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RESEARCH ARTICLE

Collagen modules for *in situ* delivery of mesenchymal stromal cell-derived endothelial cells for improved angiogenesis

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Abstract

Modular tissue engineering is a strategy to create scalable, self-assembling, three-dimensional (3D) tissue constructs. This strategy was used to deliver endothelial-like cells derived from bone marrow mesenchymal stromal cells (EL-MSCs) to locally induce vascularization. First, tissue engineered modules were formed, comprising EL-MSCs and collagen-based cylinders. Seven days of module culture in a microfluidic chamber under continuous flow resulted in the formation of interstices, formed by random packing of the modules, which served as channels and were lined by the EL-MSCs. We observed maintenance of the endothelial phenotype of the EL-MSCs, as demonstrated by CD31 staining, and the cells proliferated well. Next, collagen modules covered with EL-MSCs, with or without embedded MSCs, were implanted subcutaneously in immune-compromised SCID/Bg mice. After 7 days, CD31-positive vessels were observed in the samples. These data demonstrate the feasibility of EL-MSCs coated collagen module as a strategy to locally stimulate angiogenesis and vasculogenesis. Copyright © 2013 John Wiley & Sons, Ltd.

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

Keywords mesenchymal stromal cell; collagen modules; modular tissue engineering; angiogenesis; endothelial differentiation; vasculogenesis

1. Introduction

There is a great need for technology to enhance the formation of blood vessels, such as in peripheral vascular disease (PVD) or in tissue engineering of large three-dimensional (3D) tissues. As such, engineering of small-diameter blood vessels is one of the major challenges in current tissue-engineering and revascularization strategies. Numerous trials have been performed to discover the optimal way of restoring or introducing vascular networks in the tissues that require such treatment. Delivery of cells of endothelial nature *in situ*, either in the tissue-engineered construct or in the human body, has been one of these strategies. There are various types of endothelial cells (ECs) that are able to improve tissue vascularization, either by providing signals that induce angiogenesis (Byrne *et al*., 2005) or by contributing to the new vessel system by self-organization into capillaries that then become connected to the surrounding vascular network (Rouwkema *et al*., 2006). The usage of these cells in therapy is unfortunately hampered due to problems with their isolation, expansion capacity and the lack of autologous sources (Tiwari *et al*., 2001; Salomon *et al*., 1991; Nerem and Seliktar, 2001). As an alternative, we and others have investigated the possibility of using another cell type, i.e. mesenchymal stromal cells (MSCs), in therapies that require vessel regeneration.

MSCs are adult cells that can easily be isolated from many sources, including adipose tissue, tibia, femur, lumbar spine and trabecular bone (Siddappa *et al*., 2007; Cowan *et al*.,

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2004; Oreffo *et al.*, 1998; D'Ippolito *et al.*, 1999). MSCs can be expanded *in vitro* and have the capacity to differentiate into the adipogenic, osteogenic and chondrogenic lineages (Banco and Gehron, 2000) as well as towards endothelial cells, skeletal muscle cells and neural cells (Dezawa *et al.*, 2005; Jiang *et al.*, 2011; Janeczek Portalska *et al.*, 2012). The differentiation of MSCs is influenced by soluble factors (Pittenger *et al.*, 1999), oxygen tension (Lennon *et al.*, 2001) and mechanical stimuli, such as flow or sheer stress (O'Cearbhaill *et al.*, 2008). Another important feature of MSCs is that they secrete various growth factors and cytokines, by which they can influence both cells within tissue-engineered constructs and cells from the surrounding host tissues. It was shown that, by this trophic effect, MSCs can inhibit apoptosis, fibrosis and stimulate angiogenesis, among other things (Caplan and Dennis, 2006; Khan *et al.*, 2011), which is beneficial for the survival of cells within the graft.

The first experiments in which MSCs were applied in vascular therapies were performed by simple injection of the cells into the vein (Kamihata *et al.*, 2001). The assumption was that injected MSCs would reach the tissue where vascularization was needed through the regular blood flow. The observed improvement of vascularization was very limited but was seen as proof of principle, and it encouraged further research in this area. It was hypothesized that simple injection of cells might not be the best way of delivering them at the place of injury. The potential of MSCs in vascular network formation could be improved by providing a matrix support that would direct these cells towards the formation of capillaries able to connect with the recipient's vascular network.

One approach to creating scalable, self-assembling, 3D tissue constructs is modular tissue engineering. This technique allows the fabrication of tissue building blocks with specific features and then these modular units are used to engineer biological tissues bottom-up (Nichol and Khademhousseni, 2009). This is of crucial importance, because the tissues to be engineered (e.g. fragments of bone) are often in the centimeter range and, thus, are too large to be generated *in vitro* in one piece. There are several ways to create modular blocks, including assembly of hydrogel layers (Yeh *et al.*, 2006), aggregation of microtissues (Dean *et al.*, 2007), direct gel printing (Mironov *et al.*, 2003) and using cell sheets (L'Hereux *et al.*, 1998). In order to build larger tissues, such blocks are then assembled by random packaging (McGuigan and Sefton, 2006, 2007; McGuigan *et al.*, 2006) or layer stacking (L'Hereux *et al.*, 1998). Such systems can be used, among others, to improve vascular network formation within an engineered construct by providing channels that can serve as a route for both implanted cells and in-growing host vessels.

Cooper and Sefton (2011) showed that coating collagen modules with human umbilical vein endothelial cells (HUVECs) prevented loss of shape of these modules and allowed for vessel ingrowth, in contrast to the situation when uncoated modules were implanted; the latter led to creation of an indistinguishable mass of collagen and limited vessel penetration. Chamberlain *et al.* (2012) showed that endothelial cell-coated collagen constructs with embedded MSCs can be used for the formation of functional blood vessels. They used MSCs and ECs isolated from Sprague–Dawley rats (ECs from aorta and MSCs from bone marrow). To obtain clinically relevant data, we decided to use human MSCs and ECs in the following experiments.

