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Impact of Endothelial Cells on 3D Cultured Smooth Muscle Cells in a Biomimetic Hydrogel

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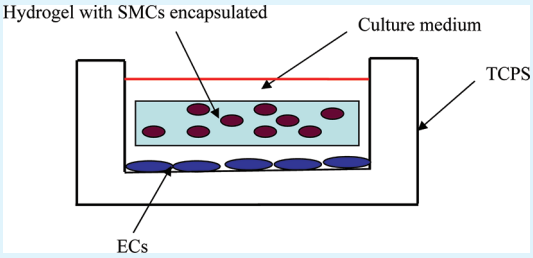
ABSTRACT: For the development of vascular tissue engineering, the impact of endothelial cells (ECs) on smooth muscle cell (SMC) spreading, proliferation, and differentiation is explored in the current study using a coculture model. In this coculture model, SMCs were encapsulated in a biomimetic hydrogel based on methacrylated dextran-graft-lysine (Dex-MA-LA) and methacrylamide-modified gelatin (Gel-MA), and exposed to a monolayer of ECs. With EC coculture, SMC proliferation in 3D hydrogel was promoted at initial period, and the formation of denser cellular networks was enhanced. ECs dynamically modulated SMC phenotype by promoting a more contractile SMC phenotype initially (on day 2), indicated by the upregulated expression of contractile genes α-actin, calponin, smooth muscle-myosin heavy chain (SM-MHC), and smoothelin; however, the onset of maximum expressions was delayed by ECs. Full differentiation of SMCs was not obtained even with EC coculture. Higher level of platelet-derived growth factor (PDGF)-BB and latent transforming growth factor (TGF)-β1 were detected in medium of coculture. These biochemical cues together with the physical cue of tensional force within cellular networks may be responsible for the dynamic modulation of SMC phenotype in coculture. Synthesis of elastin was promoted by ECs at transcriptional level. The formation of denser cellular networks and synthesis of elastin suggest that coculture with ECs is a potential method to construct functional vessel media layer in vitro.



KEYWORDS: *3D coculture, hydrogels, endothelial cell, smooth muscle cell, phenotype, growth factors*

INTRODUCTION

Tissue engineering of biologically functional grafts for replacement of small-caliber (<6 mm) vessels is urgently required, since autologous tissues are limited in supply and existing synthetic materials are unsuitable.[1] In blood vessels, smooth muscle cells (SMCs) in the media layer are important cellular components. They mediate vasoactivity by cellular contraction and relaxation, and regulate the balance of extracellular matrix (ECM) secretion and degradation, all of which are important in vascular development, maintenance, and remodeling.[2,3] Because of the key role of SMCs in vessel physiology, controlling the spreading, proliferation and phenotype of SMCs is paramount in tissue engineering of small-caliber blood vessel substitute.

Endothelial cells (ECs), which form a monolayer on the luminal surface of blood vessels---the intima, have marked impacts on SMC behaviors. The EC-produced vasoactive molecules, including endothelin, platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, prostacyclin etc., influence SMC behaviors such as spreading, proliferation, migration, differentiation, and secretion of ECM proteins.[4−9] Communication between SMCs and ECs through direct physical contact or synthesis and diffusion of specific mediators regulates the behaviors of both ECs and SMCs[4,5,10−12] and the structural and functional maintenance of vessels is dependent on such communications.[13−15] For the development of tissue engineered blood vessels, there is a need to study the interactions between ECs and SMCs.

Much work has been done to determine the regulatory effects of ECs on SMCs and vice versa. These investigations were performed in various ways: direct coculture in which ECs were seeded directly on top of SMCs,[4] bilayer coculture in which ECs and SMCs were seeded on opposite sides of a porous membrane[5−7] and SMC or EC culture using EC- or SMC-conditioned medium.[5] Although these coculture methods have resulted in many important findings regarding EC-SMC interactions, they mostly experimented 2D cultures, whereas the behaviors of the cells in 3D cultures could resemble the in vivo condition more closely.

Several coculture models using 3D cultured SMCs have also been investigated thus far.[9,16−19] A coculture model with SMCs sandwiched between two layers of collagen gel and ECs seeded on the top surface has been used to explore the effects of SMCs on ECs.[16] Although approximating the vessel wall architecture, this coculture model has the limitation that SMCs were essentially presented in a monolayer. Bilayered poly(ethylene glycol) diacrylate (PEGDA) hydrogel constructs composed of SMCs and ECs encapsulated in two different layers were developed as a 3D coculture model.[9] However, the spherical

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morphology of SMCs within PEGDA hydrogels and the 3D encapsulated rather than the 2D monolayer arrangement of ECs are limitations of the system in mimicking the native condition. In another coculture model, SMCs were embedded in collagen gel, and ECs were cultured directly on the surface of the cell-laden gel.[17−19] ECs did not show significant proliferation on collagen gel, not to mention the formation of monolayer.[19] Also the growth of SMCs was suppressed in 3D collagen gels compared to 2D culture.[20]

We have reported a biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin which promoted 3D SMC spreading and proliferation.[21] The excellent SMC proliferation in this hydrogel offers the possibility to study the effect of ECs on 3D-cultured SMCs. In the present work, we reported an EC/SMC coculture system using SMCs encapsulated inside the dextran- and gelatin-based hydrogel. Our initial results showed that it was difficult to obtain an integrated monolayer of ECs on the surface of hydrogels, and during longer time cultures, the ECs detached from the surface, which may be due to hydrogel compaction. So in this coculture system, ECs were cultured on surface of tissue culture polystyrene (TCPS) and allowed to form a subconfluent monolayer prior to coculture with SMCs, which were encapsulated in hydrogel (Figure 1).

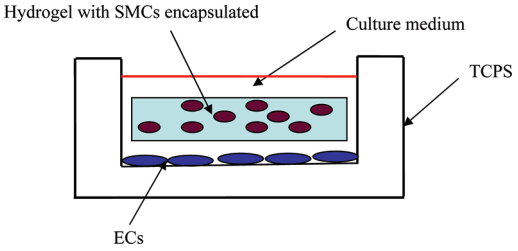

Figure 1. EC/SMC coculture model. SMCs were encapsulated in hydrogels and exposed to a monolayer of ECs.

Hydrogels with encapsulated SMCs and cultured in the presence of ECs are denoted "EC$^+$ constructs". Cell/hydrogel constructs were also cultured without the EC monolayer as the control group; these are referred to hereafter as "EC$^-$ constructs". Using this coculture model, the effect of ECs on SMC spreading and proliferation in a 3D environment was investigated. The transcriptional expressions of SMC-specific smooth muscle (SM) α-actin, calponin, smooth muscle-myosin heavy chain (SM-MHC), smoothelin, and nonmuscle-myosin heavy chain B (SMemb) were measured in both EC$^+$ and EC$^-$ constructs to investigate the impact of ECs on SMC differentiation. The ECM production of SMCs was also measured at transcriptional level.

