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Endothelial Cell–Smooth Muscle Cell Co-Culture in a Perfusion Bioreactor System

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Abstract—Vascular endothelial cells (EC) are exposed to a complex biomechanical environment *in vivo* and are responsible for relaying important messages to the underlying tissue. EC and smooth muscle cells (SMC) communicate to regulate vascular development and function. In this work, a vascular perfusion bioreactor is used to grow tubular constructs seeded with EC and SMC under pulsatile shear stress in long-term co-culture to study the effects of EC on SMC function. SMC seeded into porous poly(glycolic acid) tubular scaffolds are cultured in the bioreactor for 25 days. Constructs are seeded with EC on day 10 or day 23 creating 2-day (short-term) or 15-day (long-term) EC and SMC co-cultures. Long-term EC–SMC co-culture significantly increases cell proliferation and downregulates collagen and proteoglycan deposition compared to short-term co-culture. After 25 days of culture, 15-day co-culture constructs have a more uniform cell distribution across the construct thickness and SMC express a more contractile phenotype compared to 2-day co-culture constructs. These data demonstrate strong interactions between SMC and EC in the bioreactor under physiologically relevant conditions. Thus, the vascular construct perfusion bioreactor is an important tool to investigate cell–cell and cell–extracellular matrix interactions in vascular cell biology and tissue engineering.

Keywords—Vascular grafts, Bioreactor, Tissue engineering, Endothelial cells, Smooth muscle cells, Co-culture, Arteries, Blood vessel substitute.

INTRODUCTION

Endothelial cells (EC) were once solely regarded as a blood vessel lining that forms a barrier to bulk blood flow into tissue. However, it is now known that EC play an important role in tissue homeostasis, coagulation and fibrinolysis, regulation of vascular tone, growth regulation of other cell types, and blood cell activation and migration during physiological and pathological processes.[10,25,27] EC–SMC (smooth muscle cell) interactions are critical and effect blood vessel development and function.[3] EC are in close proximity to and communicate with SMC via heterocellular junctions and signaling molecules.[9,19] Intermittent fenestrations in the internal elastic lamina of 0.5–1.5 μm in large vessels and 0.1–0.45 μm in capillaries allow direct contact between the two cell types.[26]

In vitro experiments incorporating EC–SMC co-culture demonstrate an effect of EC on SMC proliferation,[31] migration,[6] phenotypic expression,[15] and extracellular matrix (ECM) production.[20,21,24] In those studies, SMC and EC are typically plated on opposite sides of a porous membrane, such as polycarbonate, polyethylene terephthalate, or dialysis membrane, or are plated together in a tissue culture flask. Those experiments demonstrate that EC increase SMC expression of vascular endothelial cell growth factor (VEGF), platelet-derived growth factor (PDGF-AA and PDGF-BB), and transforming growth factor (TGF-β) genes, and decrease basic fibroblast growth factor (bFGF) gene expression compared to pure SMC cultures.[15] EC also significantly upregulate SMC proliferation[31] in a time-dependent manner.[12]

Studying EC and SMC structural and metabolic interactions is essential to understand vascular disorders such as atherosclerosis and intimal hyperplasia[29] and to successfully engineer artificial tissues. However, EC and SMC are not separated by a synthetic biomaterial *in vivo*, but rather by ECM proteins that are deposited by the cells. Furthermore, in many experimental systems developed to date,[13,18] SMC and EC are cultured under static conditions that neglect biomechanical stimulation, an important regulator of vascular cell behavior. Several previous studies have incorporated laminar flow conditions by exposing EC to steady shear stress. In those studies, EC are cultured either directly on SMC embedded in a collagen gel[16,34] or across a porous membrane seeded with SMC[7,8] and have focused on SMC effects on EC. Proliferation of sheared (10 dynes/cm^2) EC cultured on SMC-seeded collagen gels is significantly reduced compared to unsheared constructs.[16] These results indicate that activated mechanosensing pathways affect EC

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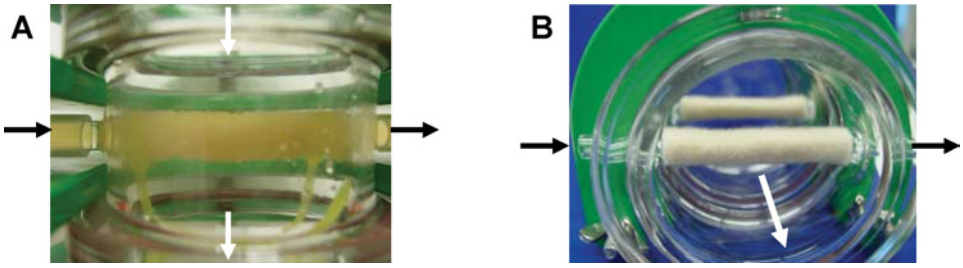

FIGURE 1. Flow configuration and vascular construct placement in the perfusion bioreactor. (A) Side view of construct module shows cell-seeded PGA scaffold mounted in the bioreactor. (B) Top view of opened two-module bioreactor without the head plates shows two unseeded scaffolds mounted in adjacent modules. Black arrows indicate lumen flow and white arrows show flow direction on the external surface of the constructs.

proliferation and suggest a level of SMC–EC communication. However, the co-culture model with slab geometry (instead of tubular) and laminar flow (instead of pulsatile) mimic the *in vivo* conditions only to a small extent.

