come mainly from studies on cancer cells, similar interaction might positively or negatively regulate cardiac stem cells where both β-catenin and TAZ have been demonstrated to be instrumental to the self-renewing and differentiation processes.

Another example of how the interaction of known signaling pathways can affect the regulation of cardiac progenitors is given in mouse embryos, where the ablation of Notch1 in Islet1+ CPCs

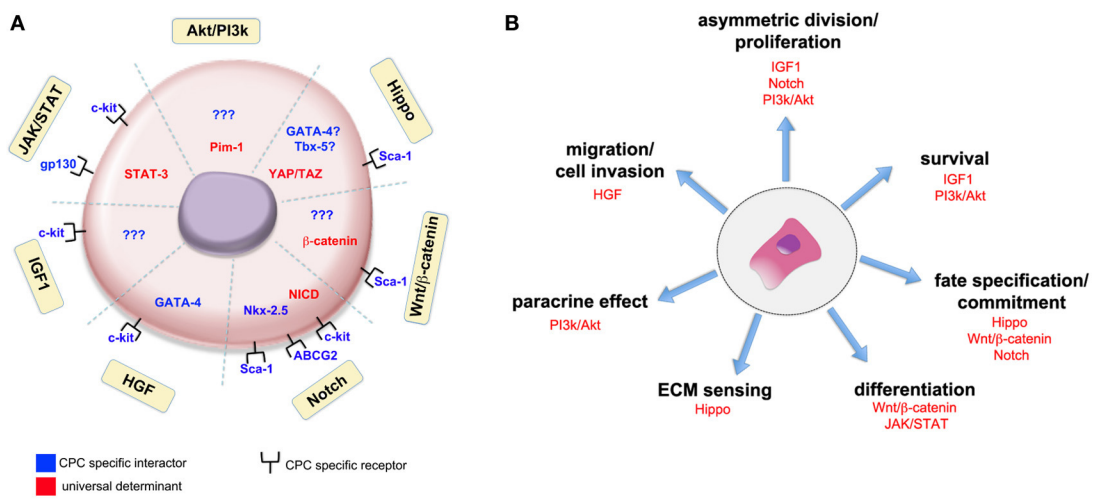

FIGURE 1 | Universal signaling pathways and cell-specific actuators interact in cardiac stem cells to induce given biological responses. The interaction of universal determinants (in red) and stage-specific actuators (in blue) in adult cardiac progenitor cells (CPCs) is schematically represented **(A)**. Yellow boxes identify the signaling pathways so far recognized to participate in CPCs function. The association with CPC specific receptors has been found for some of the pathways, while for others no clear evidence of any interaction with cell-specific determinants has been given so far (indicated by question marks). The participation of well-known signaling pathways to given activities in adult cardiac progenitor cells is shown in **(B)**. Please refer to the main text for the meaning of the abbreviations.

triggers their expansion by negatively regulating Wnt canonical signaling and thus increasing the levels of active β-catenin protein. The subsequent inhibition in their cardiac differentiation is reported (Kwon et al., 2009; Andersen et al., 2012). Further studies will be required to confirm such data in adult cardiac stem cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, studying the regulation of adult cardiac stem cell homeostasis and differentiation could require a higher degree of complexity. Few studies have highlighted a complex network of interactions among several signaling cascades contributing to the control of cardiac specification.

Therapeutic approaches trying to enhance the impact of cardiac regeneration or involving the differentiation of resident cardiac progenitor cells will also benefit from the knowledge produced. A graphical representation of such interactions occurring in the context of adult cardiac progenitor cells is shown in **Figure 1**.

In fact, investigations aimed at highlighting the interaction between universal signaling pathways and stage-specific determinants are overseen as a promising perspective to design novel approaches to the treatment of cardiac diseases. A context-specific system is already considered a dogma as referred to the nucleus of different cell types, where pleiotropic transducers physically interact with cell-specific transcription factors (*adaptors*) to evoke unique responses in terms of gene program activation (Andel et al., 1999).

While apparently multiplying the complexity of the regulatory mechanisms presiding at cardiac stem cell homeostasis, the idea that such a modular interaction exists provides a hub for biochemical and high-throughput studies to unveil the nature and function of such mechanisms and help to design strategies to control cardiac stem cell function in a clinical perspective.

For example, the context-specific activity of intracellular effectors can be selectively tuned by changes in the mechanics of tissue-specific extracellular milieu, like in the case of Hippo pathway transducers affecting cell responses (growth, proliferation and differentiation) through the modification of the cellular genetic program (Mendez and Janmey, 2012; Mosqueira et al., 2014).

Understanding and reproducing this extremely sophisticated level of control will be vital to the success of cardiac regenerative therapies in the next future (Lutolf et al., 2009).

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Skeletal muscle tissue engineering: best bet or black beast?

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Skeletal muscle possesses a remarkable self-repair capacity whose underlying mechanisms have been thoroughly investigated (Vandenburgh et al., 1988; De Arcangelis et al., 2003; Musaro et al., 2007; Moresi et al., 2008, 2009) with a view to stimulating *in situ* regeneration. It is, however, unable to restore volumetric tissue loss as a consequence of trauma, congenital defects, ablation, or denervation. This is the rationale behind the creation of new skeletal muscle through tissue engineering (TE). While most review articles announce that skeletal muscle TE is advancing and is readily translatable, it seems clear that engineered skeletal muscle is still lagging far behind other tissues if placed within a clinical practice context. Why is it not yet possible to transplant off-the-shelf, functional muscles into patients?

Major difficulties encountered in skeletal muscle TE on the whole organ scale are currently being addressed. These include the following: the large size of human organs has been overcome by studies on rabbit, dogs and humans (Badylak et al., 1998; Rossi et al., 2010; Badylak et al., 2013); numerous myogenic cell populations of muscle and non-muscle origin (Rossi et al., 2010) are now available as cell sources; scaffolds with a specific 3D orientation are obtained through organ decellularization, freeze drying and electrospinning of synthetic or natural materials (Klumpp et al., 2010); function has been proven for several of these engineered muscle constructs (Mudera et al., 2010; Carosio et al., 2013). Even some of the interactions between muscle and other organs have been addressed: the muscular-tendinous junction can be restored by suturing the residual tendon proximal extremity of muscle-derived acellular scaffolds to the host tendon (Perniconi et al., 2011); the vascular bed can be reconstructed in striated muscles (Koffler et al., 2011; Carosio et al., 2013), particularly if the elegant approach proposed by Ott et al. for decellularized heart is applied (Ott et al., 2008).

Muscles, however, remain very challenging organs to rebuild. Muscle hierarchic architecture and heterogeneous cell composition have not yet been sufficiently investigated by either *in vitro* or *in vivo* studies. Moreover, innervation by the somato-motor system has not been addressed at all. We believe that the latter issue will be the hardest to address; since we are still unable to induce normal re-innervation following motor neuron injury, the successful innervation of a new, pre-built organ transplanted *in vivo* is unlikely to be straightforward. Lastly, even some basic aspects of muscle TE, such as graft bioactivity and integration, which are often claimed to be established, are actually still poorly understood; for instance, the novel idea of cryptic peptides released by the extra-cellular matrix (ECM) while it is being biodegraded and remodeled (Grayson et al., 2009) opens new avenues for the exploration of ECM component bioactivity. This need to gain a better knowledge of the properties of the ECM and bioactive molecules used in TE applications is indeed the rationale of dedicated publications, such as this Frontiers in Muscle Physiology Special Issue.

While obtaining a fully functional, innervated human skeletal muscle from TE that may be used for clinical purposes remains a long-term goal, here we propose two applications for engineered muscle that are no less ambitious and that may be achieved and exploited in the short term. As stated by Grayson et al., the biomimetic effort being made within the context of skeletal muscle TE is mostly aimed at: (a) the creation of functional tissue grafts for regenerative medicine applications *in vivo*; (b) the generation of experimental models *in vitro* for studies on stem cells, development, and disease (where engineered tissues can serve as advanced 3D models).

(a) As far as organ replacement *in vivo* is concerned, we expect very specific TE-based interventions, such as the application of muscle flaps and the generation of minute individual muscles, to be immediately successful, whereas the mass production of large muscles involved in chronic and global muscle wasting diseases is less unlikely to be so. One example of organ replacement that is likely to be successful is the *Stapedius*, which is the smallest and weakest skeletal muscle in the human body. It stabilizes the stapes, a very small bone in the inner hear, and is innervated by the facial nerve. Dysfunctions in the *Stapedius* induce hyperacusis or other sound perception defects that are clinically relevant. While the problem of innervation is likely to persist, the engineering and grafting of this small and simple muscle to replace the diseased one (often due to a local defect) appears to be highly feasible.

(b) As far as *in vitro* studies are concerned, it is self-evident that bidimensional

cultures are very limited insofar as the physiological 3D tissue organization they yield is somewhat approximate. Muscle TE was initially designed for *in vitro* studies, when Vandenburgh et al. introduced the 3D cultivation of primary myoblasts in collagen gel and generated contracting muscle tissue *in vitro* for the first time in 1988 (Vandenburgh et al., 1988). A progressive increase in the architectural complexity of ECM and cells in tissue-culture grade constructs is likely to provide adequate experimental models for the *in vitro* study of phenomena that are specific to the *in vivo* situation [e.g., stem cell niche, tissue regeneration, aging (Sharples et al., 2012)].

With regard to the two applications described above, it should be borne in mind that the goals and approaches involved in building engineered muscle tissue may not be the same owing to the significant differences between *in vitro* and *in vivo* TE strategies (reviewed by Rossi et al., 2010).

For all these reasons, we believe that the best bet for skeletal muscle TE is to focus on specific, anatomically defined solutions or on 3D *in vitro* modeling of muscle tissue for basic and applied research. We are confident that we will eventually be able to transform the black beast (i.e., striated muscle tissue engineering) into the best bet (i.e., a successful clinical practice based on engineered muscles). However, for more ambitious muscle TE applications, there may still be a long way to go.

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Skeletal muscle tissue engineering: strategies for volumetric constructs

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Skeletal muscle tissue is characterized by high metabolic requirements, defined structure and high regenerative potential. As such, it constitutes an appealing platform for tissue engineering to address volumetric defects, as proven by recent works in this field. Several issues common to all engineered constructs constrain the variety of tissues that can be realized *in vitro*, principal among them the lack of a vascular system and the absence of reliable cell sources; as it is, the only successful tissue engineering constructs are not characterized by active function, present limited cellular survival at implantation and possess low metabolic requirements. Recently, functionally competent constructs have been engineered, with vascular structures supporting their metabolic requirements. In addition to the use of biochemical cues, physical means, mechanical stimulation and the application of electric tension have proven effective in stimulating the differentiation of cells and the maturation of the constructs; while the use of co-cultures provided fine control of cellular developments through paracrine activity. This review will provide a brief analysis of some of the most promising improvements in the field, with particular attention to the techniques that could prove easily transferable to other branches of tissue engineering.

Keywords: skeletal muscle, tissue engineering, physical stimulation, mechanobiology, vascularization

INTRODUCTION

Skeletal muscle tissue represents the most abundant tissue type in the human body, amounting to 60% of the average weight. It is a metabolically active tissues requiring a constant flow of nutrients and metabolites, provided by an extensive capillary network forming an organized branching pattern throughout the fibers (Dennis and Kosnik, 2000; Liu et al., 2012).

Skeletal muscle tissue engineering (SMTE) aims to replicate the structure and function of skeletal muscle tissue *in vitro* and *in vivo*, to obtain valid models and functional constructs whose ultimate goal is the implantation as a therapeutic device (Ostrovidov et al., 2014). This discipline presents unique challenges compared to other tissue engineering strategies that have shown promise in clinical applications (Naito et al., 2003; Atala et al., 2006; Macchiarini et al., 2008; McAllister et al., 2009), in that small lesions and defects in skeletal muscle tissue seldom, if at all, require chirurgical intervention and transplantation; injuries heal spontaneously through an inflammation related mechanism (Ciciliot and Schiaffino, 2010; Turner and Badylak, 2012) involving resident stem cells, named satellite cells (SCs) (Pannérec et al., 2012).

Lesions so extensive that the function of the muscle is impaired are commonly designated volumetric muscle losses (VMLs) (Grogan et al., 2011); these lesions, mainly due to traumatic injury and chirurgical resection of tumors, are treated using free standing flaps as gold standard, a procedure that is inevitably associated with morbidity at the donor site and limited successful outcomes (Agostini et al., 2013). The use of flaps, rather than grafts, is a necessity because transplants of this kind cannot survive without an independent blood supply Similarly, clinically relevant SMTE constructs would require autonomous vascular networks to avoid central (Griffith et al., 2005; Bae et al., 2012; Shandalov et al., 2014).

However, in a clinical setting the goal of an SMTE device is the reestablishment of function rather than the original homeostasis of the tissue, there have been efforts to engineer constructs in which the contractile ability of the muscles is restored through functional fibrotic structures (Corona et al., 2013b); as long as there is a continuity in muscle architecture that allows the force transmission even a fibrotic structure formed from and acellular scaffold will ameliorate VML (Aärimaa et al., 2004).

Given the unique requirements in the treatment of the lesions in musculoskeletal tissues, SMTE constructs are designed to ameliorate large tissue defects and can only have limited clinical application without a vascular system to support the cellular component; similar considerations must be undertaken in the case of preclinical constructs, as large three dimensional structures have proven to replicate more closely the physiology of the original tissues (Rouwkema et al., 2011). It is necessary to mention that lesions occurring in craniofacial skeletal muscles (Garland and Pomerantz, 2012) must be set apart from the previous generalizations, as these tissues present a different cellular milieu compared to the muscles of the limbs and trunk (Kelly, 2010; Lemos et al., 2012); as such, it could be argued that different models should be produced to study these pathologies, though discipline may still prove of use for

clinically applicable constructs due to the relatively restricted size of the lesions and the difficult application of flaps in this region.

SKELETAL MUSCLE STRUCTURE

The structure of skeletal muscle is inherently correlated to its function; this tissue is characterized by a highly ordered structure composed of parallel elements that can be summarily divided in myofibrils, muscle fibers and fascicles. A myofibril is the cytoplasmic molecular machinery capable of actuating muscle contraction through the relative movement of two interlocking macrostructures, the thin actin filaments and thick myosin filaments (Huxley and Hanson, 1954). Myofibrils are bundled within the massive cytoplasm of a multinucleated syncytium, the muscle cell or myofiber; motoneuron connections in the cell membrane (the sarcolemma) regulate the flow of calcium ions through the sarcoplasmic reticulum, necessary for the contraction of the myofibrils.

Myofibers constitute the parenchyma of skeletal muscle, and are bundled in a complex ECM structure which connects them to the muscle-tendon junction through three fibrous layers: the endomysium, surrounding individual myofibers, the perimysium, found over fascicles and the epimysium which cover the entire muscle.

The fine structure is particularly evident in decellularized muscles, where the bare extracellular matrix (ECM) forms structures not unlike bundles of flexible straws (Lieber and Fridén, 2000), which is the principal component of the muscle's anisotropic response to stress (Takaza et al., 2013). Blood vessels run along the fascicles in the perimysium and penetrate into the endomysium, forming capillaries that project around the myofibers; due to the elevated metabolic need of the skeletal muscle tissue, each myofiber is connected to a capillary (**Figure 1**). At the same time, the axons of motor neurons, which transmit the synaptic signal to the myofibers. Following the route traced by the blood vessels, motor neurons terminate into neuromuscular junctions in the proximity of sarcolemma-SR connections (triads); the association between the vascular and the nervous system is thought to start from the developmental phase of the organism (Carmeliet and Tessier-Lavigne, 2005) and is conserved in regenerative processes involving a common mesenchymal progenitor cell able to generate musculoskeletal, nervous and vascular tissues (Tamaki et al., 2013).