In our previous work (Janeczek Portalska *et al.*, 2012; Rouwkema *et al.*, 2008a), we showed that human MSCs can be differentiated into endothelial-like cells (EL-MSCs), which can be used to create vascular networks within grafts. We showed that EL-MSCs express characteristic endothelial markers and perform well in *in vitro* functional tests such as Ac-LDL uptake and capillary-like structure formation on Matrigel, and contribute to the vasculature upon implantation in immune-deficient mice. Importantly, MSCs used for obtaining EL-MSCs can be autologous to the patients. In our previous study (submitted for publication) we observed donor variability among MSCs obtained from various donors; all cells tested responded to an applied differentiation protocol, showing endothelial phenotype, but with varying efficiency. Therefore, to be able to compare data obtained in this study with those obtained previously, we decided to again use the immortalized clone that we tested before.

We have also tested several ways of delivering these cells *in vivo*, employing either a gel (Matrigel) plug or a PLLA–PLGL scaffold (Janeczek Portalska *et al.*, 2012). In this paper we describe how EL-MSCs can be combined with collagen modules in which a clinically approved biomaterial is used, is easy to obtain and is cheaper than Matrigel, but also can be formed *in vitro*, which would enable *in vivo* formation of channels lined with cells. We hypothesize that EL-MSCs in modules are useful for the preformation of functional vasculature in tissue-engineered constructs.

2. Materials and methods

2.1. Isolation and culture

An immortalized clone of human MSCs (referred to as iMSCs; a kind gift of Professor Ola Myklebost) was used in these studies. The cells were grown in MSC proliferation medium, which contains α-minimal essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Lonza), 100 U/ml penicillin (Gibco), 10 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp; Sigma-Aldrich) and 1 ng/ml basic fibroblast growth factor (bFGF; Fisher Scientific) at 37°C in a humid atmosphere at 5% CO_2. HUVECs (Lonza) were cultured in endothelial growth medium (EGM-2; Lonza).

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2.2. Endothelial induction of MSCs

iMSCs at passage 25 were used for endothelial induction, as described previously (Janeczek Portalska *et al*., 2012). Cells were seeded at a density of 3000 cells/cm^2 on tissue culture plastic in EGM-2 and cultured for 10 days. For induction on Matrigel, wells of six-well plates were coated with 1 ml growth factor-reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at a density of 30 000 cells/cm^2 and cultured in a humid atmosphere with 5% CO_2 for 24 h. Cells that were cultured according to this protocol will be referred to as EL-MSCs.

2.3. Collagen module fabrication

Modular collagen cylinders were fabricated as described previously (McGuigan and Sefton, 2006). In brief, Purcol-acidified collagen (type I, bovine dermal, 3 mg/ml; Cedarlane) was mixed with 10× minimum essential medium (Invitrogen); 128 µl 10× medium/ml collagen, and neutralized with 0.8 M $NaHCO_3$ (Sigma-Aldrich). Pelleted iMSCs were mixed with the neutralized collagen (1×10^6 cells/ml) and the solution was drawn into the lumen of an ethylene oxide gas-sterilized PE tube (0.76 mm i.d., 1.22 mm o.d.) connected to a syringe at one end; 1 h of incubation at 37°C was applied to allow collagen gelation. The gel-filled tubes were cut into fragments of 1.5 mm, using a custom-built automated cutter (FCS Technology). Sections were vortexed gently in cell culture medium to recover the gel modules from the tubing lumen. The collagen–cell modules were allowed to settle, separated from the PE tubing and cultured for 24 h in Petri dishes under static conditions. Collagen-only modules were fabricated identically (same collagen concentration) without the addition of the cells.

2.4. Cell-seeding

HUVECs (5×10^6 cells/pack of modules; 1 ml collagen) or EL-MSCs (2.5×10^6 cells/pack of modules; 1 ml collagen) were added to modules with or without encapsulated MSCs and incubated for 1 h with gentle shaking. The modules were transferred into Petri dishes and cultured for another 24 h. Four module systems were used for *in vitro* study: surface-seeded EL-MSCs only; surface-seeded EL-MSCs with embedded MSCs; surface-seeded HUVECs with embedded MSCs; and embedded MSCs only. The conditions 'surface-seeded EL-MSCs' and 'surface-seeded EL-MSCs with embedded MSCs' were also used for *in vivo* studies.

2.5. Chamber assembly and flow circuit construction

Modules were loaded in chambers (3D KUBE, Kiyatec) on 0.45 µm polyethersulphone membranes (Figure 1A). These