To address these limitations, we have developed a vascular construct perfusion bioreactor that allows direct contact between SMC and EC while providing a biomechanical environment that simulates the *in vivo* hemodynamics.[32] In this bioreactor, tubular poly(glycolic acid) (PGA) nonwoven felts are seeded sequentially with SMC and EC under dynamic flow conditions. SMC populate the porous biomaterial and EC are seeded on the lumen surface allowing the two cell types to interact as they do in native tissues. The lumen is perfused with culture medium, and EC are exposed to pulsatile shear stress. Dynamic culture under pulsatile flow conditions promotes cell proliferation and matrix deposition leading to the development of tissue-engineered vascular constructs.[32]

The goal of the present work was to determine the effect of EC on SMC expression of differentiated function when the two cell types are co-cultured for different times in a physiologically relevant environment. This study evaluated the effect of EC–SMC co-culture time on SMC proliferation, ECM deposition, and phenotypic expression in tubular arterial constructs exposed to pulsatile shear stress. This paper demonstrates that the vascular construct perfusion bioreactor is a useful tool to study SMC–EC interactions under well-controlled conditions that mimic the architecture and biomechanical environment of native blood vessels.

MATERIALS AND METHODS

Unless otherwise stated, reagents and chemicals were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO).

Co-Culture Model

SMCs were isolated from the media layer of thoracic aortas of young calves and cultured in supplemented MCDB 131 (Mediatech Inc., Herndon, VA) medium as previously described.[32] Bovine aortic EC were generously provided by Dr. Robert M. Nerem (Georgia Institute of Technology, Atlanta, GA). EC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Mediatech) and 1% penicillin-streptomycin.

Biodegradable PGA nonwoven felts (97% porosity) obtained from Albany International (Mansfield, MA) were sutured into 4.5-mm inner diameter (ID) tubes using 6-0 PGA (Dexon) sutures (Davis and Geck Inc., Manati, Puerto Rico), sterilized, and prewetted in culture medium overnight to increase protein adsorption and hydrophilicity. PGA scaffolds (50-mm long) were mounted onto hollow posts that penetrate the bioreactor wall (Fig. 1). These posts were connected to a medium perfusion loop for delivery of cell suspensions to the construct lumen during seeding and to provide pulsatile flow during culture.[32] The unsupported length of the construct between the hollow posts was approximately 35 mm, and this section of the constructs was used for analysis. Culture medium or cell suspensions were also delivered to the external construct surface via additional ports to the module. All constructs were cultured for a total of 25 days.

Cell seeding occurred under dynamic flow conditions in the bioreactor. Constructs were seeded with SMC and cultured for 25 days under pulsatile flow conditions. The construct lumen was subsequently seeded with EC on day 10 or day 23 and experiments were completed on day 25 to create 15-day or 2-day EC–SMC co-culture constructs, respectively. Two different SMC seeding protocols were investigated in two-module bioreactors (Fig. 1) to enhance SMC distribution within polymeric constructs: lumen only and dual seeding. In lumen only seeding, 47×10^6 SMC were perfused reciprocally through the scaffold lumen at a low flow rate (4 ml/min) by a dual syringe pump (Harvard Apparatus Inc., Holliston, MA) for 24 h, followed by perfusion of another 47×10^6 SMC through the lumen under the same conditions for an additional 24 h. During these 48 h, the external surface of the scaffolds was supplied with culture medium by a peristaltic pump. In dual (lumen and external surface) seeding, 47×10^6 SMC were perfused through the lumen for 24 h (as described above), followed by perfusion of an additional 47×10^6 SMC on

the external surface of the scaffold for an additional 24 h. During cell seeding of the external scaffold surface, culture medium was perfused through the lumen by a peristaltic pump. During seeding in these two-module bioreactors, a separate syringe pump was used to deliver cell suspensions to each construct. After seeding, medium was delivered through the lumen and to the external surface of the scaffold at approximately 40 ml/min and at a pulse frequency of 1.5 Hz by a peristaltic pump.

Lumen only and dual-seeded constructs were endothelialized by perfusing 5×10^6 EC through the construct lumen using a reciprocating dual syringe pump at 4 ml/min 2 days prior to harvest (2-day co-culture) for 24 h as described above for SMC suspension. In a separate set of experiments with dual-seeded constructs, EC were seeded for 24 h after 10 days of culture (15-day co-culture) to quantify the effect of EC on SMC proliferation and matrix production.

Constructs were cultured under sterile conditions in a 37°C, 95% air/5% CO_2 cell culture incubator. During bioreactor operation, 1 L of medium was replaced in the reservoir every 5 days in a sterile flow hood, and the bioreactor was reperfused with fresh medium. Culture medium consisted of MCDB 131 (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Mediatech), 1% penicillin–streptomycin, 0.5 μg/ml fungizone (Gibco, Grand Island, NY), 25 mM HEPES buffer, 10 ng/ml human epidermal growth factor (EGF), 2 ng/ml bFGF (PeproTech, Inc., Rocky Hill, NJ), 160 μg/ml L-ascorbic acid, 1% of 200 mM L-glutamine, 92 μg/ml L-proline, 60 μg/ml glycine, 71 μg/ml L-alanine, and 2.9 ng/ml cupric sulfate. Freshly prepared ascorbic acid was added to the bioreactor medium daily and externally added growth factors (i.e. bFGF and EGF) were removed from the medium after 10 days of culture.

