TISSUE ENGINEERING
Volume 12, Number 4, 2006

Valvular Endothelial Cells Regulate the Phenotype of Interstitial Cells in Co-culture: Effects of Steady Shear Stress

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ABSTRACT

Valvular endothelial cells interact with interstitial cells in a complex hemodynamic and mechanical environment to maintain leaflet tissue integrity. The precise roles of each cell type are difficult to ascertain in a controlled manner *in vivo.* The objective of this study was to develop a three-dimensional aortic valve leaflet model, comprised of valvular endothelium and interstitial cells, and determine the cellular responses to imposed lumenal fluid flow. Two leaflet models were created using type I collagen hydrogels. Model 1 contained 1 million/mL porcine aortic valve interstitial cells (PAVICs). Model 2 added a seeding of the lumenal surface of Model 1 with approximately 50,000/cm^2 porcine aortic valve endothelial cells (PAVECs). Both leaflet models were exposed to 20 dynes/cm^2 steady shear for up to 96 h, with static constructs serving as controls. Endothelial cell alignment, matrix production, and cell phenotype were monitored. The results indicate that PAVECs align perpendicularly to flow similar to 2D culture. We report that PAVICs in model 1 express vimentin strongly and α-smooth-muscle actin (SMA) to a lesser extent, but SMA expression is increased by shear stress, particularly near the lumenal surface. Model 1 constructs increase in cell number, maintain protein levels, but lose glycosaminoglycans in response to shear. Co-culture with PAVECs (Model 2) modulates these responses in both static and flow environments, resulting in PAVIC phenotype that is more similar to the native condition. PAVECs stimulated a decrease in PAVIC proliferation, an increase in protein synthesis with shear stress, and reduced the loss of glycosaminoglycans with flow. Additionally, PAVECs stimulated PAVIC differentiation to a more quiescent phenotype, defined by reduced expression of SMA. These results suggest that valvular endothelial cells are necessary to properly regulate interstitial cell phenotype and matrix synthesis. Additionally, we show that tissue-engineered models can be used to discover and understand complex biomechanical relationships between cells that interact *in vivo*.

INTRODUCTION

THE AORTIC VALVE THRIVES in a complex and dynamic mechanical environment while maintaining unidirectional circulation and preventing left ventricular overload. Many pathologies and congenital heart defects adversely affects the aortic valve, compromising its function and ability to repair itself,[1,2] Homograft or prosthetic (mechanical or biological) valve replacements can provide approximately 10 to 20 years of function before failing in a number of ways, mainly associated with the fact that they are nonliving substitutes.[3–5] Tissue engineering

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shows great potential to develop a valve substitute that can grow and remodel similar to the success reported with pulmonary autografts in the Ross procedure.[6] Attempts to engineer heart valve tissue have focused on the maintenance of mechanical integrity using a variety of matrix scaffolds.[7–10] One of the most successful animal trials using engineered valvular substitutes, reported by Hoerstrup and colleagues, used biodegradable polymer scaffolds seeded with vascular cells. They reported that after 20 weeks' implantation in a sheep model, the engineered valves exhibited similar tissue structure, cytoskeletal markers, and mechanical properties as those normal valves.[11] Unfortunately, these valves maintained persistent nontrivial regurgitation that may preclude their use in the clinical setting. Although the reasons for these shortcomings are unclear, they may be due to the fact that nonvalvular cells were used to initially populate the matrices, which perhaps miss key phenotypic behaviors. The future success of engineered heart valves may therefore depend ultimately on the ability of the cells populating the scaffolds to thrive within the dynamic and complex mechanical environment, remodel the tissue matrix, and resemble native cell phenotypes.

Surprisingly, very little is known about native valvular cells and their interactions *in vivo*. Advances in the understanding of valvular cell biology have not kept up with the understanding of valve tissue mechanics and scaffold design. Indeed, most tissue-engineering applications have used cells isolated from blood vessels[8,12,13] or stem cells[14] to populate their matrices. The aortic valve leaflet is populated with interstitial cells, while the surfaces are lined with endothelial cells. Valvular endothelial dysfunction has been implicated as the initiator of many clinical sequelae, including inflammatory reactions, calcification, and blood clots.[1,15,16] We have previously demonstrated that aortic valve endothelial cells respond differently to shear stress than aortic endothelial cells do. Aortic valve endothelial cells align perpendicularly to the direction of flow, in contrast to aortic endothelial cells, which align parallel, and this morphology change may be associated with unique mechanotransduction pathways.[17] Transcriptional profiling also indicates that valvular endothelial cells may be different from aortic endothelial cells, most notably that aortic valve endothelial cells are more proliferative than aortic endothelial cells.[18] *In vivo* evidence suggests that valvular interstitial cells are highly dynamic producers of protein and glycosaminoglycans, unlike smooth-muscle cells,[19] and express markers that suggest a hybrid myofibroblast-like phenotype.[20] Cultured interstitial cells express α-smooth-muscle actin to a variable degree, as well as fibroblast surface antigen, and this was not mimicked by cells from other sources.[21] Taylor and colleagues demonstrated that interstitial cells in three-dimensional culture *in vitro* expressed markers similar to interstitial cells *in vivo*, and the relative proportions of specific marker expressing cells was maintained.[22] We have previously shown that interstitial cells expressed α-smooth-muscle actin *in vitro* to a similar degree as smooth-muscle cells, desmin to a lesser degree, and that three-dimensional culture also influences interstitial cell phenotype.[23]