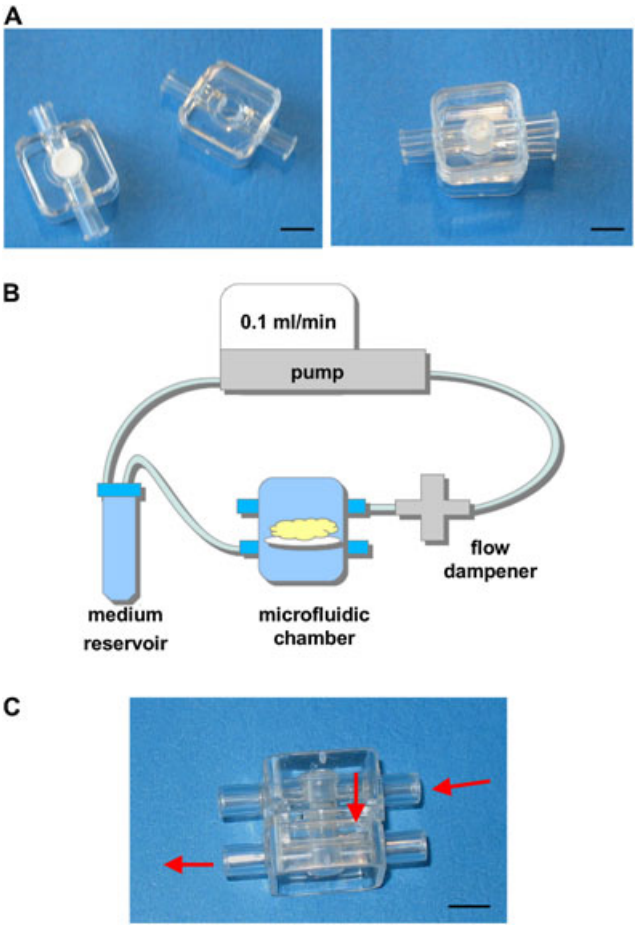

Figure 1. Experimental set-up: (A) microfluidic chamber before and after assembly; (B) flow circuit; (C) scheme of medium flow through the chamber. Scale bar = 1 cm

chambers were connected to a multichannel peristaltic pump and EGM-2 medium was circulated through the chamber in a closed loop, as described previously (Khan and Sefton, 2011; Khan *et al*., 2011). Medium was pumped using a Masterflex L/S Digital standard drive with a four-channel L/S multichannel pump head (Cole-Parmer, Vernon Hills, IL, USA). 50 ml BD Falcon™ polystyrene conical tubes (Becton-Dickinson) were used as a reservoir for cell culture medium, and tubing was a combination of L/S 13 Tygon® laboratory tubing and L/S 13 Tygon® two-stop tubing with connections made via Luer lock connectors (Cole-Parmer). Flow dampeners (fabricated from Nalgene Cryogenic Vials) were used to reduce pulsation and remove air bubbles from the system (Figure 1B, C). 35 ml EGM-2 was circulated from separate reservoirs at a flow rate of 0.1 ml/min and was changed twice a week. Samples were maintained for 7 days at 37°C and 5% CO_2. After 7 days, the remodelling chambers were perfused with 5% formalin for 30 min. The chambers were opened and the samples recovered and embedded in blocks of 5% agarose (Roche Diagnostics), and then fixed again in 10% formalin. These samples were embedded in paraffin and sectioned at 4 µm (two levels of sections, 50 µm apart) before analysis.

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2.6. Module implantation

Male 6 week-old Fox Chase severe combined immunodeficient/beige mice (SCID/Bg; Charles River) were anaesthetized with a mixture of isofluorane and oxygen. Approximately 200 modules in 0.2 ml phosphate-buffered saline (PBS) were injected subcutaneously, one injection per mouse. After implantation the mice were individually housed. One and three weeks after implantation, the mice were sacrificed and the implants were recovered. Samples were fixed in 10% formalin, embedded in paraffin and sectioned at 4 μm (two levels of sections, 50 μm apart) before staining. The study was approved by the University of Toronto Animal Care Committee.

2.7. Histochemical analysis

Haematoxylin and eosin (H&E; Sigma-Aldrich) and Masson's trichrome (Merck Chemicals) stainings were performed according to the manufacturers' protocols. Immunohistochemical analyses were used to detect and characterize the endothelium. Sheep anti-von Willebrand factor (vWF; Dako), rabbit anti-CD31 (Santa Cruz Biotechnology), mouse anti-desmin (Dako) and rabbit anti-SMA (Vector Laboratories) antibodies were used. All primary antibodies recognize both mouse and human antigens. All secondary antibodies were obtained from Vector Laboratories. To detect endothelium of human origin, biotinylated *Ulex europaeus* agglutinin 1 (UEA-1; Vector Laboratories) was used, after which anti-biotin secondary antibody (Vector Laboratories) was applied. All slides were developed with DAB chromogen and weakly counterstained with Mayer's haematoxylin (Sigma-Aldrich). Normal mouse and human tissues served as positive controls. As described previously (Janeczek Portalska *et al*., 2012), vessels were counted manually (without considering their size), based on Masson's trichrome staining by three observers blinded to the sample composition. Three areas of each sample (three samples/condition) were used for this quantification.

2.8. Statistical analysis

Each experiment was performed in triplicate. Data that required multiple comparison testing were analysed using SPSS (PASW statistics), using one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

3. Results

3.1. Collagen module assembly

To examine EL-MSC behaviour under flow conditions and relate those observations to our previous results (Janeczek Portalska *et al*., 2012), four types of modular constructs were assembled. We used collagen modules with surface-seeded EL-MSCs only, surface-seeded EL-MSCs with embedded MSCs, surface-seeded HUVECs with embedded MSCs and embedded MSCs alone. The last two conditions were nearly the same as those used by Khan *et al*. (2011), with in our case human instead of rat cells, and were used as positive control for the system. The first two conditions were chosen to show the potential of EL-MSCs to replace endothelial cells. Collagen module preparation (Figure 2A) and the efficiency of coating with cells was assessed 24 h after cell seeding. As expected, MSCs within the modules were uniformly distributed (Figure 2B). The sizes of the collagen modules, with or without embedded MSCs, were similar, with a diameter of ca. 0.7 mm and a length of ca. 1.5 mm (estimation based on microscopic observations). Coating with HUVECs resulted in an approximately two-fold shrinkage of the modules (Figure 2C), whereas coating with EL-MSCs induced even more shrinkage, both with empty and MSC-embedded modules, with a four-fold size reduction (Figure 2C, D); 24 h after cell seeding, all modules were uniformly coated with cells.