DNA, Collagen, and GAG Content

After 25 days of culture, constructs were harvested from the bioreactor and sectioned into rings 5 mm in length. One to three construct rings were rinsed in phosphate-buffered saline solution, frozen at −20°C, and lyophilized, and construct dry weight was measured. ECM was digested by overnight incubation with proteinase K at 55°C.[17] Cell number was quantified by DNA measurement using Hoechst 33258 dye in a microplate spectrofluorometer (Spectra Max Gemini Dual-Scanning Microplate Spectrofluorometer, Molecular Devices Corp., Sunnyvale, CA). Cell number of co-culture constructs included both SMC and EC. Total collagen content was assessed by hydroxyproline measurement after acid hydrolysis in 6 N HCl for 3 h at 120°C and reaction with chloramine-T and *p*-dimethylaminobenzaldehyde.[33] Sulfated glycosaminoglycan content was measured using the dimethylmethylene blue (DMMB) spectrophotometric assay.[11]

Histological and Immunohistochemical Analyses

One to three construct rings from each module were fixed in 10% formalin, paraffin embedded, cut in 5-μm thick cross-sections, and stained with hematoxylin and eosin (H&E) for cells and Masson's Trichrome for collagen. Elastin, smooth muscle α-actin, calponin, and myosin heavy chain were visualized through immunohistochemical analysis by antibody binding (protocol provided by Vector Laboratories Inc., Burlingame, CA). Sections were deparaffinized and immunostained with mouse anti-elastin (1:5000), mouse smooth muscle α-actin (1:800), mouse calponin (1:50), and myosin heavy chain (1:50), respectively, as previously described.[32]

Scanning Electron Microscopy

One construct ring from each module was fixed in 4% gluteraldehyde overnight, dehydrated in a graded series of ethanol/water solutions (25–100% ethanol), dried with hexamethyldisilazane (Electron Microscopy Sciences, Ft. Washington, PA), and sputter coated with gold. A scanning electron microscope (Hitachi S800 FEG scanning electron microscope, Hitachi Ltd., Tokyo, Japan) operated at 15 kV was used to image samples.

Experimental Design and Statistical Analysis

All experiments were run in triplicate and performed in two-module bioreactors with one construct per module. One to three 5-mm long construct rings were used for the biochemical analysis of each construct. In this study, N indicates the number of independent experiments performed, and n equals the number of constructs per data point. For example, lumen only and dual seeding bioreactor experiments were run in triplicate with two constructs in each bioreactor. Therefore, for these experiments $N = 3$ and $n = 2$. For each data set representing 3 or more independent experiments, values are presented as mean ± standard deviation. Comparisons were made using the Student's t test and one factor ANOVA.[14] Differences in the content of the constructs were considered significant when $p < 0.05$. The p values of all comparisons are noted in Table 1.

RESULTS

Prior to long-term SMC–EC co-culture studies, SMC lumen only and dual seeding protocols were evaluated for the development of a media layer tissue analog. In both cases, SMC-seeded constructs were seeded with EC on day 23 and harvested on day 25, generating 2-day co-culture constructs. In all experiments, 5-mm long construct rings were used for biochemical analysis. After 25 days of culture, dual seeding of SMC through the lumen and on the external surface of the scaffolds resulted in arterial

TABLE 1. Summary of SMC–EC co-culture construct biochemical analysis.

	2-day co-culture constructs	15-day co-culture constructs	Difference (%)	*p* value
SMC seeding number[a]	1.6×10^6	1.6×10^6	n/a	n/a
EC seeding number	0.7×10^6	0.7×10^6	n/a	n/a
Construct dry weight at harvest (g)	0.021 ± 0.0099	0.026 ± 0.0036	+27	0.087
Cell number at harvest	$(16 \pm 9.2) \times 10^6$	$(27 \pm 3.6) \times 10^6$	+68	0.001
Cell number at harvest per g dry weight	$(780 \pm 120) \times 10^6$	$(1100 \pm 44) \times 10^6$	+32	0.107
Collagen (μg)	740 ± 360	640 ± 250	−13	0.477
Collagen per dry weight (%)	3.6 ± 0.81	2.4 ± 0.33	−32	0.049
Collagen per cell (pg)	0.046 ± 0.011	0.024 ± 0.0032	−48	0.049
Glycosaminoglycans (GAG) (mg)	9.2 ± 4.1	9.6 ± 3.6	+5	0.804
GAG per dry weight (%)	44 ± 10	37 ± 4.2	−17	0.804
GAG per cell (pg)	0.57 ± 0.13	0.36 ± 0.043	−38	0.028

Note. All constructs are seeded with SMC through the lumen and on the external surface (dual seeding protocol). Short-term co-culture constructs are endothelialized on day 23 (2-day co-culture) and long-term co-culture constructs are endothelialized on day 10 (15-day co-culture). All constructs are harvested on day 25. Construct rings 5-mm long are sectioned from the 35-mm long constructs and used for analysis. Cell seeding numbers are also reported for 5-mm long construct rings for comparison. Data are mean ± standard deviation for $N = 3$ independent experiments with $n = 2$ constructs per experiment.