What has not been addressed by any of the previously mentioned studies is the influence that native valvular endothelium may exert on the underlying interstitial cells. It is well known that proper interaction between vascular endothelial and smooth-muscle cells is paramount to normal vessel function. Vascular endothelial stimulation of underlying smooth-muscle cells is critical for the maintenance of vessel tone and inhibition of pathological smooth-muscle cell differentiation. *In vitro* co-culture models have shown that endothelial cells inhibit smooth-muscle proliferation under flow.[24,25] Gene expression of endothelial cells is also altered when soluble factors are allowed to exchange with smooth-muscle cells.[26] It is likely that interactions between valvular endothelial cells and interstitial cells are similarly important.

The objective of this study therefore was to use tissue-engineering techniques to develop a three-dimensional co-culture model of an aortic valve leaflet. By entombing native aortic valve cells in a known amount of relevant biological matrix protein, much more experimental control can be gained over biological variations, and perhaps a more clear understanding of the cell behaviors. Valvular endothelial-interstitial cell co-cultures were also exposed to steady laminar shear for 48 or 96 h to investigate the role of hemodynamics in the interaction between the cells as determined by morphological, biochemical, and immunohistochemical assays.

MATERIALS AND METHODS

Cell culture and model creation

Porcine aortic valve endothelial cells (PAVECs) and aortic valve interstitial cells (PAVICs) were isolated using collagenase digestion as previously described,[17,23] and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Gibco). For these experiments, PAVECs were used at passage 5, whereas PAVICs were used between passage 5 and 8. Two tissue models were created for this study: Model 1 contained only PAVICs entombed in collagen, whereas Model 2 was additionally seeded with PAVECs. Tubular molds were created using glass rods and rubber tubing as previously described,[27] and shown in Fig. 1. A collagen gel suspension (2 mg/mL) containing 1 million PAVICs/mL was created as described previously,[23] and 5 mL of suspension inoculated into a sterile test tube. The tubular molds were then inserted into the test tubes, and

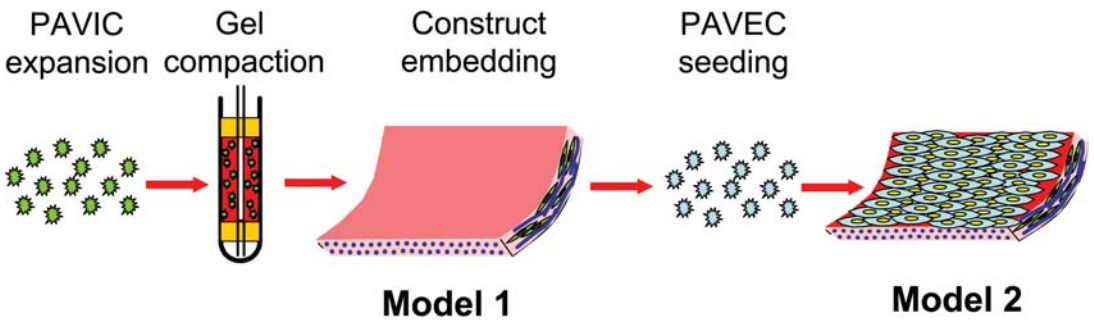

FIG. 1. Schematic of valvular co-culture model creation. (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

the constructs were allowed to gel for 1 h. The molds were then removed from the tubes, placed in a sterile dish containing 150 mL of culture media, and incubated at 37°C, 5% CO_2. The constructs were then allowed to compact circumferentially around the mandrel for 6 days, increasing the density and strength of the tissue so that it could be manipulated. Each construct was then gently removed from the mold and sectioned longitudinally to create a rectangular tissue structure. The surface contacting the glass mandrel was placed face down on a glass microscope slide, and any media were aspirated from under the tissue to ensure complete surface contact. A rectangular polycarbonate mold was placed around the construct, and 6 mL of a 3.5% agar solution at 47°C was inoculated over the construct and immediately sealed with another glass slide. The agar solidified after 1 min at room temperature. The sandwich was flipped over and the slide covering the flat surface was removed to expose it to media. The resulting embedded construct was then placed in 35 mL of culture media. To create the co-culture model (Model 2), Model 1 constructs were additionally seeded with 50,000 PAVECs/cm^2 for 1 h. Both models were then cultured for an additional 48 h to accommodate PAVEC monolayer formation in Model 2 constructs.