EXPERIMENTAL SECTION

Hydrogel Preparation. Methacrylated dextran-graft-lysine (Dex-MA-LA) and methacrylamide-modified gelatin (Gel-MA) were synthesized as described in the previous paper.[21] Hydrogel precursor solution used for SMC encapsulation was prepared with 80 mg/mL of Dex-MA(9)-Ly (with 9 methacrylate groups per 100 glucopyranose residues for dextran), 40 mg/mL of Gel-MA(51) (with 51% of ε-amino groups been modified for gelatin), and 0.1 wt % of Irgacure 2959 (purchased from Ciba). This hydrogel was donated as L-G-2A hydrogel.

Cell Culture. Human umbilical artery endothelial cells (HUAECs, cryopreserved) and human umbilical artery smooth muscle cells (HUASMCs, cryopreserved) were purchased from commercial sources (Lonza), and were thawed and expanded at 37 °C in a humidified atmosphere containing 5% CO_2. During expansion, HUAECs and HUASMCs were cultured in EGM MV Microvascular Endothelial Cell Growth Medium Bulletkit (Lonza) and SmGM Smooth Muscle Growth Medium-2 Bulletkit (Lonza), respectively. Cells were passaged before reaching confluency by trypsinization using 0.05% (w/v) Trypsin-0.02% (w/v) EDTA solution. Cells within passage 7 were used in coculture study.

Coculture of ECs and SMCs. HUAECs were cultured on the surface of 24-well TCPS using EGM MV Microvascular Endothelial Cell Growth Medium Bulletkit (Endothelial Basal Medium supplemented with 2.5% fetal bovine serum (FBS), Bovine Brain Extract, hydrocortisone, human epidermal growth factor (hEGF), and gentamicin/amphotericin-B (GA)). Depending on the seeding density, ECs were cultured for 2−3 days to subconfluence and then subjected to coculture with SMCs as described below.

SMCs were encapsulated in L-G-2A hydrogel as follows: SMCs were suspended in hydrogel precursor solution; unless otherwise indicated, 80 μL of cell-laden precursor solution was deposited onto the wells of 48-well nontreated TCPS (BD Falcon), and subjected to low intensity UV illumination (365 nm, 20 mW/cm^2) for 5 min under

Table 1. Primers Used for Real-Time RT-PCR[a]

gene	primer sequence (5′-3′)	A.T. (°C)	P.S. (bp)	ref
α-actin	F:CATCACCAACTGGGACGA	58	85	24
	R: GGTGGGATGCTCTTCAGG			
calponin1, basic	F: GAGTCAACCCAAAATTGGCAC	58	138	23
	R: GGACTGCACCTGTGTATGGT			
collagen type I	F: ATGTGGCCATCCAGCTGAC	58	75	24
	R: TCTTGCAGTGGTAGGTGATGTTCT			
elastin	F: GAGCTTTTGCTGGAATCCCA	62	130	24
	R: GGCAGTTTCCCTGTGGTGTAG			
GAPDH	F: ATGGGGAAGGTGAAGGTCG	58	108	23
	R: GGGGTCATTGATGGCAACAATA			
myosin-heavy chain10, nonsmooth muscle (SMemb)	F:CCCATGAAGAGATTCTGTCAATGC	60	151	22
	R: ACATTCATCCCAAGAAGATGGC			
myosin heavy chain11, smooth muscle (SM-MHC)	F: CTGCAGCTTGGAAATATCGT	52	136	23
	R:GAGTGAGGATGGATCTGGTG			
smoothelin	F: CCCTGGCATCCAAGCGTTT	62	137	23
	R: CTCCACATCGTTCATGGACTC			

[a]A.T., P.S., and ref refer to annealing temperature, product size, and references, respectively.

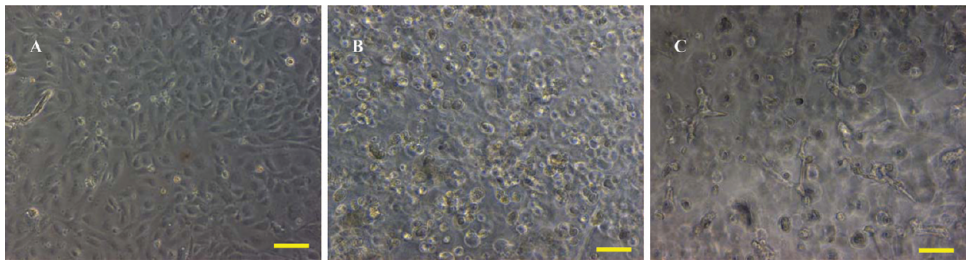

Figure 2. Phase contrast microscopic images of (A) EC monolayer on TCPS after 2−3 days of culture, (B) SMCs just after encapsulation and (C) cultured for 1 day within L-G-2A hydrogel. Scale bar is 100 μm. SMC seeding density in hydrogel: 1.8×10^6 cells/ml.

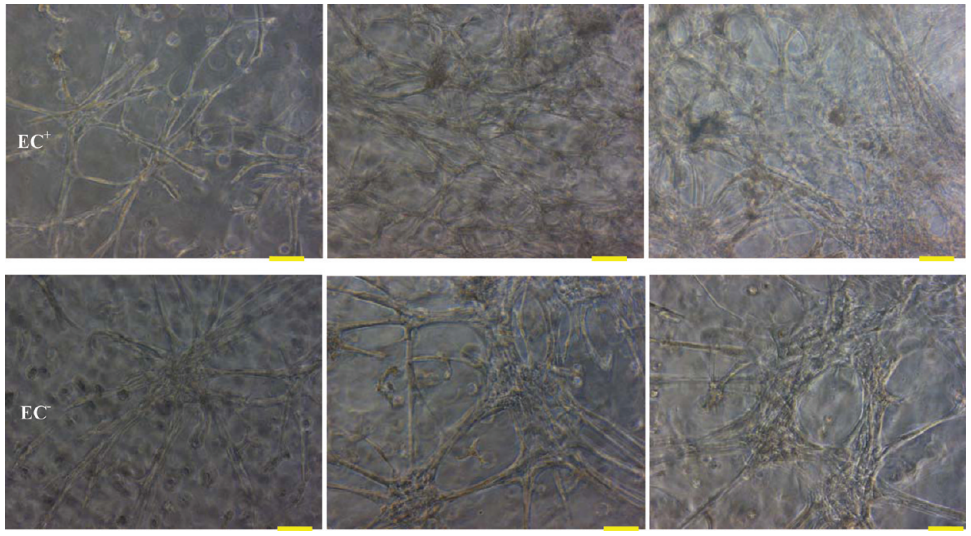

Figure 3. Morphology of SMCs encapsulated within L-G-2A hydrogels and cultured for 2 days (left column), 4 days (middle column), and 7 days (right column) with ECs (top row) and without ECs (bottom row). Scale bar is 100 μm. SMC seeding density: 1.8×10^6 cells/mL.

an argon atmosphere. After UV irradiation, the cell/hydrogel constructs were washed with PBS and supplied with Dulbecco's Modified Eagle' Medium (DMEM) containing 10% FBS. One day after cell encapsulation, the constructs were transferred to the wells of 24-well TCPS which had a monolayer of ECs and supplied with DMEM containing 10% FBS (Figure 1). The medium was refreshed every 2 days. To avoid problems associated with long time culture of ECs, such as detachment, we relocated the SMC-laden hydrogel constructs to other TCPS wells with freshly cultured EC monolayers every 3−4 days.