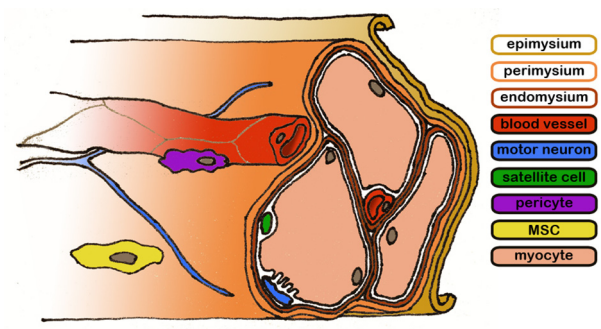

FIGURE 1 | Representative diagram of the skeletal muscle structure. Connective layers and cell populations of particular interest are evidenced. The biological components are not drawn to scale.

The cellular machinery involved in the maintenance and regeneration of skeletal muscle tissue is the same responsible for. the delicate balance between hypertrophy and catabolism; these processes has been studied in detail (Joe et al., 2010; Ten Broek et al., 2010; Dellavalle et al., 2011; Turner and Badylak, 2012; Saclier et al., 2013), and three cellular populations have proven especially relevant to the regeneration process. SCs (Mauro, 1961), located between the basal lamina and the sarcolemma, are the tissue resident progenitor cells responsible for the maintenance and the continual production on myoblasts. Once activated, SCs will reveal heterogeneity in their population and will start dividing and producing muscle progenitor cells (MPCs) that eventually will become myoblasts and fuse to the existing myofibers (Boldrin et al., 2010); pathological conditions such as Duchenne muscular dystrophy (DMD) are known to deplete the reservoir of available SCs, leading to fibrosis and adipose tissue inclusion in muscular tissues.

SC pools can be replenished by muscular tissue pericytes (MPs), a population of cells found in close proximity to blood vessels and difficult to identify, as they bear no unique CD set or biochemical markers' (Dellavalle et al., 2011). These cells are capable of directing the behavior of endothelial cells (ECs), acting as guides during the formation of new blood vessels (Fuoco et al., 2014). Here, these cells have been shown to lose in part their ability to differentiate into myogenic lineage and to induce angiogenesis in nearby ECs as their host ages; nevertheless, the "myogenic/vasculogenic ability" appears to be restored after the cells are seeded on a polyethylene glycol-fibrinogen (PEG-F) surface. The mechanism underlying this behavior is yet to be elucidated, although a comparison with previous experiments by Discher et al. (2005) and Engler et al. (2006), might suggest a sensitivity to the mechanical stimuli of cellular environment.

Finally, fibro/adipogenic progenitors (FAPs) are a PDGFRα^+ myogenic cell population, found in state of quiescence near blood vessels, which activates in case of injury to the muscle (Joe et al., 2010). FAP cells are known to promote the differentiation of MPCs but do not have any capability to generate muscle tissue on their own; on the contrary, they appear to be the main source of the fibrotic and adipose tissues found in pathological muscles (Uezumi et al., 2011). As such, these cells have been studied as a target for with microRNA (miRNA) treatments intended to reduce the symptoms of the DMD syndrome through the upregulation of the FAP cells myogenic programming, inducing a "compensatory regeneration" (Mozzetta et al., 2013; Saccone et al., 2014).

The interplay between these cell populations is fundamental for the continuous renewal of skeletal muscle tissue, required to compensate the damage occurring in day-to-day activities (Järvinen et al., 2013); however, pathological states also require the activation of the same cells in different processes that can result in the regeneration of the tissue or the formation of a fibrotic scar.

SKELETAL MUSCLE REPAIR PROCESS

The regenerative process of muscle tissue is complex, and not yet understood in its entirety; different cell populations interact with each other and their environment to progress into outcomes which span from the reestablishment of function to fibrosis to pathological states of chronic inflammation (Koopman et al., 2014). A brief recapitulation of the phases of this process and the cellular populations involved will provide a background for the *in vivo* implantation of SMTE constructs, and will define some of the challenges involved in the development of *in vitro* devices. Skeletal muscle regeneration can be summarily divided into three overlapping phases: inflammation and activation of stem cells, differentiation and deposition of a provisional ECM and finally maturation of the tissues and remodeling of the ECM (Ciciliot and Schiaffino, 2010).

In the first phase (the peak is reached in 2 h) of the regeneration process neutrophil granulocytes, innate immune response cells with antimicrobial activity, enter the lesion from the blood stream; their function is disputed but it has been proven that they possess antimicrobial activity, exert phagocytosis on tissue debris (Teixeira et al., 2003) and that they can stimulate vascularization through production of VEGF-A (Christoffersson et al., 2012); at the same time, however, this cell population releases cytotoxic compounds that can interfere with the regeneration process (Tidball and Villalta, 2010).

Another population of immune cells, the eosinophils, invade the tissue in this phase and activate FAP cells through the release of IL-4 (Heredia et al., 2013); after this initial stage the macrophages enter the lesion site: pro-inflammatory CD68+ CD163− macrophages (M1), which reach maximal concentration 24 h after the injury, followed by a switch in polarization leading to alternative activated, CD68− CD163+ macrophages (M2) with anti-inflammatory activity (Tidball and Villalta, 2010; Mantovani et al., 2013; Tidball et al., 2014). This activity bridges the inflammatory-proliferative phase with the following one, anti-inflammatory and differentiative; the shift is driven by the interaction between the macrophages and the SCs, which activate in response to the tissue injury and start an asymmetric replication cycle to produce myogenic progenitors (MP): simplistically, it can be said that during the initial phase SC division is stimulated by M1 macrophages via TNFα, then differentiation occurs through IL-4 and IL-10 intervention by M2 cells. Whereas quiescent SC express a characteristic set of markers including Pax-7, M-Cadherin and CD 34, activated SC rapidly change this configuration as they proliferate; diminishing Pax -7 expression and producing basic helix-loop-helix (bHLH) transcription factors as they differentiate into committed MPs; these cells form multinucleated myotubes that express neonatal MyHC (Tidball et al., 2014). It is important to mention that only a fraction of the SC population possesses stemness and is capable of perpetuate itself and generate daughter cells of different phenotypes (Wang and Rudnicki, 2012).

At the same time, FAP cells rapidly multiply under IL-4 stimulation, enforcing an ambivalent role in the context of wound healing: while they provide MPs with growth factors necessary for their differentiation in myocytes, they are also the main origin of fibrosis in tissue repair and adipose tissue in pathological conditions (Uezumi et al., 2011; Heredia et al., 2013). In parallel, a population of connective tissue fibroblasts counterbalances FAPs' activity by dampening SC differentiation through the formation of a muscle connective tissue (MCT) and reciprocated paracrine signals (Murphy et al., 2011).

The final phase in muscle regeneration there is the resolution of the provisional ECM and the formation of a definitive structure that connects the stumps of the damaged fibers; in this process the basal lamina acts as a guide for the growth of the myofibers (Schmalbruch, 1976). The remodeling of the fibrotic tissue and the subsequent regeneration of the skeletal muscle, or lack thereof, is dependent on a variety of factors, including the vascularization and the innervation of the healing area. Generally, two positive outcomes can be envisioned as the resolution of the third phase: the regeneration of the muscle tissue, with the original architecture, or the formation of scar tissue and the separation of the muscle fibers (Ciciliot and Schiaffino, 2010; Turner and Badylak, 2012).

VASCULARIZATION

Vascular supply in the muscle is provided by a highly organized network of vessels, which are for the most part aligned with the direction of the muscle fibers. The organization of the structure is highly hierarchical an provides each myofiber with blood supply; the primary arteries run along the direction of the muscle fibers, and diverge inside the muscle through the epimysium via feed arteries; those are short branches that penetrate inside the muscle at an angle, or perpendicularly. Secondary arteriolar branches run again parallel to the muscle fibers, and then arterioles, through the perimysium, and finally connect to a microvascular unit composed of arterial and venous capillaries that follows the endomysium.

SMTE constructs, as well as any other tissue engineering construct which exceeds in any dimension the diffusion limit for oxygen and nutrients (Karande et al., 2004; Griffith et al., 2005; Radisic et al., 2006), require a specialized mean of delivery of such substances and removal of metabolic byproducts if they are to maintain the viability of seeded cells. Without these structures the constructs are likely to form necrotic cores (Radisic et al., 2004), compromising the regeneration process.

An arbitrary divide can be made between the techniques that see the formation of vascular structures *in vitro* and those that are implanted and develop vessels *in vivo*: in the first case various avenues have been explored to generate patterns within the volume of the engineered material so that the definitive shape of the network will be informed beforehand, whereas in the second case there is no specific structural conformation before implantation. This discrimination is necessary as current techniques are unable to produce mature blood vessel networks *in vitro*, meaning that a maturation process will always be present in the development of such constructs, if they are to be used as implants. Constructs intended as a vascularization models are subjected instead to different degrees of maturation *in vitro*, which determine the characteristic of their vascular structures, and as such will be discussed in the first group.

It can be argued that no engineered construct can be produced that will not incur into substantial remodeling upon

implantation: even an hypothetical vascularized flap undistinguishable from the host's tissue would be colonized and altered by the surrounding capillaries, meaning that vascularization of implanted devices will always comprise an *in vivo* phase. According to this reasoning, the processes described here will be grouped according to whether they focus on the production of vascular structures within the constructs or the realization of constructs capable of allowing and increasing the penetration of the host vessels upon implantation.

IN VITRO VASCULARIZATION

Approaches for *in vitro* vascularization focus on the production and maturation of structured constructs before an eventual implantation; this is realized by producing an environment favorable to the formation of vascular compartments by vasculogenic cell populations, either by mechanical placement or through directed self-assembly (**Table 1**).

Constructs belonging to the first group are arguably the most numerous: they comprise those devices which implement structural and mechanical cues to direct the growth of vascular cells, most often ECs, endothelial progenitor cells (EPCs) and vascular smooth muscle cells (VMSCs). The methods used to produce the patterns include, most notably, sacrificial three-dimensional patterns (Miller et al., 2012; Hooper et al., 2014), direct deposition of cellular suspensions (Kolesky et al., 2014), self-assembly of polyelectrolyte solutions (Leong et al., 2013) or patterned cellular sheets (Muraoka et al., 2013), and usage of decellularized scaffolds, although the best known example refers to cardiac tissue (Ott et al., 2008; Koffler et al., 2011). In particular, the cell sheet techniques recently developed (Haraguchi et al., 2012; Nagamori et al., 2013; Sakaguchi et al., 2013; Sekine et al., 2013) resulted in the production of *in vitro* vascularized tissues using culture dishes coated with poly-N-isopropylacrylamide (PNIPAM) polymer derivatives that are able to modify the cellular adhesion by changing their hydrophilicity in a temperature range compatible with cell survival (Yamato et al., 2007). Interestingly, the interposition of a collagen sheet traversed with microchannels parallel to its surface through which was flowing medium was sufficient to generate the growth of vascular structures. These results indicate that ECs are able to respond to the mechanical cues generated by shear stress in adjacent vessels, even if these vessels are simply cavities in collagen; this is in accordance with findings in the field of vascular tissue engineering (Feaver et al., 2013) which describe the result of the mechanical stimulation on the phenotype of ECs. The complex effects resulting from cyclic mechanical stimuli are a valid tool in directing the behavior of vasculogenic cells and the generation of blood vessels (Kilarski et al., 2009; Boerckel et al., 2011), but the resulting vessels lack the order and pervasiveness of the natural vasculature; a better understanding of the cellular response to mechanical stress is therefore necessary to obtain mature, lifelike constructs.

On the other hand, devices that stimulate vascular growth *in vitro* without offering a predetermined pattern are not as common since scaffolds permitting unimpeded development of vascular structures are typically hydrogels, whose plastic qualities and wide range of possible biochemical and structural modifications make them more adapt for *in vivo* settings. Nonetheless, similar scaffold have been used to obtain networks either from preexistent structures (angiogenesis) (Chiu et al., 2012), or from isolated precursors (vasculogenesis) (Raghavan et al., 2010), demonstrating a better performance upon implantation compared to non-prevascularized constructs. This is also true for non-woven polymeric scaffolds, where the random structure of the scaffold limits the control over the growth of vessels (Levenberg et al., 2005); these devices have also profited from the application of biologically-derived hydrogel coatings, which provided a more permissive to vascular growth (Lesman et al., 2011; Sadr et al., 2012). Finally, "spontaneous" *in vitro* vascularization is fundamental in engineering "myooids" (Dennis and Kosnik, 2000), self-aggregated constructs based on the mixed muscle cell population. These constructs are based on provisional ECM scaffolds synthesized by the fibroblast population present in the extract from minced muscle, and present a self-organizing vascular layout derived from the EC and the SMC population (Carosio et al., 2013).

Construct which do not present geometrically defined cues for the vascular development such as these rely instead on the vasculogenic properties of EPCs and their development in correlation with VSMCs, myoblasts and fibroblasts to obtain a viable network of vessels (Lesman et al., 2011; Alekseeva et al., 2014); however, these networks are rapidly restructured *in vivo*, and substituted with mature vessel which conform to the structure of the implant (Bae et al., 2012; Hanjaya-Putra et al., 2013). This means that these implants will undergo a vascularization process *in vivo* that will largely supersede the one completed in the *in vitro* phase; in the case of more mature vessels, the construct will connect to the existing vasculature *in vivo* through "wrapping and tapping" anastomoses (Cheng et al., 2011); after this passage, the construct will be replace with cells and matrix from the host's tissues, in an inflammation-like process requiring the chemotaxis of progenitors cells (Roh et al., 2010).

IN VIVO VASCULARIZATION

The development of blood vessels *in vivo* takes advantage of the host's regenerative capabilities, which can be regulated varying the cellular and chemical composition of the implanted device. The different phases that follows implantation recapitulate, optimally, those observed in regeneration, steering the wound healing process from a fibrotic/scarring outcome to a functional one (de Jonge et al., 2014).

While regenerative processes are necessary for the maturation of the former constructs into functional tissue, the immediate reaction to the engineered device is fundamental for the generation of a viable vessel network, since the *in vivo* vascularization requires a delicate balancing of the different phases of wound healing and regeneration following the implantation, so to achieve the necessary chemotaxis of progenitor cells and paracrine regulators without incurring in a state of prolonged inflammation (Gurtner et al., 2008).

There are two main tools with which to achieve this goal: the cellular component of the device and the structure and functionalization of the scaffold. The cellular components have been selected from autologous (Conconi et al., 2005), syngeneic, allogeneic (Corona et al., 2014) or xenogeneic (Kang et al., 2011)

Table 1 | *In vitro* models of interest.

