

# Silk fibroin scaffolds enhance cell commitment of adult rat cardiac progenitor cells

Valentina Di Felice<sup>1\*</sup>, Claudia Serradifalco<sup>1</sup>, Luigi Rizzuto<sup>1</sup>, Angela De Luca<sup>1</sup>, Francesca Rappa<sup>1</sup>, Rosario Barone<sup>1</sup>, Patrizia Di Marco<sup>2</sup>, Giovanni Cassata<sup>2</sup>, Roberto Puleio<sup>2</sup>, Lucia Verin<sup>3,4</sup>, Antonella Motta<sup>3,4</sup>, Claudio Migliaresi<sup>3,4</sup>, Annalisa Guercio<sup>2</sup> and Giovanni Zummo<sup>1</sup>

<sup>1</sup>Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Italy

<sup>2</sup>Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy

<sup>3</sup>Department of Materials Engineering and Industrial Technologies and Biotech Research Centre, University of Trento, Italy

<sup>4</sup>European Institute of Excellence in Tissue Engineering and Regenerative Medicine and INSTM Research Unit, Trento, Italy

## Abstract

The use of three-dimensional (3D) cultures may induce cardiac progenitor cells to synthesize their own extracellular matrix (ECM) and sarcomeric proteins to initiate cardiac differentiation. 3D cultures grown on synthetic scaffolds may favour the implantation and survival of stem cells for cell therapy when pharmacological therapies are not efficient in curing cardiovascular diseases and when organ transplantation remains the only treatment able to rescue the patient's life. Silk fibroin-based scaffolds may be used to increase cell affinity to biomaterials and may be chemically modified to improve cell adhesion. In the present study, porous, partially orientated and electrospun nanometric nets were used. Cardiac progenitor cells isolated from adult rats were seeded by capillarity in the 3D structures and cultured inside inserts for 21 days. Under this condition, the cells expressed a high level of sarcomeric and cardiac proteins and synthesized a great quantity of ECM. In particular, partially orientated scaffolds induced the synthesis of titin, which is a fundamental protein in sarcomere assembly. Copyright © 2013 John Wiley & Sons, Ltd.

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Supporting information may be found in the online version of this article.

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## 1. Introduction

Cardiovascular disease is the leading cause of death worldwide in both low-income and middle-income countries (Nabel and Braunwald, 2012) and approximately 1900 patients/million population are hospitalized for acute myocardial infarction (AMI) in Europe (Widimsky *et al.*, 2010). With larger infarcts, the non-infarcted myocardium remodels over time, becoming hypertrophic and eventually progressing into failure. When the pharmacological approach no longer arrests disease evolution, organ transplantation remains the

only treatment able to rescue the patient's life. However, recent studies have suggested the possibility of replacing the injured cells with stem cells. This approach would circumvent many of the limitations of organ transplantation, such as the low availability of organs, major surgical procedures, high costs and long-term immunosuppression.

As discussed in a recent paper published in *Nature* (Laflamme and Murry, 2011), cardiac progenitor cells (CPCs) are among the cells that are closest to clinical trials of AMI. CPCs are partially differentiated cells located in the adult myocardium that can differentiate into fibroblasts, endothelial cells, cardiomyocytes of the conduction system and working cardiomyocytes (Bernstein and Srivastava, 2012; Di Felice *et al.*, 2009b). In 2003, Piero Anversa's group isolated and characterized these cells for the first time (Beltrami *et al.*, 2003). We now know that there is most likely a unique population of c-Kit<sup>+</sup> cells and

\*Correspondence to: V. Di Felice, Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Università degli Studi di Palermo, Via del Vespro 129, 90127 Palermo, Italy. E-mail: vdfelice@inwind.it

that the most important surface markers of CPCs are c-Kit, MDR-1 and Sca-1 (Bernstein and Srivastava, 2012). The origin of these cells is unknown, although data suggest that they are present in the myocardium during human heart development and persist after birth (Di Felice and Zummo, 2009; Serradifalco *et al.*, 2011).

Currently, two clinical trials are in progress in the USA: one trial is implementing intracoronary injection of CPCs in coronary heart disease and congestive heart failure [phase I; NCT00474461 (Bolli *et al.*, 2011)] and the other is a dose escalation study of the safety and efficacy of intracoronary delivery of cardiosphere-derived CPCs in patients with ischaemic left ventricular dysfunction and a recent myocardial infarction [phase I; NCT00893360 (Makkar *et al.*, 2012)]. In these two clinical trials, stem cells are delivered by intracoronary injection. However, the delivery of stem cells alone to infarcted myocardium may provide no structural support while the myocardium heals, and the injected stem cells may not properly integrate into the myocardium because they may not be subjected to the mechanical forces that are known to drive myocardial cellular physiology.

Tissue-engineering strategies are a promising therapeutic approach, in which new matrices are being developed to support cellular activity. Tissue engineering for cardiac muscle is possible, even though the construction of this highly organized tissue is very difficult to achieve. The main goals are to select the proper cell source and to reach a high degree of differentiation through a bioactive 3D scaffold (Pelacho *et al.*, 2007; Singh and Williams, 2008).

Scaffolds may reduce the number of cells needed for each implantation via driving cell fate by mimicking the specialized micro-environment (niche) where CPCs are located *in vivo* and resembling the elasto-mechanical forces of the heart wall. The chemistry, geometry and mechanical properties of scaffolds for tissue engineering should be designed so that the scaffolds act as templates for cell adhesion, activation and extracellular matrix (ECM) production, which will lead to the regeneration of damaged tissues (Stoppato *et al.*, 2011). Considering natural ECM as a model, biopolymer matrices and particularly proteins are attracting the interest of many researchers. Like many natural polymers, silk is a promising candidate for various medical applications. The uniqueness of silk is derived from its multi-functional nature; it possesses adaptable mechanical performance coupled with controllable degradation and excellent biocompatibility. Fibroin-based materials can be obtained by directly treating degummed fibres or after fibroin dissolution and dialysis. Active efforts have been made to develop silk for the production of scaffolds, such as films, hydrogels, nano- and micro-nets and sponges, that can be tailored and designed to specific applications (Fini *et al.*, 2005; Wang *et al.*, 2006). The potential of applying silk-based materials to induce the regeneration of various mammalian tissues, including bone, cartilage, tendon and skin, is increasingly reported (Bondar *et al.*, 2008; Unger *et al.*, 2007). Silk fibroin can be used as a polymer on its own or with other molecules to increase cell affinity to biomaterials (Cai *et al.*, 2002; Ghanaati *et al.*, 2010) and may be

chemically modified to improve cell adhesion (Morgan *et al.*, 2008). We have recently demonstrated that porous fibroin scaffolds may be used for 3D chondrocyte culture, and these cells adhere to the structure using the  $\alpha 5$ ,  $\beta 1$  and  $\beta 3$  integrin subunits (Wang *et al.*, 2010).

Integrins are the main constituent of costameres, which are peripheral Z-disk and subsarcolemmal proteins that transduce force from the sarcomere to the sarcolemma and anchor cardiomyocytes to the ECM (Danowski *et al.*, 1992).

Cardiac stem cells must organize costameres, titin anchors and sarcomeres to be considered fully differentiated. Titin is a giant protein that connects each costamere to a peripheral Z-disk and is one of the proteins that transduces biomechanical stress signals from the Z-disk to the nucleus (Frank and Frey, 2011).

Whether a polymer can induce the correct expression of sarcomeric proteins and promote the Z-disk signalling network is not known, but for its particular interactions with integrin subunits, silk fibroin may be a good candidate. In the present study, we evaluated whether the properties of silk fibroin scaffolds influence CPC differentiation and integrin, cardiac and sarcomeric protein expression *in vitro*. Specifically, three different geometries were designed for the scaffolds, which were obtained using a fibroin–water solution, two sponges with different pore sizes and distributions and an electrospun nanometric net.

## 2. Materials and methods

### 2.1. Material preparation

#### 2.1.1. Fibroin–water solution

*Bombyx mori* cocoons (kindly provided by Socio Lario, Cassina Rizzardi, Como, Italy) were boiled for 1.5 h in an aqueous solution containing 1.1 g/l  $\text{Na}_2\text{CO}_3$  (10 g silk/l solution) and then for another 1.5 h in bath of water containing 0.4 g/l  $\text{Na}_2\text{CO}_3$ . The cocoons were rinsed thoroughly with distilled warm water to extract the glue-like sericin proteins and finally air-dried.