Characterization of SMC Spreading and Proliferation. SMC spreading in the hydrogel was monitored after seeding using an Axiovert 200 Motorized Inverted Microscope System (Carl Zeiss Vision GmbH) and recorded with a digital CCD camera. SMC proliferation was tested via quantification of cell viability using Cell Proliferation Reagent WST-1 (Roche Diagnostics). Following incubation for various periods, EC^+ and EC^- constructs (fabricated from 40 μL of precursor solution) were transferred to the wells of a new 24-well TCPS and supplied with 400 μL of fresh DMEM without phenol red or FBS. Forty microliters of WST-1 reagent was added to each well. After incubation for an additional 4 h, the absorbance of the formazan dye solution in culture medium at 440 nm was recorded. Six parallels were averaged for each hydrogel sample, and data are expressed as mean ± standard deviation values.

Gene Expression Analyze. RNA was extracted from cell/hydrogel constructs using the RNeasy Mini Plant Kit (Qiagen) following the manufacturer's protocols. cDNAs were generated from 200 ng of total extracted RNA using Omniscript Reverse Transcription Kit (Qiagen) with oligo-dT primer. Gene expression levels were determined via real-time PCR analysis using iQ SYBR Green reagent on an iQ qPCR system (Biorad). Melt curve analysis was performed to ensure a single amplification for each gene. The ΔC_T method as normalized to glyceraldehyde-2-phosphate dehydrogenase (GAPDH) was applied to calculate the relative gene expression. Gene specific primers used were listed in Table 1.[22−24]

Degradation of Hydrogel Constructs. At various time points during culture, cell/hydrogel constructs were washed with PBS and freeze-dried. The dry weight of the constructs was measured. Hydrogels without SMC encapsulation or exposure to ECs were prepared as the control group. They were also incubated in culture medium (DMEM with 10% FBS) at 37 °C in a humidified atmosphere containing 5% CO_2, and the degradation was monitored by measuring the dry weight. Six parallel samples were averaged for each construct group at each measuring time point. The raw data was normalized by the dry weight of hydrogels after 12 h of incubation in culture medium.

Quantitative Analysis of PDGF-BB and TGF-β1. Enzyme-linked immunosorbent assay (ELISA) was used to measure the amount of PDGF-BB and TGF-β1 in culture medium. At various time points during culture, conditioned medium for both EC^+ and EC^- constructs was collected and stored at −80 °C. Quantitative measurement was performed using Quantikine human PDGF-BB and TGF-β1 ELISA kits (R&D Systems). The minimum detectable dose for PDGF-BB and TGF-β1 is 15 and 1.7−15.4 pg/mL (mean 4.61 pg/mL), respectively.

Both the active and latent TGF-β1 concentrations in culture medium were measured. For measurement of active TGF-β1, we directly added culture medium to 96-well plates precoated by the manufacturer. For measurement of total TGF-β1, we treated culture medium with an acid activation step that coverts latent TGF-β1 to the active form before adding to the precoated 96-well plates. For activation, twenty microliters of 1N HCl was added to 100 μL of cell culture supernate and mixed well; the mixture was incubated at room temperature for 10 min. The acidified sample was then neutralized by adding 20 μL of 1.2 N NaOH/0.5 M HEPES. The amount of latent

TGF-β1 was calculated by subtracting the amount of active TGF-β1 from that of total. All ELISA measurements were performed triplicate.

Statistical analysis. All data are reported as mean ± standard deviation. Comparisons between two different groups were carried out using Student's *t*-test. A value of $p < 0.05$ was considered to be significantly different.

RESULTS

Impact of ECs on SMC Spreading and Proliferation. ECs were cultured on TCPS for 2−3 days (Figure 2A) before the beginning of coculture with SMCs. The ECs were subconfluent at the beginning of coculture. SMCs were encapsulated in L-G-2A hydrogel. Initially, SMCs had a round morphology (Figure 2B). After 1 day of culture, some of the cells started to spread out (Figure 2C). At this time point, the SMC/hydrogel constructs were transferred to TCPS with a precultured monolayer of ECs.

To examine the effect of EC monolayer on SMC spreading, phase contrast images of SMCs within EC^+ and EC^- constructs were taken during 7 days of culture (corresponding to 6 days of exposure of SMCs to EC monolayer in the EC^+ group). After 4 days of culture, more spreading SMCs and denser cellular network were present in EC^+ constructs than in EC^- constructs (Figure 3).

The proliferation of SMCs in EC^+ and EC^- constructs during 2 weeks of culture was quantified via analyzing the number of viable cells using WST-1 reagent. As shown in Figure 4, the general trend of cell proliferation with culture time in EC^+ and EC^- constructs were similar: the number of viable cells initially increased, but decreased in longer cultures. In EC^+ constructs, SMC proliferation was stimulated by the EC coculture initially (at fourth day and shorter period of culture), as indicated by the significantly higher WST-1 absorbance compared with EC^- constructs. However, this stimulatory effect disappeared with longer cultures: the numbers of viable cells in EC^+ and EC^- constructs were not significantly different on and after 7 days of culture.

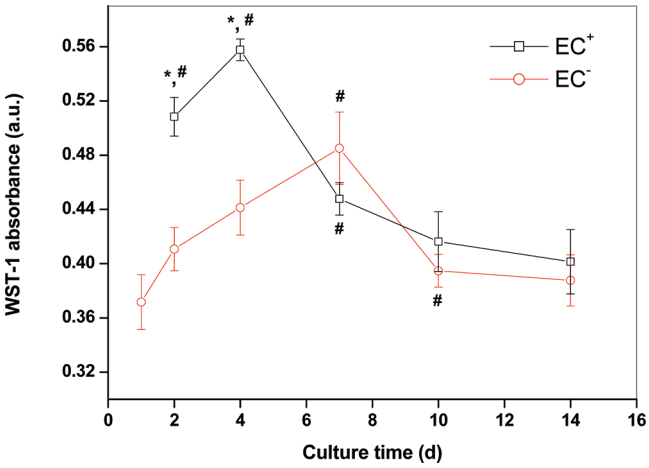

Figure 4. Measurement of cell proliferation, using WST-1 assay, of SMCs encapsulated within L-G-2A hydrogels with and without the presence of ECs (EC^+ and EC^- constructs). *: $p < 0.05$, EC^+ vs EC^- constructs at the same time point; #: $p < 0.05$, vs prior time point (comparison was performed within EC^+ and EC^- groups respectively, except that the 2 days result of EC^+ constructs was compared with the 1 day result of EC^- constructs).