References	Model	Scaffold	Construct type	Relevant details
Salimath and García, 2014	C2C12	PEG-MAL hydrogel	*In vitro* model	3D model; variable stiffness; chemical functionalization
Egusa et al., 2013	Mouse BMMSC; C2C12	Fibrin on silicone	*In vitro* model	Differentiation due to mechanical stimulation, myogenic medium
Morimoto et al., 2013	C2C12, primary mNSC	Matrigel	*In vitro* model	Self tension, NMJ formation
Nunes et al., 2013	hPSC differentiated in cardiomyocites	Autologous ECM; collagen	*In vitro* model	Self assembly; electrical stimuli; wire structure
Palamà et al., 2013	C2C12	–	*In vitro* model	μtopography; LbL polyelectrolyte on glass
Shah et al., 2013	Human masseter muscle cells	Biodegradable bacteriostatic phosphate glass fibers; collagen	*In vitro* model	μpatterning; self contraction
Snyman et al., 2013	C2C12; pHMB	Neutralized collagen I hydrogel	*In'vitro* model	Self contraction; reproducibility
Wang et al., 2013	GFP-C3H myoblasts	Fibrin gel	*In vitro* model	Laminin, agrin formation; formation of NM receptors
Bian et al., 2012	Neonatal sprague-dawley rat SM myoblasts	Matrigel-fibrin mesoscopically structured hydrogel	*In vitro* model	Self contraction; analysis of SM networks; electrical stimuli
Hosseini et al., 2012	C2C12	μstructured gelatin methacrylate	*In vitro* model	μpatterning; size analysis; electrical stimuli
Monge et al., 2012	C2C12	Patterned film (polyelectrolyte)	*In vitro* model	μpatterning; stiffness modification
Sharples et al., 2012	Aged C2C12 (54x)	Collagen	*In vitro* model	Self contraction; reduced contractile force
Weist et al., 2012	Primary cells from F344 soleus	Self produced ECM	*In vitro* model	TGF-β1 effect evaluation; electrical stimuli; self contraction into 3D
Elmer et al., 2011	C2C12; C3H10T1/2	Electrospun PS	*In vitro* model	Chemical functionalization; μtopography
Li et al., 2011	Primary myoblasts from C57/B6 mice; embryonic fibroblasts from E13/CF1 mice	Self produced ECM; fibrin	*In vitro* model	Self contraction and generation of 3D structures; cocolture
Pennisi et al., 2011	C2C12	Collagen I	*In vitro* model	Mechanical conditioning, uniaxial vs. multiaxial
van der Schaft et al., 2011	H5V EC; C2C12	Collagen I; self produced ECM	*In vitro* model	Self contraction; cocolture; effect of paracrine VEGF on vascularization and alignment
Lam et al., 2009	Spreague-dawley rat soleus SC	Self-produced ECM	*In vitro* model	μpatterning; self assembly into 3D (roll-up)
Riboldi et al., 2008	C2C12	Degrapol	*In vitro* model	μpatterning
Engler et al., 2004	C2C12; primary human fibroblasts	Patterned polyacrylamide gel	*In vitro* model	Mechanoregulation; cellular substrate
Dennis et al., 2001	Primary sprague-dawley rat SC and FBs	Self produced ECM	*In vitro* model	Self contraction; self structuring 3D (roll up)

BMMSC: Bone Marrow Mesenchimal Stem Cell. EC, Endothelial Cell; FB, FibroBlast; GFP, Green Fluorescent Protein; hPSC, human Pluripotent Stem Cell; mNSC, mouse Neural Stem Cell; NM, NeuroMuscular; NMJ, NeuroMuscular Junction; PEG-MAL, PolyEthylene Glycol-MALeimide; pHMB, primary Human MyoBlast; SC, Satellite cells; SM, Skeletal Muscle.

sources, and have been used directly or subsequently to genetic modification intended to increase their effectiveness through augmented expression of growth factors and cytokines (De Coppi et al., 2005). As for the scaffold, its structure can retain chemical signals and modify their local concentration, either geometrically or by surface modification which bind and/or release these factors at a controllable rate; similarly, scaffold derived from the decellularization of tissues are treated to minimize the immune reaction of a xenogeneic host (Badylak, 2014). Moreover, the rate of degradation, and the mechanical and topographical characteristics of the scaffold modify the behavior of the surrounding cells (Zhang et al., 2014), and therefore of the vascular compartment; uniform, randomized geometry, such as interconnected pore networks, facilitate the vascularization process, without imparting a definitive structure of the vascular compartment (Levenberg et al., 2005). However, since a functional design is proved to be advantageous in the integration of SMTE constructs (Koffler et al., 2011), and given that the process of vascularization is inextricably bound to the structural development of living tissues (Tirziu and Simons, 2009; Lesman et al., 2010), it is reasonable to assume that the presence of a defined architecture prior to implantation will inform the structure of the resulting implant, facilitating its integration in tissues characterized by a high degree of anisotropy such as skeletal muscle (Takaza et al., 2013).

MECHANICAL AND PHYSICAL STIMULI

Cells are sensitive to mechanical and physical changes in their environment, such as the bidimensional or tridimensional structure of the scaffold, its stiffness, or the presence of a voltage across the culture medium; recently, the effect induced by these parameters on cellular behavior have been studied *in vitro*, in the hope to obtain versatile tools for tissue engineering not subjected to the drawbacks suffered by biochemical signals, namely difficult application, limited availability and restrictive regulations.

The cellular mechanisms responsible for the translation of these signals into complex behavior are still being studied, notably in the engineering of tissues with load-bearing and dynamic functions (Guilak et al., 2014). Cells are able to feel and interact with their environment through specialized clusters of transmembrane proteins forming focal adhesions (FAs) (Eyckmans et al., 2011). These structures connect extracellular proteins and other surfaces viable to attachment to the cytoskeleton, the complex system of structural proteins that functions as an actuator of mechanical actions and as a sensor to the environment (Discher et al., 2005), translating its deformation into transcription factors headed for the nucleus; moreover, this structure forms a mechanical connection the nucleus and the Golgi Apparatus to the exterior of the cell (Wang et al., 2009), suggesting a close relationship between the shape of nucleus and the behavior of the cell (Nava et al., 2014).

In particular, the cytoskeletal architecture of the skeletal muscle is intrinsically related to its function and mediates the mechanical stimuli required in the maturation of tissue engineering constructs (Vandenburgh et al., 1988, 1991, 1999). In the original work of Vandenburgh et al. chicken myoblasts seeded on a collagen gel were exposed to stimuli meant to simulate processes observed in embryos, in accordance with the generally agreed principle that regeneration of tissue recapitulates embryonic development (Chargé and Rudnicki, 2004); the protocol, with some variations (Boonen et al., 2010), consists in an initial stretching corresponding to the elongation of the bone, followed by intermittent contractions similar to the impulses derived from postnatal stimuli (Riboldi et al., 2008). Skeletal muscle cells exposed to the correct stimuli show increased expression of differentiation markers compared to static cultures (Candiani et al., 2010), and progress into the formation of polynucleate syncytia; the reaction to cyclic strain, however, depends heavily on the amplitude and frequency of the stimuli: the same line of myoblasts have shown different reactions to alternate protocol and, interestingly, to alternate topographies. It has been shown (Ahmed et al., 2010) that myoblast cell lines tend to align to grooves in the substrate, but they respond to cyclic tension by forming striates stress fibers angled at 45° with respect to the direction of the deformation; finally, on flat surfaces, cyclic mechanical stretching induces fiber angled ad 70° to the stretching axis, attributed to the perpendicular contraction due to the Poisson's ratio of the material. Given the complex behavior exhibited by the cells in response to mechanical stimuli, a reliable protocol for cellular alignment would prove of great value in the engineering of tissues with specific geometric requirements (Li et al., 2013b).

An interesting interpretation has been proposed recently Equation (1) (Livne et al., 2014), which considers the dissipation of cellular elastic energy as the principal reorientation factor The resulting model predicts the orientation angle of the cells θ based on a dimensionless parameter specific of the cell line and the ratio between principal strain and the Poisson ratio of the support:

$$\overline{\theta} = \arccos\left(\sqrt{b + \frac{1-2b}{r+1}}\right) = \arctan\left(\sqrt{\frac{r + b\cdot(1-r)}{1 - b\cdot(1-r)}}\right) \quad (1)$$

where r is the negative inverse of the ratio between the principal strain and its perpendicular counterpart, and b is a dimensionless parameter that depends from the ratio between the cellular Young modulus along its main orientation axis ($E_{\theta\theta}$) and that along the perpendicular axis ($E_{\rho\rho}$). Although this model is capable of predicting the orientation angle well within a significant confidence interval in a bidimensional setting, it is yet to be tested with multiple cell lines or in a three dimensional environment, a setting that is known to influence cell behavior in a complex manner (Boonen et al., 2010). Nevertheless, it provides a relatively straightforward interpretative key to the disposition of cells in load bearing and dynamic tissues, provided of course that the parameter *b* could be used in cells with high aspect ratio such as myocytes.

While the disposition of the cells in response to mechanical stimuli can be approximated to a purely physical process, growth, differentiation and function are far more complex behaviors; a viable avenue for this kind of research lies in the automated analysis of the cellular response to every possible combination of multiple factors. This technique can provide a unifying research framework and it can be used to rapidly individuate unexpected cellular responses to specific sets of stimuli. Similar approaches have been used to determine the reactions of undifferentiated cells to 2D topography (Unadkat et al., 2011), biomaterial composition (Mei et al., 2010), stiffness in 3d scaffolds (Sala et al., 2011)

and a combination of 3D scaffolds and growth factors (Ranga et al., 2014). On a final note, it is worthwhile to mention that these combinatorial studies are also limited in that they expose the cell to repetitive stimuli that do not accurately replicate the environment: the ECM is characterized by a controlled randomness, which has proven to influence cell behavior in bidimensional studies (Dalby et al., 2006; Biggs et al., 2010); it can be argued then that these studies can only provide a framework for the identification of complex stimuli to be analyzed subsequently with more accurate techniques.

CLINICAL APPLICATIONS—PRESENT AND FUTURE

As of now, SMTE constructs are either intended as *in vitro* skeletal muscle disease models (Gayraud-Morel et al., 2009; Vandenburgh, 2010; Kelc et al., 2013) (**Table 1**) or as *in vivo* preclinical research tools (Turner et al., 2012; Carosio et al., 2013; Mertens et al., 2014) (**Table 2**).

The only regenerative medicine devices to have reached clinical application are acellular compounds derived from animal ECM used *in loco* to improve the motility and strength of skeletal muscles subjected to traumatic injury (Mase et al., 2010; Sicari et al., 2014), or cell therapy strategies that are injected locally and systematically to improve syndromes stemming from genetic diseases (Tedesco et al., 2010; Tedesco and Cossu, 2012). While these therapies use tissue engineering techniques, they do not fall into the classic definition of TE constructs (Langer and Vacanti, 1993), since they lack either a cellular component or a scaffold and are not subjected to maturation in a bioreactor. Nonetheless, it is likely that future TE devices will refer to these forerunners as a model on which to improve, and that further early approaches in this field will implement methodologies that have proven most effective on their own in clinical trials.

TOWARD A HUMAN HOST

In the translation to the clinic, TE devices face a unique set of challenges due to their being artificial constructs made of synthetic and biologic material, the latter being of autologous, syngeneic or even xenogeneic origin (Mertens et al., 2014); setting aside the regulatory aspects of this progression, which have been recently described in details (Pashuck and Stevens, 2012; Fisher and Mauck, 2013; Martin et al., 2014) there are some aspects in the development of a preclinical engineered skeletal muscle device that are of particular relevance when considering clinical applications.

Most of the SMTE constructs that are currently being developed rely on small mammals as animal models to study their effects on muscle repair (**Table 2**); these models are reliable, easily available and provide comparable result between different laboratories, yet they also suffer from substantial limitations in the translation to the clinic (Boldrin et al., 2010; Seok et al., 2013; Bareja et al., 2014). In most cases, human cell lines and primary cells are used to develop constructs that are subsequently implanted in athymic models, lacking therefore the ability to mount an adaptive immune response; these models allow for the maturation of the implant in a living subject, under the reasoning that subsequent human trials would rely on autologous cells and scaffolds proven to be clinically compatible. However, the reaction to the human molecular apparatus found in the cellular compartment of the devices may differ from that originating from the host (Borisov, 1999). The predictive capability of these models in regard to cellular behavior under growth factor stimulation was put into question in a recent paper (Mujagic et al., 2013), where the authors suggested that the effects of different species' isoforms of the growth factor VEGF vary greatly depending on the recipient cells: this would explain the apparent inability of human VEGF to cause angiomas in mouse models, which could lead to an underestimation of the side effects of modified cells in autotransplantation cases. As a consequence, other studies which propose the use of genetically modified cells to improve the vascularization of regenerating muscles (Gianni-Barrera et al., 2013; Shevchenko et al., 2013) may have to compensate the effects observed in animals as to avoid the formation of irregular vasculature in human hosts; moreover, these uncertainties are also observed in clinical cases involving novel combinations of accepted materials and cellular populations, particularly undifferentiated staminal cells, which, if unregulated, could lead to ectopic tissue formation and neoplastic lesions (Amariglio et al., 2009; Thirabanjasak et al., 2010).

There are currently few ways to solve this problem, as the understanding of the interaction between host and implant is still largely incomplete, particularly in regard to the role of the immune system (Bohgaki et al., 2007); however, given the urgent need for reliable constructs capable of supplementing and replacing tissue transplants, alternative solutions to this problem could be found in therapies that do away entirely with the cellular component of the graft (Burdick et al., 2013), or in the development of cellular populations such as autologous induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), which could solve the problems correlated with allotransplantation (Araki et al., 2013). Nevertheless, as these cells are capable of forming teratomas (Knoeplfer, 2009), their usage in clinical setting is problematic; protocols intended to produce differentiated cells from iPSCs have proven effective in limiting the occurrence of tumors both in mice (Liu et al., 2013; Suzuki et al., 2013) and in non-primate models that more closely resemble human physiology (Hong et al., 2014); as this line of research progresses, it will be feasible to use this technology to develop constructs from the patient's own differentiated cells, in a clinical testing framework offsetting potential benefits from limited risks (Goldring et al., 2011).

Another topic that will need to be addressed before the clinical application of SMTE constructs is also correlated to the immune reaction to the devices and to the conditions present at the site of implantation: these devices are *in situ* implants in recently wounded sites which do not replicate the conditions of VML encountered in clinical setting (Grogan et al., 2011; Li et al., 2013a): it is not common practice to develop animal models of VML in which the site of implantation had already undergone a normal repair process which resulted in the formation of scar tissue. As the homeostasis of the site of injury is much different compared to that of uninjured tissues, the predictive value of the animal models is diminished, and even more so when the damage is compounded by genetic mutations leading to defects in the repair process (Bosurgi et al., 2011).

Table 2 | *In vivo* models of interest.

References	Model	Scaffold	Construct type	Relevant details
Corona et al., 2014	Heterologous MDCs	BAM	TA VML; Lewis rats	Uniaxial mechanical strain
Juhas et al., 2014	GCaMP3+ Sprague-Dawley muscle tissue	Fibrin/Matrigel	*In vitro* model; dorsal skinfold window chamber in nude mice	Self contraction; *in vitro* maturation; *in vivo* vascularization; *in vivo* CTX damage regeneration
Carosio et al., 2013	Autologous muscle tissue	Autologous ECM	EDL VML; C57BL6 WT, MLC/hAP	Self assembly, self contraction; response to electrical stimuli; vascularization
Corona et al., 2013a	Autologous muscle tissue	Autologous ECM	TA VML; Lewis rats	*In vivo* filler
Criswell et al., 2013	GFP-FVB MPCs; HUVECs; 10T1/2 cells	Matrigel	Subcutaneous insertion in nude mice	*In vivo* activity of ECs and pericytes on SMTE constructs
Haraguchi et al., 2012	Rat cadiac cells; HUVECs; HSMMCs	PNIPAAm substrate; fibrin, gelatin substrate; self produced ECM	Subcutaneous dorsal insertion, transplantation onto infarcted heart in F344 nude mice	Cell sheet stacking; electrical stimuli maturation; *in vitro* vascularization
Williams et al., 2012	Explanted soleus muscle cells	Autologous ECM on fibrin	Implantation along the VL, near the sciatic nerve. Innervation with sural nerve in F344	Self contraction and effect of innervation with host's nerve; shift in myosin type
Koffler et al., 2011	C2C12; HUVECs; human foreskin fibroblasts	Surgisis SIS	Full thickness abdominal wall replacement in nude mice	Cocolture; variable *in vitro* conditioning period
Levenberg et al., 2005	C2C12; HUVECs; mouse EFs	PLLA-PGA porous scaffold	Dorsal midline subcutaneous implantation in CB17 SCID	Coculture; prevascularization
Sicari et al., 2014	–	SIS	ATC (3), quadriceps (2) VML	Clinical trial
Mase et al., 2010	–	BAM	Quadriceps femori VML	Clinical case

BAM, Bladder Acellular Matrix; CTX, CardioToXin; EDL, Extensor Digitorum Longus; EF, Embryonic Fibroblasts; HSMMC, Human Skeletal Muscle Myoblast Cells; HUVEC, Human Umbilical Vascular Endothelial Cell; MPC, Muscle Progenitor cell; PGA, PolyGlicolic Acid; PLLA, Poly-L-Lactic Acid; PNIPAAM, poly-N-IsoPropylAcrylAmide; SIS, Small Intestine Submucosa; TA, Tibialis Anterior.