The fibroin–water solution was prepared by dissolving fibroin in an aqueous solution containing 9.3 M LiBr (10% w/v; Fluka Chemical) at 65 °C for 2 h, followed by dialysis (for 3 days) against distilled water with a 3500 Da MWCO membrane (Slyde-A-Lyzer, Pierce) to eliminate the salt. The resulting solution was concentrated by dialysing against a PEG–water solution (25% w/v) for 5 h and filtered through a 160–250 mm filter (Duran Group). The final concentration of silk fibroin in the aqueous solution was approximately 15% w/v, as determined using a NanoDrop ND-1000 Spectrophotometer (A280).

#### 2.1.2. Freeze-dried sponges

3D porous fibroin scaffolds were prepared using a freeze-drying technique with different parameters to obtain

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different pore sizes and orientations. The aqueous fibroin solution was diluted to 5%, poured into polystyrene Petri dishes, frozen at  $-80^{\circ}\text{C}$  and freeze-dried (sample RP).

Sponges with an orientated structure were obtained using the 5% fibroin solution, but the Petri dish was positioned vertically to induce a temperature gradient (sample O).

All obtained samples were stabilized by treating with a methanol:water solution (80:20) for 10 min, rinsed with distilled water to eliminate the solvent and freeze-dried again.

### 2.1.3. Electrospun nets

The aqueous fibroin solution was frozen at  $-20^{\circ}\text{C}$ , lyophilised at  $-50^{\circ}\text{C}$  and stored in a desiccator until use. The freeze-dried fibroin was dissolved in formic acid (98–99%) at 15% w/v. A 3 ml syringe with a metal needle was filled with the solution and mounted in a programmable syringe pump (Model 11 Plus, Harvard Apparatus), providing a constant flow rate of 0.002 ml/min. A positive voltage (15 or 20 kV) was applied to the solution by the needle, while a rotational mandrel, which was covered with aluminium, was connected to the ground electrode. The deposition was performed at room temperature at constant rotational speed (3250 rpm) for 8 h. The resulting non-woven nets were stabilized in methanol/ $\text{H}_2\text{O}$  (80/20 v/v) for 10 min and washed in distilled water for 2 days to remove the residual solvent (sample F).

## 2.2. Sample characterization

### 2.2.1. Field-emission scanning electron microscopy (FE-SEM)

All scaffolds were examined with a field-emission scanning electron microscope (FE-SEM; Supra 40, Zeiss). Samples were observed after coating with gold in a reduced argon atmosphere. SEM photos, fibre diameters of the nanofibrous membranes and pore sizes of the sponges were analysed using the image visualization software ImageJ, which was developed by National Institutes of Health (<http://rsb.info.nih.gov/>).

### 2.2.2. Water content

The water uptake of the three different sponges was measured by weighing the samples in the dry state and then in the wet state. Water was added one drop at a time onto the surface of the sample, avoiding the entrance of air into the pores. Finally, the water content percentage was calculated as follows:

$$\text{Water Uptake}(\%) = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100$$

## 2.3. Cardiac progenitor cell isolation, culture and seeding into 3D cultures

Adult Sprague–Dawley rats (up to 8 months old) were anaesthetised with vaporized isofluran, 0.20 mg/kg

intramuscular (i.m.) Zoletin 20, 0.25 mg/kg i.m. medetomidine and 0.0025 mg/kg subcutaneous (s.c.) atropine. The hearts were excised from the chests while they were still beating. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Italian Ministry of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Istituto Zooprofilattico Sperimentale Sicilia. All efforts were made to minimize suffering. Each excised heart was placed directly into a Falcon tube with 50 ml Hank's balanced salt solution (HBSS; Invitrogen, Life Technologies, Carlsbad, CA, USA) with 50 U/ml collagenase II (Life Technologies) and 3 mM  $\text{CaCl}_2$  to prevent blood coagulation and to allow for the rapid penetration of the collagenase solution directly into the coronary vessels. Under a laminar flow hood, the atria were separated from the ventricles. The ventricles were cut into four pieces and these (1 heart/tube) were placed into 20 ml fresh HBSS with 50 U/ml collagenase II and 3 mM  $\text{CaCl}_2$ . After a 10 min incubation at  $37^{\circ}\text{C}$  in a rotating dry incubator, the solution was collected and pieces were left on the bottom of the 50 ml Falcon tube. Isolated cells were removed from the collagenase solution by centrifugation and put into fresh M-199 medium (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 20% fetal bovine serum (FBS; Biolife Italiana, Milano, Italy), 3 mg/ml fungizone, 300 mg/ml streptomycin and 300 U/ml penicillin (Antibiotic–antimycotic 100 $\times$ , cat. no. 15240-062, Invitrogen). Another 20 ml HBSS with 50 U/ml collagenase II and 3 mM  $\text{CaCl}_2$  were added to the 50 ml Falcon tube with the four pieces, which was incubated for another 10 min at  $37^{\circ}\text{C}$ . We then performed repeated centrifugations, as previously described (Di Felice *et al.*, 2009a, 2010).

Only the second fraction (which was enriched in c-Kit<sup>+</sup>/Sca-1<sup>+</sup>/MDR-1<sup>+</sup> cells; Di Felice *et al.*, 2009a) was plated into 75 cm<sup>2</sup> poly-D-lysine-coated BD flasks with fresh M-199 medium supplemented with 20% FBS. The first fraction was discarded. After letting the cells grow, they were mildly harvested with a solution of 0.05% porcine trypsin and 0.02% EDTA (Trypsin–EDTA solution 10 $\times$ , T4174, Sigma-Aldrich) in PBS or HBSS for no more than 2 min; cells that did not detach from the plasticware were cardiac fibroblasts and were discarded (Di Felice *et al.*, 2009a). To maintain an undifferentiated state, the cells were cultured at 600 000 cells/25 cm<sup>2</sup> in M-199 supplemented with 20% FBS and harvested 2 days/week with a trypsin–EDTA solution (Sigma-Aldrich).

For two-dimensional (2D) cultures, the plasticware was coated with 0.1 mg/ml poly-D-lysine in PBS.

For three-dimensional cultures, the cells were cultured inside a 50  $\mu\text{m}$  thick collagen I gel rat tail (diluted 1:8; BD Biosciences), with or without scaffolds, inside the inserts in 24-well plates ( $2 \times 10^5$  cells/insert; BD Biosciences). The OPLA scaffold (BD Biosciences) has been previously used as a positive control of differentiation in 3D cultures (Di Felice *et al.*, 2009a). Scaffolds with cells were placed in 300  $\mu\text{l}$  diluted collagen, covered with M-199-20% FBS medium and incubated in a cell culture incubator for



21 days, with medium changes twice per week. After incubation, the 3D cultures were fixed with acetone:methanol:water (2:2:1), for immunofluorescence analysis, or 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 100 mM sodium cacodylate buffer (Sigma-Aldrich) for transmission electron microscopy (TEM) to enhance visualization of the cell membrane in 3D cultures, as previously described (Di Felice *et al.*, 2007).

## 2.4. Flow cytometry

After 10 days of tissue digestion, cells were detached from the flasks, counted and placed into FACS tubes (200 000 cells/sample). Cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS. After washing, the cells were blocked with incubation buffer (IB; PBS: M-199 supplemented with 10% FBS, 1:9 v/v), placed into the primary antibody solution in IB for 45 min at room temperature (RT) [anti-c-Kit, KAP-TK005 (Stressgen Bioreagents, Ann Arbor, MI, USA); anti-Ly-6A/E (Sca-1), clone E13-161.7ne V9 (Santa Cruz Biotechnology, Heidelberg, Germany); anti-MDR-1, SC-71557 (Santa Cruz Biotechnology); normal mouse IgG, SC-2025 (Santa Cruz Biotechnology); normal rabbit IgG, SC-3888 (Santa Cruz Biotechnology); each diluted 1:200], rinsed twice in PBS and then incubated in the secondary antibody solution (diluted 1:200 in IB for 45 min RT). After rinsing with PBS, the cells were analysed using a FACSCalibur Flow Cytometer (BD Biosciences).