The comparable cell viability in EC^+ and EC^- constructs with longer (day 7 and beyond) culture periods seems not to agree with higher density of cellular networks in EC^+ constructs (Figure 3). However, significantly higher degree of compaction was observed in EC^+ constructs compared with EC^- constructs during culture (data not shown), which may be due to the promoted SMC proliferation in the early culture periods in the presence of ECs. We propose that the reduced gel volume resulted in denser cellular networks in EC^+ constructs.

Impact of ECs on SMC Differentiation. It has been well demonstrated that SMC differentiation occurs during normal development, under pathophysiological conditions, as well as during in vitro culture.[2,3,25−28] SMC differentiation is ultimately modulated at transcriptional level.[26−28] In order to investigate the effect of ECs on SMC differentiation, the expressions of a number of genes were analyzed during 2 weeks of culture. These genes included SMC contractile marker genes (specifically α-actin, calponin, SM-MHC, and smoothelin), synthetic marker gene (SMemb), and ECM protein genes (collagen type I and elastin).

α-Actin, calponin, SM-MHC, and smoothelin are contractile apparatus proteins. They participate in the contraction function of SMCs. In EC^- constructs, the trends of transcriptional expressions of these contractile proteins, except smoothelin, showed a similar pattern with culture time (Figure 5A−C): there were significant increases during initial culture; maximum expressions were reached at fourth day; the expression levels declined thereafter. For smoothelin, in EC^- constructs (Figure 5D), the expression had a maximum level at the second day. Also, the 1.2-fold maximum increase (by comparing expression on the second day with that on the first day) of smoothelin was considerably less than the maximum increase of other contractile marker genes (7.8 fold for α-actin, 14.5 fold for calponin, and 2.6 fold for SM-MHC obtained by comparing day 4 expressions with day 1 expressions).

In EC^+ constructs, the general trend of expressions of α-actin and calponin with culture time resembled the corresponding ones in EC^- constructs (Figure 5A, B): the gene expression increased initially, and then declined, though the maximum expressions were achieved at 10th day instead. There was no significant difference between the maximum expressions of α-actin, as well as calponin, of SMCs in EC^+ and EC^- constructs ($p = 0.44$ for α-actin; $p = 0.27$ for calponin). The α-actin and calponin curves for the EC^+ constructs seemed to be merely shifted to the later time compared to the corresponding ones for the EC^- constructs.

Once exposed to ECs, SM-MHC expression of SMCs significantly increased ($p = 0.04$, EC^+ constructs second day vs EC^- constructs first day) (Figure 5C). Thereafter, the expression decreased significantly and remained at a low level until the 10th day, after which a sharp increment led to the maximum expression. The 14th day maximum expression level was slightly higher than the second day expression in EC^+ construct, and was comparable to the maximum in EC^- constructs.

Smoothelin expression in both EC^+ and EC^- constructs had a similar dynamic trend (Figure 5D). There seems to be an oscillation of some kind. However, in general, the expression level was higher at the beginning and lower with longer culture periods. The EC^+ constructs had a consistently higher expression level than EC^- constructs, except at the fourth day.

The EC^+ and EC^- constructs also had similar dynamic expression patterns of SMemb (Figure 5E), which was lower at