CURRENT APPROACHES

There are numerous hurdles to be overcome before SMTE constructs will have a use in clinical settings; to the best of our knowledge, there have only been two clinical applications of regenerative medicine devices for VML in skeletal muscle, both involving *in situ* implantation of decellularized porcine small intestinal submucosa (SIS). In the first instance, a clinical case, a large volumetric defect in the quadriceps femori, treated 3 years before with a latissimus dorsi muscle flap (Mase et al., 2010), was treated with an acellular SIS scaffold. Following the positive outcome of this experiment a clinical trial involving five patients and a different scaffold (porcine bladder matrix) was recently published (Sicari et al., 2014), showing that the treatment did not induce an adverse effect [as xenogeneic ECM would if not completely decellularized (Keane et al., 2012)] and improvement in strength and mobility in three cases, compared to a patient-specific baseline. While this device cannot be considered the result of tissue engineering according to the usually accepted definition (Langer and Vacanti, 1993), it provides evidence that a commonly used tissue engineering scaffold can improve skeletal muscle injuries; moreover, acellular scaffolds such as SIS have shown performance comparable to decellularized muscle tissue in SMTE treating of VML (De Coppi et al., 2006; Wolf et al., 2012), which could obviate the need for human tissue as a starting material for scaffold development.

These results, and other reports analyzing the effect of empty scaffolds on cell homing (Ju et al., 2014), suggest that SMTE materials may indeed constitute a valid clinical options for the treatment of VML. A better understanding of the role of the cellular component of the devices will be required, as evidence in other engineered tissues have proven a limited structural role for the seeded cells, which are rapidly overcome and substituted by the host's own (Hibino et al., 2011).

FUTURE DIRECTIONS

Tissue engineering and regenerative medicine treatment of skeletal muscle VML is intrinsically dependent on the dimensions of the devices meant to substitute the missing tissues: the lesions to be treated are extensive by their very own nature, and require thick flaps that are beyond what is currently possible to reproduce through TE constructs. Given the unique characteristics of skeletal muscle tissue, clinically relevant SMTE constructs will require techniques aimed at the development of functional structures of large scale in the three dimensions. The techniques that are going to see clinical application in the most immediate future are those which are derived from accepted clinical methodologies, which allow the introduction of fewer variables to be tested in the preclinical phases.

In this respect, decellularized acellular devices possess many characteristics that make them suitable for a rapid translation to a clinical application: the ECM derived from large mammals has seen ample use as grafts in vascular settings (Breymann et al., 2009), and the structure and composition of the decellularized material is capable of promoting the vascularization of the structure as it is assimilated in the body (Burdick et al., 2013; Teodori et al., 2014); nonetheless, concerns about the complete decellularization of the materials are still present, as residual traces of cellular material can lead to the failure of the device (Keane et al., 2012). An alternative to the use of heterologous material is the production of *in vitro* ECM from cell cultures derived from the host: this approach allows for less stringent decellularization parameters, and provide a molecular milieu closely resembling that of the original tissue, lacking however its highly organized architecture, as these devices are produced with synthetic scaffolds (Lu et al., 2011) or directly from connected cell sheets (Dahl et al., 2011). For this methodology to be applicable in VML treatment, the *in vitro* maturation should produce either a single construct with patent vessels (Gualandi et al., 2013) or multiple layers complete of vascular structure which would anastomose when placed in close contact. The latter approach has met with some results in recent reports (Sakaguchi et al., 2013; Sekine et al., 2013), where multiple cell sheets have been stacked to achieve multiple layer thickness to produce cellular constructs that were directly implanted in animal models. While the constructs obtained through this methodology are still far from the requirements of skeletal muscle VML treatments, they have been proven advantageous in the treatment of a case of dilated cardiomyopathy (DCM) (Sawa et al., 2012); although the dimensions of the implants (40 mm of diameter and a thickness of four cell layers) were scarcely comparable to those required for VML, this case constitutes an important precedent for the application of cell sheet engineering in the treatment of striated muscle disorders.

Therefore, it is foreseeable that clinical SMTE will proceed gradually incrementing the complexity of the implanted devices, giving preference to methodologies that implement cellular populations subjected to minimal *in vitro* treatment, or none at all.

CONCLUSIONS

SMTE has seen a rapid development in the last decade, facilitated by the growing understanding of the processes underlying the regeneration of this tissue and the concomitant formation of vascular networks. The latter topic is of particular interest for the production of clinically relevant constructs, as the treatment of VMLs requires large, mature engineered tissues with stable vessel networks. The structure of the scaffold and mechanical stimuli applied during the *in vitro* phase of development have emerged as viable tools for the production of ordered, vascularized tissues with significant prospective clinical applications. As decellularized materials are undergoing clinical trials, it is foreseeable that clinical devices composed of ordered scaffolds and autologous vascular compartments will also be used in the treatment of VMLs.

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Cardiac tissue engineering: a reflection after a decade of hurry

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The heart is a perfect machine whose mass is mainly composed of cardiomyocytes, but also fibroblasts, endothelial, smooth muscle, nervous, and immune cells are represented. One thousand million cardiomyocytes are estimated to be lost after myocardial infarction, their loss being responsible for the impairment in heart contractile function (Laflamme and Murry, 2005).

The potential success of cardiac cell therapy relies almost completely on the ability of the implanted cells to differentiate toward mature cardiomyocytes. These cells must be able to reinforce the pumping activity of the injured heart in the absence of life-threatening arrhythmias due to electrophysiological incompatibility.

These conditions can only be met if the newly formed cardiomyocytes can integrate electromechanically with the host tissue while getting appropriate vascularization from host- or donor-derived vessels.

Together with the regeneration of the contractile component of the heart, attention must be paid to the search for novel methods to deliver appropriate vascularization to the ischemic areas and to the preservation of structures controlling the efficiency of blood pumping to the organism: the heart valves. Although fetal, neonatal, and adult cardiomyocytes have been tested for their actual ability to engraft diseased heart and improve its function (Dowell et al., 2003), most of the hopes in cardiac tissue regeneration relies on the possibility to use pluripotent and adult stem cells in targeted tissue engineering applications.

In this respect, the set-up of protocols to obtain reprogrammed pluripotent stem cells (Takahashi and Yamanaka, 2006), a discovery awarded with the Nobel Prize in Physiology or Medicine in 2012, represents the demonstration that such advances in knowledge and technology will eventually lead to novel approaches to the treatment of incurable diseases.

Unfortunately, notwithstanding a decade of intense investments, the results in terms of knowledge transferred to the clinical practice are very limited.

The present issue addresses the topic of cardiac tissue regeneration from the viewpoint of stem cell biologists and tissue engineers, seeking for the most appropriate source of cells to replace dead myocardium, improve the organ structure and function, as well as exploring the most suitable delivery system for such cells.

In the last couple of years a number of papers have been published on the ability of biomaterials and bio-constructs to drive the differentiation of stem cells for cardiac tissue engineering applications. Most of these studies used mesenchymal stem cells (MSCs) from bone marrow, adipose tissue or induced pluripotent stem cells (iPSCs). On the other hand, the contribution of cells derived from the heart tissues is here considered of outmost importance since these cells are believed to retain the potential to become highly differentiated cardiomyocytes.

LOOKING FOR SOURCES OF CONTRACTILE CELLS

Studies on the differentiation of embryonic cells into cardiomyocytes started in 1989 (Rudnicki et al., 1989). From that time, many candidates have been taken into consideration to substitute dead contractile cells, as myoblasts, hematopoietic lineages, and cardiac side populations.

While skeletal myoblasts can generate contractile cells that cannot couple electromechanically with the host myocardium (Forte et al., 2013), the other two stem cell subsets, hematopoietic stem cells (HSCs) and MSCs, are now universally recognized to retain no capacity to acquire the contractile phenotype (Balsam et al., 2004; Murry et al., 2004; Gnecchi et al., 2005; Siegel et al., 2012).

In the case of HSCs, after contrasting papers were published by the group of Piero Anversa and Charles E. Murry, the compelling conclusion that this bone marrow subset is not suitable for contractile cell regeneration was agreed (Orlic et al., 2001; Balsam et al., 2004; Murry et al., 2004).

The resistance of the other bone marrow-derived stem cell subset (bone marrow MSCs) as candidate to produce new cardiomyocytes has been even longer: after a seminal paper published by the group of Keiichi Fukuda in 1999 (Makino et al., 1999) it took more than 10 years to clarify that bone marrow mesenchymal progenitors have no cardiogenic potential.

A convincing investigation by Siegel and collaborators finally clarified that

human MSCs are indeed able to acquire specific cardiomyocyte markers *in vitro*, while failing to show contractile functional features. When implanted in an *in vivo* model of rodent myocardial infarction, MSCs do not completely differentiate into functional cardiomyocytes, proving that BM-derived MSCs may not be used in cardiac regeneration (Siegel et al., 2012).

Additionally, the work of the group of Massimiliano Gnecchi recently explained the positive outcome of many pre-clinical and clinical trials entailing MSC administration, by showing that the predominant effect of implanted MSCs in the heart consists in the secretion of anti-apoptotic signals for the resident cells (Gnecchi et al., 2005).

The same mechanism has been recalled to explain the modest but positive results obtained by vessel-associated mesangioblast implantation in infarcted heart (Galli et al., 2005) and may be responsible of the positive outcome of the implantation of epicardially delivered adipose tissue-derived stromal cell (ADSC) sheets in a mouse model of dilated cardiomyopathy (Hamdi et al., 2013).

Other sources of mesenchymal progenitors are still credited of cardiogenic potential: the process of adipocyte dedifferentiation to produce mesenchymal progenitors has been known for some years, while only recently evidence that dedifferentiated adipocytes and adipose-derived mesenchymal cells can spontaneously acquire the contractile phenotype *in vitro* and help tissue healing *in vivo* was given (Jumabay et al., 2009). Interestingly, the use of this cell source for cardiac regeneration therapy would pose no availability issue, nor any immunological concern.

The choice of the cell and the construct to implant is of outmost importance. With the usefulness of MSCs as an easy source of cells for autologous implantation being questioned, the search for the best candidate as contractile cell substitute is still open. The use of cardiac resident progenitor cells—firstly being identified by Quaini et al. (2002) based on c-Kit+ expression and already being tested in a clinical trial (Bolli et al., 2011)—appears to be a promising alternative for cardiac tissue engineering. Nonetheless, compelling evidence of their efficacy *in vivo* still needs to be provided by independent research groups.

No matter the route of delivery of cells to the cardiac tissue and the cell source, small but significant improvements in terms of cardiac function have been repeatedly reported mainly through a paracrine effect (Mirotsou et al., 2011), while the goal of regenerating massive portions of cardiac tissue still remains elusive.

CARDIAC PROTO-TISSUES: TRYING TO REPRODUCE THE COMPLEXITY OF THE MYOCARDIUM

The complexity of the myocardium resides in the number of cell types represented in it and in its highly hierarchical organization.

The ideal cardiac-specific scaffold would comply with cardiac muscle architecture, be deformable enough to sustain cardiac contraction, while being colonized by host vasculature and resorbed/degraded in due time. The biomaterials and scaffolds so far proposed for cardiac tissue engineering can be grouped into three categories: (1) scaffolds mimicking extracellular matrix (ECM) structure and/or the elasto-mechanical properties of the myocardium, (2) *in vitro* engineered cardiac patches, (3) three-dimensional (3D) scaffolds reproducing myocardial structural properties.

Culturing cells in three dimensions or on substrates displaying the mechanical properties of the myocardium has been deemed sufficient to prompt cardiac commitment but not complete maturation *per se* (Engler et al., 2006; Di Felice et al., 2013).

The attempts at designing scaffolds mimicking ECM structure and replicating the *in vivo* structural and mechanical features of the ECM, in order to provide stem cells with meaningful cues and comply with the architecture of the host tissue are far from being successful.

In an attempt to reproduce cardiac ECM stiffness, polyurethane acrylate (PUA) mold was recently used to pattern polyurethane (PU) and poly(lactide-co-glycolide) (PLGA) hydrogels via UV-assisted or solvent-mediated capillary force lithography (CFL) (MacAdangdang et al., 2014).

Alternatively, the combination of cell electrospraying and electrospinning to generate a solution of hydrogel and polyurethane blend was also proposed (Xu et al., 2014).

A natural approach to the issue of reproducing the ECM mechanics and topography was proposed by adopting human pericardium membranes to create 3D microporous scaffolds (Rajabi-Zeleti et al., 2014), or by using decellularized human myocardium as a biomaterial for cardiac tissue regeneration and engineering (Oberwallner et al., 2014).

Finally, hybrid biomimetic membranes were developed for cardiac tissue regeneration by binding the epitope sequences of laminin and fibronectin to poly(glycerol sebacate) (PGS) films (Rai et al., 2013). The same polymer has been successfully manufactured to obtain scaffolds displaying the anisotropic elasticity typical of the myocardium and promoting neonatal cardiomyocyte alignment (Engelmayr et al., 2008).

An example of cardiac constructs displaying the elasto-mechanical properties of the myocardium is given by the incorporation of thiophene-conjugated carbon nanotubes (T-CNTs) into polycaprolactone (PCL) electrospun meshes (Wickham et al., 2014) and using the polymer 3-hydroxybutyrate and 3-hydroxyvalerate (PHBHV). The combination of PHBHV with gelatin resulted in a 3D scaffold resembling the physico-chemical properties, micro-topography, and mechanics of the myocardium (Cristallini et al., 2014).

Given the superior performance in terms of survival and function of 3D co-cultures including endothelial cells (or their progenitors) and embryonic fibroblasts in skeletal muscle engineering (Levenberg et al., 2005), few attempts have been taken to create complex, multicellular engineered cardiac tissue constructs (proto-tissues) *in vitro* to be applied on damaged cardiac tissues.

For example, in this issue of Frontiers in Physiology, human MSCs have been used to provide interconnected vessel-like structures onto gelatin scaffolds, where pre-committed human cardiomyocyte progenitor cells (CMPCs) were then seeded (Pagliari et al., 2014).

Alternatively, a heterogeneous neonatal cardiac cell preparation was seeded onto fibrin gels until endothelial cells grew

and an adequate contractile activity was registered (Tao et al., 2014).

Scaffolds may represent a very useful device to deliver cardiac stem cells to the ischemic or damaged heart. Besides the dream of generating personalized prototissues with finely controlled size and shape to match the particular need of the patient, the formulations proposed are far from reproducing the complexity of the cardiac tissue.

The use of medical devices may help researchers to reduce the number of implanted cells and to drive their differentiation through the use of embedded molecules or growth factors. And while the physico-chemical features of the scaffold proved to be important in prompting stem cell alignment and commitment, the role of the immune response in cardiac tissue engineering deserves more attention.

Although promising in terms of technology advancement, the extent to which these studies address the hierarchical organization of the myocardium is still far from being relevant. Cardiac tissue engineering holds the promise to revolutionize the future of heart disease treatment and the use of scaffolds may provide valuable 3D cues for stem cells to grow, differentiate, and distribute into an organized tissue.