[a]Construct SMC seeding number is based on 12% seeding efficiency measured after 2 days of SMC perfusion.

constructs with increased cell number, collagen, and GAG as percentages of construct dry weight as compared to constructs seeded through the lumen only (Fig. 2). Construct dry weights were 0.025 ± 0.0078 g and 0.021 ± 0.0099 g in lumen only and dual-seeded constructs, respectively. Higher cell density was observed close to the free surfaces that were in direct contact with culture medium compared to regions inside the vessel wall as shown previously.[32] Collagen and GAG deposited per cell increased from 0.03 ± 0.01 pg to 0.05 ± 0.01 pg and from 0.46 ± 0.08 to 0.57 ± 0.13 in dual-seeded compared to lumen only seeded constructs. Collagen and GAG content also increased from $590 \pm 380\,\mu$g to $740 \pm 360\,\mu$g and from 8.5 ± 2.7 mg to 9.2 ± 4.1 mg, respectively, in dual-seeded compared to lumen only seeded constructs. Amorphous elastin was deposited in a similar fashion in lumen- and dual-seeded arterial substitutes (data not shown). SMC stained positively for smooth muscle α-actin, calponin, and myosin heavy chain (Fig. 3) indicating a highly differentiated cell phenotype.

Cell seeding efficiency was quantified for both lumen only and dual SMC seeding protocols. In these experiments, scaffolds were seeded with SMC and harvested after 2 days (at the end of the seeding phase). Cell counts were made in the culture medium and in the constructs. On the basis of cell numbers in the constructs, cell-seeding efficiency was 13% in lumen only seeding and 12% in dual seeding.

In vivo, SMC deposit large amounts of ECM proteins such as collagen, elastin, and GAG. Since dual seeding of SMC through the lumen and external surface resulted in higher ECM deposition than lumen only seeding, the dual seeding protocol was used in subsequent experiments that compared the effect of SMC–EC co-culture time on construct development. In these experiments, EC were seeded directly on the construct lumen 10 days or 23 days after SMC seeding and harvested on day 25 to compare 15-day and 2-day SMC–EC co-cultures, respectively. Fifteen-day co-culture constructs had significantly higher cell number on day 25 compared to 2-day co-culture constructs (Fig. 4). Cell number per gram of dry weight was $(1100 \pm 44) \times 10^6$ and $(780 \pm 120) \times 10^6$ in 15-day and 2-day co-culture arterial constructs. Collagen content ($640 \pm 250\,\mu$g vs. $740 \pm 360\,\mu$g) was lower and collagen as percentage of dry weight (Fig. 4) and collagen deposited per cell (0.024 ± 0.0032 pg vs. 0.046 ± 0.011 pg) were significantly lower in the 15-day compared to the 2-day co-culture constructs. Proteoglycans deposited per cell were significantly lower in 15-day than in 2-day co-culture constructs (0.36 ± 0.043 pg vs. 0.57 ± 0.13 pg). However, GAG content (9.6 ± 3.6 mg vs. 9.2 ± 4.1 mg) and GAG as a percentage of the construct dry weight (Fig. 4) were similar in 15-day and 2-day co-culture constructs. The biochemical analysis data of short- and long-term co-culture constructs are summarized in Table 1. SMC seeding number was estimated from the initial cell seeding number by assuming 12% cell seeding efficiency. EC seeding number is the initial cell seeding number. Elastin immunostaining was sparse and comparable for the two co-culture methods (data not shown).

Cell distribution across the cross-section was more uniform in 15-day compared to 2-day co-culture constructs (Fig. 3). Two-day co-culture constructs had higher cell densities near the construct lumen and external surfaces, whereas 15-day co-culture constructs had a more uniform cell distribution across the construct wall. SMC in 15-day co-culture constructs expressed smooth muscle α-actin and calponin and stained more intensely for myosin heavy chain than 2-day co-culture constructs (Fig. 3). Therefore, the presence of EC in direct contact with SMC for 15 days compared to 2 days had a pronounced effect not only on