3.2. Cell characterization in microfluidic chamber

Cell survival under flow conditions was assessed after 7 days. Collagen modules with embedded MSCs formed a single mass of collagen over that time period (Figure 3A). MSCs could be easily observed within the mass; however, we did not witness evidence of proliferation (only single cells were observed, with a lack of cell colonies within the collagen). Similarly, MSC proliferation was also not identified in modules with embedded MSCs coated additionally with HUVECs (Figure 3B). As shown previously for rat endothelial cells (Khan *et al*., 2011), the endothelial coating of the collagen modules was still intact after 7 days of culture of HUVECs, but here also no increase in the amount of cells was observed. In contrast, we observed a large increase in the amount of EL-MSCs on the surface, but not in the interior, of the modules (Figure 3C, D). Several markers were examined to characterize the phenotype of MSCs and EL-MSCs under flow conditions. First, we stained the modules for the endothelial marker CD31. No staining was observed in samples with embedded MSCs only (Figure 4A), whereas staining was observed in the HUVEC-coated samples (Figure 4B). In samples with EL-MSC coating (Figure 4C, D), the cells present in the intermodular region were CD31-positive, which indicates that EL-MSCs kept the phenotype acquired after Matrigel induction (Janeczek Portalska *et al*., 2012). UEA-1-positive cells were found uniformly distributed throughout the whole sample only in constructs containing HUVECs (Figure 4F). In samples with EL-MSC coating but without embedded MSCs, several regions were found, in which cells were UEA-1-positive, but those regions were not uniformly spread through the sample (Figure 4G). No UEA-1-positive cells were observed in modules with embedded MSCs, either in modules without coating or modules with EL-MSC coating (Figure 4E, H).

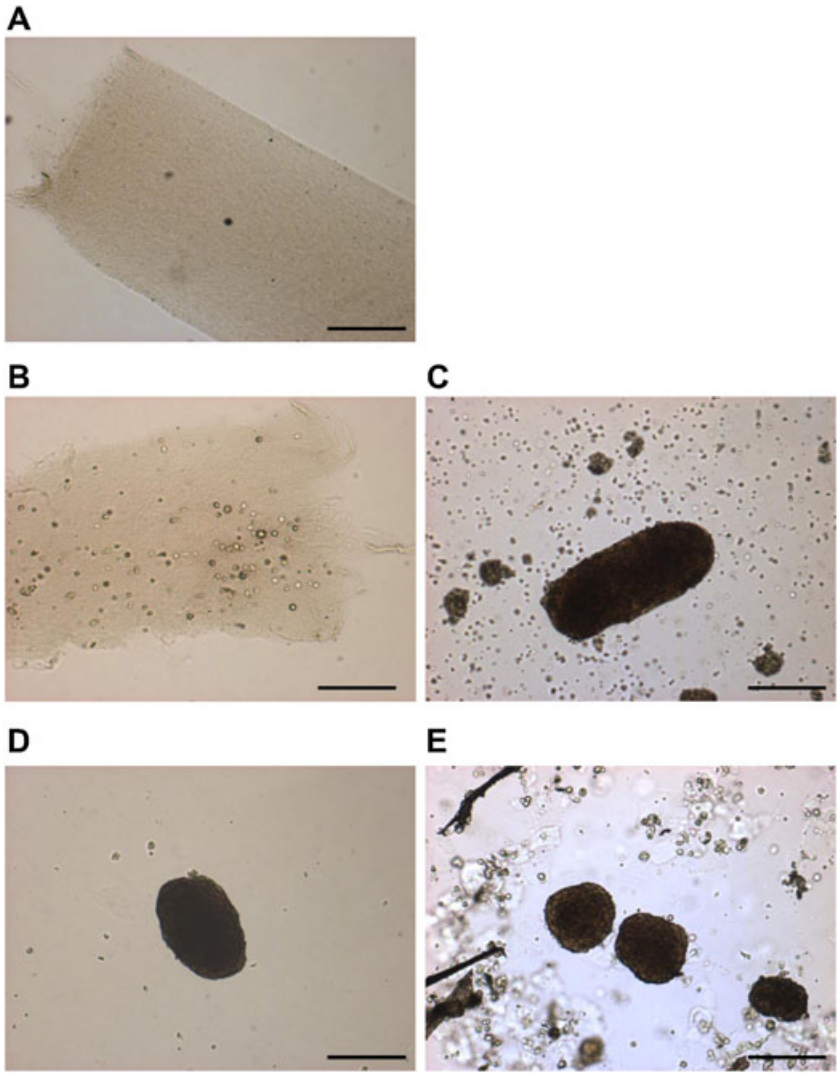

Figure 2. Collagen modules 24 h after assembly: (A) empty collagen modules; (B) modules with embedded MSCs; (C) modules with embedded MSCs and surface-seeded HUVECs; (D) modules with surface-seeded EL-MSCs; (E) modules with embedded MSCs and surface-seeded EL-MSCs. Scale bar = 500 μm

To observe whether differentiation towards smooth muscle cells occurred in the system as described previously (Khan *et al*., 2011), we performed smooth muscle actin (SMA) and desmin staining as smooth muscle cell and pericyte markers, respectively (see Supporting information, Figure S1); however, no staining was observed. Therefore we can conclude that culture in EGM-2 supported the endothelial phenotype of EL-MSCs, whereas it did not induce MSC or EL-MSC differentiation towards a smooth muscle phenotype.