Shear stress experiments

The embedded constructs were placed into a modified version of the parallel plate flow chamber previously described.[17] A smaller rubber spacer was used to accommodate the glass coverslip and rectangular mold containing the embedded construct. A larger spacer was used to create the flow channel and to accommodate the increased variation in channel height due to any undulations in the construct surface. The lumenal surfaces were then exposed to 20 dynes/cm^2 steady laminar shear stress for 48 or 96 h. The hemodynamics of the native valve are very complex and difficult to mimic *in vitro*, but previous studies have shown that 20 dynes/cm^2 approximates the mean wall shear stress averaged over a normal cardiac cycle.[28] This value was used both as a first approximation of the hemodynamic environment and to compare with our previous results using valvular cells.[17] Nonsheared Model 1 and Model 2 constructs at 96 h, as well as immediately before the application of flow (0 h), served as controls.

Endothelial morphology

Upon completion of the experimental time points, the constructs (Model 2) were removed, and the agar mold was cut away and then fixed in 3.7% paraformaldehyde for 24 h. The constructs were then gently peeled away from the agar and placed in phosphate-buffered saline solution (Gibco). The constructs were then permeabilized with 0.1% Triton X-100 for 5 min, followed by blocking in 1% neonatal goat serum for 1 h. Endothelial cell phenotype was then labeled by incubation with anti-human von Willebrand factor (vWF) (Sigma F-3520, 1:100, in rabbit) for 1 h, secondary antibody incubation (goat anti-rabbit, FITC, Molecular Probes A-11008, 1:100, 1 h) and counterstained for f-actin (rhodamine phalloidin, Sigma R-418, 1:400) and cell nuclei (Hoechst, Sigma F-32258, 1:1000) for 30 min, followed by additional rinsing and coverslipping. Constructs were then imaged using laser scanning confocal microscopy (Zeiss LSM 510). The lumenal surface of the constructs was placed face down (closest to the objective) on a glass coverslip and five representative pictures were taken of each construct. Morphological parameters (shape index and orientation angle) were assessed as previously described using image analysis software (LSM Image, Zeiss).[17] Measurements were then compared with the morphology of PAVEC monolayers on glass slides. Eight constructs per condition were used for statistical analyses. Two-factor analysis of variance was used to determine significance between cell type and condition, with $p < 0.05$ considered significant.

Biochemical assays and histology

Constructs were sectioned in half widthwise: half of the construct was fixed for histology, and half was im-

mediately frozen at -80°C until analyzed biochemically. PAVIC only constructs (Model 1) and co-cultures (Model 2) were analyzed for cell number, total protein content, and sulfated glycosaminoglycan content as previously described.[23] Cell number was normalized to dry weight to account for differences in size of the samples, while matrix production was normalized to DNA content to approximate per cell values. Additionally, all construct values were normalized by day 0 averages to account for variations between batches of constructs. Samples from six constructs in each condition were used to determine significant differences. Statistical significance was determined using analysis of variance for time duration, and T tests for differences between flow condition and cell type. $p < 0.05$ was considered significant for these tests. PAVIC phenotype was qualitatively assessed using immunohistochemistry. Construct samples were fixed overnight in 3.7% paraformaldehyde and placed in 70% ethanol. Constructs were then paraffin embedded and sectioned at 5 μm. Slides were deparaffinized, washed twice in phosphate-buffered saline, and blocked in 1% bovine serum albumin for 30 min. Antibodies to vWF (Sigma F-3520, 1:600), vimentin (Cy3 conjugate, Sigma V2228, 1:200), α-smooth-muscle actin (Cy3 conjugate, Sigma 0-6198, 1:400), or myosin heavy chain (Sigma, M-7786, 1:100) were incubated singly for 1 h. Slides were then washed, followed by incubation in secondary antibody for vWF (Molecular Probes, A-11008, 1:100) for 40 min. Slides were then washed twice and coverslipped with a DAPI counterstain for cell nuclei. Slides were viewed using a fluorescent microscope (Nikon E600), and several representative pictures were taken from each construct stained.

RESULTS

Co-culture model characterization

PAVICs compacted the collagen hydrogels over the 6-day period (Fig. 2A), with a majority of the compaction occurring by day 3, similar to our previous data.[23] Imaging by polarized light showed the collagen fibril alignment through the thickness of the construct (Fig. 2E). Highly aligned fibers were concentrated near the surface of the tissue closest to the glass mandrel, and more randomly oriented near the opposite surface. It is likely that the developed tissue anisotropy is due to the physical constraint placed at this surface.

The surface with highly aligned underlying matrix became the "lumenal" surface upon longitudinal sectioning, which was then seeded with PAVECs, creating the Model 2 co-culture. Immunofluorescent staining for vWF indicated that the entire surface of the leaflet was not completely covered with endothelial cells, but large patches as shown in Fig. 2C existed throughout the surface, leading us to estimate the surface to be approximately 70% covered with endothelial cells, with some variation between individual constructs. Z-stacked confocal microscopy showed a contact-inhibited endothelial monolayer on top of a closely apposed interstitial cell population (Fig. 2D). Some PAVICs were closely apposed to surface PAVECs in the model, suggesting that these cells could readily interact with each other in the co-culture model, but it was unclear whether these represented junctional contacts.