## 2.5. Haematoxylin/eosin staining

To assess the aspect of the constructs, paraffin sections were stained with haematoxylin and eosin (H&E). Scaffolds with cells were fixed in a solution of acetone, methanol and water (2:2:1) for 12 h, washed in tap water and dehydrated with ethanol at 30%, 50%, 70%, 95% and 100% v/v. After dehydration, the tissue pieces were placed in xylol for 1 h and embedded in paraffin. The paraffin-embedded tissue samples were cut into 5  $\mu$ m sections. The sections were deparaffinized with xylene for 10 min and hydrated with a decreasing ethanol gradient. They were stained with haematoxylin for 4 min (Merck KGaA, Darmstadt, Germany), blocked for 15 min in tap water, treated with eosin (Merck KGaA) for 1 min and rinsed in water. The sections were dehydrated and mounted using Canada balsam (Panreac Química S.L.U., Barcelona, Spain). Images were captured using a Leica DM5000 microscope.

## 2.6. Reverse transcription–polymerase chain reaction (RT–PCR)

CPCs were cultured into BD or fibroin scaffolds inside inserts ( $2 \times 10^5$  cells/insert; BD Biosciences) and allowed to grow and differentiate for 21 days in M-199 medium

supplemented with 20% FBS in a cell culture incubator. Total RNA was extracted from the untreated cell culture (considered as a tissue biopsy) using the ChargeSwitch<sup>®</sup> Total RNA Cell Kit (Life Technologies) according to the manufacturer's instructions. Total RNA was extracted from cardiac tissue using the QuickPrep Total RNA Extraction Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Total RNA from both extractions was then quantified with the Qubit<sup>®</sup> RNA Assay Kit (Life Technologies Corp.).

## 2.7. RT–PCR for cardiac protein expression

RT–PCR was performed in a two-step reaction, first using the ImProm-II Reverse Transcriptase Kit (Promega, Madison, WI, USA) and then using the GoTaq Flexi DNA Polymerase Kit (Promega), according to the manufacturer's instructions; 5 ng total RNA/reaction were used. cDNA was amplified using the primers listed in Table S1 (see Supporting information).  $\beta$ -actin was used as a positive control. RT–PCR products were separated on a 3% agarose gel. The gel was stained with SYBR SafeDNAgel stain (Life Technologies) and visualized with UV light. PCR fragments purified using the Nucleospin PCR and Gel Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) were sequenced by the MWG Biotech Sequencing Service (Edersberg, Germany). Sequences were then analysed with the BLASTn Web Tool on the NIH website (<http://www.ncbi.nlm.nih.gov/BLAST/>). All PCR-amplified fragments corresponded to the desired target.

## 2.8. Real-time quantitative RT–PCR analysis

Quantitative RT–PCR (qRT–PCR) analysis was performed using GoTaq qPCR Master Mix (Promega). mRNA levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), ribosomal protein S6 kinase (*S6*) and  $\beta$ -glucuronidase (*GUSB*). Changes in the transcript level were calculated using the  $-\Delta\Delta C_t$  method [20]. cDNA was amplified using the primers indicated in Table S2 (see Supporting information). cDNA was amplified using the StepOnePlus<sup>™</sup> Real-Time PCR System (Life Technologies). PCR fragments purified using the Nucleospin PCR and Gel Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) were sequenced by the MWG Biotech Sequencing Service (Edersberg, Germany). Sequences were then analysed with the BLASTn Web Tool on the NIH website (<http://www.ncbi.nlm.nih.gov/BLAST/>). All PCR-amplified fragments corresponded to the desired target.

## 2.9. Immunofluorescence

Cells cultured on poly-D-lysine (Sigma-Aldrich)-coated chamber slides were fixed first with 4% paraformaldehyde for 30 min and then with ice-cold methanol for 30 min. After deparaffinization and rehydration, the tissues were rinsed in PBS. Antigen retrieval was performed

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using 10 mM citrate buffer, pH 6.0, with 0.05% Tween 20 for 10 min. After incubation with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 30 min, the cells and tissues were incubated with primary antibodies overnight at 4 °C [anti-Troponin T cardiac isoform, clone C-19, sc-8122 (Santa Cruz Biotechnology); anti-c-Kit, KAP-TK005, (Stressgen Bioreagents, Ann Arbor, MI, USA); anti-Ly-6A/E (Sca-1), clone E13-161.7ne V9 (Santa Cruz Biotechnology); anti-MDR-1, SC-71557 (Santa Cruz Biotechnology); each diluted 1:100]. The linked primary antibodies were detected with FITC- or TRITC-conjugated secondary antibodies (Sigma-Aldrich) at a 1:50 dilution. The nuclei were stained with Hoechst 33342 staining in PBS (Invitrogen, Carlsbad, CA, USA) for 5 min. Images were captured using a Leica CTR5000 fluorescent microscope.

### 2.10. Preparation for TEM

The 3D cultures were grown in M-199 medium (Life Technologies) supplemented with 20% FBS (Life Technologies) for 21 days and then fixed twice. In the first step, scaffolds were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in 100 mM sodium cacodylate buffer, pH 7.4 (Life Technologies), for 30 min and rinsed three times with 100 mM sodium cacodylate buffer. A second fixation step was performed with 2% OsO<sub>4</sub> (Electron Microscopy Sciences) in 100 mM sodium cacodylate buffer. After fixation, the samples were rinsed twice with 100 mM sodium cacodylate buffer, pH 7.4, and dehydrated with an ethanol series (25%, 50%, 70%, 95% and 100%). After dehydration, the scaffolds were embedded in EPON resin (Electron Microscopy Sciences) with passages of 1:3 resin:70% ethanol for 2 h, 3:1 resin:70% ethanol for 2 h, pure resin overnight, pure resin with 0.1 ml DMP-30 for 3 h before inclusion and for 48 h at 65 °C. Semi-thin sections were prepared and stained with methylene blue (Sigma-Aldrich) or used for immunohistochemistry.

### 2.11. Immunohistochemistry on EPON semi-thin sections

EPON-embedded TEM samples were cut into 1 µm semi-thin sections and dried over poly-D-lysine-coated slides unstained. The slides were then treated with 10% sodium ethoxide for 6 min at room temperature under a laboratory chemical hood to dissolve the EPON resin. After two 5 min rinses in 100% ethanol, the sections were rehydrated with an ethanol series (95%, 70%, 50%, 30%) and distilled water. After rehydration, the sections were rinsed in Tris buffer saline (TBS), pH 7.3, for 5 min and heated in a microwave oven at 700 W for 5 min in a hot unmasking sodium citrate buffer, pH 6.

After 30 min at room temperature, the sections were rinsed twice for 5 min in TBS and endogenous peroxidase blocked, treating the slides with 3% H<sub>2</sub>O<sub>2</sub> for 5 min. Then the sections were blocked with 3% BSA in TBS for 30 min and incubated for 1 h at room temperature with the anti-

titin antibody (SC-8724, Santa Cruz Biotechnology; diluted 1:50 in 1% BSA in TBS). After two rinses in TBS, the sections were incubated with Goat Probe (GP613G, Goat AP-Polymer Kit, Biocare Medical LLC, Concord, CA, USA) for 15 min, rinsed twice in TBS and incubated with Goat Polymer AP (GAP628G, Goat AP-Polymer Kit, Biocare Medical) for 15 min.

After three 5 min rinses in TBS, the sections were incubated with Warp Red<sup>TM</sup> chromogen (WR806 H, Biocare Medical) for 10 min and the reaction was stopped under tap water. The sections were then counterstained with haematoxylin for 30 min at 60 °C; the reaction was stopped with tap water and samples kept at 60 °C for 20 min. The slides were mounted with Vectamount permanent mounting medium (H-5000, Vector Laboratories, Burlingame, CA, USA).

### 2.12. Immunocytochemistry

Cells cultured on poly-D-lysine (Sigma-Aldrich)-coated chamber slides were fixed first with 4% paraformaldehyde for 30 min and then with ice-cold methanol for 30 min. Immunocytochemistry was performed using a MACH 1 Universal HRP-Polymer Detection kit (M1U539 G, Biocare Medical) for rabbit primary antibodies and Goat AP-Polymer Kit (GAP514 G, Biocare Medical) for goat primary antibodies, according to the manufacturer's instructions. Betazoid DAB chromogen (BDB2004 H, Biocare Medical) and Warp Red<sup>TM</sup> chromogen (WR806 H, Biocare Medical) were used as chromogens. The cells were counterstained with haematoxylin for 2 min. Anti-c-kit, KAP-TK005 (Stressgen Bioreagents) and anti-titin, SC-8724 (Santa Cruz Biotechnology) were used as primary antibodies.