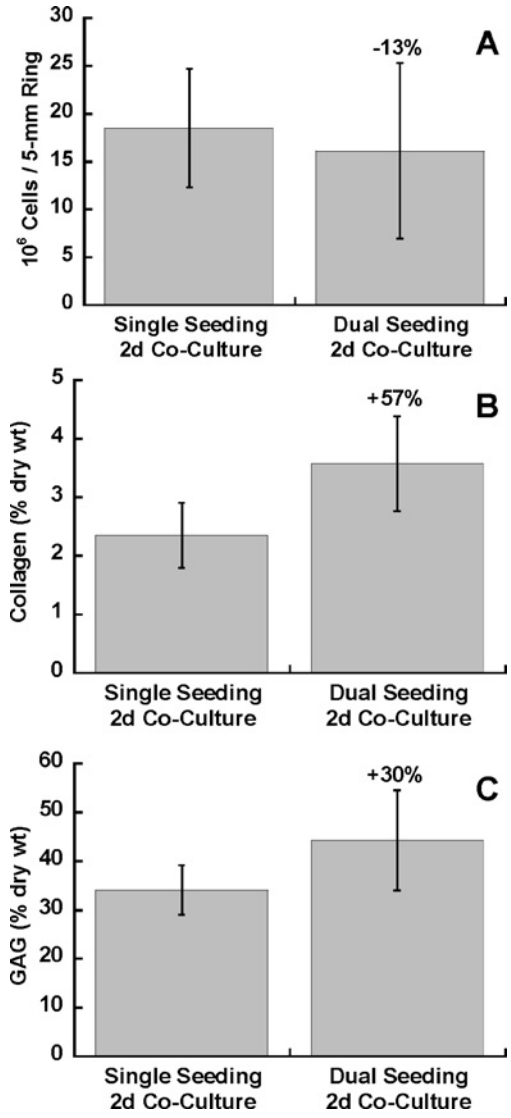

FIGURE 2. Construct composition for lumen and dual SMC seeding protocols. Biochemical analysis of 25-day constructs seeded with SMC through the lumen only (lumen seeding) or through both the lumen and the external surface (dual seeding). All constructs are seeded with EC on day 23 and harvested on day 25 (2-day co-culture). Construct rings 5-mm long are sectioned from the 35-mm long constructs and used for analysis. Data are mean ± standard deviation for $N = 3$ independent experiments with $n = 2$ constructs per experiment ($n = 1$ in one lumen only SMC seeding experiment). (A) Cell number, (B) total collagen as percentage of construct dry weight, and (C) GAG as percentage of construct dry weight. Values of percent change in construct cell and matrix content with dual seeding compared to single seeding are shown above the bars.

cell proliferation and ECM deposition but also on cell distribution and differentiation.

Scanning electron microscopy demonstrated that constructs were populated with cells that deposited ECM proteins and filled the space between the PGA fibers (Fig. 5). A confluent cell monolayer was present on the lumen surface of both 2- and 15-day co-culture constructs. Notably, 15-day co-culture constructs contained elongated cells aligned in the flow direction consistent with *in vivo* vascular EC morphology (Fig. 5). PGA fibers were occasionally observed on the lumen surface of 2-day but not in 15-day co-culture constructs.

An important characteristic of bioreactors for tissue engineering is the ability to develop multiple constructs with similar composition at the end of *in vitro* culture. To address this point, two constructs were cultured in adjacent modules of the bioreactor in each experiment to quantify inter- and intra-construct variability. Inter-construct (construct to construct) analysis addresses variability between constructs cultured in different modules, whereas intra-construct analysis evaluates composition across the construct length (middle vs. edge). Figure 6 compares cell number, collagen, and GAG content in the middle section of constructs harvested from adjacent modules of the same bioreactor for 15-day co-culture constructs. There were no statistically significant differences between the two constructs in either case. Similarly, no statistically significant difference was found in cell number, collagen, and GAG content between the middle and edge sections of the constructs for 2-day and 15-day co-culture constructs (data not shown). Together, these data demonstrate the reproducibility of multiple module experiments for the production of several vascular constructs simultaneously.

DISCUSSION

In vivo, each cell type of the vascular wall is "dependent" on its neighboring cells, and all act synergistically toward the development, maintenance, remodeling, and regulation of the tissue under physiological and pathological conditions. EC affect SMC proliferation, migration, differentiation, and ECM production.[25,27] Most studies to date investigate EC–SMC interactions under 2D co-culture conditions. In this work, the two cell types were seeded in a 3D tubular scaffold and exposed to pulsatile shear stress to create a more physiologically relevant co-culture environment.

The vascular construct perfusion bioreactor was used to compare the effect of short-term EC and SMC co-culture (2 days) versus long-term co-culture (15 days) on cell proliferation, ECM production, and expression of differentiated function after 25 days of construct growth. Construct cell number was significantly increased in 15-day compared to 2-day co-culture constructs. Although 15-day co-culture constructs generally contained less ECM than 2-day co-culture constructs, 15-day co-culture constructs exhibited more uniform cell and ECM protein distribution across the construct thickness. Most importantly, SMC in 15-day co-culture constructs were in a more differentiated state, and EC in the construct lumen were more strongly aligned in the fluid flow direction compared to 2-day co-culture constructs. Collectively, these results demonstrate that EC have