Endothelial cell alignment under flow

Fig. 3 shows that on an aligned three-dimensional matrix in co-culture with PAVICs, the alignment tendencies of PAVECs were not significantly different overall from culture on coated slides. Valvular endothelial cells in both conditions decreased their Shape Indexes from approximately 0.8 to 0.6 under flow, and changed orientation from 45° (random) to 75° (perpendicular to flow). The larger standard deviation in orientation angle for PAVECs in co-culture was due to slight variations in underlying matrix and interstitial cell orientations, which somewhat influenced endothelial cell alignments.

Cell organization and proliferation

Model 1 and Model 2 constructs were exposed to 20 dynes/cm^2 steady shear with static cultures of both conditions serving as controls. Fig. 4 shows the histologically determined cellular arrangement in seeded and nonseeded constructs over time. Interstitial cells are randomly oriented within the Model 1 constructs, with slight circumferential alignment near the lumenal surface. The application of flow enhances this alignment somewhat, but only right at the surface. Model 2 constructs had cells throughout the construct, but there were preferentially more cells near the lumenal surface and opposite edge of the constructs. This may be due to diffusion constraints imposed by the addition of PAVECs, limiting perfusion through the collagen matrix. Biochemical analysis of cell number (DNA content) across experimental conditions is shown in Fig. 5A. Model 1 constructs increase cell number over time, with shear stress having no effect. This was in contrast to the Model 2 constructs, which maintained cell number during static culture, and decreased cell number under flow. By day 4, cell content in sheared Model 1 constructs was significantly higher than sheared Model 2 constructs.

Matrix production

Fig. 5B and 5C show the matrix production of the Model 1 and Model 2 constructs under static and fluid flow conditions. Protein content was regulated in a manner opposite to that of cell content. Model 1 constructs

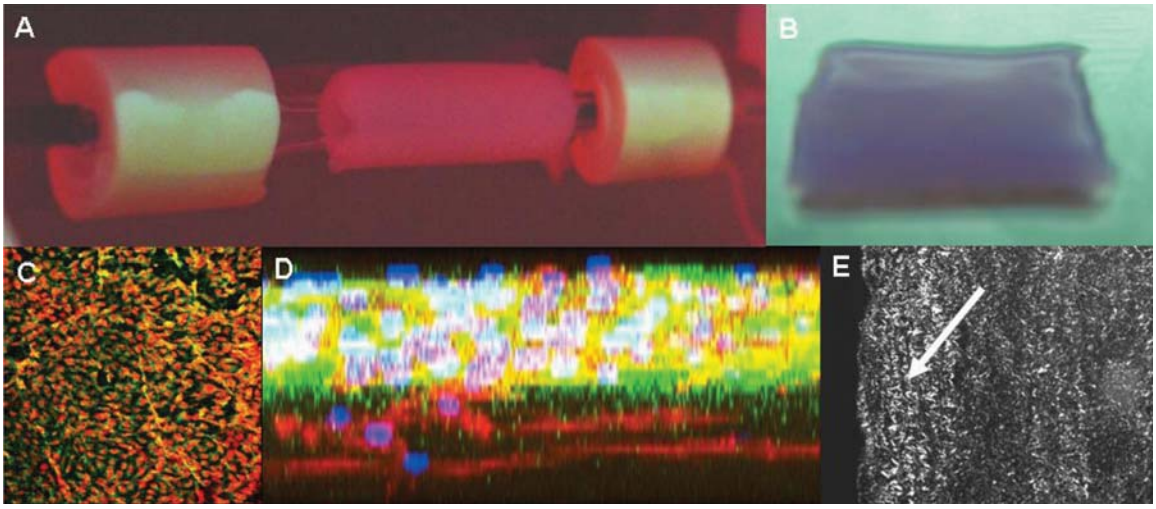

FIG. 2. Co-culture model characterization. **(A)** Tubular mold. **(B)** Embedded construct. **(C)** Confocal microscope image indicating endothelial monolayer. **(D)** Three-dimensional confocal image indicating cellular arrangement. **(E)** Polarized light image indicating matrix alignment (bright field). Confocal staining: green (von Willebrand factor), red (f-actin), blue (cell nuclei). Scale bar = 1 cm (A,B), 100 μm (C–E). (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