### 2.13. Statistical analysis

All data are expressed as mean ± standard deviation (SD). qRT-PCR data in the form of cycle thresholds (C) were exported to Microsoft Excel. The data were analysed as  $2^{-\Delta C_T}$  and  $2^{-\Delta\Delta C_T}$  compared to the level of the same mRNAs in the heart tissue. Value comparisons of the genes between treatments were calculated with one-way ANOVA with Bonferroni's multiple comparison test. Values were considered significantly different at  $p < 0.05$ . These analyses were performed using GraphPad Prism<sup>®</sup> software (GraphPad Software, La Jolla, CA, USA).

## 3. Results

### 3.1. Material characterization

Silk porous structures with different morphologies were prepared using freeze-drying and electrospinning techniques (Figure 1). To obtain porous sponges, a silk fibroin-water solution (5% w/v) was frozen under different conditions, thus affecting pore size and distribution.



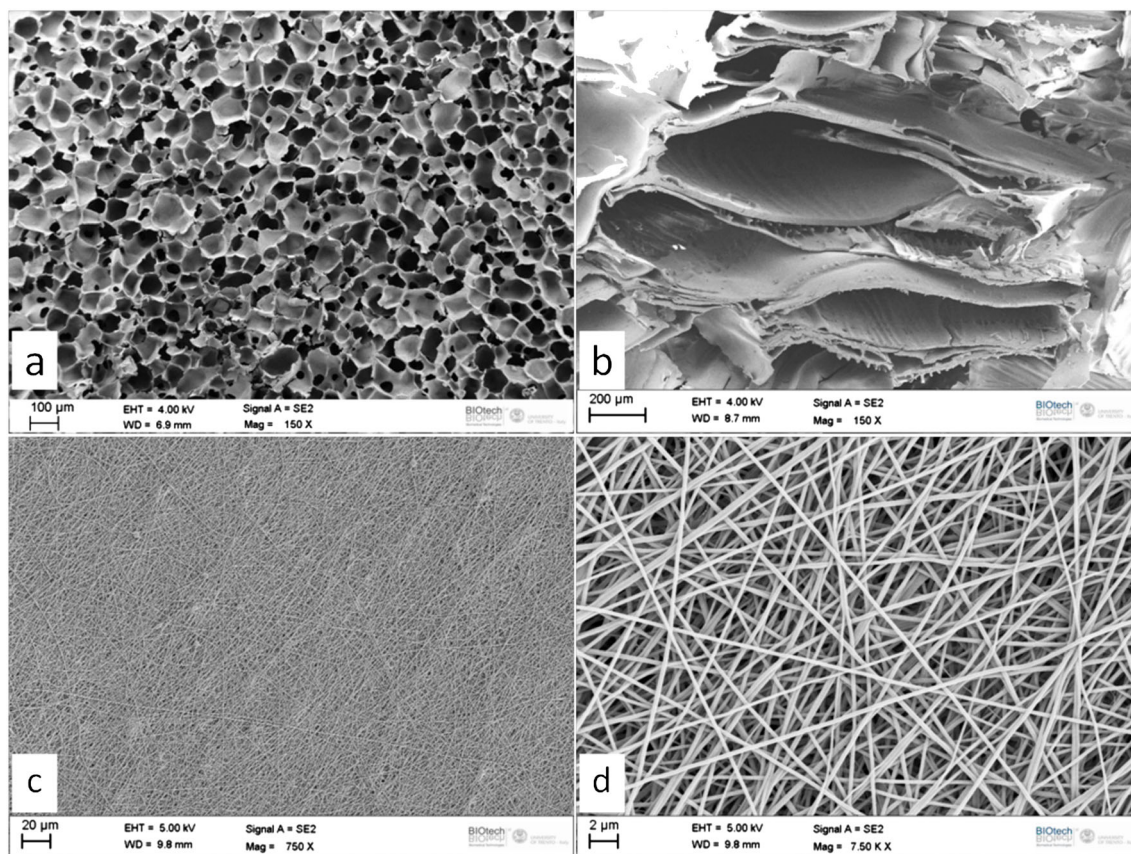


Figure 1. SEM micrographs of silk fibroin scaffolds used in the *in vitro* and *in vivo* experiments: (a) scaffolds with random porosity (RP), magnification =  $\times 150$ ; (b) partially orientated scaffolds (O), magnification =  $\times 150$ ; electrospun nets (F); magnifications = (c)  $\times 5000$ ; (d)  $\times 7500$

A relatively uniform pore morphology and distribution were observed in the porous structure obtained by freezing at  $-80^{\circ}\text{C}$  (Figure 1a), with pore sizes in the range 28–85  $\mu\text{m}$ . The scaffold produced by inducing a temperature gradient demonstrated a well-orientated sheet-like structure, with a bimodal pore size distribution in the range 200–400  $\mu\text{m}$  (largest pores), as shown in Figure 1b.

The electrospun net showed fibres that were randomly distributed in the mats, with an average fibre diameter of  $332 \pm 74$  nm (Figure 1c, d).

The water up-take capability of the porous scaffolds was also evaluated. The scaffold with orientated lamellae showed higher water uptake (728%) compared to the sample with randomly distributed pores (503%).

### 3.2. Cell isolation, characterization, seeding and distribution

We isolated immature cells from mature female rats using a differential adhesion method, as previously described (Di Felice *et al.*, 2009a). Using poly-D-lysine and a mild digestion with trypsin–EDTA solution, 2 weeks after tissue digestion, we obtained a good amount of highly proliferating cells. The isolated cells were then characterized by FACS analysis (Figure 2a): the fraction of c-Kit<sup>+</sup> cells varied (61.0–96.7%, mean  $83.23 \pm 19.3\%$ ), as previously described (Di Felice *et al.*, 2010); Sca-1-positive cells varied (74.6–98.2%, mean

$86.93 \pm 11.83\%$ ); MDR-1-positive cells varied (61.1–72.3%, mean  $63.13 \pm 5.68\%$ ). Immunofluorescence experiments on chamber slides confirmed the expression of these three markers on the surface of the isolated cells (Figure 2b–d). Cells selected with anti-Sca-1 antibody-coated magnetic beads showed the same expression levels of Sca-1, c-Kit and MDR-1 surface markers as non-selected cells. The surface markers CD73, CD14 and CD90 were tested by RT-PCR for each experiment to verify that there were no mesenchymal stem cells in the preparation, as previously described (Chimenti *et al.*, 2011; Di Felice *et al.*, 2009a). These three markers were not expressed in our samples.

Cells were seeded by capillarity in porous scaffolds and layered on electrospun nets. For porous scaffolds, a small drop of medium–collagen–CPCs (600 000 cells/20  $\mu\text{l}$ ) was added to two small dry pieces of the matrices (two samples with approximately the same drop volume). For electrospun nets, the same drop was put on the top of the sheet and layered with 150  $\mu\text{l}$  collagen I diluted in M-199 medium (1:8). Both porous and electrospun nets were placed into BD inserts and covered with medium. This method insured a homogeneous distribution of cells inside the scaffolds (Figure 3b).