3.3. *In situ* delivery of endothelial cells

To evaluate whether the collagen module system can be used to deliver EL-MSCs in the place of interest and whether a vascular network will be created at the place of administration, one package of modules was injected subcutaneously in immunocompromised SCID/Bg mice. We implanted collagen modules coated with EL-MSCs with or without embedded MSCs. Embedded MSCs were included in this experiment because these cells were required to support HMECs in the study bt Butler and Sefton (2012). Samples were explanted at 7 and 21 days after implantation. After both 7 and 21 days, we observed that the collagen mass was separated by layers of cells (Figure 5A, B) and the collagen modules were uniformly infiltrated by host blood vessels. Quantification based on Masson's trichrome staining (Figure 5C) showed that 7 days after injection, modules with and without embedded MSCs were penetrated by similar numbers of vessels. The results were different when the quantification was performed on samples explanted 21 days after injection. In the case of collagen modules coated with EL-MSCs without embedded MSCs the number of vessels was the same as at day 7, but in samples with embedded MSCs a significant, two-fold decrease in vessel number was observed.

To confirm our results and further study the maturity of in-growing vessels, we performed several additional stainings, including markers such as CD31, UEA-1, SMA and desmin (Figure 6). Our results showed that the vessels

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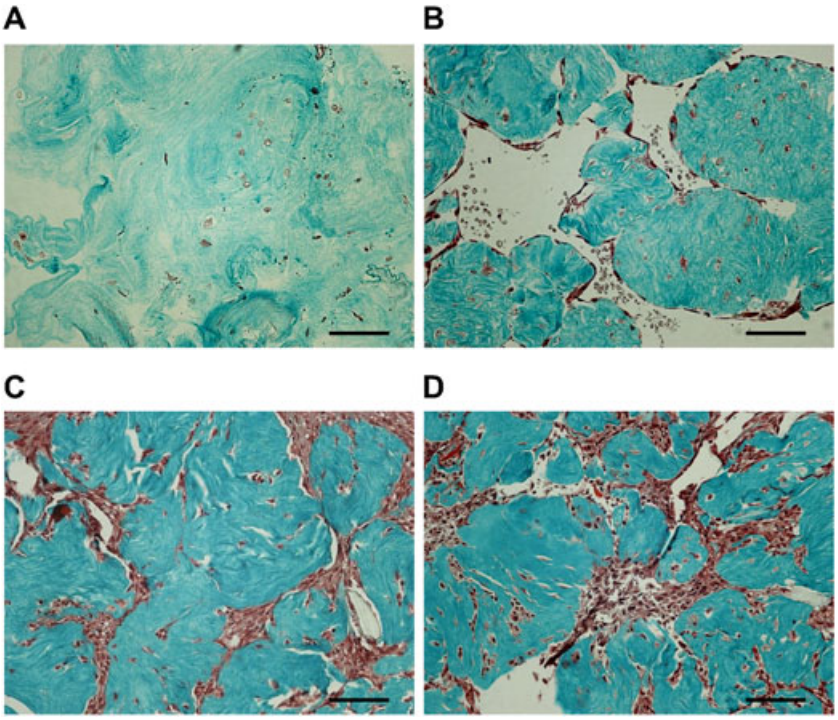

Figure 3. Cell migration and proliferation over time. Masson's trichrome staining was performed on samples subjected to flow conditions for 7 days. Collagen appeared blue, while nuclei appeared dark brown. (A) Modules with embedded MSCs; (B) modules with embedded MSCs and surface-seeded HUVECs; (C) modules with surface-seeded EL-MSCs; (D) modules with embedded MSCs and surface-seeded EL-MSCs. In samples with embedded MSCs, flow did not cause a migration of cells from the interior of the modules towards the outer surfaces and the intermodular regions. EL-MSC-coated modules showed a greater number of cells in the intermodular regions and a higher number of total cells, as compared to uncoated modules and modules coated with HUVECs. Scale bar = 100 μm

were CD31- and SMA-positive, which indicates mature blood vessels with a smooth muscle cell layer. Surprisingly, we did not observe desmin-positive cells in the samples. The vessels present in the samples were also UEA-1 negative.

4. Discussion

Modular tissue engineering allows for the formation of 3D tissue constructs that can be scalable and have self-assembly abilities. Such systems can be used to improve the vascularization of implanted tissues by providing a route for host vessel ingrowth (McGuigan and Sefton, 2006). It can also be implemented as a system to guide angiogenesis in host ischaemic tissues by the supply of cells that will secrete pro-angiogenic factors.

Several *in vitro* and *in vivo* studies have shown the potential of the modular tissue-engineering concept in enhancing modular construct remodelling upon implantation (McGuigan and Sefton, 2008; Cooper and Sefton, 2011; Butler and Sefton, 2012), which enables faster tissue and vessel ingrowth. In this paper we describe modular tissue constructs with various cell types that were assembled and subjected to flow for 7 days to observe cell self-organization, with the focus on the performance of EL-MSCs. We examined whether these cells could be used in prevascularization strategies. Additionally, the aspect of dedifferentiation of EL-MSCs was analysed. As observed previously, cell coating influences the size of collagen modules, possibly by traction forces, and this effect is dependent on the cell type (Khan *et al.*, 2011; Leung and Sefton, 2007). Interestingly, covering the modules with EL-MSCs caused higher shrinkage than coating with HUVECs, which is most likely due to the faster spreading of EL-MSCs on the surface of the modules. Faster spreading resulted in an increased area subjected to the forces influencing the contraction of the modules (Ferrenq *et al.*, 1997). Cells embedded within the modules did not cause any change in the module volume, as the 24 h period was too short a time for the embedded MSCs to conduct significant matrix remodelling.