CONCLUDING REMARKS

The common limitation of the studies so far proposed in cardiac tissue engineering remains the lack of a credible signature of complete cell maturation toward the cardiomyocyte phenotype. Most of the investigations showcased the expression of proteins typical of the cardiac tissue, and a spatial distribution or alignment of the cells inside the scaffolds. Some research groups were able to show a striation typical of myofibrils (Engelmayr et al., 2008), others the massive expression of cardiac troponins, formation of intercalated disks, and functional gap junctions (Di Felice et al., 2013; Cristallini et al., 2014). Unfortunately, the extent of differentiation shown is very far from the actual maturation required to rebuild the cardiac tissue complexity. Reproducing such complexity would require the formation of blood vessels, the connection of nerve terminations, the participation of cells from the immune system, fibroblasts, enterochromaffin cells, cardiomyocytes of the conduction system.

While waiting for bio-instructive scaffolds displaying a modular distribution of differentiative cues, the possibility to combine different cell subsets with distinct potential with 3D scaffolds reproducing the hierarchical structural complexity of the native tissue represents a valuable approach to generate complex cardiac constructs to treat cardiopathic patients.

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Muscle acellular scaffold as a biomaterial: effects on C2C12 cell differentiation and interaction with the murine host environment

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The extracellular matrix (ECM) of decellularized organs possesses the characteristics of the ideal tissue-engineering scaffold (i.e., histocompatibility, porosity, degradability, non-toxicity). We previously observed that the muscle acellular scaffold (MAS) is a pro-myogenic environment *in vivo*. In order to determine whether MAS, which is basically muscle ECM, behaves as a myogenic environment, regardless of its location, we analyzed MAS interaction with both muscle and non-muscle cells and tissues, to assess the effects of MAS on cell differentiation. Bone morphogenetic protein treatment of C2C12 cells cultured within MAS induced osteogenic differentiation *in vitro*, thus suggesting that MAS does not irreversibly commit cells to myogenesis. *In vivo* MAS supported formation of nascent muscle fibers when replacing a muscle (orthotopic position). However, heterotopically grafted MAS did not give rise to muscle fibers when transplanted within the renal capsule. Also, no muscle formation was observed when MAS was transplanted under the xiphoid process, in spite of the abundant presence of cells migrating along the laminin-based MAS structure. Taken together, our results suggest that MAS itself is not sufficient to induce myogenic differentiation. It is likely that the pro-myogenic environment of MAS is not strictly related to the intrinsic properties of the muscle scaffold (e.g., specific muscle ECM proteins). Indeed, it is more likely that myogenic stem cells colonizing MAS recognize a muscle environment that ultimately allows terminal myogenic differentiation. In conclusion, MAS may represent a suitable environment for muscle and non-muscle 3D constructs characterized by a highly organized structure whose relative stability promotes integration with the surrounding tissues. Our work highlights the plasticity of MAS, suggesting that it may be possible to consider MAS for a wider range of tissue engineering applications than the mere replacement of volumetric muscle loss.

Keywords: extracellular matrix, niche, osteogenic differentiation, myogenic differentiation, tissue engineering, regenerative medicine

INTRODUCTION

A niche is composed of elements that surround stem cells: tissue specific cells, extracellular matrix and local growth factors (Yin et al., 2013). These elements determine the local microenvironment that supports the maintenance of stem cell identity and regulates the function of stem cells (Kuang et al., 2008). In addition, the niche supports stem cells and controls their self-renewal *in vivo* (Spradling et al., 2001) by modulating the asymmetric cell division insomuch as it ensures stem cell renewal and production of a sufficient number of committed daughter cells for tissue homeostasis and repair (Kuang et al., 2008). It is worth bearing in mind that the local microenvironment affects not only stem cell behavior (particularly the stem cell specific auto-renewal feature) but also the differentiation potential and cell division of committed daughter cells deriving from stem cell asymmetrical division. Indeed, a fibroblast-specific niche has been described for cell culture purposes (Sivan et al., 2014), while the bone marrow niche that regulates hematopoietic stem cells is also reported to be necessary for B-cell commitment (Adler et al., 2014). Future challenges involved in the recreation of cell niches as platforms

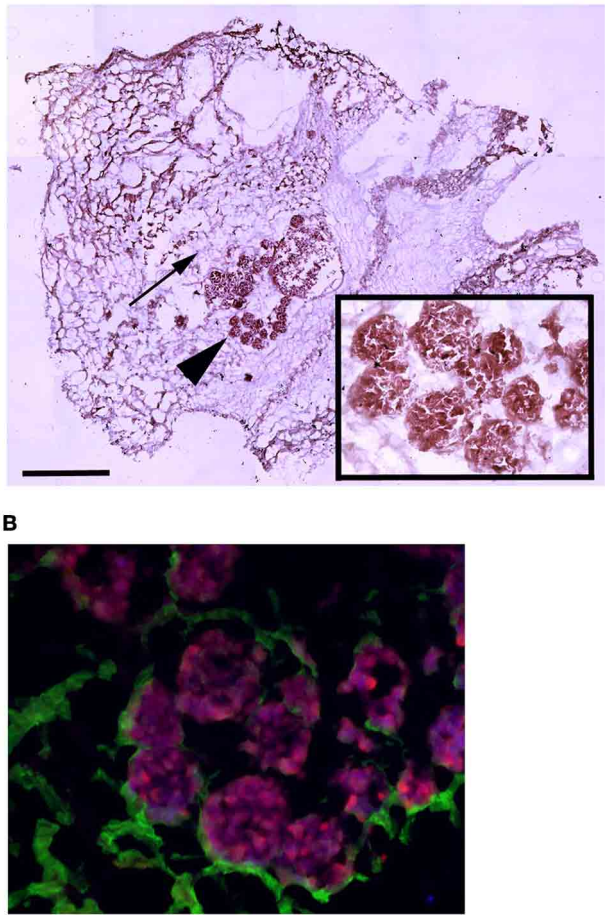

FIGURE 1 | MAS is suitable for 3D myogenic cultures *in vitro*. (A) Hematoxylin and eosin staining of C2C12 cells cultured in MAS in myogenic differentiation medium for 1 week, showing both the sponge-like MAS network (arrow) and cell aggregates (arrowhead); the latter are also visible at a higher magnification in the inset. **(B)** Immunofluorescence localization of laminin (green) and myosin (red) on a serial section demonstrates that well differentiated muscle cells lie in aggregates on base membrane-derived laminin sheets. The image is representative of a triplicate experiment. Bar: 200 μ.

for culture models, which will allow the true *in situ* regenerative niche to be investigated, have been reviewed by Kirkpatrick (Kirkpatrick, 2014).

The definition of the microenvironment affecting both stem cell renewal and committed daughter cell differentiation is of particular relevance to tissue engineering (TE). TE represents an innovative approach based on the emulation of neo-organogenesis aimed at recreating a wide range of tissues to be used to replace lost tissues (Klumpp et al., 2010). A commonly applied definition of TE, provided by Langer and Vacanti, is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer and Vacanti, 1993). For TE purposes cells are often transplanted or seeded into a structure capable of supporting three-dimensional tissue formation. These structures, referred to as scaffolds, are critical, both *ex vivo* and *in vivo*, to recapitulate the niche and to support cell adhesion, survival and differentiation. Indeed, not only do scaffolds allow cell migration and/or attachment, but they also deliver and retain cells and biochemical factors, permit the diffusion of vital cell nutrients and exert mechanical and biological influences that control cell behavior (Macchiarini et al., 2008; Whitney et al., 2012). Scaffolds may be made of either natural or synthetic materials. Indeed, various derivatives of the extra cellular matrix (ECM) have been studied because they possess all the features of the ideal tissue-engineered scaffold or biomaterial, which include histocompatibility, bioactivity, porosity, degradability, non-toxicity and mechanical properties that match those of the original tissue (Borschel et al., 2004).

An alternative to the production of constructs composed of cells seeded into scaffolds is ECM. ECM can be used to support *in situ* regeneration, thereby relying on the bioactivity of autologous or heterologous biomaterial on autologous cells. Indeed, ECM is manufactured by the resident cells of each tissue and organ and is in a state of dynamic equilibrium with its surrounding microenvironment. We may assume, even without deciphering the complex three-dimensional organization of the structural and functional molecules of which it is composed, that ECM is biocompatible because cells produce their own matrix (Badylak, 2007). Recently, an increasing amount of attention has been paid to the use of ECM-based scaffolds for TE interventions. ECM-based scaffolds not only preserve the structure and molecular features of the native ECM, but also release matricryptic peptides during degradation. Matricryptic peptides affect cell motility, proliferation and differentiation, thereby greatly influencing the constructive remodeling of new tissue (Faulk et al., 2013). For these reasons, various forms of intact ECM have been used as biological scaffolds to promote the constructive remodeling of tissues and organs (Dahms et al., 1998; Meyer et al., 1998), with many of these ECM materials being marketed for a variety of therapeutic applications (Perniconi and Coletti, 2014; Teodori et al., 2014). Intact ECM is typically obtained by means of decellularization from explanted tissue in such a way as to create scaffolds that maintain the original spatial organization and biochemical composition. Tissue decellularization may be achieved in various ways, all of which eliminate the cellular compartment and leave a spatially and chemically preserved ECM (Crapo et al., 2011; Teodori et al., 2014).

We previously produced muscle acellular scaffolds (MAS) by means of decellularization at the whole organ scale of murine skeletal muscles. We characterized the *in vivo* response to grafted MAS and observed that such a construct provides a pro-myogenic environment (Perniconi et al., 2011). In particular, we reported that MAS orthotopically transplanted in mice was colonized by both inflammatory and stem cells and supported *de novo* muscle fiber formation (Perniconi et al., 2011). By definition MAS possesses only one component of the niche, i.e., the muscle ECM, being deprived of tissue specific cells and growth factors. In our previous experimental settings (Perniconi et al., 2011), as MAS was orthotopically grafted to replace a *Tibialis anterior* muscle (TA), the relative contribution to muscle formation by

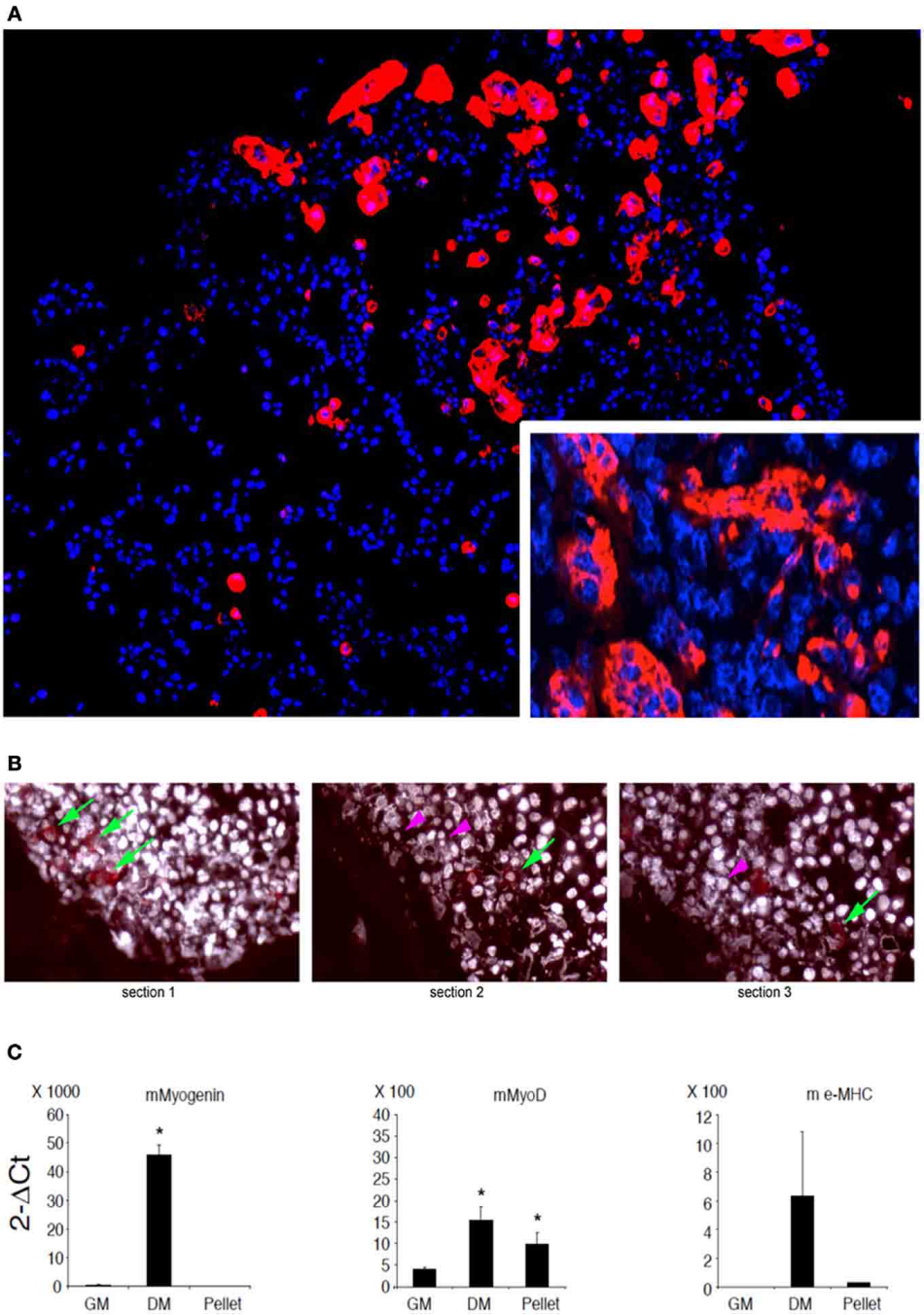

FIGURE 2 | C2C12 3D culture myogenic differentiation. (A) Immunofluorescence analysis of all myosin expression (MF20 antibody, red) in pellets of C2C12, whose nuclei are counterstained by Hoechst (blue), cultured for 5 days in a minimal medium consisting of DMEM supplemented with 1% BSA. The inset at a higher magnification shows myosin positive syncytia. **(B)** Serial sections of C2C12 pellets (distance between section = 15 μm) following immunofluorescence analysis for myosin showing the appearance (green arrows) and disappearance (purple arrowheads) of the signal, suggesting that the dimension of the objects is of the order of magnitude of 30 μm. Nuclei are counterstained by Hoechst and post-processed to withe to ameliorate contrast. **(C)** Q-PCR of muscle markers expressed by C2C12 pellets cultured in 1% BSA for 5 days (pellet) as compared to C2C12 2D cultures in growth medium (GM) and differentiation medium (DM), used as a negative and positive control, respectively. From left to right: murine (m) myogenin, MyoD and embrionic (e) myosin heavy chain (MHC). The mean ± s.e.m. of triplicate samples is shown. $*p < 0.05$ vs. GM, by Student's *t*-test. Culturing myogenic cells in 3D aggregates is sufficient to trigger a certain extent of myogenic differentiation. Therefore, whether MAS promotes muscle differentiation in serum-free medium (i.e., in the absence of pro-myogenic stimuli) cannot be clearly ascertained due to lack of proper 3D culture controls.

the graft and the surrounding environment could not be fully assessed because both MAS and TA were of muscular origin. The aim of the present work was to investigate whether MAS *per se* is exclusively a pro-myogenic environment (which would mean its use is limited to muscle tissue engineering applications) or is compatible with other differentiation pathways. In order to achieve this aim, we analyzed, *in vitro* and *in vivo*, the interaction between MAS and both muscle and non-muscle cells and tissues, to determine whether MAS is necessary or sufficient to induce myogenesis regardless of its location.