### 3.3. Cells partially differentiated *in vitro*

To understand the behaviour of these cells inside BD OPLA and customized scaffolds over time, we grew the

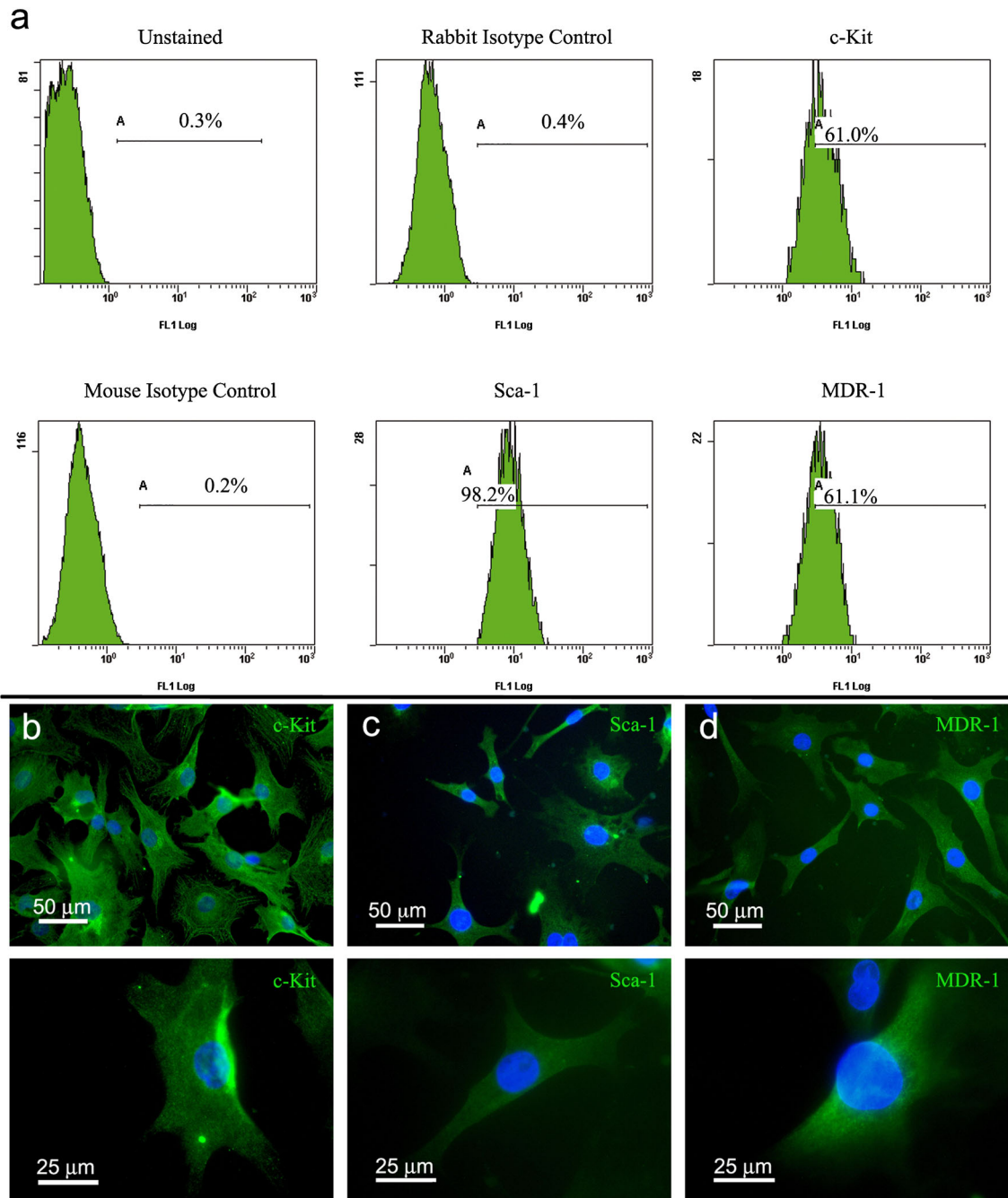


Figure 2. FACS analysis of isolated cardiac stem cells. (a) Percentage of cells expressing c-Kit, Sca-1 and MDR-1 10 days after tissue digestion. (b–d) Immunofluorescence, demonstrating the expression of these three surface markers in unselected cells. C-kit pattern of expression looks like very small dots following actin filaments, probably because c-Kit receptor may mediate F-actin assembly and cytoskeletal rearrangements (Vosseller *et al.*, 1997)

cells inside the scaffolds for 7, 14 and 21 days under their normal conditions (M199 supplemented with 20% FBS).

After 21 days, these cells grew inside the scaffold, were uniformly distributed and synthesized a great quantity of ECM (Figure 3b, c). They changed their morphology, become full with material that was similar to unorganized sarcomeric or cytoskeletal proteins and had intact cell membranes and caveolae (Figure 3d, e).

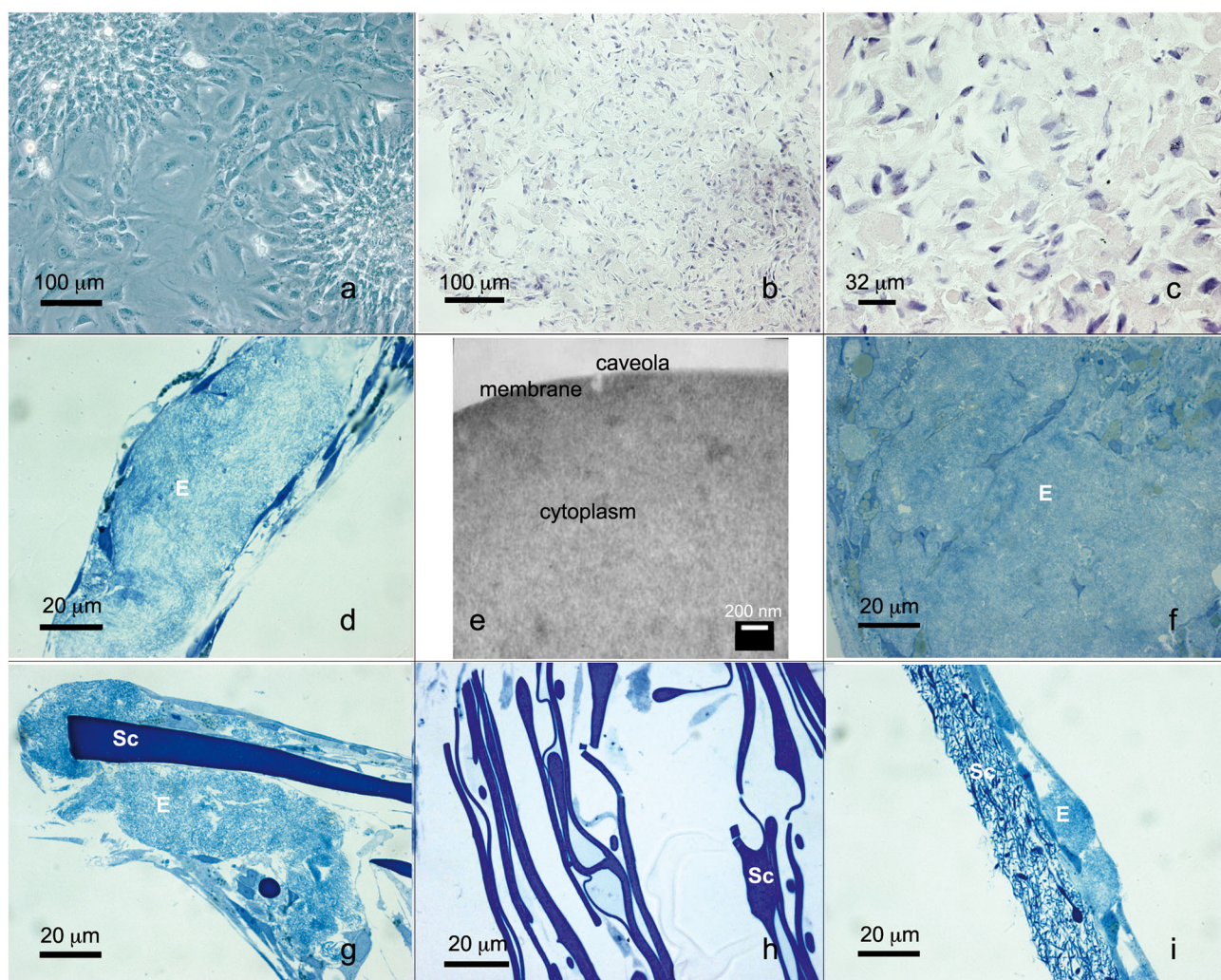
To determine whether or not the morphology of the 3D cultures was dependent on the presence of the scaffold, we cultured the same cells inside inserts with collagen and medium alone for 21 days (Cell + coll, Figure 3f).

These four features (increased cardiac troponin T expression, change in morphology, the presence of unorganized material in the cytoplasm, and the accumulation of native ECM) were the same in all studied conditions and scaffolds (Figure 3d, f–i). The optimal time point to study the differentiation of these cells appeared to be 21 days.