After 7 days of culture in the microfluidic chamber, the effect of flow on cell behaviour and phenotype was assessed. First of all, contrary to a previous study with MSCs isolated from rat bone marrow (Khan *et al.*, 2011), flow did not noticeably affect the differentiation of the embedded MSCs. We did not observe MSC migration towards the surface of the modules, or differentiation towards SMCs or endothelial cells. This was unexpected, as many previous reports have shown that growth factors and cytokines present in the medium, together with applied mechanical forces, can stimulate MSC differentiation (O'Cearbhaill *et al.*, 2008; Zhao *et al.*, 2007). A possible explanation is that in our system, MSCs were not directly exposed to shear forces. Also, the flow rate was lower than previously tested by Khan *et al.* (2011). Another observed difference was the absence of MSC migration from the

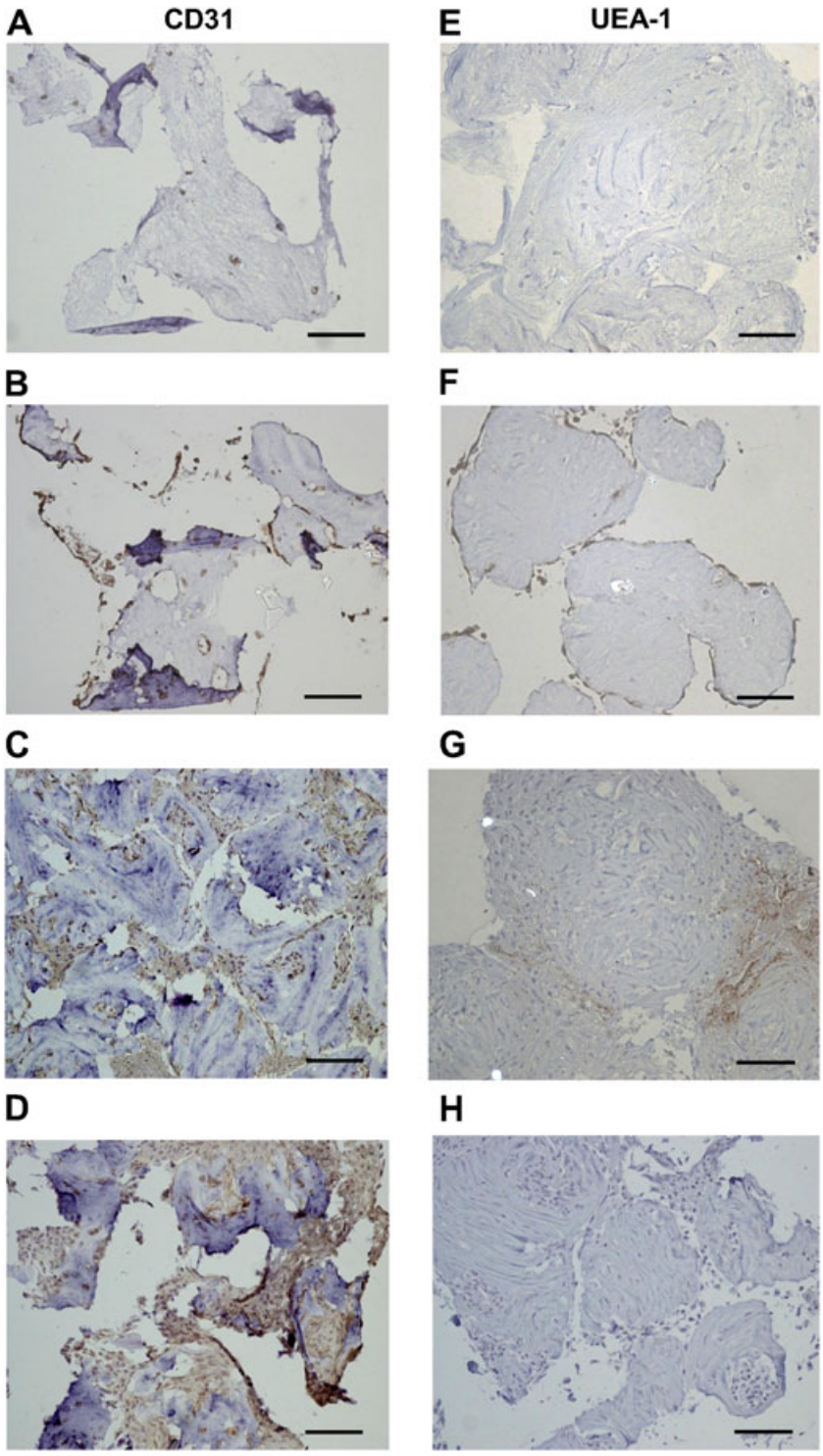

Figure 4. CD31 and UEA-1 staining. Staining was performed on samples subjected to flow conditions for 7 days. Cells were stained for CD31 (brown) and nuclei appeared blue. (A) Modules with embedded MSCs; (B) modules with embedded MSCs and surface-seeded HUVECs; (C) modules with surface-seeded EL-MSCs; (D) modules with embedded MSCs and surface-seeded EL-MSCs: MSCs did not stain positive for CD31. In samples containing HUVECs or EL-MSCs, CD31-positive cells can be observed. Cells were stained for UEA-1 (brown) and nuclei appeared blue. (E) Modules with embedded MSCs; (F) modules with embedded MSCs and surface-seeded HUVECs; (G) modules with surface-seeded EL-MSCs; (H) modules with embedded MSCs and surface-seeded EL-MSCs: MSCs did not stain positive for UEA-1. In samples containing HUVECs or EL-MSCs without embedded MSCs, UEA-1-positive cells can be observed. Scale bar = 100 μm

interior of individual modules towards the perimeter, and lack of SMA expression, indicating that MSCs did not differentiate towards a smooth muscle cell phenotype. MSCs embedded in the modules also did not show any expression of endothelial-specific markers. These results confirm our previous findings, where Matrigel stimulation was necessary for the induction of endothelial differentiation of MSCs (Janeczek Portalska *et al.*, 2012).