MATERIALS AND METHODS

CELL CULTURES

C2C12 mouse myoblasts were cultured in growth medium (GM), composed of Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l Glucose, L-Glutamine (Sigma), supplemented with 15% fetal bovine serum (FBS), and 100 U/ml penicillin/100 microg/ml streptomycin (Invitrogen). For differentiation experiment positive controls, C2C12 were cultivated in GM until they reached 80–90% of confluence on plastic Petri dishes, then were shifted to 2% horse serum (HS) medium (DM). Alternatively, 2×10^6

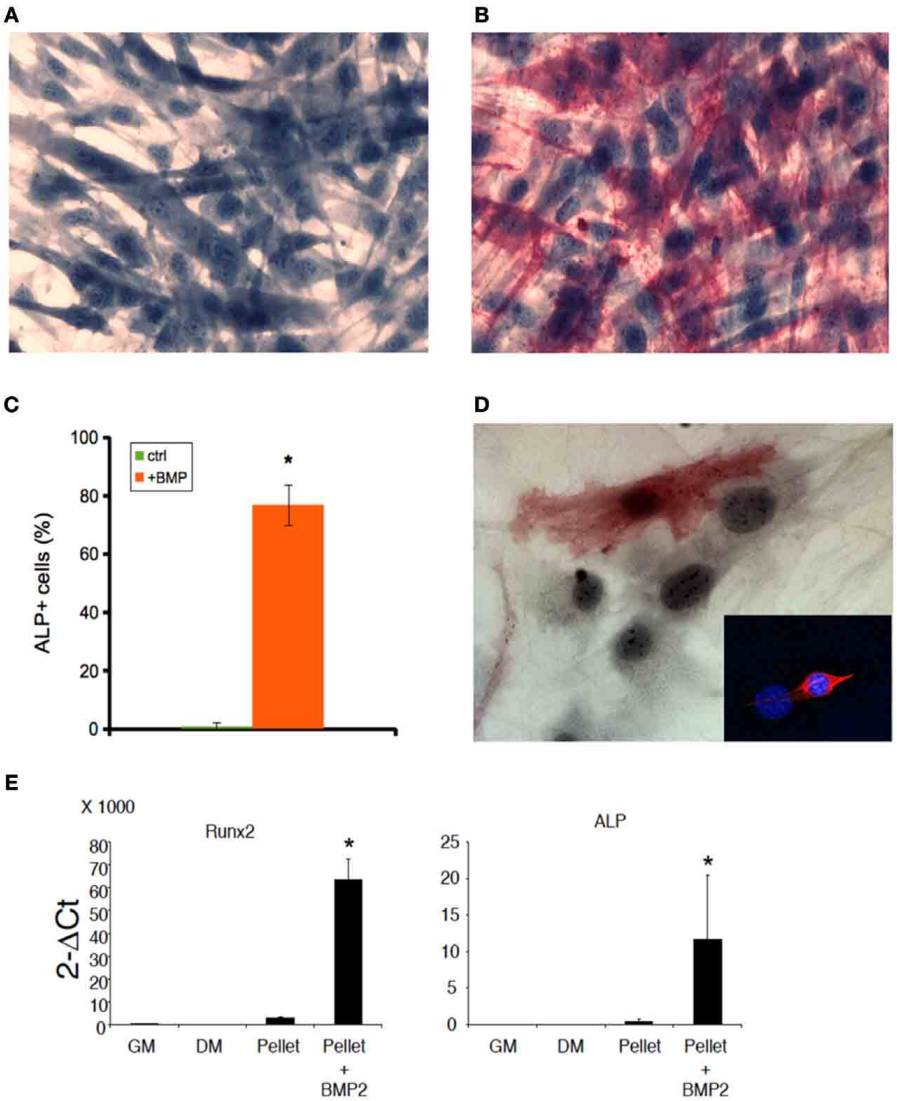

FIGURE 3 | BMP induces osteogenic differentiation both in 2D and 3D C2C12 and primary cultures. ALP staining of C2C12 cultures on plastic in the absence **(A)** or presence **(B)** of 300 ng/ml BMP-2 for 5 days, showing that C2C12 possess osteogenic potential when cultured in the presence of BMP. The cells were counterstained with Hematoxylin and the osteoblasts (red cells) were quantified **(C)**. The mean ± s.e.m. of quadruplicate samples is shown. $*p < 0.05$ vs. GM, by Student's *t*-test. **(D)** A similar myogenic to osteogenic conversion upon BMP treatment occurs in myogenic cell primary cultures: an ALP+ cell is visible in the photomicrograph; the inset shows the myogenic lineage of primary cultures from skeletal muscle by desmin expression (red), while nuclei were counterstained by Hoechst (blue). Nuclear size and shape discriminate myoblasts/osteoblasts from fibroblasts. Bar: 50 μ. **(E)** Q-PCR of bone markers expressed by C2C12 pellets cultured in 1% BSA for 5 days (pellet), in the absence of presence (+BMP-2) of 300 ng/ml BMP-2, as compared to C2C12 2D cultures in growth medium (GM) and differentiation medium (DM), both used as a negative controls for osteogenic markers. From left to right: murine runt-related transcription factor 2 (Runx2) and alkaline phosphatase (ALP). The mean ± s.e.m. of triplicate samples is shown. $*p < 0.05$ vs. GM, by Student's *t*-test.

C2C12 cells were resuspended in 50 microL of GM and injected within a MAS derived from a murine TA (see the paragraph Decellularization of skeletal muscle, below). The cells were treated for 5–7 days with 2% HS (horse serum) in the absence or presence of BMP-2.

DECELLULARIZATION OF SKELETAL MUSCLE

For decellularization of skeletal muscle we dissected TA or *Extensor digitorum longus* (EDL) and immediately incubated them in sterile 1% SDS in distilled water for 48 and 24 h, respectively, at RT under slow rotation. At least 10 ml of SDS solution

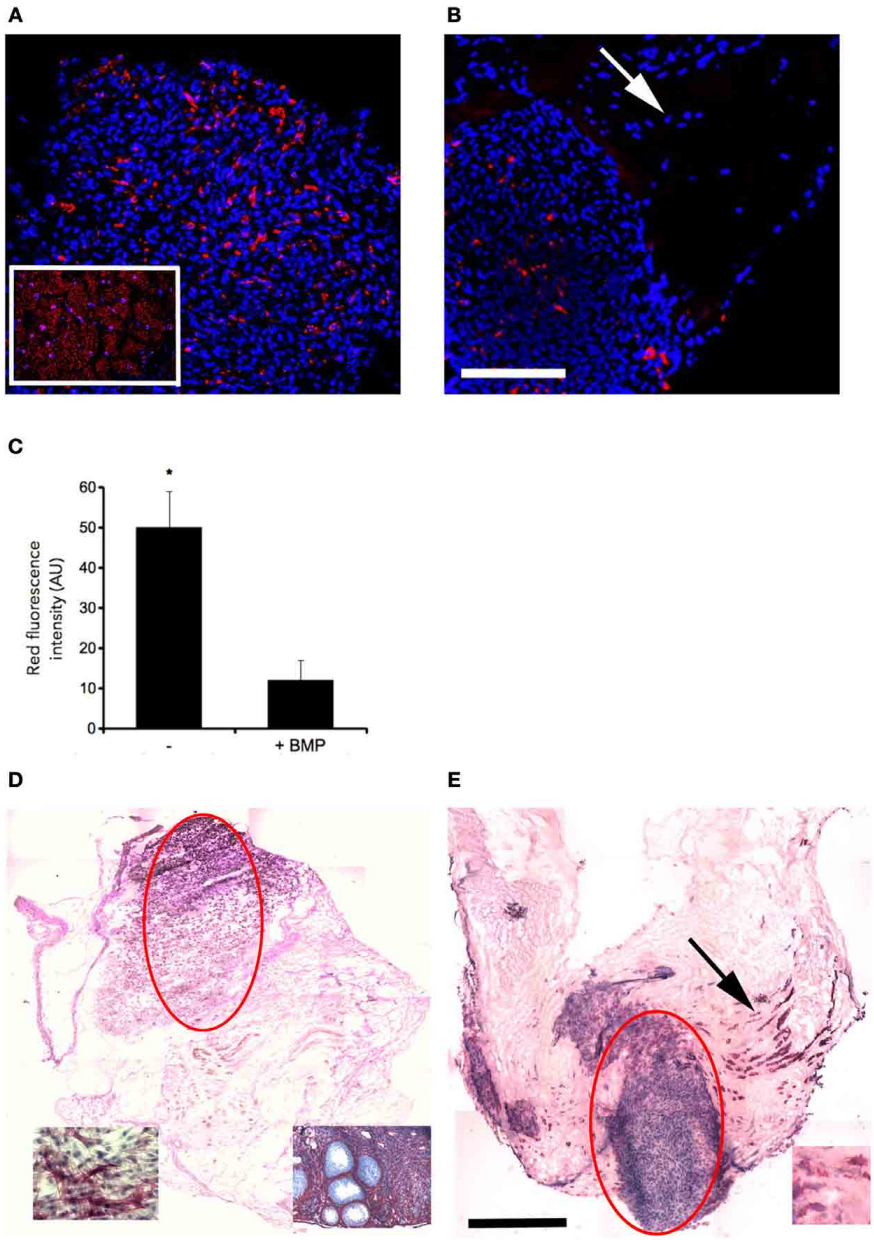

FIGURE 4 | MAS does not irreversibly commit C2C12 cells to a myogenic fate. Immunofluorescence analysis of all myosin expression (MF20 antibody, red) in C2C12 cells, whose nuclei are counterstained by Hoechst (blue), cultured for 5 days in MAS in a medium consisting of DMEM supplemented with 2% HS in the absence **(A)** or presence **(B)** of 300 ng/ml BMP-2. The inset in **(A)** shows a muscle section used as a positive control. Myosin positive cells (red) are visible in both cases; however, myogenic differentiation seems to occur predominantly in tightly aggregated cells, since the scattered, ALP positive cells do not express myosin in serial sections (see the arrows in **B,E**). **(C)** Quantification of myosin (red fluorescence) from quadruplicate samples as above. Mean ± s.e.m. $*p < 0.05$ vs. GM, by Student's *t*-test. As shown by ALP staining, and hematoxylin counterstaining, of C2C12 cells cultured in MAS in the absence **(D)** or presence **(E)** of BMP, C2C12 do express ALP but only in the presence of BMP. The bulk cell aggregates deriving from the original injection of cell suspension into the MAS are indicated by light ellipses. The insets in **(D)** represent positive controls: an ovary section (left) and BMP-treated C2C12 cells (right); the inset in **(E)** shows ALP expressing cells at a higher magnification. The latter are abundant in the region in which MAS was colonized by cells (arrow in **E**), which are likely to have migrated out of the cell aggregate visible in the center of the MAS. Black bar = 500 μ, white bar = 100 μ.

was used for each pair of muscles. After the decellularization procedure, the muscles were thoroughly washed by means of 3 incubations lasting at least 30 min each in sterile PBS. Decellularized scaffolds were used on the same day as they were produced or were stored for specific experiments.

ANIMALS AND SURGICAL PROCEDURES

Adult sex-matched BALB/C mice were used throughout this study as both donors and hosts. Mice were treated according to the guidelines of the Institutional Animal Care and Use Committee. Donor animals were sacrificed before skeletal muscle removal, while host animals were anaesthetized before muscle dissection and MAS engraftment. The transplantation procedure is described in detail below. TA acellular scaffolds were used to replace TA of inbred, age- and sex-matched wild type mice. The grafts were subsequently dissected from the host 2 weeks following transplantation. The surgical procedures have been described previously (Perniconi et al., 2011).

Scaffold grafting within the renal capsule. Animals were weighed and anaesthetized. After a small incision had been made in the body wall, the kidney popped out of the hole in the body wall when pressure was applied on either side of the kidney using the forefinger and thumb. An incision of approximately 5 mm was made in the renal capsule. Then a glass Pasteur pipette, which had been drawn thin and fire-polished with a rounded closed end, was used to obtain a capsule pocket by manipulating the pipette point tangential to the kidney in such a way as to detach the renal capsule. The scaffold was inserted into the pocket under the capsule using the polished glass pipette. After replacing the kidney within the peritoneal cavity, the body wall was sutured with 1 or 2 stitches of silk thread.

Scaffold grafting under the xiphoid process. Animals were weighed and anaesthetized. A small incision was made under the sternum. Without touching the muscle diaphragm, the scaffold was attached with a suture to the xiphoid process in order to suspend it within the peritoneal cavity in close contact with cartilage tissue. The body wall was sutured with 1 or 2 stitches of silk thread.

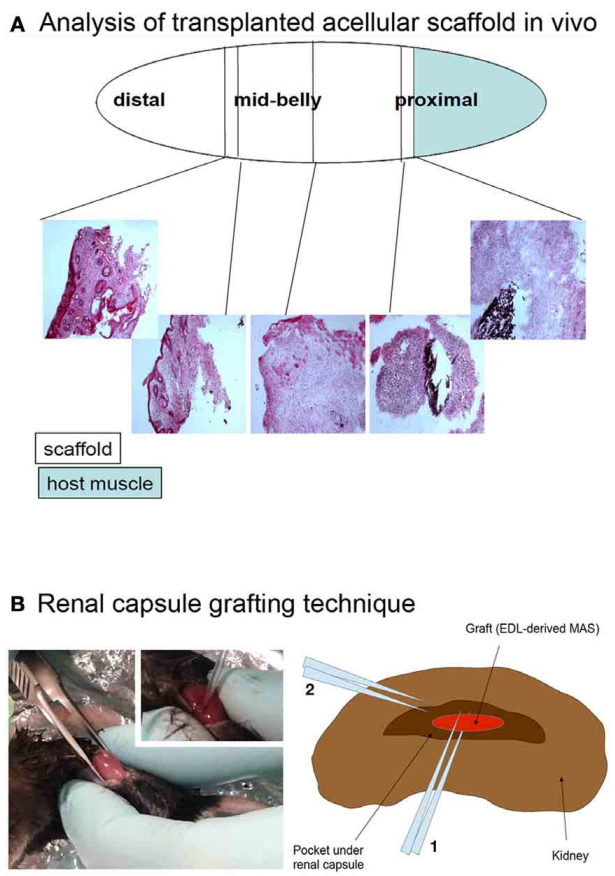

FIGURE 5 | (A) Analysis of transplanted acellular scaffold *in vivo*. The *Tibialis anterior* muscle was removed, with the exception of a proximal fragment (light blue) and the MAS (open ellipse) was sutured to the muscle at the proximal end and to the tendon at the distal end. The black silk suture knots, visible in the hematoxylin and eosin stained cryosections, were used as a reference of the graft extremities. The first third, second third and last third of the graft, starting from the proximal region, were defined as the proximal, mid-belly and distal parts of the grafted MAS, respectively. **(B)** Renal capsule grafting technique. The kidney was exposed by dorsal skin incision (picture) and the renal capsule cut lengthwise for about 5 mm; a pocket was created under the connective tissue renal capsule by using a fire polished Pasteur pipet (picture inset). The graft was inserted into the pocket by mean of forceps and the tip of the pipet (2 and 1 in the drawing, respectively). For this specific experiment, the grafted MAS was obtained from an *extensor digitorum longus* muscle (*Graft, EDL-derived MAS*) because of the size of the latter was compatible with the grafting technique.

HISTOLOGICAL ANALYSIS

At the end of the experimental period the pellets within the scaffolds were frozen within OCT mounting medium (Leica) in liquid nitrogen-cooled isopentane. Cryosections (8 μm) were obtained using a Leica cryostat. For histological analysis, the sections were stained with hematoxylin and eosin using standard methods (Sigma). Alternatively, cryosections were stained with 0.05% Toluidine blue (BDH) for 30 min. Photomicrographs were obtained using an Axioscop 2 plus system equipped with an Axiocam HRc (Zeiss) at 1300 × 1030 pixel resolution.