### 3.4. Expression of sarcomeric proteins

To understand the degree of differentiation in the different constructs, we analysed the gene expression of 10





**Figure 3.** Partial differentiation of CPCs *in vitro* inside porous scaffolds and collagen gel. (a) Phase-contrast image of isolated cells in 2D cultures. (b, c) H&E staining of isolated cells cultured for 21 days in M199 medium enriched with 20% FBS inside BD scaffolds. (d) Methylene blue-stained semi-thin section of cells cultured for 21 days in BD scaffolds. (e) TEM image of (d), showing the cellular membrane, cytoplasm and one caveola. Methylene blue-stained semi-thin sections of cells, 3D cultured for 21 days in collagen I alone (f), RP (g), O (h) and over F electrospun nets with collagen I (i). Sc, scaffold; E, extracellular matrix synthesized by the same CPCs

known rat sarcomeric proteins, comparing two-dimensional (2D) samples and/or heart tissue to Cell + coll, BD OPLA (BD, our control), random porosity (RP) and partially orientated (O) fibroin porous matrices, and fibroin electrospun nets (F) (Figure 4).

The data were analysed as  $2^{-\Delta C_T}$  and  $2^{-\Delta\Delta C_T}$ . With respect to  $2^{-\Delta C_T}$ , only cardiac  $\alpha$ -actinin 1 was considerably expressed in the 2D sample. When BD was the scaffold, the lower expression of all 10 mRNAs was observed. Silk fibroin scaffolds induced good expression of all the mRNAs, while Cell + coll expressed higher levels of titin and cardiac troponin T type 2 (Figure 4a). Telethonin-like mRNA was expressed only by O. Cardiac troponin I type 3 was expressed only by silk fibroin sponges (Figure 4a).

For the  $2^{-\Delta\Delta C_T}$  method, only muscle Z-line capping protein, myosin-binding protein H and titin showed a level of expression comparable to that of the heart tissue sample. Among these three mRNAs, titin was well expressed in silk fibroin scaffolds and Cell + coll constructs (Figure 4b). In the samples where titin was abundantly expressed, dense bodies with aligned fibres (forming Z-bodies) were visible

inside the cytoplasm of the cells (Tokuyasu and Maher, 1987). An example of forming Z-bodies is represented in Figure 4d. In 21-day samples (Figure 4d), forming Z-bodies with aligned and thick fibres were visible; while in 72 h samples (Figure 4c) only disorganized fibres were observed. The observation of thick and well-aligned fibres let us suppose that titin was effectively present, was located between thick and thin filaments and started sarcomere assembly, as shown in Figure 5.

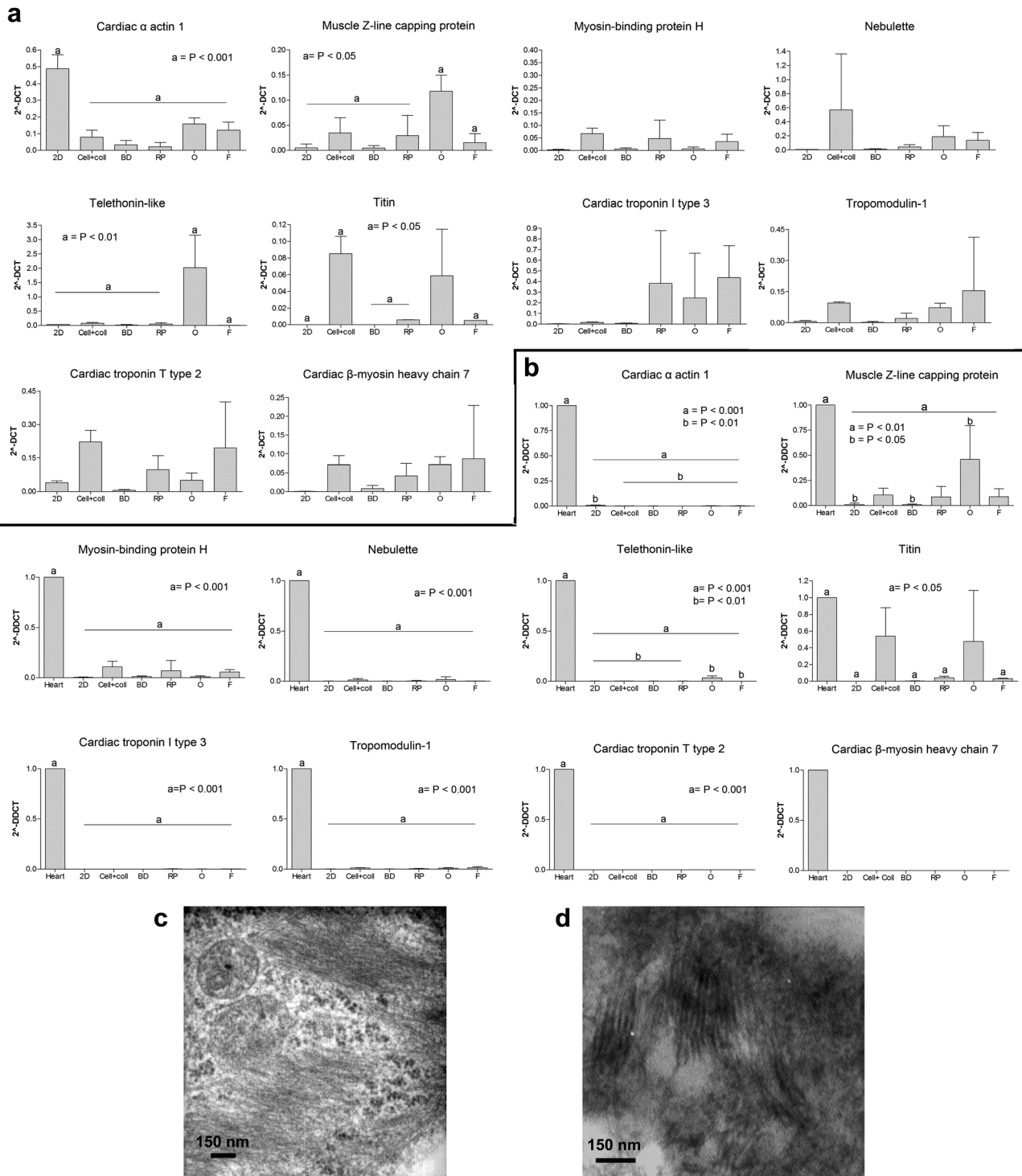
To confirm the expression of titin in Cell + coll, RP, O and F, we performed immunohistochemistry on TEM-EPON-embedded samples. As Figure 6 shows, titin was not expressed in 2D samples compared to c-Kit (our positive control), while it was expressed in Cell + coll, RP, O and F samples. Figure 6 confirms real-time PCR data.

### 3.5. Expression of cardiac proteins

To evaluate the differentiation state and the level of expression of mRNAs encoding ECM molecules, adhesion



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**Figure 4.** Real-time PCR analysis and differentiation evidences. The figure shows the expression of sarcomere genes in CPCs cultured on plasticware inside BD scaffolds, fibroin porous scaffolds and partially orientated fibroin scaffolds, and over electrospun nets after 21 days. (a) The graph shows normalization with the reference genes only ( $2^{-\Delta C_T}$ ). (b) The graph shows normalization with the reference genes and the calibrator (heart tissue), Livak Method ( $2^{-\Delta\Delta C_T}$ ). Heart, rat cardiac tissue; 2D, 2D culture on plasticware; Cell + coll, CPCs cultured inside inserts embedded in collagen I rat tail; BD, BD OPLA scaffolds; RP, fibroin porous scaffolds with 5% fibroin; O, partially orientated scaffolds with 5% fibroin; F, fibroin electrospun nets. A significant difference from 2D was observed; (b) from O, (c) from Cell + coll; (d) from F; (e) from Heart. (c) TEM image of disorganized fibres in 72 h Cell + coll samples. (d) TEM image of forming Z-bodies in 21 day Cell + coll samples. Fibres similar to (c) and (d) were also visible in 72 h and 21 day O samples

proteins, eNOS pathway members and structural proteins typically expressed in cardiomyocytes, we performed several RT-PCR reactions. As shown in Table 1, CPCs cultured in flasks expressed mainly fibronectin, calmodulin

and heat shock protein 90 (HSP90), while CPCs cultured in collagen I alone expressed many proteins at a level similar to the cardiac tissue. CPCs cultured inside the different scaffolds expressed several mRNAs, but the O