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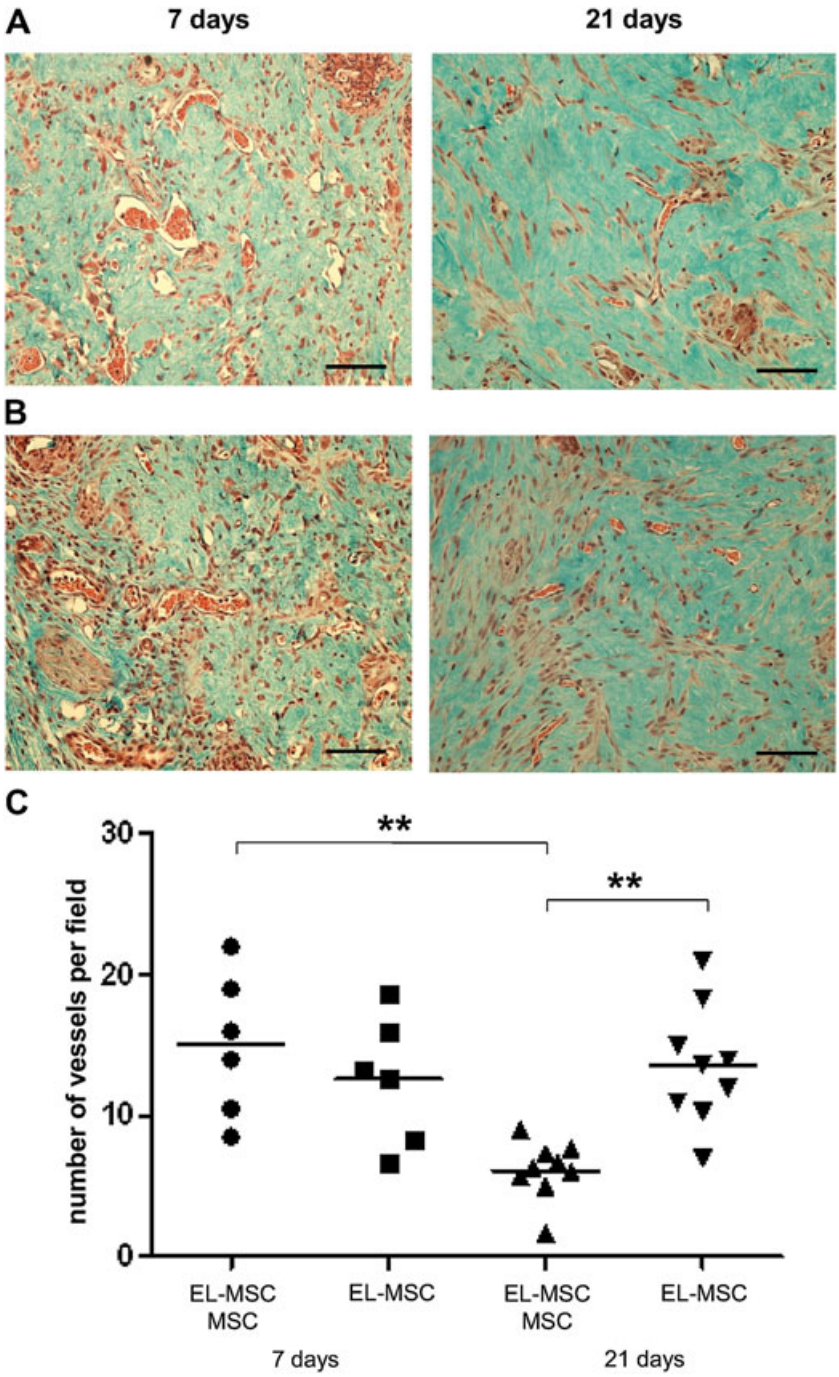

Figure 5. Blood vessel formation *in vivo*: (A) Masson's trichrome staining of modules with embedded MSCs and surface-seeded EL-MSCs and (B) modules with surface-seeded EL-MSCs after 7 and 21 days *in vivo*: collagen appears blue, erythrocytes are bright red and nuclei appear brown; the presence of erythrocytes indicates that vessels within the sample are functional; scale bar = 100 μm. (C) Quantitative analysis of vessels in polymeric constructs: 7 days after injection, modules with and without embedded MSCs were penetrated by similar amounts of vessels; 21 days after injection, the number of vessels within samples coated with EL-MSCs without embedded MSCs was the same as at day 7, but in samples with embedded MSCs a significant, two-fold decrease in vessel number was observed. Numbers of vessels/sample were quantified by three people blinded for the conditions. **Statistically significant ($p < 0.01$)

EL-MSCs that were subjected to flow conditions for 7 days increased in number in samples both with and without embedded MSCs. This was not observed with HUVEC-coated modules or in the study of Khan *et al.* (2011), which used rat aortic endothelial cells. It remains to be assessed whether this is due to the difference in the type of cells used, the differentiation protocol to which the EL-MSCs were exposed or whether the immortalized nature of the EL-MSCs plays a role. Proliferating EL-MSCs were expressing the endothelial marker CD31, which indicates that, even 7 days after Matrigel induction, these cells maintained their endothelial phenotype. This clearly shows that no dedifferentiation occurs under the conditions tested, and we speculate that EL-MSCs, upon implantation