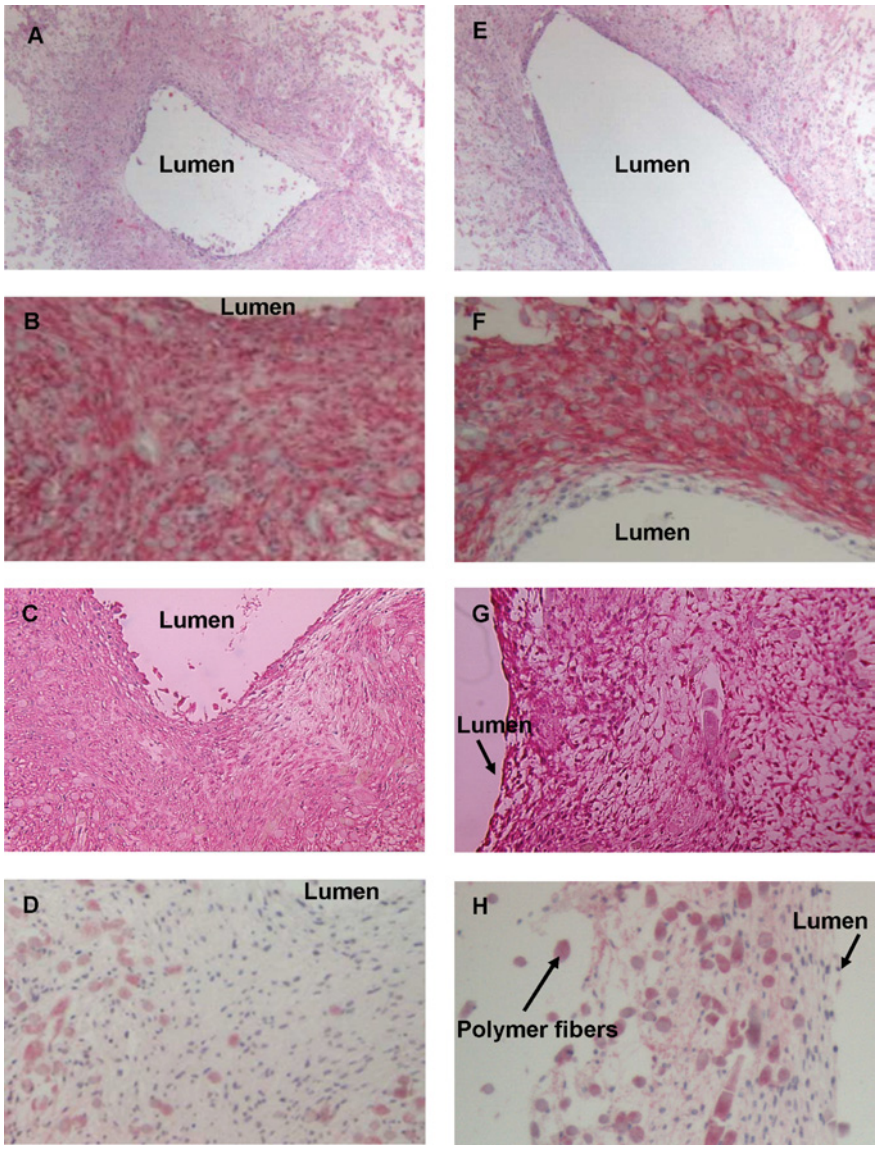

FIGURE 3. Cell distribution and SMC differentiated expression in 2-day (A-D) and 15-day (E-H) co-culture constructs. SMC and EC distribution (hematoxylin and eosin staining) in (A) 2-day and (E) 15-day co-culture constructs. Higher cell density is observed near the lumen surface in (A), whereas cells are more uniformly distributed across the construct wall in (E). (B and F) Smooth muscle α-actin, (C and G) calponin, and (D and H) myosin heavy chain expressions (red) are robust near the lumen surface in dual-seeded (2-day and 15-day) co-culture constructs indicating SMC expression of differentiated function. Original objective magnification: 10×.

a significant effect on SMC proliferation and ECM deposition and that longer term SMC–EC co-culture enhances the development of differentiated 3D arterial constructs.

SMC proliferation depends on the EC proliferative state, and EC can either promote or inhibit SMC proliferation.[5,6] Synthetic SMC in the presence of proliferating endothelium have an increased proliferation rate, whereas confluent, quiescent endothelium inhibits SMC proliferation. The effect of EC on SMC proliferation may depend upon cell separation distance[12,23,30,31] or other culture conditions[2] when membranes are used to separate the two cell types. The vascular construct perfusion bioreactor allows the characterization of EC effects on SMC proliferation and matrix deposition for cells cultured in a spatially correct orientation in 3D tissue constructs. The overall increase in construct cellularity in 15-day co-culture constructs suggests that EC enhance SMC proliferation in bioreactor culture. Although SMC proliferation could not be distinguished from EC proliferation in our co-culture constructs, there is evidence that EC migrate to the construct lumen and form a monolayer when they are seeded on scaffolds as a mixed cell population with other cell types.[28] In our experiments, EC were seeded on the lumen surface only and since they are contact-inhibited cells, the large increase in