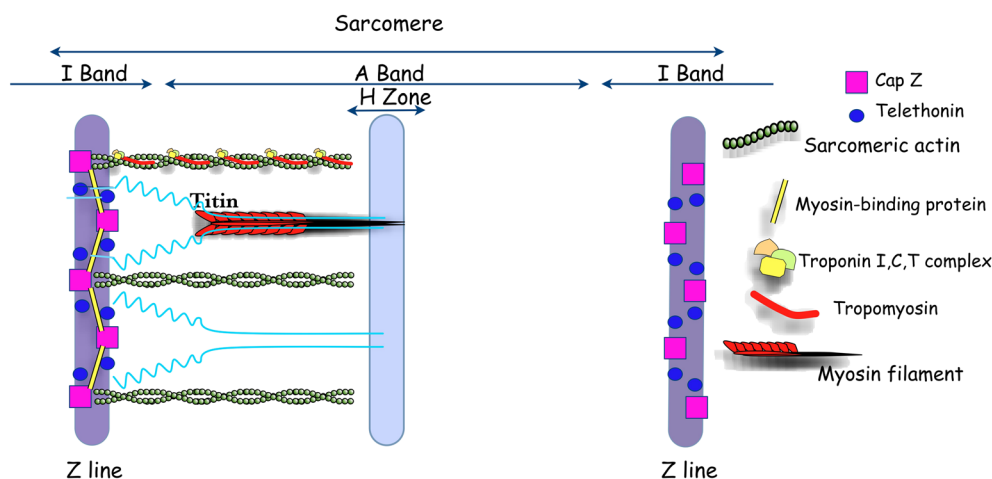


Figure 5. Role of titin in sarcomere assembly

sample expressed the most mRNAs compared to the other constructs. Samples with many positive signals are most likely the samples with more committed cells.

## 4. Discussion

The heart is an organ with a reduced regeneration capacity and a complex architecture, which is difficult to achieve from isolated stem cells. Although it is difficult to obtain a real myocardium *in vitro* from progenitor or stem cells, many research groups continue their studies on cardiac tissue engineering, because heart transplantation is the only possibility when the myocardium is highly damaged.

To use progenitor or stem cells to repair a damaged myocardium, it is necessary to find a surgical protocol that reduces the number of cells used, which maximizes the efficiency of the differentiation process and can be easily applied by surgeons. Progenitor and stem cells were delivered to the damaged myocardium, both in animals and in the first clinical trials, by intracoronary injection (Bolli *et al.*, 2011; Bui *et al.*, 2010; Makkar *et al.*, 2012; Smith *et al.*, 2007). This method reduces the efficiency of implantation and increases the risk of thrombi.

Scaffolds may increase the efficiency (fewer cells are needed) and may reduce the risk of thrombi (the construct may be sutured onto the damaged area). Unfortunately, a scaffold with low reactivity that can be used to deliver progenitor or stem cells to the myocardium is not known.

The complete differentiation of CPCs and the formation of organized myofibrils are difficult to achieve *in vitro*, and the integration of these cells into a damaged myocardium may be only an effect of fusion with cells that are already present *in vivo*, instead of a real regeneration of the myocardium. CPCs reach complete differentiation and form organized sarcomeres only in co-culture with a feeding layer of neonatal cardiomyocytes, as previously demonstrated (Pagliari *et al.*, 2011). It has also been suggested that a scaffold may only drive cell commitment, but that the concomitant effect of soluble factors, cell–cell interaction signals

and 3D structure may induce the complete differentiation of progenitor cells (Pagliari *et al.*, 2011).

In the present study, we investigated the possibility of using fibroin-based substrates, two 3D porous matrices and an electrospun net to deliver CPCs *in vivo* and as a substrate to drive progenitor cell commitment. The pore size, pore distribution and water uptake capability of the obtained sponges were affected by the freezing temperature and freezing rate. The electrospun net can be considered a nanopatterned 2D structure, due to its pore and fibre diameter size.

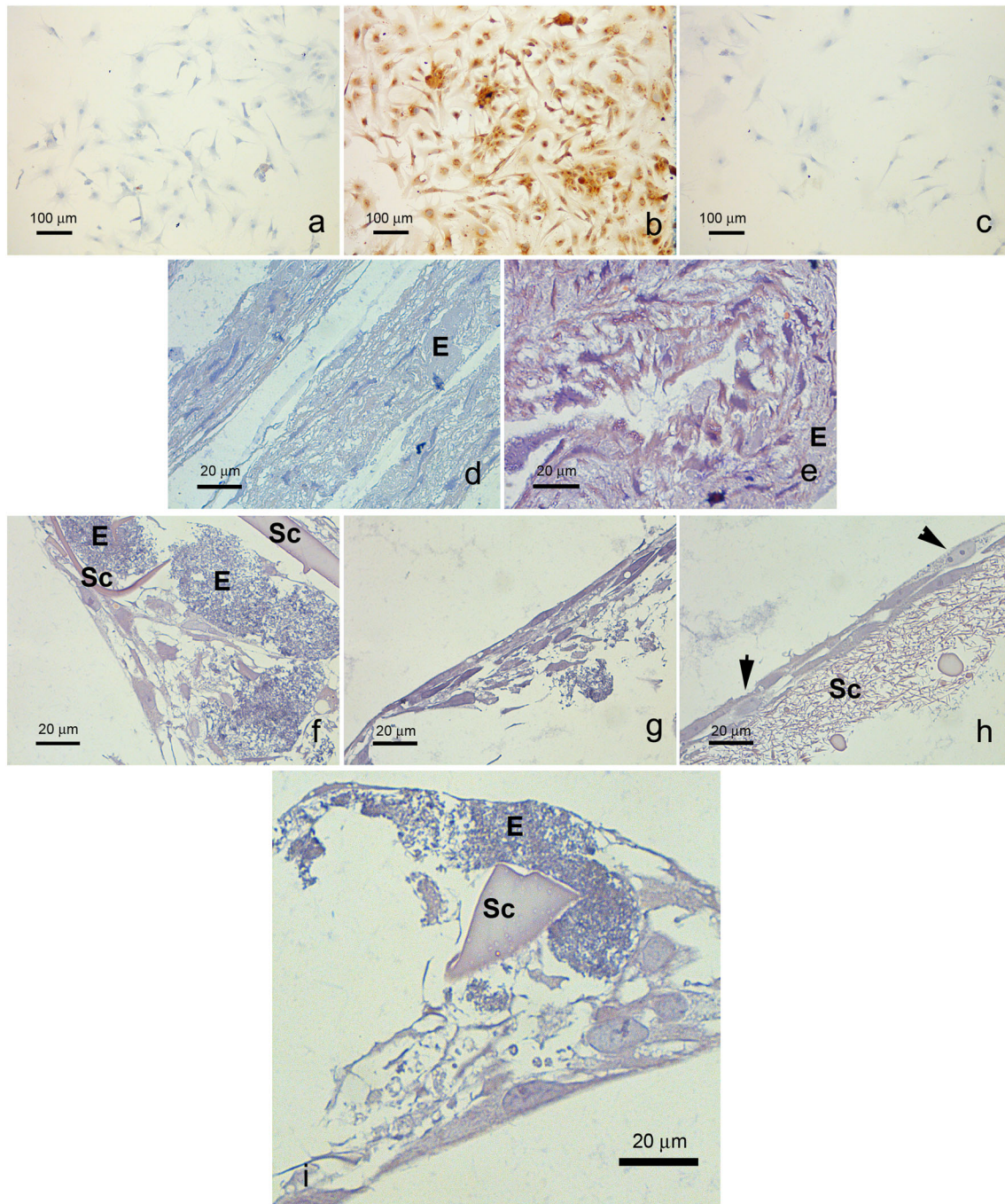
In the literature, only a few papers have been published on the use of polymeric scaffolds to enhance the cardiogenic differentiation of progenitor or stem cells. It is widely accepted that the physicochemical, mechanical and bioactive properties of scaffolds may control stem cell differentiation (Gupta *et al.*, 2011). A low fibre density 4% PEG 86%–PCL 10% CPCL electrospun co-polymer enhanced the cardiogenic differentiation of ESC-derived EBs in terms of  $\alpha$ -MHC expression in the presence of differentiation stimuli (Noggin or the BMP type-1 inhibitor DMH1).