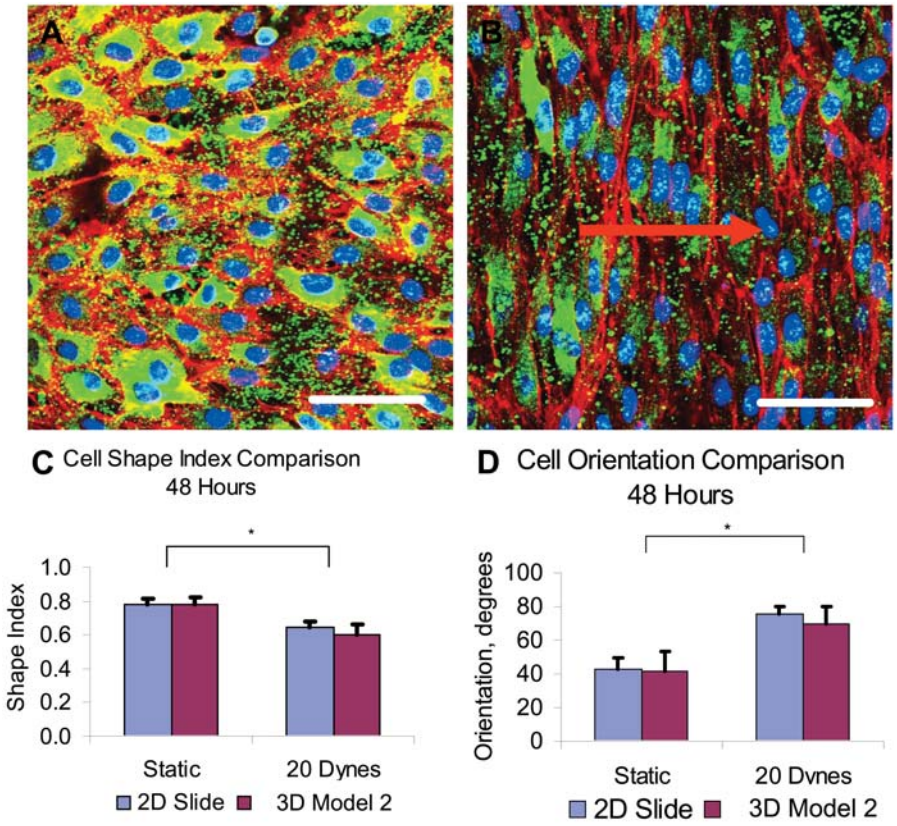

FIG. 3. Endothelial cell alignment on valve leaflet co-culture models. **(A)** Static culture. **(B)** Forty-eight-hour flow (20 dynes/cm^2). **(C)** Shape index comparison. **(D)** Cell orientation angle comparison. Green = von Willebrand factor, red = f-actin, blue = cell nuclei. Scale bar = 50 μm. N = 8 for each condition. (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

tended to have less protein over time, with no effects of shear. Model 2 constructs, on the other hand, had no change in protein content in static culture, but increased protein content under flow. At day 4, sheared Model 2 constructs had significantly more protein than Model 1 constructs. Sulfated glycosaminoglycan (sGAG) content regulation was much different, however. sGAG content was decreased in Model 1 constructs over time and with shear. Both day 2 and day 4 sheared Model 1 constructs had significantly less sGAGs than day 0, but no significant difference between static sheared constructs at day 4. In Model 2 constructs, no significant difference in sGAG content was detected in static culture or under flow ($p = 0.055$). There was significantly more sGAG content in day 4 sheared Model 2 constructs compared to sheared Model 1 constructs, but no significant difference between statically cultured models ($p = 0.18$).

Changes in interstitial cell phenotype

Interstitial cell phenotype was determined through the expression of vimentin, α-smooth-muscle actin (α-SMA), and smooth-muscle myosin. Fig. 6 shows the vimentin expression in the Model 1 and Model 2 constructs. Vimentin expression is maintained throughout the time points investigated in both models, with little differences between flow and static culture. The cells appear more aligned after 4 days of flow, with vimentin expression throughout the cytoplasm. Fig. 7 shows the expression of α-SMA in both models and flow conditions. In VIC constructs, only a basal level of expression is observed in static culture throughout the time period investigated. The application of flow, however, increases expression of α-SMA, especially near the lumenal surface. The expression of α-SMA is also regulated with the addition of PAVECs. At day 0, a basal level of expression is noted, but this expression drops off during the culture period. By day 4, virtually no expression is detectable in static culture, and only slightly more with the application of flow, again in the cells closest to the lumenal surface. These results suggest that the PAVECs decrease the expression of α-SMA in PAVICs in static and fluid flow conditions. No expression of smooth-muscle myosin was observed in either model under any culture condition (data not shown). PAVEC phenotype on the lumenal surface of the Model 2 constructs was confirmed through vWF staining, as shown in Fig. 7. A confluent monolayer of endothelial cells was more difficult to demonstrate through immunohistochemistry, because these constructs were extremely fragile, and the handling necessary for paraffin processing likely detached some of the cells. It was interesting to note that on rare occasions, a few cells expressing vWF were detected in the subendothelial layers of the constructs, suggesting that some migration may have occurred (arrow), but not to any significant degree.

DISCUSSION

This study is the first expose an engineered co-culture model of a valvular leaflet to a defined hemodynamic environment. Native aortic valve endothelial cells were placed in close proximity to valvular interstitial cells, which were homogeneously entombed in an anisotropic biological collagen matrix. This model was then incorporated into a steady shear flow system to investigate interactions between fluid flow, endothelial cells, and interstitial cells.