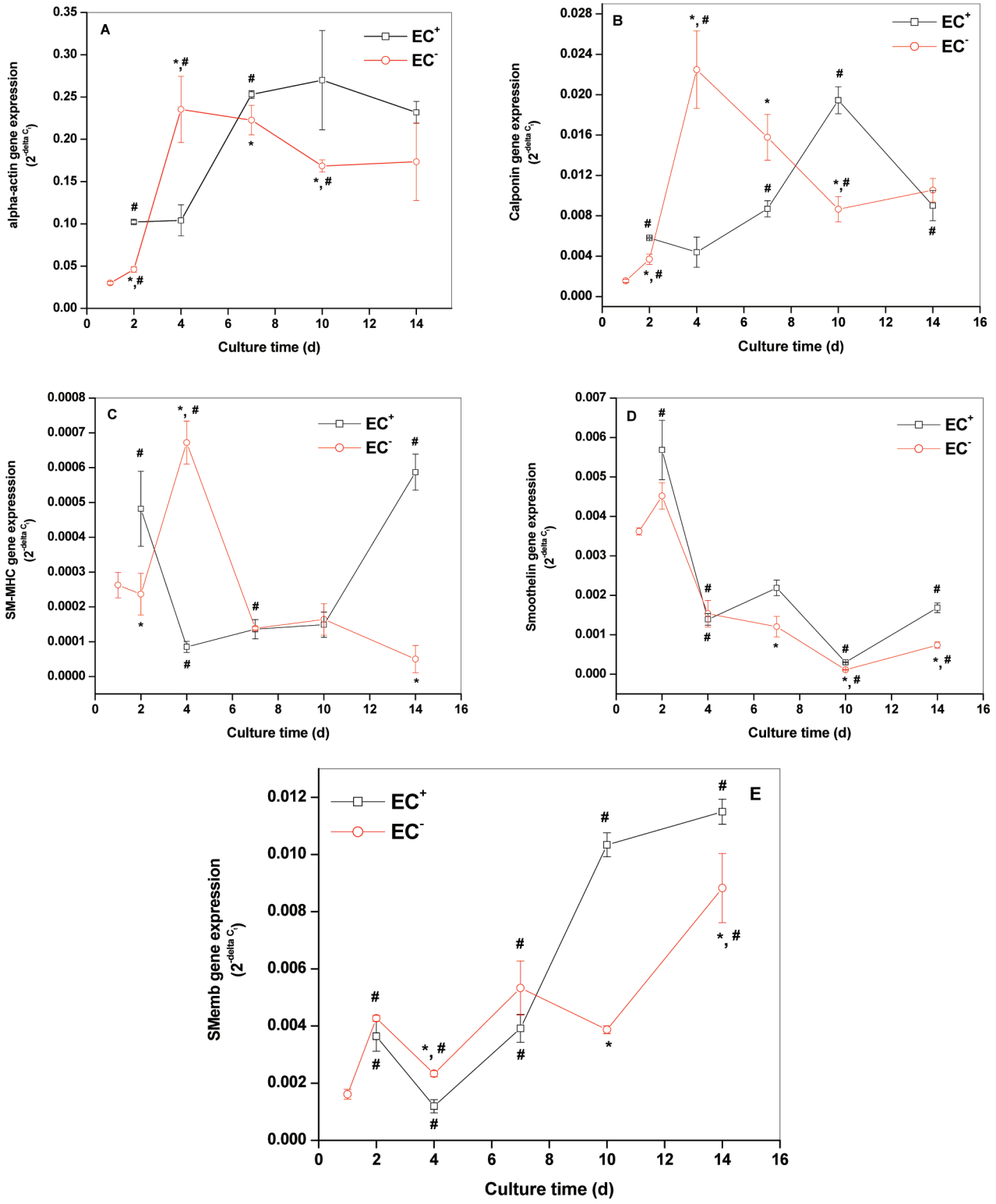

Figure 5. Expression of (A) α-actin, (B) calponin, (C) SM-MHC, (D) smoothelin, and (E) SMemb over 14 days of culture in EC^+ and EC^- constructs. Expression levels shown are relative to GAPDH expression in the same sample. *: significant difference ($p < 0.05$) between EC^+ and EC^- constructs at the same time point; #: significant difference ($p < 0.05$) vs prior time point (within the same group of constructs except that the 2 day result of EC^+ constructs was compared with the 1 day result of EC^- constructs).

the beginning (on day 7 and before) but increased thereafter. Initially, the expression levels in EC^+ constructs were lower than in EC^- constructs. It was after the seventh day that this trend was reversed with EC^+ having higher level of SMemb expression. There is a hint of an oscillation about the trend in these curves.

Impact of ECs on ECM Production of SMCs. In vascular tissue engineering, ECM synthesis is required to replace the degrading scaffold. Synthesis of ECM is also indicative of SMC

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phenotype state. It is generally considered that SMCs in a more synthetic phenotype increase their synthesis of ECM components.[3] We have investigated the synthesis of collagen type I and elastin at transcriptional level. As shown in Figure 6A, EC$^+$ and EC$^-$ constructs had similar expression dynamics of

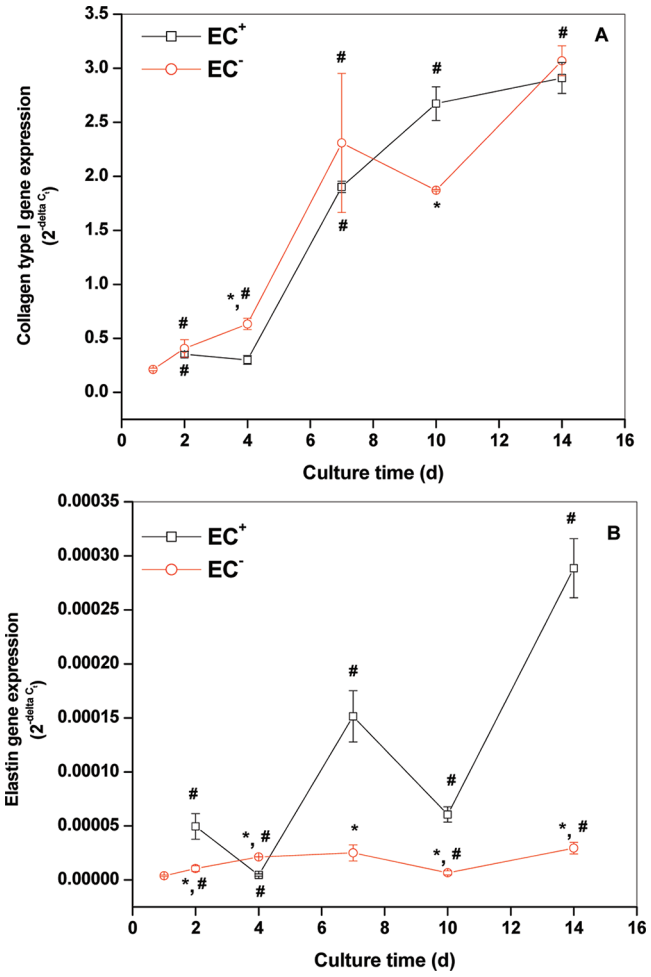

Figure 6. Gene expressions of ECM proteins: (A) collagen type I and (B) elastin, over 14 days of culture in EC$^+$ and EC$^-$ constructs. Expression levels shown are relative to GAPDH expression in the same sample. *: significant difference ($p < 0.05$) between EC$^+$ and EC$^-$ constructs at the same time point; #: significant difference ($p < 0.05$) vs prior time point (within the same group of constructs except that the 2 day result of EC$^+$ constructs was compared with the 1 day result of EC$^-$ constructs).

collagen type I: the levels increased with time. The expression of collagen type I in EC$^+$ constructs was lower than in EC$^-$ constructs at all the evaluated time points except on the 10th day. However, the difference between these two groups was not significant at most of the measured time points.

There was significant difference in the expression patterns of elastin in EC$^+$ and EC$^-$ constructs (Figure 6B). In EC$^-$ constructs, SMCs had considerably lower and stable elastin transcriptional expressions during 2 weeks of culture. EC$^+$ constructs had significantly higher levels of elastin expression than EC$^-$ constructs at the evaluated time points except on the fourth day. However, there was a large oscillation in elastin synthesis of SMCs in EC$^+$ constructs.

Impact of ECs on Hydrogel Degradation. The degradation of hydrogels with encapsulated SMCs was analyzed via measuring the dry weight of constructs during 2 weeks of in vitro culture. Because of the incorporation of natural gelatin, the hydrogels based on dextran and gelatin are expected to be degraded by cell-secreted enzymes, such as matrix metalloproteinases (MMPs). This has been verified by degradation test. As shown in Figure 7, the dry weight of both EC$^+$ and EC$^-$

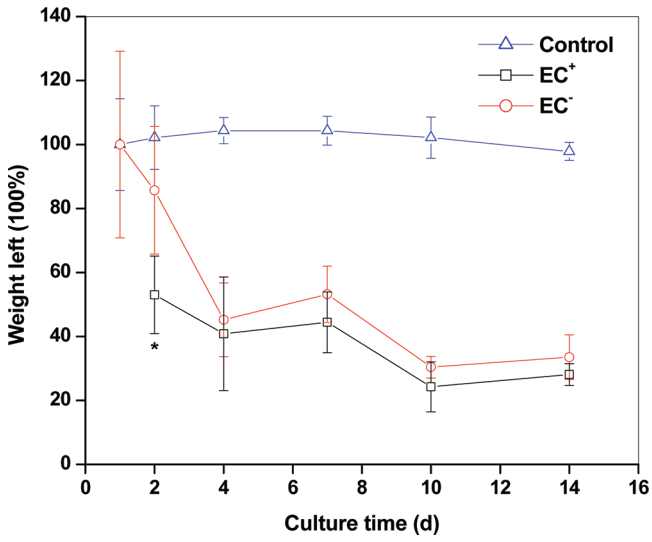

Figure 7. Weight loss of cell-laden hydrogel constructs (EC$^+$ and EC$^-$) during 14 days of culture. Control samples are hydrogels without SMC encapsulation or EC coculture. *: significant difference ($p < 0.05$) between EC$^+$ and EC$^-$ constructs at the same time point. ($n = 6$).

constructs decreased with time, whereas control hydrogels (without SMC encapsulation or EC coculture) showed no significant weight loss.