ALKALINE PHOSPHATASE (ALP) ASSAY FOR THE CHARACTERIZATION OF OSTEOBLASTIC PHENOTYPES

A leukocyte ALP kit (Sigma-Aldrich) was used for ALP staining according to the recommended protocol. Cells were counterstained with 0.05% neutral red (Sigma). They were then fixed by immersion in fixative solution (citrate-acetone-formaldehyde) for 30 s, gently rinsed with water and put in an alkaline-dye mixture (sodium nitrite solution, FRV-Alkaline solution, deionized water, Naphthol AS-BI Alkaline solution) for 15 min. The samples were then rinsed for 2 min in deionized water and counterstained for 2 min with Hematoxylin solution Gill N.3, rinsed in tap water and air dried.

IMMUNOFLUORESCENCE ANALYSIS

Transverse cryosections were rinsed in PBS for 5 min at RT and then incubated with Blocking minBuffer (1%BSA, 10% Goat Serum in PBS) for 1 h at RT. The samples were washed in PBS and incubated with primary antibody (Ab) MF20 (Mouse

IgG2b myosin Hybridoma bank) at a 1:50 dilution in PBS, and polyclonal anti-laminin Ab (policlonal Rabbit Sigma) at a 1:50 dilution in PBS. The sample were incubated with the secondary Ab anti-mouse-Dylight 549 and anti rabbit AlexaFluor 488 a 1:400 dilution in PBS for 1 h. Alternatively, laminin Ab was detected by anti-rabbit-Alexa 568 Ab. Secondary Abs were used to detect endogenous IgG on cryosections of the grafted material using anti-mouse-Alexa 488. Pre-immune serum was used for the negative control. Finally, 0.5 ug/ml Hoechst 33342 (Sigma) was used to counterstain cell nuclei. Photomicrographs were obtained by means of an Axioskop 2 plus system (Zeiss) or a Leica Leitz DMRB microscope fitted with a DFC300FX camera for confocal analysis (Leica).

Quantitative analysis was performed on fluorescence images of 10 randomly chosen microscopic fields acquired in the red channel. Post-processing of the images was performed using Adobe Photoshop and Scion Image Softwares (the latter, a software originally developed with the name of NIH Image at NIH, Bethesda,

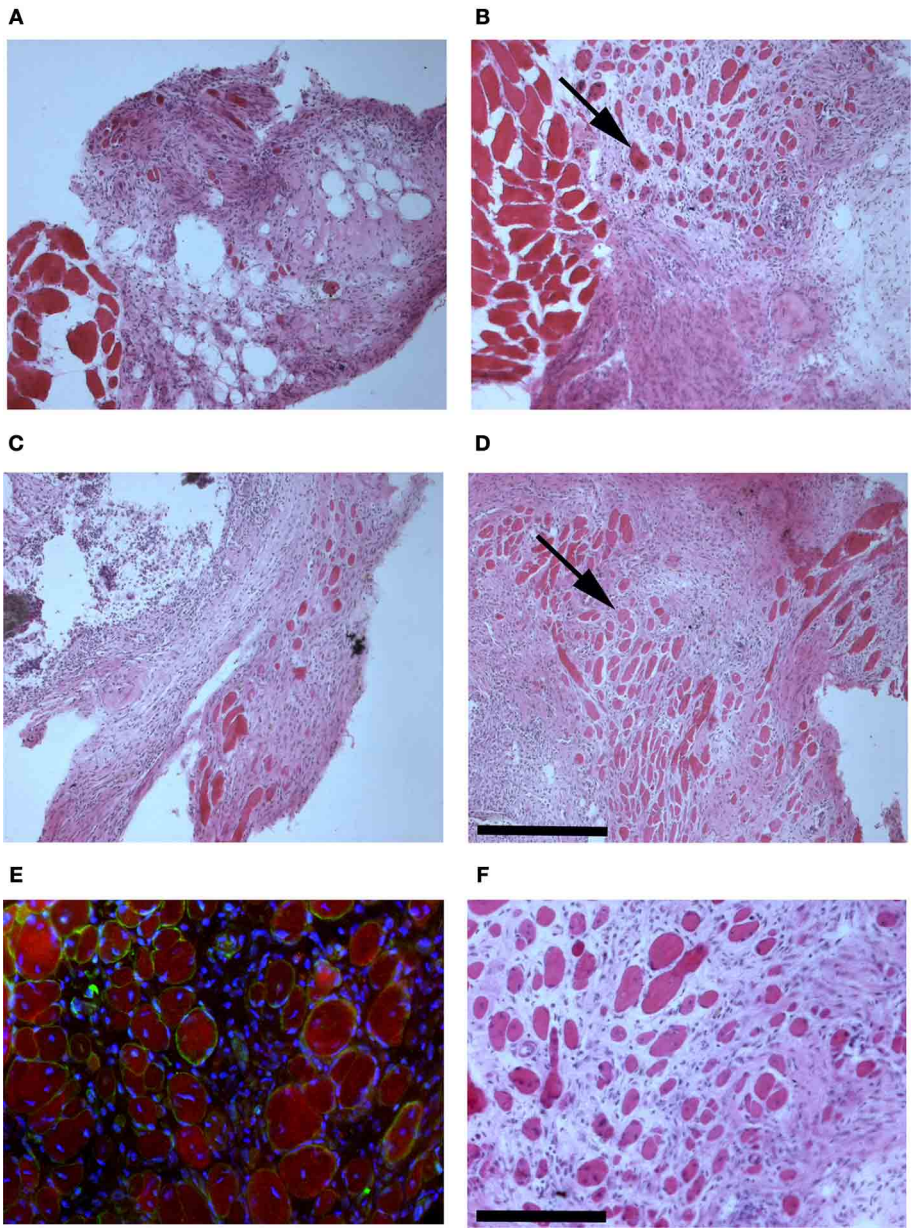

FIGURE 6 | MAS is sufficient to support muscle regeneration in volumetric muscle loss. Hematoxylin and eosin staining of cross-cryosections of MAS grafted in the place of the *Tibialis anterior* in a syngeneic mouse and analyzed at the level of distal **(A,C)** and mid-belly **(B,D,F)** muscle sections (for the definition of these positions, refer to **Figure 5A**), 2 **(A,B)** and 3 **(C,D,F)** weeks following transplantation. Numerous regenerating muscle fibers (arrow), characterized by centrally located nuclei, are visible within the inflammatory infiltrate. Multiple and/or centrally located nuclei in the same rose cytoplasm indicate bona fide nascent muscle fibers **(F)**, as confirmed by sarcoglycan (green) and myosin (red) expression around centrally located nuclei (blue) shown by immunofluorescence in **(E)**. The picture is representative of triplicate experiments. Bar = 200 μ.

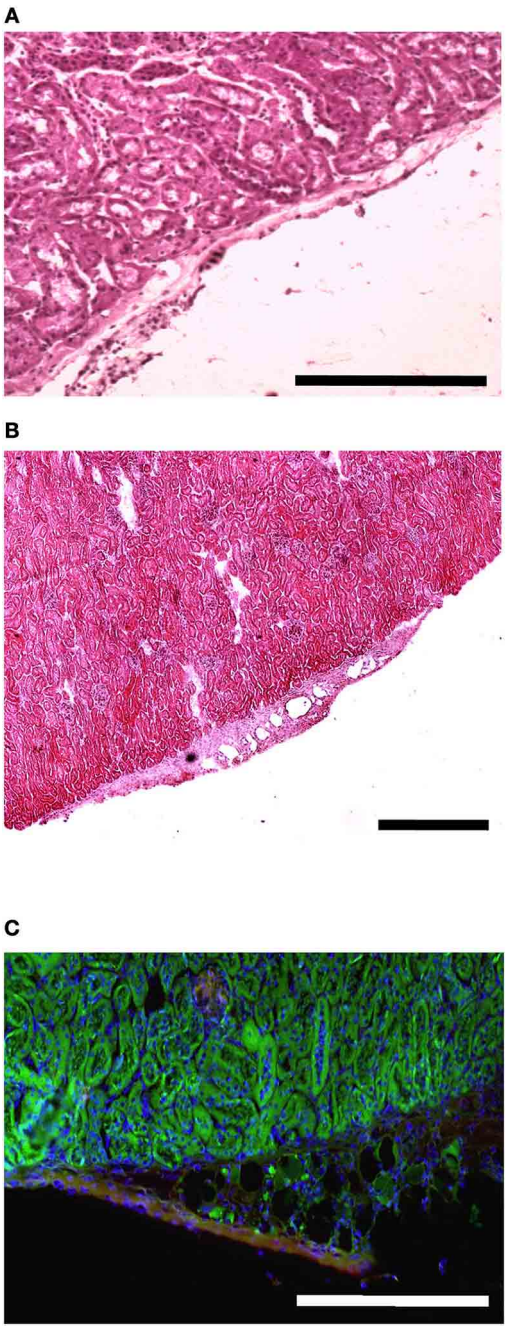

FIGURE 7 | MAS is not sufficient to promote myogenesis within the renal capsule. Hematoxylin and eosin staining of cross-cryosections of a peripheral region of the kidney and its renal capsule in the absence **(A)** or in the presence **(B)** of grafted MAS, 2 weeks following transplantation. The immunofluorescence analysis **(C)** for laminin (green) and myosin (red) shows the absence of muscle fibers in the grafted MAS. Note that the glomeruli are strongly autofluorescent. Nuclei are stained by Hoechst (blue). Bar = 200 μ. The picture is representative of triplicate experiments.

is freely downloadable at http://rsb.info.nih.gov/ij/index.html). Modifications were the same for all images and consisted in a conversion to gray scale, followed by the measure of the mean fluorescence calculated on the whole field. The values were normalized by the area examined and expressed as arbitrary units (AU). Values deriving from the 10 sampled fields were averaged and the result was considered representative of one sample. At least four independent replicates were analyzed and averaged.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-qPCR)

Total mRNA was isolated using an RNeasy mini kit (Qiagen, Courtaboeuf, France) or a TRIzol® reagent method (Invitrogen) as described by the manufacturers. DNAse-treated RNA was reverse transcribed with SuperScript® III RT (Invitrogen), the cDNA obtained was amplified using TaqMan® or SYBR®Green chemistries (Applied Biosystems by Life Technologies, Courtaboeuf, France), and monitored with the 7500HT Fast Real-Time PCR System (Applied Biosystems). Primers used for RT-qPCR are listed below. Amounts of cDNA of interest were normalized to that of GAPDH (ΔCt = Ct gene of interest—Ct GAPDH). Results were reported as relative gene expression (2-ΔCt). The following couples of primers were used for murine (m) or human (h) genes: (1) glyceraldehyde 3-phosphate dehydrogenase (mGAPDH: forward CTGAGCAAGAGAGGCCCTA; reverse TATGGGGGTCTGGGATGGAA; (2) runt-related transcription factor 2 (mRUNX2: forward TTGACCTTTGTCCCAATGC; reverse AGGTTGGAGGCACACATAGG); (3) alkaline phosphatase (mALP: forward TGTCTGGAACCGCACTGAACT; reverse CAGTCAGGTTGTTCCGATTCAA); (4) glyceraldehyde 3-phosphate dehydrogenase (GAPDH: forward CCAGCAAGAGCACAAGAGGA; reverse AGATTCAGTGTGGTGGGGG); (5) runt-related transcription factor 2 (hRUNX2: forward GAATCCTCCACCCACCCAAG; reverse AATGCTGGGTGGCCTACAAA); (6) bone sialoprotein 2 (hIBSP: forward CCATTCTGGCTTTGCATCCG; reverse GACAAGAAGCCTATTACTTTGC); (7) bone sialoprotein 2 (hOC: forward GTGCAGAGTCCAGCAAAGGT; reverse TCCCAGCCATTGATACAGGT); (8) alkaline phosphatase (hALP: forward GTGCAGAGTCCAGCAAAGGT; reverse TCCCAGCCATTGATACAGGT); (9) myogenin (forward GCACTGGAGTTCGGTCCCAA; reverse TATCCTCCACCGTGATGCTG); (10) Myo D (forward ACCCAGGAACTGGGATATGGA; reverse AAGTCGTCTGCTGTCTCAAA); (11) embryonic myosin heavy chain (e-MHC: forward CGTCTGCTTTTGGCAA; reverse TGGTCGTAATCAGCAGCA). RUNX2 and ALP represent classic markers of osteogenic differentiation (Coquelin et al., 2012; Leotot et al., 2013), while MyoD, myogenin and myosin represent the standard markers for myogenic differentiation (De Arcangelis et al., 2003, 2005; Naro et al., 2003; Musaro et al., 2007).

RESULTS

MAS IS SUITABLE FOR 3D MYOGENIC CELL CULTURES

To assess whether MAS supports cell attachment, survival and differentiation, we cultured myogenic C2C12 cells in MAS in DM for 1 week. C2C12 is a multi-potent cell line derived from muscle satellite cells, and is thus primarily myogenic. On the other hand, MAS is a muscle-derived biomaterial which we had previously demonstrated is pro-myogenic *in vivo*, without knowing whether it retained the same properties *in vitro*. In these

conditions, C2C12 cells showed the tendency to aggregate in a cell-cell fashion (**Figure 1A**), even though they clearly came into contact with the MAS laminin (**Figure 1B**), when cultured within MAS. The cells survived for several days in culture and were able to differentiate, i.e., to express markers of fully differentiated muscle fibers (**Figure 1B**). Therefore, we considered that MAS was sufficiently stable to support cell cultures and was compatible with myogenic differentiation.