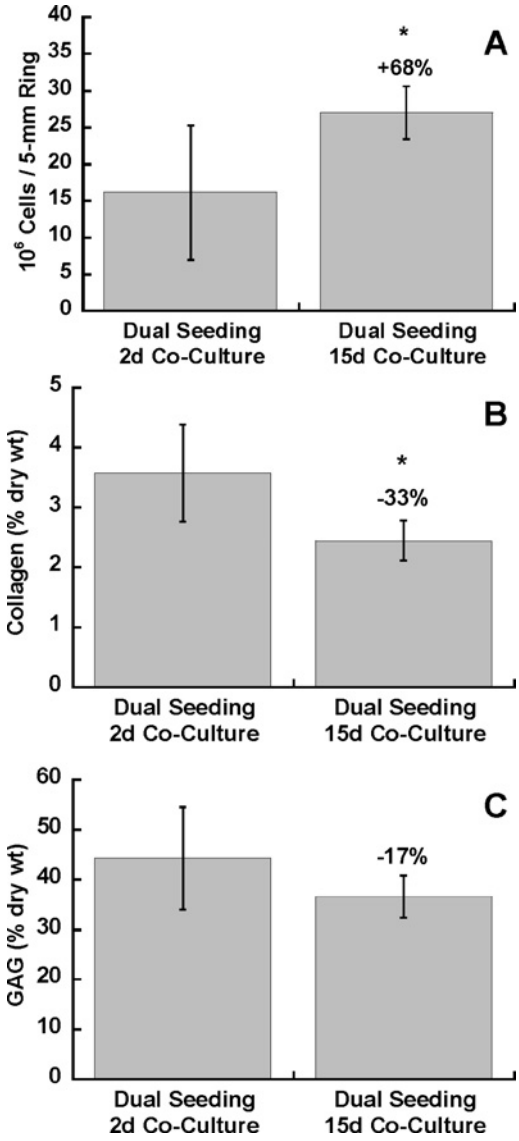

FIGURE 4. Construct composition for short- and long-term SMC–EC co-culture. All constructs are seeded with SMC through the lumen and on the external surface (dual seeding protocol). Short-term co-culture constructs are endothelialized on day 23 (2-day co-culture), and long-term co-culture constructs are endothelialized on day 10 (15-day co-culture). All constructs are harvested on day 25. Construct rings 5-mm long are sectioned from the 35-mm long constructs and used for analysis. Data are mean ± standard deviation for $N = 3$ independent experiments with $n = 2$ constructs per experiment. (A) Cell number, (B) total collagen as percentage of construct dry weight, and (C) GAG as percentage of construct dry weight ($^*p < 0.05$).

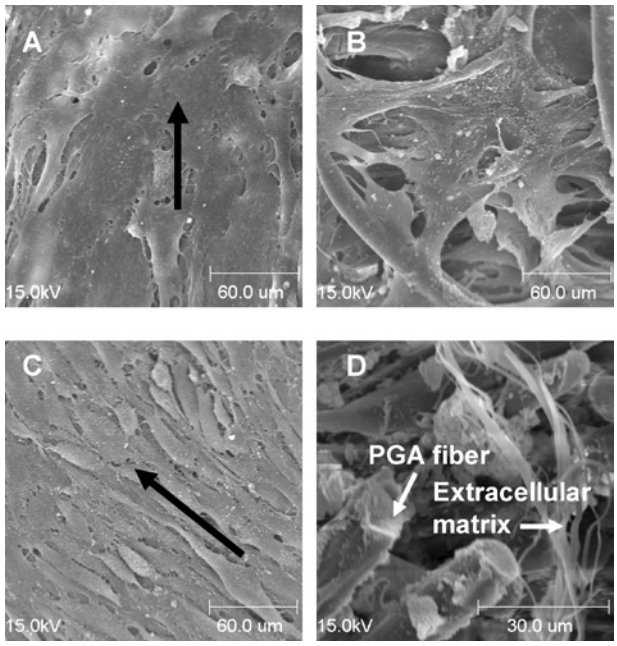

FIGURE 5. Construct ultrastructure. Scanning electron microscopy images show ultrastructure of (A and B) 2-day and (C and D) 15-day co-culture constructs. Lumen surface was confluent in both (A) short and (C) long-term co-culture constructs, and ECM proteins surrounded the PGA fibers on (B) the external surface and in (D) the construct wall. Black arrows indicate fluid flow direction.

cell number that we observed is most likely due to SMC proliferation.

SMC deposition of collagen and GAG in the arterial constructs is also affected by the early presence of EC. Long-term co-culture constructs contained significantly less collagen and GAG per cell compared to short-term co-culture constructs. Additionally, collagen per dry weight is significantly lower in 15-day than in 2-day co-culture constructs. These results are consistent with another study showing lower collagen synthesis and collagen type I expression in an EC–SMC co-culture model compared to SMC single cultures.[24]

A hypothesis for lowered ECM deposition in the long-term co-culture constructs could be linked to SMC differentiation. Indeed, decreased matrix production could be associated with a switch in SMC phenotypic expression toward a more contractile phenotype that is characterized by decreased ECM production compared to a more synthetic phenotype.[1,4] Even though smooth muscle α-actin and calponin expression were similar in both short- and long-term co-culture constructs, myosin heavy chain expression was more intense in 15-day compared to 2-day co-culture constructs (Fig. 3), suggesting that SMC are in a more contractile phenotype in 15-day compared to 2-day co-culture constructs. This reduction in SMC matrix deposition concomitant with increasing SMC phenotypic expression of differentiated function in long-term co-culture suggests a role for EC–SMC co-culture time as a process variable for vascular construct development.