With the presence of ECs, the EC$^+$ constructs seemed to degrade faster than EC$^-$ constructs. At all the evaluated time points, the dry weights of EC$^+$ constructs were lower than that of EC$^-$ constructs. However, the differences were not significant except at the second day. The presence of ECs could enhance the degradation by secretion or activation of MMPs such as MMP-1 and MMP-2.[29]

PDGF-BB and TGF-β1 in Culture Medium. The amounts of PDGF-BB and TGF-β1 in the culture medium were measured by ELISA for both EC$^+$ and EC$^-$ constructs. During 2 weeks of culture, PDGF-BB was undetectable in medium from EC$^-$ constructs (Figure 8A) at all the evaluated time points. Medium from cocultures (EC$^+$ constructs) showed detectable PDGF-BB at day 2, 4, and 10, with the amount peaked at day 4 to be 26.7 pg/mL. The PDGF-BB in the coculture medium may be attributed to the secretion of ECs. It was also likely that SMCs were stimulated to secrete PDGF-BB in the presence of ECs, although no detectable PDGF-BB was found in medium of homotypic SMC culture. It has been shown that ECs increased the expression of PDGF-BB gene in cocultured SMCs.[4]

Active TGF-β1 was detectable in medium of neither EC$^+$ nor EC$^-$ constructs (data not shown). Latent TGF-β1 was detected under both conditions (Figure 8B). Medium from cocultures had significantly higher level of latent TGF-β1 than medium from EC$^-$ constructs at all the measured time points. The secretion of ECs may be accounted for the increased amount of LTGF-β1 in coculture model. It was also possible that in

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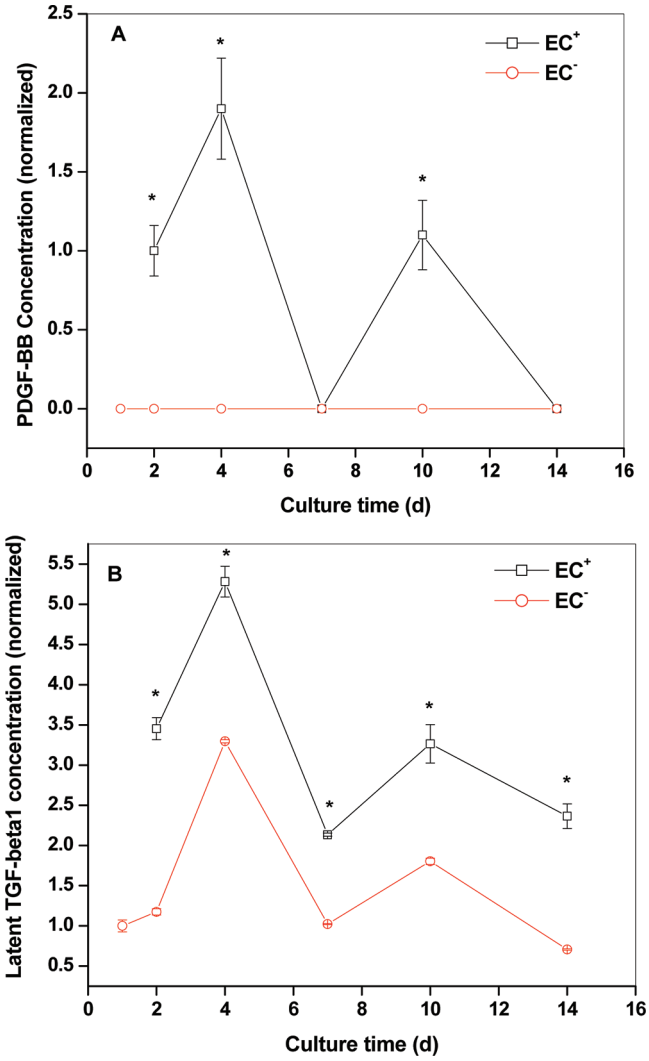

Figure 8. ELISA of medium from EC^+ and EC^- constructs reveals the dynamic changes of (A) PDGF-BB and (B) latent TGF-β1 production during 14 days of culture. No detectable PDGF-BB was found in medium from EC^- constructs. The measured PDGF-BB concentrations for EC^+ constructs were normalized to that at day 2. All measured latent TGF-β1 concentrations were normalized to the 1-day concentration of EC^- constructs. *: significant difference ($p < 0.05$) between EC^+ and EC^- constructs at the same time point.

coculture model SMCs were stimulated to upregulate LTGF-β1 production. Coculture with ECs has been demonstrated to increase the expression of TGF-β gene in SMCs.[4] For both EC^+ and EC^- constructs, the amount of latent TGF-β1 increased during initial culture and peaked at day 4. The maximum amount of TGF-β1 detected in medium of EC^+ constructs was 2595 pg/mL, whereas in medium of EC^- constructs, the maximum value was 1619.3 pg/mL.

DISCUSSION

An EC/SMC coculture model is presented here in which SMCs encapsulated in 3D hydrogels were exposed to a monolayer of ECs. This coculture model is superior in that the SMCs were kept in a 3D microenvironment mimicking the native condition. Although no direct EC-SMC physical contact was present, the exposure of SMC/hydrogel constructs directly to EC monolayer is expected to be superior to the model using EC-conditioned medium,[5] in which the effect of EC-secreted regulators on SMCs may be neglected because of their short life times. Additionally, SMCs may also influence EC secretion of regulators. This bidirectional communication is absent in cultures with EC-conditioned medium.

Coculture Effect on SMC Proliferation. Upon exposure to ECs, the initial SMC proliferation was promoted within 3D hydrogels (Figure 4). The EC regulatory effect, either inhibitory or stimulatory, on SMC proliferation is known to be dependent on the culture state of ECs. Proliferating ECs have been reported to stimulate SMC proliferation whereas no such stimulatory effect was evident with confluent quiescent ECs.[15,30,31] The EC monolayer in our model at the beginning of coculture was actually subconfluent, as shown in Figure 2A: gaps existed between cells, and formation of tight cell−cell junctions was limited. These subconfluent ECs might have been still in a proliferative state, so the observed stimulatory effect of ECs on SMC growth was consistent with the reported results.[31] The initial (2 d) faster hydrogel degradation of EC^+ constructs might have also been responsible for the enhanced SMC proliferation (Figure 7). Creation of space by hydrogel degradation would promote SMC proliferation. This was consistent with the reported observation that in 3D hydrogels, the "steric hindrance" effect of the dense matrix would suppress cell growth because of the restricted space for cell division.[20]