C2C12 are cells that fuse with each other during differentiation to form myotubes *in vitro*. As expected, cell-cell contact represents a condition leading to fusion for these cells (Rochlin et al., 2010). However, this is not a sufficient trigger when C21C2 are cultured in a minimal medium supplemented with 1% BSA in 2D cultures, a condition that is not associated with their myogenic differentiation and cell fusion. On the other hand, 3D cultures of C2C12 are still poorly characterized (Carosio et al., 2013). Therefore, we decided to characterize the effects of 3D culturing *per se* on C2C12 differentiation with the aim to better interpret the C2C12 3D cultures within MAS. We noticed that 3D C2C12 aggregates obtained by pelleting C2C12 before placing them in culture, displayed several markers of myogenic differentiation both in 2% HS in 1% BSA (**Figure 2** and data not shown). In particular, C2C12 2D cultures in 2% HS (the gold standard myogenic differentiation for this type of cells) were compared to C2C12 pellets cultured in

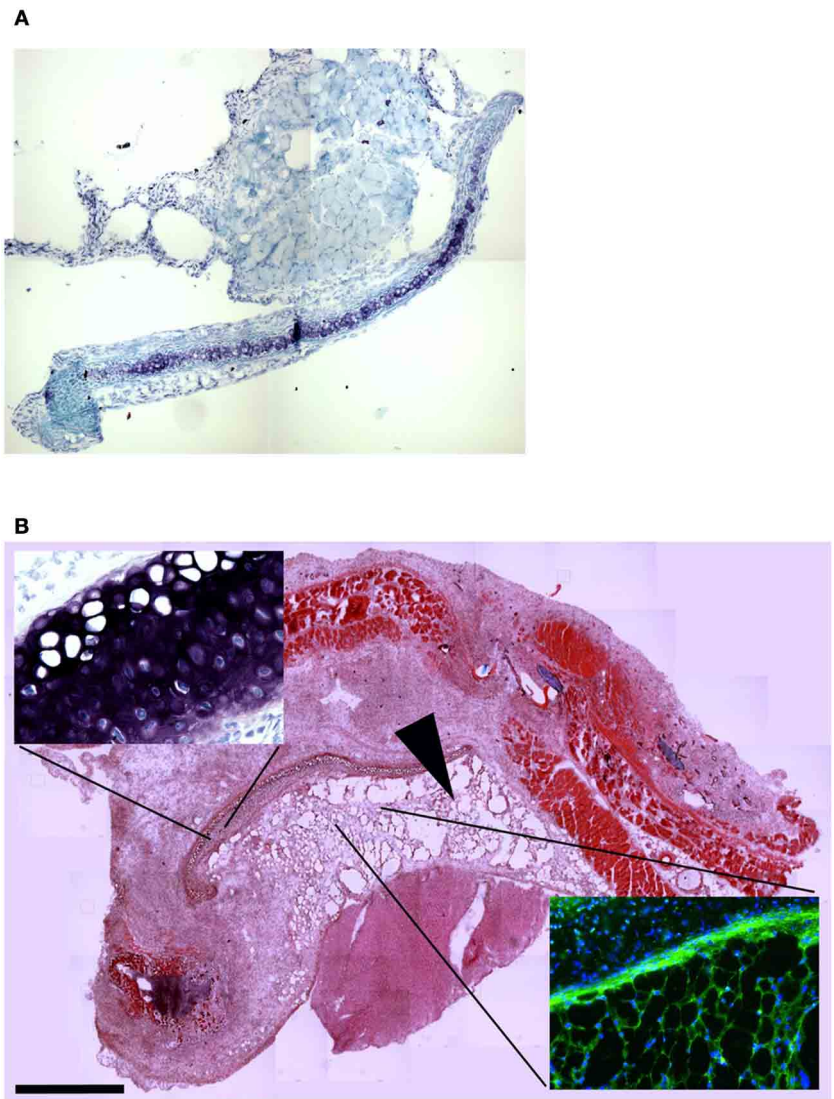

FIGURE 8 | MAS grafted under the xiphoid process. (A) Toluidine blue staining of the region of the xiphoid process without grafting. Cartilage tissue (arrow) is strongly metachromatic. **(B)** Hematoxylin and eosin staining of the same region including grafted MAS, 2 weeks following transplantation. The absence of muscle fibers in the grafted MAS (arrowhead) is evident when the latter is compared with skeletal muscles of the pectoral muscles in the upper region of the image. The insets, at a higher magnification, show: (bottom right) immunofluorescence analysis for laminin (green) and myosin (red)—nuclei are stained by Hoechst (blue)—on a serial section demonstrating the absence of muscle fibers in the grafted MAS; (upper left) toluidine blue staining showing the position of the xiphoid process, whose matrix is highly metachromatic. The picture is representative of triplicate experiments.

1% BSA. The latter showed: not detectable myogenin but significant MyoD expression (**Figure 2C**), which was sufficient to trigger myosin expression (detectable with an anti-pan-myosin antibody such as MF20, **Figure 2A**). Among the myosin isoforms possibly expressed by C2C12, we found low embryonic myosin expression (**Figure 2C**), suggesting that other isoforms typical of more mature fibers are expressed, in spite of the absence of elongated myotube formation (**Figure 2B**). Such aberrant differentiation resembled that of rhabdomyosarcomas and the Rb cell line (Wang et al., 2010). However, since a 3D organization was *per se* myogenic to a certain extent, a 3D C2C12 culture in MAS in 1% BSA was considered to be unsuitable to test whether MAS was sufficient to induce differentiation under minimal medium, conditions owing to the lack of an adequate negative control *in vitro*.

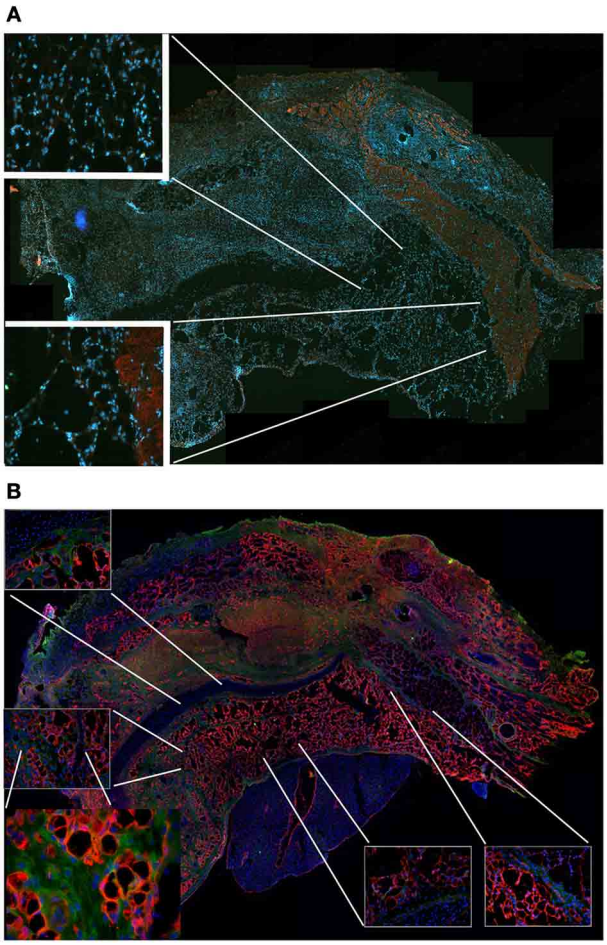

FIGURE 9 | MAS is not sufficient to promote myogenesis under the xiphoid process. (A) The panel represents a reconstruction (with insets at a higher magnification) of the whole region, following an immunofluorescence analysis for myosin (red) and nuclei (blue), once again demonstrating the absence of muscle markers in the spongy structure of MAS; this is in contrast to the pectoral muscles in the upper region of the image, which represent a positive control for the immunofluorescence reaction. **(B)** Reconstruction (with insets at a higher magnification) of the whole region, following an immunofluorescence analysis for laminin (red), immunoglobulins (green) and nuclei (blue), which demonstrates MAS colonization by cells during an inflammatory process. All the analyses in this figure were performed 2 weeks following transplantation, which is a sufficient amount of time for muscle regeneration in MAS grafted to replace a *Tibialis* muscle. The picture is representative of triplicate experiments. Bar = 400 μ.

MAS DOES NOT IRREVERSIBLY INDUCE 3D CELL CULTURES TO A MYOGENIC FATE

Both C2C12 cells and muscle primary cell cultures, while committed to a myogenic lineage, differentiate into osteoblasts in the presence of BMP (Katagiri et al., 1994; Friedrichs et al., 2011). We confirmed this notion for 2D C2C12 and primary satellite cells obtained from murine skeletal muscle and cultured for 5 days in 2%HS supplemented with 300 ng/ml BMP-2 (**Figures 3A–D**). BMP-treated cells displayed loss of myogenic differentiation, such as multinucleated myotube formation (**Figure 3A**) in favor of a potent pro-osteogenic conversion as demonstrated by the presence of non-fused, ALP expressing cells (**Figures 3B–D**). Worth noting BMP-2 effects on standard osteogenic markers (Coquelin et al., 2012; Leotot et al., 2013) were still potent in 3D C2C12 cultures (**Figure 3E**), indicating that 3D cultures are not impermeable nor insensitive to BMP treatment and suggesting to use BMP as a non-myogenic cue to test whether MAS irreversibly commit C2C12 to a non-myogenic fate.

To this purpose, we treated C2C12 cultured in MAS for 5 days within 2%HS in the absence or presence of 300 ng/ml BMP-2. While C212 in MAS accumulated myosin and did not express ALP (**Figures 4A,D**), the same cells showed significantly reduced myosin expression and differentiated in ALP-expressing osteoblasts in the presence of BMP (**Figures 4B,C,E**). Interestingly, ALP expression was stronger in those cells that displayed migratory activity by leaving the bulk cell aggregate and becoming isolated cells (**Figure 4E**, inset). Thus, MAS supports at least two differentiation pathways for cells of mesenchymal origin and, while participating in the muscle environment *in vivo*, does not suppress the pro-osteogenic stimulus induced by BMP-2 *in vitro*.

MAS PROMOTES MUSCLE REGENERATION IN VOLUMETRIC MUSCLE LOSS

To assess *in vivo* whether MAS itself is inherently myogenic, we grafted the MAS both orthotopically (i.e., replacing a *Tibialis anterior*) and ectopically (i.e., within the renal capsule or underneath the xiphoid process). The **Figure 5** illustrates these procedures, that are described in detail elsewhere and represent standard *in vivo* approaches to test xeno- or auto-grafts (Mericskay et al., 2004; Perniconi et al., 2011).

The presence of muscle fiber formation within orthotopically grafted MAS (**Figures 6A–D**) confirmed our previous *in vivo* results (Perniconi et al., 2011). Within 2 weeks from transplantation MAS was colonized by cells and showed the presence of nascent muscle fibers characterized by centrally located nuclei and muscle fiber specific markers such as myosin and sarcoglycan (**Figures 6E,F**), thereby demonstrating that MAS represents an environment that is compatible with neo-myogenesis. We found it striking that MAS alone, i.e., an empty scaffold even though of muscle origin, was colonized by cells and hosted new muscle

formation, suggesting that it represents a pro-myogenic niche *per se*. To verify whether MAS was sufficient to induce myogenesis, we grafted MAS in anatomically different regions, i.e., the renal capsule and the peritoneal cavity under the xiphoid process. The renal capsule represents the gold standard for *in vivo* transplantation and assessment of allografts survival, bioactivity and function, because it is a highly vascularized environment that lends itself to the rapid integration and nutrition of the grafts. Two weeks following transplantation, we found grafted MAS (derived from EDL muscle for this specific experiment) within the renal capsule, which retained its sponge-like, laminin-based structure. While we observed that numerous cells came into contact with and colonized MAS, we did not detect any overt differentiated muscle fiber, by either the morphological or immunofluorescence analysis of muscle fibers (**Figure 7**).

To confirm this result, we grafted MAS under the xiphoid process by suturing it to the latter. In physiological conditions under the xiphoid process, which is a cartilaginous organ enveloped by connective tissue, there is the empty intraperitoneal space and the liver (**Figure 8A**). When grafted in this position, MAS was recognized by its sponge-like structure but could not be confirmed as a site of neo-myogensis, even though many cells did colonized the MAS and interacted with its laminin matrix (**Figure 8B**). Most of the cells migrating within the MAS and along the laminin are, in fact, likely to have been inflammatory cells, as demonstrated by the presence of abundant immunoglobulins (**Figure 9A**), which is consistent with our previously published findings. As a result, while myosin positive muscle tissue is visible in the analyzed sections, this is clearly not deriving from the grafted scaffold, on the basis of its histology and anatomical localization, which is distant and not in continuity with the grafted MAS (**Figure 9B**).

All together, the aforementioned results indicate that MAS *per se* is not sufficient to induce myogenesis *in vivo*, but it requires the presence of surrounding muscles to show robust myogenesis, an effect further increased if MAS is seeded with myogenic cells before engraftment.

DISCUSSION

In tissue engineering interventions, grafting of decellularized tissues or organs, such as MAS, is an increasingly widespread approach in pre-clinical and clinical settings (Badylak et al., 1998, 2013; Perniconi and Coletti, 2014; Teodori et al., 2014). In particular, ECM of muscle origin has been proposed as en efficient scaffold for organ-scale reconstruction following volumetric muscle loss (Ott et al., 2008; Perniconi et al., 2011). When MAS is orthotopically grafted in mouse to replace a skeletal muscle, *de novo* myogenesis is observed within a few weeks, probably as a result of MAS colonization by stem cells of host origin with myogenic potential (Perniconi et al., 2011). However, the relative contribution to muscle formation by the graft and the surrounding environment cannot be fully understood using this approach, because both are of muscular origin. In order to verify whether MAS possesses pro-myogenic properties *per se*, i.e., regardless of the site of transplantation, we grafted MAS in a heterotopic position in syngeneic mice. Using this approach, here we demonstrate that MAS is neither sufficient nor necessary for myogenesis. Indeed, when MAS is transplanted within the renal capsule or the peritoneal cavity under the xiphoid process it is colonized by an abundant cell infiltrate but does not display any regenerating fiber within its laminin network. These results demonstrate that MAS is stable in anatomical sites other than the skeletal musculature, but does not provide enough signals to trigger myogenesis by the colonizing cells. By cultivating C2C12 cells in 3D aggregates, we showed that the 3D culture condition is a potent pro-myogenic cue, that facilitates the formation of fully differentiated myotubes, which is in agreement with other reports (Carosio et al., 2013); 3D cell-cell contacts bypass inhibitory signals for muscle differentiation, such as culture in minimal, serum-free medium (Minotti et al., 1998; De Arcangelis et al., 2003), and promote the formation of a functionally active construct capable of contraction. From these results, we conclude that MAS is not necessary for muscle formation in 3D cultures. We do, however, confirm the results of our previous study (Perniconi et al., 2011), demonstrating that MAS is an excellent support for 3D myogenic cultures, both *in vitro* and *in vivo* (in the latter case, when orthotopically grafted). In addition, MAS is compatible with the differentiation toward the osteogenic lineage, obtained by BMP-2 treatment of the C2C12 cell line, which thus demonstrates that MAS does not represent a signal that fully and irreversibly commits cells to a myogenic fate. In conclusion, MAS has scaffold properties insofar as it supports cell attachment, migration, survival and differentiation. Whether MAS is a good support for cells of non-muscle origin and whether it can be exploited in trans-species experiments, such as cultivating human cells in murine MAS, remains to be addressed. However, accumulating evidence suggests that acellular scaffolds of biological origin are multipurpose and may be exploited for cell culture and tissue engineering of different tissue types regardless of their origin (Badylak et al., 1998; Conconi et al., 2005; Wolf et al., 2012).

It is widely accepted that the niche supports stem cells and controls their self-renewal *in vivo* (Spradling et al., 2001) by modulating asymmetric cell division and ensuring stem cell renewal and the production of a number of committed daughter cells that is sufficient for tissue homeostasis and repair (Kuang et al., 2008). The notion of the niche appears to be closely linked to that of the stem cell; however, in addition to affecting stem cell renewal, the microenvironment also controls commitment and differentiation of daughter cells deriving from asymmetrically dividing stem cells (Zhang et al., 2009; Bhattacharyya et al., 2012). Intriguingly, the niche itself is not always indispensable for asymmetric division and stem cell renewal, as shown by the case of lymphocyte differentiation in the absence of a permanent niche (Chang and Reiner, 2008). Cell-intrinsic factors, such as DNA strand asymmetric division and segregation, play additional, important roles in determining the asymmetric fate of daughter cells (Shinin et al., 2006). A very recent report established a novel paradigm for stem-cell maintenance according to which a dynamically heterogeneous cell population functions long term as a single stem-cell pool (Ritsma et al., 2014). Our findings suggest that this degree of plasticity might be extended to committed cells and be inherent to ECM components.

The notion of the microenvironment is of paramount importance when dealing with tissue engineered constructs aimed at regenerative medicine applications. Although some features of

regeneration are shared by different tissues whereas others are tissue-specific, all those features are ultimately controlled by the tissue microenvironment. Understanding the mechanisms underlying tissue regeneration, particularly the choice between tissue restoration and reconstruction (two different strategies following tissue injury), is essential for regenerative medicine interventions (Coletti et al., 2013). The microenvironment (including the stem cell niche) is determined by three major components: the ECM, the cells and the local growth factors. In our work, we investigated whether MAS (which is basically composed of muscle ECM) is sufficient to recapitulate the effects of the muscle microenvironment on cell differentiation. We found that MAS displays features that are typical of a scaffold, though it is not strictly limited to muscle applications since it does not irreversibly commit cells to a myogenic fate. We conclude by saying that since MAS does not fully recapitulate muscle-specific microenvironment properties, it may be more plastic, and consequently easier to exploit, than has previously been believed.

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