Cell distribution across the construct wall varied significantly between the two EC seeding protocols. Short-term co-culture constructs were characterized by higher cell density close to the lumen and the external surface,

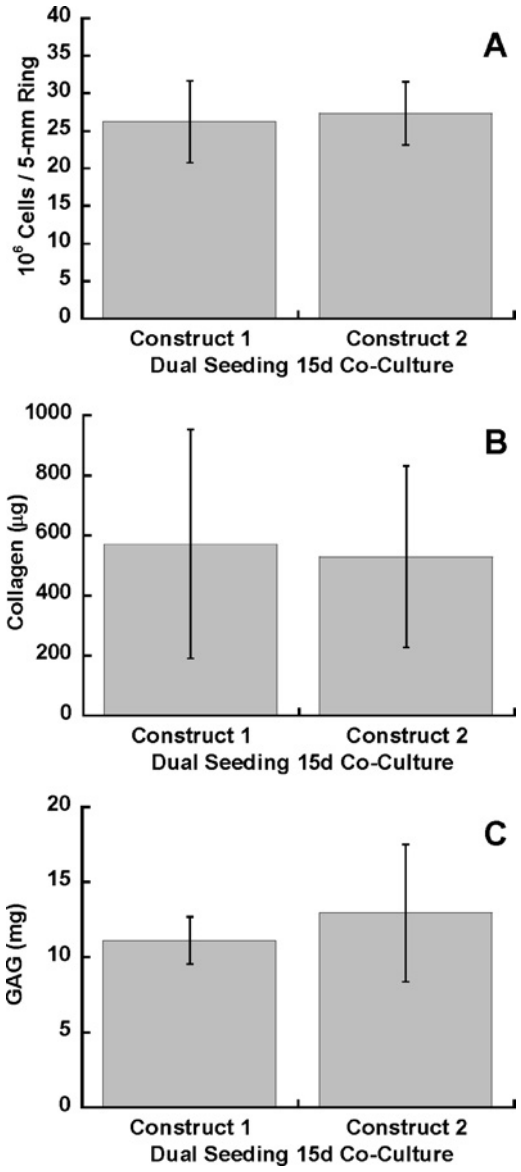

FIGURE 6. Assessment of inter-construct variability in bioreactor culture. Biochemical analysis of constructs seeded with SMC through both the lumen and the external surface (dual seeding), endothelialized on day 10, and harvested on day 25 (15-day co-culture). Construct rings 5-mm long are sectioned from the 35-mm long constructs and used for analysis. Data are mean ± standard deviation for $N = 3$ independent experiments with $n = 1$ construct per experiment. Comparison of inter-construct (construct to construct) composition in terms of (A) cell number, (B) collagen, and (C) GAG content shows no significant variability between constructs cultured in adjacent modules of the bioreactor ($p > 0.05$).

whereas long-term co-culture constructs had a more uniform cell distribution across the wall. This uniform cell distribution could be due to EC forming a monolayer on the lumen surface, thereby preventing excessive SMC proliferation in the lumen. Alternatively, EC could increase SMC migration, leading to a more uniform cell distribution. Although native vessels are highly anisotropic with nonuniform cell and ECM patterns, tissue-engineered arterial constructs most likely require uniform cell and ECM distribution to create patent tissue that can withstand *in vivo* pressures. This study shows that longer term SMC–EC co-culture can enhance construct development by distributing cells uniformly across the wall thickness.

The data presented here provide insight into SMC–EC interactions when the two cell types are seeded in a PGA scaffold and co-cultured in a vascular construct bioreactor. Long-term EC and SMC co-culture results in significant changes in SMC proliferation, collagen, and GAG deposition per cell, and cell distribution and differentiation. Dynamic cell seeding in one bioreactor allows for the introduction of different cell types at different times in culture with few handling steps and without compromising sterility. These important bioreactor features can be used for the seeding of scaffolds with different cell types toward the development of tissues with complex architecture. In vascular tissue engineering, tubular arterial constructs cultured *in vitro* are typically seeded with EC shortly before harvesting so that EC and SMC are co-cultured for short times.[18,22] This study demonstrates that co-culturing SMC and EC for 15 days results in uniform cell distribution across the construct thickness and enhances SMC expression of contractile phenotype. Key findings of this work allow for precise control of arterial construct development *in vitro* by selection of EC–SMC co-culture conditions. If the main objective is significant SMC proliferation and ECM deposition, EC need to be introduced at later culture times. However, if SMC differentiated function and uniform distribution is critical, EC should be co-cultured with the SMC for longer times. Therefore, EC–SMC co-culture time is an important process variable for vascular graft tissue engineering.

In summary, EC–SMC co-culture studies in a perfusion bioreactor revealed strong interactions between EC and SMC and showed significant upregulation of cell proliferation, more uniform cell distribution, more contractile SMC phenotype, and downregulation of ECM deposition in long-term compared to short-term co-culture constructs. The bioreactor incorporated pulsatile shear stress, tubular construct geometry, and allowed the long-term study of a physiologically relevant co-culture model. Therefore, the vascular construct perfusion bioreactor is an important tool for the investigation of cell–cell and cell–ECM interactions in vascular cell biology and tissue engineering.

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