Detachment of ECs from TCPS was observed during coculture. This could be caused by a lack of exogenous growth factors in the coculture medium: after the establishment of coculture, endothelial cell growth medium that contains bovine brain extract and hEGF was replaced by DMEM with 10% FBS. It is also likely that the proliferative SMCs caused the detachment of ECs. When ECs were seeded on proliferative SMCs, the number of ECs was found to decrease significantly over time.[32] The detachment of ECs could also promote SMC proliferation, similar to the in vivo situation where damage to the endothelium of vessels often induces the proliferation and migration of SMCs.[33]

Higher compaction of SMC-laden hydrogels was also observed with EC^+ constructs (data not shown), which might have been the result of higher rate of SMC proliferation in the early culture period. The compaction led to the formation of much denser cellular networks in EC^+ constructs (Figure 3). The stimulation of SMC growth and formation of denser cellular networks favor the use of hydrogels for vascular tissue engineering, where high cell density and deposition of ECM are required to replace and remodel the degradable hydrogel scaffold.

Phenotype Modulation of SMCs. In vascular tissue engineering, suitably timed SMC phenotype modulation is believed to be the key to success:[34] a synthetic phenotype is required initially for vessel remodeling, whereas a contractile phenotype is required ultimately for vasoactivity. We have shown that SMCs in 3D hydrogels without EC coculture were generally in a more synthetic phenotype.[21] Others have reported that ECs promoted the contractile phenotype of SMCs in 2D cultures.[35] In this work, the impact of ECs on SMC phenotype modulation in 3D hydrogels was investigated. SMC phenotype in EC^- constructs was investigated as control.

SMC Phetotype in EC^- Constructs. SMC contractile markers including α-actin, calponin, SM-MHC, and smoothelin are often used to define the phenotype of SMCs. The expressions of these markers are downregulated when SMC phenotype is switched from contractile to synthetic.[2,3,25] In EC^- constructs, expressions of contractile genes α-actin,

calponin, and SM-MHC were found to have similar dynamics (Figure 5A–C): they first increased and peaked at fourth day, but declined thereafter. The initial (4 days and before) increase of gene expression levels of these contractile proteins may be due to the establishment of increased cell–cell contact (Figure 3) which increases the tensional force imposed on cells as SMCs spread and form 3D cellular networks.[36,37] The same phenomenon has been shown in our previous work in 2D SMC cultures.[21] The general trends of α-actin and SM-MHC for the EC$^-$ constructs (Figure 5A and C) were consistent with our previous results.[21] However, the increase here was much more significant, which may be due to the higher seeding density here (1.8×10^6 cells/mL) compared to that used in our previous work (1.0×10^6 cells/mL).

Stegemann et al.[20] have also shown that the expression of α-actin protein in 2D-cultured SMCs initially increased and then dropped. However, in the same study, no such an increase was observed when SMCs were 3D cultured inside collagen gel. Instead, the expression of α-actin protein was downregulated significantly and rapidly during 3D culture. This may be caused by the suppression of SMC growth and hence the establishment of cell–cell contact in collagen gel. Compared with collagen gel, our dextran and gelatin based gels promoted SMC growth and formation of 3D cellular networks. As a result, upregulation in the expressions of contractile genes resembling that in 2D cultures was observed.

Distinct from α-actin, calponin, and SM-MHC, smoothelin transcription level in EC$^-$ constructs only marginally increased during the initial 2 days of culture. A significant decline was observed thereafter. It seems that the establishment of cell–cell contact has less influence on smoothelin transcription than on α-actin, calponin, and SM-MHC transcription. In contrast to α-actin, calponin, and SM-MHC, it is known that smoothelin is a more sensitive and advanced SMC contractile marker.[38] Its expression is downregulated rapidly when SMCs are switched to a more synthetic phenotype.[3] Once its expression ceases, it is thought to be impossible to trigger its re-expression in vitro.[38] In our hydrogels, although the increased cellular tensional force has induced the upregulation of contractile markers α-actin, calponin, and SM-MHC, it was not sufficient to induce the expression of smoothelin. Much denser cellular networks may be needed for the complete differentiation of SMCs in hydrogels.

SMC Phetotype in EC$^+$ vs EC$^-$ Constructs. Comparing the EC$^+$ and EC$^-$ constructs (Figure 5A, B, and C), the plots of α-actin, calponin, and SM-MHC for the coculture seem to be similar in shape to the corresponding ones for EC$^-$ except that the EC$^+$ ones were shifted to the right, i.e., delayed. However, the presence of ECs had no influence on the maximal transcription levels of any of these genes. It seems that ECs delayed the onset of maximum transcription of α-actin, calponin, and SM-MHC, although the cellular networks in EC$^+$ constructs were denser and SMC proliferation during the initial culture (before 7 days) in EC$^+$ constructs was higher. The transition toward a more contractile SMC phenotype during the formation of cellular networks in EC$^+$ constructs was delayed.

However, for the more advanced contractile smoothelin, there was no such delayed expression. Although the transcription levels were slightly higher in EC$^+$ constructs, the general dynamics of smoothelin transcription was not significantly influenced by ECs. This difference associated with impacts of ECs on transcription of smoothelin, α-actin, calponin, and SM-MHC could be attributed to different regulatory mechanisms at transcriptional level.[39,40] In the coculture medium there were upregulated secretion of PDGF-BB and latent TGF-β1 (Figure 8). We postulate that besides the cellular network-offered physical stimulation, these biochemical stimulants may also modulate contractile protein expression at transcriptional levels in EC$^+$ constructs.

We first describe the difference in transcriptional regulations of α-actin, calponin, SM-MHC, and smoothelin. It is generally believed that α-actin, calponin, and SM-MHC genes have multiple CArG elements (i.e., a CC(AT-rich)(6)GG motif) in their promoter-enhancer regions.[26,27,41] Serum response factor (SRF) binds to the CArG elements and regulates the transcription of these genes.[39,40,42] Although smoothelin (which refers to smoothelin-B in this work) has 2 CArG-like elements in the promoter, its expression is believed to be CArG-independent.[43] Myocardin has been identified as a potent SRF coactivator, which activates the transcription of these CArG-containing SMC marker genes.[26,27,39,40,42] Overexpression of myocardin has been shown to induce expression of α-actin, calponin, and SM-MHC genes but not smoothelin gene in SMC precursor cells.[40]

The expression of myocardin is downregulated by PDGF-BB, and PDGF-BB also inhibits myocardin-SRF interaction as well as SRF/myocardin association with CArG elements in SMC marker genes.[28,44] Through combinatorial mechanisms, PDGF-BB induces the repression of numerous CArG-containing SMC marker genes including α-actin, SM-MHC, and calponin.[28,45] In our coculture model, PDGF-BB secretion was stimulated especially during initial culture (Figure 8A). We postulate that PDGF-BB secretion in coculture is responsible for the delayed onset of maximum transcription of α-actin, calponin, and SM-MHC. Because smoothelin does not have a myocardin-SRF-dependent regulatory pathway, its expression may be largely uninfluenced by PDGF-BB.