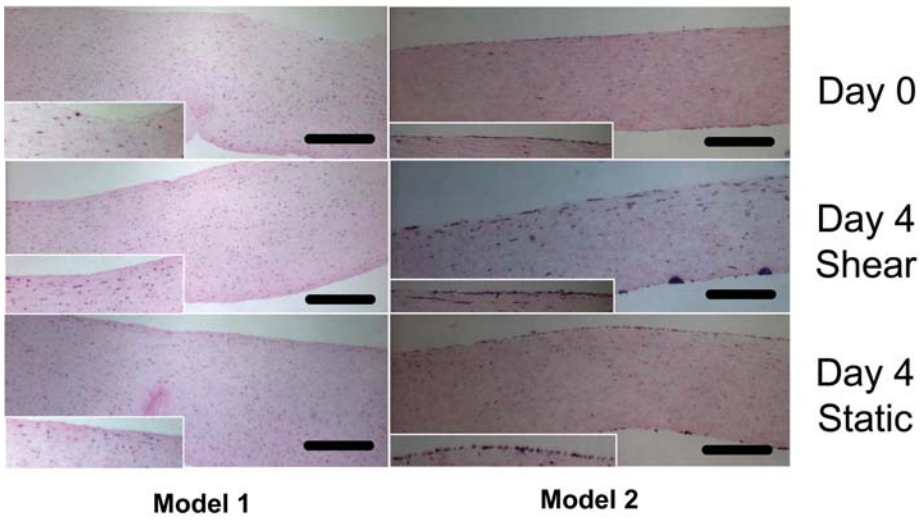

FIG. 4. Hematoxylin and eosin staining of constructs depicting matrix and cellular architecture. Left insets depict cell alignment. Right insets depict endothelial layer. Scale bar = 200 μm. (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

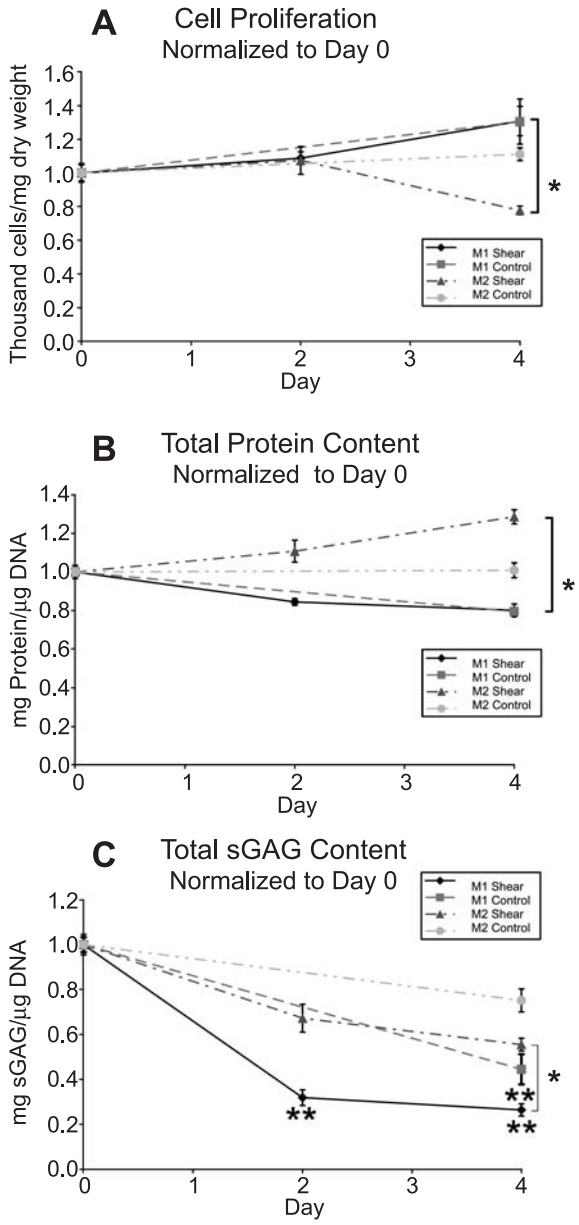

FIG. 5. Cell content **(A),** protein content **(B),** and sulfated glycosaminoglycan content **(C)** of EC and non-EC seeded constructs. Data normalized to Day 0 values and presented as mean ± SEM. Asterisk denotes significant with respect to different condition ($p < 0.05$), double asterisk denotes significant with respect to Day 0 value ($p < 0.05$). N = 6 for each condition.

Numerous studies have demonstrated the importance of appropriate vascular endothelial–smooth-muscle interactions for maintenance of blood vessel tone, and the importance of mechanical forces in regulating cell phenotype.[29–32] Surprisingly little is known about how native valvular endothelial and interstitial cells interact or how mechanical forces influence these interactions. Rothenburger and colleagues created valvular leaflet–like tissue with valvular cells co-cultured in a collagen matrix and found that these cells synthesized collagen and proteoglycans, but did not expose these to any hemodynamic forces or identify any particular aspects of endothelial regulation of interstitial cell function.[33] Weston and Yoganathan developed an organ culture model of aortic valve leaflets and exposed them to shear stress for up to 48 h.[34] They found a dramatic increase in cell number and protein and glycosaminoglycan synthesis with static incubation, but no differences with the application of any flow regimes. Our results indicated no significant changes in cell or protein content by 48 h, but a loss in sGAG content for Model 1 constructs. Weston and Yoganathan reported that their leaflet isolation protocol may have removed all viable endothelium from the leaflet surfaces, creating a model more like the VIC, and these results seem to support that notion. The native aortic valve leaflet has other matrix components besides type I collagen,[35] so some of the differences may also be a result of additional matrix signals not present in these models.