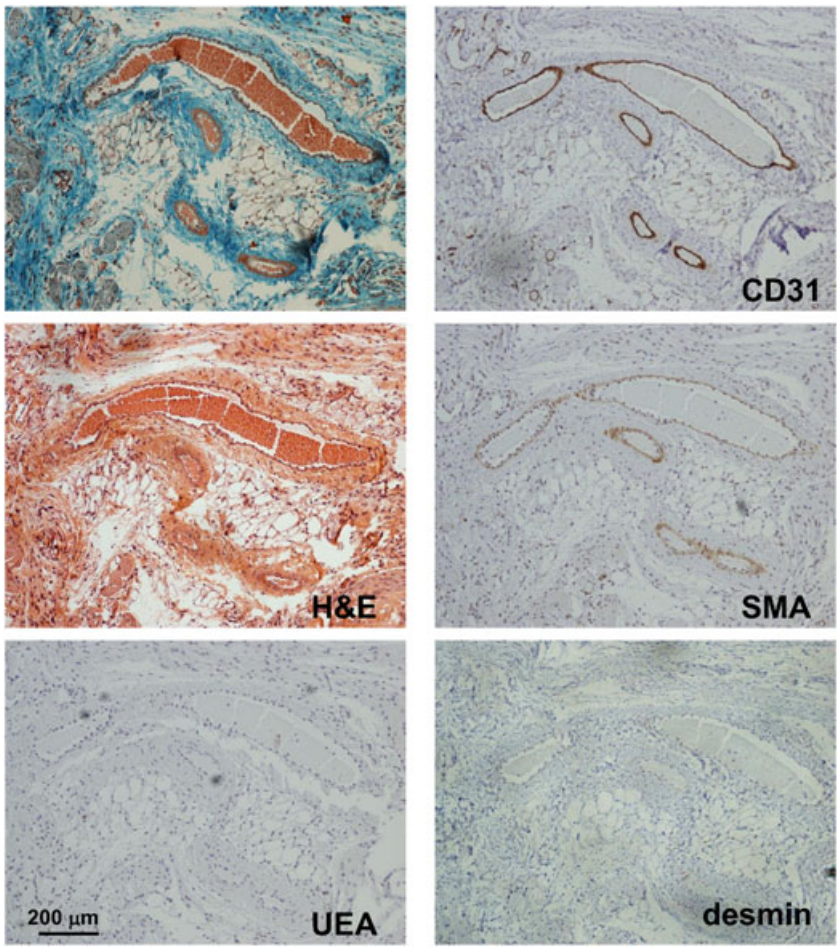

Figure 6. Immunohistochemistry of blood vessels. Modules with surface-seeded EL-MSCs were recovered after 21 days of *in vivo* culture and the following stainings were performed: Masson's trichrome, H&E, CD31, SMA, desmin and UEA-1. Observed vessels were CD31- and SMA-positive, which indicates mature blood vessels with a smooth muscle cell layer. Scale bar = 200 μm

in vivo, are able to maintain their endothelial phenotype in these conditions as well. Since one of the current challenges in applying MSCs in various therapies is to lock them in the desired differentiation stage (Dickhut *et al.*, 2009), our study provides a good example of a differentiation protocol that allows for obtaining a stable phenotype of MSCs. The lack of SMA and desmin expression indicates that EL-MSCs did not differentiate into pericytes.

Remodelling is a critical and still not fully understood parameter determining the fate of tissue-engineered constructs *in vivo*. Generally, implanted constructs are spontaneously invaded by the host vasculature as a response to hypoxia signals released by the cells within the construct (Rouwkema *et al.*, 2008b). This process, when it occurs spontaneously, is too slow to provide a proper level of oxygen and nutrients to allow cell survival in large grafts. In addition, for some patients, spontaneous vascularization will not progress well, due to the patient's age or other factors compromising normal angiogenesis, such as PVD (Franz *et al.*, 2009). Our *in vitro* results indicate that collagen modules covered with EL-MSCs can be valuable for various therapeutic applications. As shown in this *in vitro* study, EL-MSCs can proliferate on the surface of collagen modules. An increased number of cells in the place of implantation, which is hypoxic, will result in increased signals that are pro-angiogenic and, therefore, attract more vessel ingrowth. We also tested whether the presence of embedded MSCs is beneficial for EL-MSC performance. We used Masson's trichrome staining for this purpose, since this method has been considered best for visualizing functional vessels connected with host vasculature network (presence of erythrocytes). The addition of MSCs did not influence the number of vessels within the sample 7 days after implantation. The situation changed after 21 days, when significantly fewer vessels within the sample were observed. We hypothesize that this phenomenon is related to the normal healing process enhanced by the modulatory effect on the immune system by MSCs (Le Blanc, 2006). Inflammation caused by the presence of the engineered construct and by the injection itself can have pro-angiogenic effects, because proteases released by recruited inflammatory cells can release reactive ECM fragments and also activate ECM-bound pro-angiogenic growth factors (Mueller and Fusenig, 2004). Since bone marrow-derived MSCs can limit inflammation, their presence in the construct can result in a decreased density of vessels. This hypothesis can be supported by the reduced number of interstitial cells within construct with embedded MSCs. Such a situation is typical

for tissue healing, where vessel regression to the normal level is caused by apoptosis of endothelial and fibroblast cells. However, this assumption needs to be further investigated.

In conclusion, this paper presents an effective way of delivering EL-MSCs *in vivo* that can be used to improve tissue vascularization. The method is simple and can be used for several applications, including tissue-engineered scaffold vascularization and PVD treatment.

Acknowledgements

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Supporting information on the internet

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

Figure S1. SMA and desmin staining

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