It is noteworthy that initially upon exposure to ECs (at second day), there was an upregulation in the transcriptions of all the examined contractile genes (i.e., α-actin, SM-MHC, calponin, and smoothelin), and a downregulation in SMemb. This may be because of TGF-β1, which unlike PDGF-BB, can increase the expression levels of α-actin, SM-MHC, and calponin and decrease the expression of SMemb,[22,46,47] so as to induce the contractile phenotype of SMCs.[3]

TGF-β is normally secreted in an inactive latent form by various cells, including ECs and SMCs.[48,49] Latent TGF-β (LTGF-β) can be activated by plasmin.[50] In homotypic SMC culture, it is hypothesized that when plasminogen activator (PA), which converts plasminogen to plasmin, and LTGF-β are bound simultaneously to receptors on SMC surface, LTGF-β is activated.[51] Although no active TGF-β1 was detected in medium of neither EC$^+$ nor EC$^-$ constructs, it is possible that activation of LTGF-β1 occurred on the surface of SMCs inside both EC$^+$ and EC$^-$ constructs, and then active TGF-β1 may be internalized by SMCs. In the case of coculture where ECs were in direct contact with underlying SMCs, TGF-β1 was not detected in culture medium but detected in SMC lysates.[4]

In our coculture model, because of the higher level of LTGF-β1 (Figure 8B), it is possible that there was higher level of active TGF-β1 than in homotypic culture of SMCs. In EC/SMC direct coculture, the activity of PA on the surface of ECs has been demonstrated to induce LTGF-β activation.[52−55] However, this activation may require direct EC-SMC contact.[52,53,55] It is not clear whether this EC-modulated

activation pathway occurred in our coculture model. However, the increased expression of contractile genes and decreased expression of SMemb in SMCs after the establishment of coculture may be due to a higher level of active TGF-β1.

It seems that the effect of TGF-β1 on SMCs in coculture was only present transiently. On day 4, downregulated expressions of contractile genes were observed in EC^+ constructs (Figure 5A, B, C, and D). This may be due to a self-regulating system of LTGF-β activation.[54] It has been demonstrated that after the establishment of cocultures, most of the active TGF-β was produced within the first 12 h.[54] Others have observed that the difference in LTGF-β1 activation between homotypic SMC culture and EC/SMC coculture is significant only early in the experiment, and later in both culture systems the percentage of active TGF-β1 approaches zero.[56] This is because that the generated active TGF-β can stimulate the synthesis of plasminogen activator inhibitor-1 (PAI-1), which decreases the formation of plasmin, thus in turn blocks the activation of TGF-β.[54,56] Due to this self-regulating mechanism of LTGF-β activation, difference in active TGF-β1 concentration between coculture and homotypic culture may be only present during initial period, though higher level of LTGF-β1 was detectable throughout the whole culture period in medium of EC^+ constructs.

In summary, we hypothesize that the coordination of multiple biochemical factors, such as TGF-β1 and PDGF-BB, are responsible for the phenotype modulation of SMCs in coculture with ECs. It appears that the presence of ECs initially (day 2) promoted a more contractile phenotype of SMCs in 3D hydrogels compared to homotypically cultured SMCs, possibly due to more LTGF-β1 activation. However, shortly after the initial period, due to a self-regulating mechanism of LTGF-β1 activation, active TGF-β1 concentration may be decreased to a similar level in both homotypic culture and coculture. Because of the production of PDGF-BB in coculture, the phenotype of SMCs in EC^+ constructs became more synthetic than that in EC^- constructs (day 4). However, the effects of PDGF-BB in EC^+ may be abrogated due to its decreased level during later culture, so that the curves for the various gene transcription for both EC^+ and EC^- constructs (Figure 5) were similar but the EC^+ curves were delayed.

SMemb is reported to be a dedifferentiated SMC marker.[2,57,58] It is expressed in embryonic SMCs and the expression declines in adult SMCs.[58] However, in proliferating SMCs, the expression of SMemb is quickly and markedly upregulated.[3] The upregulated expression of SMemb (after day 4) collaborates with the observation of downregulated expression of smoothelin in both constructs (after day 2), and indicates a tendency toward synthetic phenotype. Considering all the five gene expressions in Figure 5, a fully differentiated SMC phenotype was not induced even in the presence of ECs, although there was increase in some contractile gene expressions.

ECM Production. SMCs encapsulated with hydrogels synthesized ECM proteins collagen type I and elastin (Figure 6). The synthesis of collagen type I was not greatly influenced by the presence of ECs. The presence of ECs significantly increased elastin transcription, which is consistent with other reported coculture works.[9] It has been reported that nitric oxide (NO) promoted elastin formation of SMCs,[59] and TGF-β1 has also been reported to enhance elastin biosynthesis of SMCs.[60,61] In our coculture model, the upregulated elastin synthesis might have been due to EC-produced NO and/or higher level of active TGF-β1 at initial culture.

In native blood vessels, elastin is not only an ECM protein which confers elasticity, but also a regulator of SMC proliferation, migration, and differentiation.[62−64] In fact, many of the current tissue-engineered blood vessels have failed due to the lack of elastin synthesis.[34,62] The synthesis of elastin in the 3D-encapsulated SMCs was quite promising for the purpose of vascular tissue engineering.

CONCLUSION

The impact of ECs on SMCs in 3D hydrogels was investigated using a coculture model in which SMCs were encapsulated in hydrogels and exposed to a monolayer of ECs. In the presence of ECs, SMC growth was enhanced initially and the formation of dense 3D cellular networks was promoted. Our hydrogel favors extensive network formation either with or without EC coculture and this led to increased cell−cell contact and cellular tensional force, which upregulated the transcription of contractile α-actin, calponin, and SM-MHC genes. Comparing SMC cultures with and without ECs, the general trends and maximum transcriptions of α-actin, calponin, and SM-MHC were similar in both cases, but the time point to reach these maximum transcriptions was delayed in the presence of ECs. The encapsulated SMCs showed some contractility which was not so usual, but full differentiation of SMCs was obtained neither with nor without EC coculture, indicated by the lower expression of smoothelin and higher expression of SMemb. The dynamic modulation of SMC phenotype by ECs is hypothesized to be associated with the release or activation patterns of signal molecules, including PDGF-BB and TGF-β1, in the coculture system. ECs also promoted the synthesis of elastin at transcriptional level. In order to obtain a functional tissue-engineered blood vessel, coculture with ECs is one promising way to promote SMC-remodeling of 3D hydrogels; however, modulation of SMC phenotype toward full differentiation is still a challenge.

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Notes

The authors declare no competing financial interest.

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