The results of our *in vitro* studies do compare well with previously reported *in vivo* and *ex vivo* observations. The static culture-induced cell proliferation in Model 1 constructs may mimic a mitogenic wound healing response as has been reported by Lester *et al*., who showed an increase in interstitial cell proliferation with endothelial denudation in an organ culture model.[36] This proliferation was not seen in organ cultures with intact endothelium, nor was it reported here in Model 2 co-culture constructs. Vascular endothelium inhibits medial smooth-muscle cell proliferation in *in vivo*[37] and *in vitro* co-culture models,[25] and this inhibition is more apparent with shear stress, similar to our results with valvular cells. In our studies, the presence of valvular endothelial cells also increased protein synthesis, and reduced in the loss of sGAGs induced by shear in comparison to controls. Short-term studies in rat aortic valves indicated more active production of proteins and GAGs in comparison to other tissues such as aortic wall,[19] suggesting that native valvular interstitial cells secrete more matrix than smooth-muscle cells. Activated interstitial cells, as determined by α-SMA expression,[38] persisted throughout the culture periods in Model 1 constructs, but was reduced in co-cultures with valvular endothelial cells (Model 2 constructs). The presence of positive α-SMA expression at day 0 may be due to interstitial cell activation resulting from *in vitro* culture,[39] which may persist through the embedding process. We previously showed high expression of α-SMA in two-dimensional culture, which was dramatically reduced in three-dimensional culture, but still positive.[23] Only interstitial cells near the lumenal surface expressed α-SMA after 4 days of steady shear stress in Model 2 constructs, which was similar to the pattern of immunohistochemi-

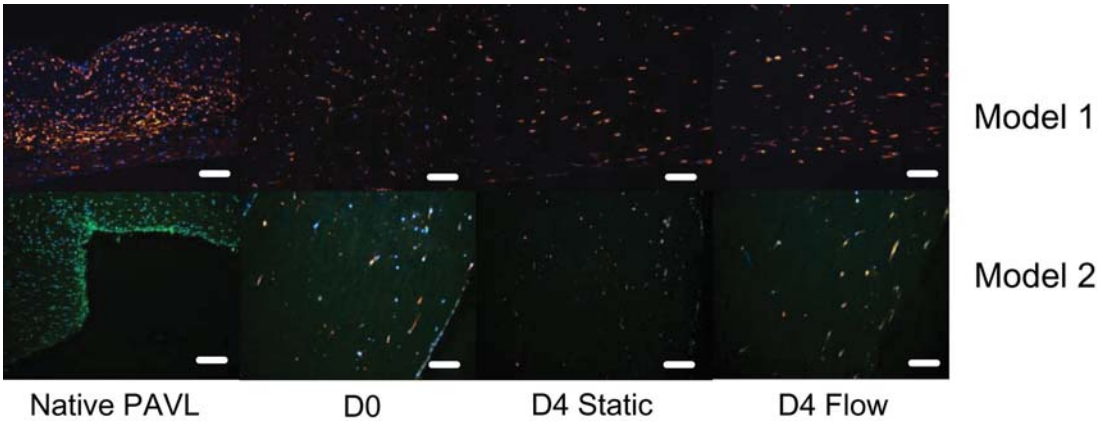

FIG. 6. Vimentin expression in PAVIC constructs and PAVEC-seeded co-cultures (TEVL). Counterstained for cell nuclei (blue). Bottom left panel indicates native leaflet endothelium (von Willebrand factor). Scale bar = 100 μm. (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

cal staining of the ventricularis side of the native leaflet shown in Fig. 7. Vascular endothelial cells, in contrast, enhance α-SMA expression in medial cells while in co-culture and in the presence of fluid shear stress.[40]

The differences in interstitial cell phenotype observed between the two culture models and reports of vascular cell interactions may suggest some important differences between valvular and vascular cells. Both interstitial cells and vascular smooth-muscle cells proliferate when not in communication with their respective endothelial cells. Valvular interstitial cells secreted matrix in a valvular endothelial– and flow-dependent manner, but smooth-muscle cell matrix synthesis is inhibited by vascular endothelial factors and flow. Valvular endothelium decreases α-SMA expression in interstitial cells, whereas vascular endothelial cells increase expression in medial smooth-muscle cells. These results suggest that the quiescent states of interstitial cells and smooth-muscle cells are different in potentially significant ways. The demanding hemodynamic and mechanical environment of the aortic valve may require a nonproliferative, nonactivated cell type capable of secreting matrix to remodel damaged matrix before it progresses to gross tissue failure. Quiescent smooth-muscle cells in this environment may remain contractile (activated) and not adequately replace matrix proteins. Synthetic smooth-muscle cells are characteristically mitogenic[41] and may cause excess tissue growth or disrupt the complex nutrient balance requirements, perhaps leading to a pathological angiogenic response as seen in diseased valves.[42] The implantation of tissue-engineered valvular conduits using vascular cells by the Mayer group[43] resulted in apparent valvular-like cell phenotypes at 20 weeks, but it was unclear whether the cells that remained were donor or host cells. The persistent transvalvular gradients and increased leaflet stiffness reported by this group[11] suggests that some phenotypic dif-