Aligned microfibrillar enzyme-sensitive biodegradable polyurethane scaffolds in combination with mouse embryonic fibroblasts have been shown to improve the differentiation of mouse embryonic stem cell-derived cardiac stem cells (mESCDCs) (Parrag *et al.*, 2012).

Very recently, an attempt was made to repair a damaged myocardium with a collagen scaffold absorbed with polyplexes encoding IL-10 and seeded with rat mesenchymal stem cells in an animal model of myocardial infarction. The authors did not find changes in the infarcted area compared to the control, but found only a significant improvement in the left ventricular ejection fraction (Holladay *et al.*, 2012).

Only a few papers have been published on the behaviour and differentiation of 3D cultured CPCs *in vitro*. Murine and human Sca-1<sup>POS</sup> CPCs may synthesize GATA-4, Cx43, cardiac troponin I and  $\alpha$ -actinin, markers of cardiac differentiation, after only 7 days in direct co-culture with neonatal cardiomyocytes (nCMy) (Pagliari *et al.*, 2011). When cultured in 3D PLA scaffolds, murine CPCs produced a massive

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**Figure 6.** Immunohistochemistry showing titin expression in CPCs and scaffold samples. The first row shows the expression of titin (c) in 2D-cultured CPCs compared to the negative control (a) and our positive control c-Kit (b). The other pictures show the expression of titin in EPON 1  $\mu\text{m}$  semi-thin sections: (d) Cell + coll, negative control without the primary antibody; (e) Cell + coll; (f) RP; (g, i) O; (h) F samples. EPON-embedded samples have been created in parallel with real-time PCR samples shown in Figure 4. Titin was stained with Warp Red (Fuchsia); c-Kit was stained with Betazoid DAB chromogen (red); nuclei were counterstained with haematoxylin. Sc, scaffold; E, extracellular matrix synthesized by the same CPCs. Black arrows, titin-negative cells in the F sample

amount of ECM molecules, and when co-cultured with nCMs they expressed an increased level of  $\alpha$ -actinin (Pagliari *et al.*, 2011). The same research group attempted to grow these human CPCs on 60 mm temperature-responsive poly-*N*-isopropyl-acrylamide (PnIPAAm)-coated dishes to obtain cell sheets of undifferentiated human CPCs to use as a delivery system. These sheets expressed proteins involved in angiogenic pathways and components of the

ECM, including GATA-4, Islet-1, TGF $\beta$ 1, myocardin and BMPR2 (Forte *et al.*, 2011).

The cardiogenic potential of human CPCs grown as secondary cardiospheres (IICSps) was evaluated in a RGD-modified collagen scaffold and its denatured counterpart. Under this condition, human IICSps expressed higher levels of GATA-4, Nkx2.5 and TnI; the expression was maximal in RGD-modified collagen structures (Chimenti *et al.*, 2011).



**Table 1.** Expression of ECM molecules, adhesion proteins, eNOS pathway components, and structural proteins typical of the cardiac tissue in all the tested 3D cultures

	Cardiac tissue	2D	Cell + coll	BD	RP	O	F
$\beta$ -Actin	+++	++	+++	+	+	+++	+
Laminin	+	-	++	-	-	-	-
Fibronectin	+	++	+++	++	+	++	+
Coll V $\alpha$	++	-	+	+	-	+	-
Vitronectin	++	-	-	-	-	-	+
Integ $\alpha$ V	+	-	+++	-	-	++	-
Integ $\alpha$ 6	++	-	++	+	-	+	-
Integ $\alpha$ 7	++	-	++	$\pm$	+	++	+
Integ $\beta$ 1	++	-	+++	$\pm$	-	$\pm$	-
ILK	++	-	+++	+	+	++	+
FAK	+	-	+++	-	-	-	-
AKT	++	+	+++	-	+	+	-
Calmodulin	+++	+++	+++	+++	$\pm$	$\pm$	-
Cav3	+++	-	+++	-	$\pm$	$\pm$	+
CgA	++	-	-	-	-	-	+
eNOS	+	-	-	-	-	++	-
HSP90	+++	++	+++	+	-	+	-
Trop T2	+++	+	+++	+++	-	+	-
Cardiac MHC	+++	-	-	-	-	-	-

These data were obtained by analysing gel bands with ImageJ to obtain a numeric value for each band intensity (mean intensity of colour  $\times$  number of pixels). Values between 1 and 15 000 were considered  $\pm$ ; values between 15 000 and 50 000 were considered +; values between 50 000 and 120 000 were considered ++; values between 120 000 and 200 000 were considered +++; 0 was considered.

Similar results were obtained for mouse and porcine cardiosphere-derived cells in hydrogels (Li *et al.*, 2011; Takehara *et al.*, 2008).

In the present study we demonstrated that a population of CPCs with a high level of the three most important surface markers, c-Kit, Sca-1 and MDR-1 (Bernstein and Srivastava, 2012), can be isolated through repeated collagenase treatments and a differential adhesion method. These c-Kit<sup>+</sup> CPCs are able to grow inside a commercially available poly-lactic acid scaffold (BD OPLA) and may synthesize cardiac proteins, such as cardiac troponin T (Di Felice *et al.*, 2009a). As observed by TEM, these cells had a cytoplasm engulfed with proteins, even though the plasma membrane was still intact.

In a 3D space that was created by scaffolds or collagen alone, CPCs were able to synthesize their own ECM molecules and organize a mass that was similar to a well-organized tissue. They could adhere to poly-lactic acid and fibroin, following the shape of the scaffolds.

Analysing the expression of ECM molecules, adhesion proteins, components of the eNOS pathway and cardiac structural proteins, we observed that CPCs expressed most of the proteins present in the myocardium only when they were cultured with collagen alone (Cell + coll) or inside partially orientated porous scaffolds (O). In particular, they expressed high levels of integrin subunits  $\alpha$ 7 and  $\beta$ 1, specific for laminin, which is one of the main constituents of the cardiac ECM (Jane-Lise *et al.*, 2000). Moreover, it is known that integrin  $\alpha$ 7 $\beta$ 1 provides a mechanical link between muscle fibres and the basement membrane in skeletal muscle, contributing to costamere assembly (Guo *et al.*, 2006).

Sarcomeric proteins were more or less expressed in all 3D cultures analysed, but when comparing the real-time data with the expression levels in the cardiac

tissue, only muscle Z-line capping protein (CapZ) and titin were expressed at considerable levels in both the Cell + coll and O samples. The presence of assembled Z-bodies of nascent premyofibrils support our hypothesis that c-Kit<sup>+</sup> CPCs 3D cultured in a collagen I gel, with or without scaffold, are committed to cardiac differentiation. Differences in the values of titin levels among samples probably depended on both the levels of the mRNA in the single cell and the number of titin-expressing cells in the sample.

Both CapZ and titin are involved in the cardiac Z-disc signalling network (Frank and Frey, 2011). CapZ is the capping molecule found on the barbed ends of F-actin filaments in the sarcomere at the Z-line. It interacts with  $\alpha$ -actinin and regulates actin dynamics (Frank and Frey, 2011). Recently, it has been demonstrated that CapZ may regulate PKC $\beta$ II binding to the myofilaments, regulating contraction (Pyle *et al.*, 2006).

Titin is a massive molecule that plays a crucial role in sarcomere assembly and the alignment of contractile filaments (Figure 5) (Miller *et al.*, 2004). It is considered a sarcomeric stretch sensor and associates with several proteins involved in force transmission and the transduction of a signal from the ECM to the nucleus (mechanotransduction) (Frank and Frey, 2011; Hoshijima, 2006).

Therefore, the present study demonstrates that physico-chemical stimuli, provided by partially orientated fibroin scaffolds and biological stimuli provided by the collagen gel and by the ECM that cells autonomously synthesize, can be properly combined *in vitro* to resemble the stem cell niche able to efficiently drive CPC commitment. Moreover, fibroin orientated scaffolds and collagen gels may help overcome the negative results and cell dispersion obtained when stem cells are directly injected in the damaged and compromised area of an infarcted myocardium.

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## Conflict of interest

The authors have declared that there is no conflict of interest.

## Supporting information on the internet

The following supporting information may be found in the online version of this article:

Table S1. Primers used for semi-quantitative RT-PCR

Table S2. Primers used for real-time quantitative PCR analysis

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