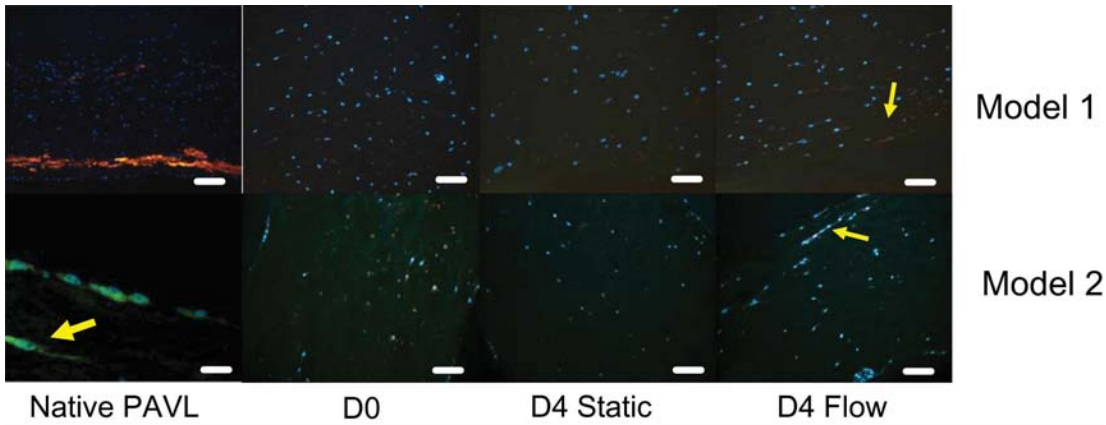

FIG. 7. α-Smooth-muscle actin expression in PAVIC constructs and PAVEC-seeded co-cultures (TEVL). Counterstained for cell nuclei (blue). Bottom left panel indicates TEVL endothelium (von Willebrand factor). Scale bar = 100 μm. (Color images are available online at ⟨www.liebertpub.com/ten⟩.)

ferences between the cells in these tissues and native valves may remain.

From these studies it is apparent that the valvular endothelial cell is the key regulator of interstitial cell phenotype and shear flow enhances this regulation, but the exact mechanisms are as yet unclear. Gotlieb *et al*. have shown that valvular interstitial cell wound repair is mediated by fibroblast growth factor 2 (FGF-2),[44] and several researchers have shown that transforming growth factor b1 (TGFβ1) expression is associated with valvular pathology.[45,46] FGF2 and TGFβ1 signaling pathways may therefore be likely candidates for the investigation of valvular endothelial cell regulation of interstitial cell phenotype. It is not currently known whether valvular endothelial cells would stimulate vascular smooth-muscle cells toward a more valvular interstitial cell-like phenotype or how vascular endothelial cells would regulate valvular interstitial cells. We previously reported that valvular endothelial cells responded to shear stress differently than vascular endothelial cells,[17] which suggests that the aforementioned pairings would interact heterogeneously.

The long-term success of a tissue-engineered valvular substitute may pivot more on the maintenance of appropriate cell phenotypes rather than adequate tissue strength, and therefore the interactions between the native cell types should serve as the "gold standard" for appropriate valvular cell biology. Given the fact that valvular cells are generally not available for isolation and repopulation of a tissue-engineered autograft[47] and that allogeneic cells elicit an immune response,[48] other cell sources may need to be explored. Recent work creating valvular conduits populated with stem cells may have great potential in that these cells might be stimulated more easily to differentiate into valvular phenotypes.[49] Much more work, however, is needed to broaden our understanding of the phenotypes of these cells and their interactions to progress further with this pursuit, and tissue engineering may serve as a useful tool to accomplish this. Valvular mechanics and hemodynamics are truly complex, as is the three-dimensional tissue structure. Attempts to expose the entire aortic root to approximate native tissue forces and flows *in vitro* may not only cause premature tissue destruction due to nutrient transport limitations, but also impairs the determination of causal parameters. Engineered tissue models provide a well-defined three-dimensional matrix environment where the interactions between relevant cell types can be investigated under well-controlled stimuli. These models can complement more involved and expensive animal trials in a positive feedback loop both as a pre-animal feasibility assessment and as a post-animal model of mechanistic understanding. The field may therefore be able to progress more quickly without sacrificing biological understanding that will become critical as these engineered conduits progress to preclinical phases.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Tracey Couse in the processing of the histology. This work was funded by the Georgia Tech/Emory Center for the Engineering of Living Tissues, an NSF-ERC (RMN), and by a predoctoral fellowship from the American Heart Association (JTB).

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