

Porcine Aortic Valve Interstitial Cells in Three-Dimensional Culture: Comparison of Phenotype with Aortic Smooth Muscle Cells

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Background and aim of the study: Recent heart valve tissue engineering efforts have involved populating scaffolds with cells isolated from vascular sources, though it is unclear whether cells from valvular origins behave similarly to vascular cells. The study aim was to compare the phenotype of porcine aortic valve interstitial cells (PAVICs) and porcine aortic smooth muscle cells (PASMCS) in two-dimensional cultures and within three-dimensional (3D) collagen gels.

Methods: PASMCS and PAVICs were isolated from fresh pig hearts, and cultured in either tissue culture flasks or collagen constructs created with 1×10^6 cells/ml. After up to 10 days of culture, gels were lysed or cells isolated with collagenase. Expression of α -smooth muscle actin (α -SMA) and desmin were determined using flow cytometry and laser confocal microscopy. Gel compaction was measured up to day 6, and lysate was analyzed for protein, glycosamino-

glycan (GAG), and cell number.

Results: PAVICs and PASMCS compacted collagen gels similarly, and expressed similar levels of α -SMA but differing amounts of desmin. PAVICs appeared to produce more protein and GAGs than PASMCS over the six-day period in 3D culture. These results agreed well with previously published observations of interstitial cell behavior in vivo.

Conclusion: PAVICs possess both contractile properties and the ability to synthesize matrix components, highlighting their unique function in the demanding environment of the leaflet. Other potential cell sources for heart valve tissue engineering may not be able to mimic adequately some of these functions, and their use may impair tissue function in the long term.

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For the past 40 years, heart valve replacement surgery using mechanical and bioprosthetic valve substitutes has shown great success in extending the life and enhancing the quality of life of patients with severe valvular disease. Current valvular substitutes can function effectively for 20 years or more in older patients (1,2). However, results in younger populations are poorer due to both the increased activity level of the patient, and the need to reoperate as the child grows (3-5). Bioprosthetic valves are generally not suitable for more active child and young adult populations, because of high probability of leaflet tears and calcification (6-8). Although the Ross procedure has shown promising results for aortic valve replacement,

it still necessitates the use of a non-viable valve in the pulmonic position (9).

One reason for the reduced long-term durability of bioprosthetic valves may be that they are non-viable, and therefore possess no ability to remodel and repair localized tissue defects as they occur. These failures will then progress unchecked until primary tissue malfunction occurs. A living cell component would provide a means for the tissue to sense and respond to changes in environment, and adapt the tissue for optimal performance.

To date, tissue engineering approaches to heart valve replacement have employed cells that are isolated from blood vessels and then seeded onto either biodegradable polymeric scaffolds (10-12) or decellularized valves (13). These can then be mechanically conditioned in vitro before implantation in vivo (14). Several small studies have demonstrated the feasibility of these techniques in heart valve replacement. In a recent study, autologous vascular myofibroblasts and endothelial cells were seeded onto decellularized allogenic valves, and these were then implanted in grow-

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ing sheep models for up to six months (15). At autopsy, histological examinations revealed that the tissue-engineered valves were almost identical to native valves in cellular restitution and phenotype, with some evidence of subvalvular inflammation. It is unclear whether similar results would be achieved using xenogenic tissue or allogeneic cells. One report of a tissue-engineered valve implanted in a human has been published, employing many of the aforementioned techniques, and the short term results were encouraging (16).

One question not addressed by the aforementioned studies is whether cells derived from blood vessels are phenotypically similar to the native interstitial cells that populate heart valves. While relatively small differences in cell phenotype may be masked in the short term, it is possible that over long-term culture, phenotypic differences may become more apparent. Valvular interstitial cells are commonly understood to be myofibroblast in nature, with certain aspects similar to both fibroblasts and smooth muscle cells (17-19). These cells have been shown to express markers such as α -smooth muscle actin (α -SMA), fibroblast surface antigen, and vimentin. Some qualitative differences in expression levels have been observed across valves, but the expression levels were dissimilar to cells from other non-vascular origins (20). These data were obtained semi-quantitatively through epifluorescence analysis. The results of one study showed that interstitial cells from aortic valves secreted various matrix molecules and contracted in response to various vasoactive agents (21). These findings suggested that valvular interstitial cells may possess a phenotype with both synthetic and contractile properties, and that smooth muscle cells may not adequately mimic this broad level of function. The present study expands on previous investigations by comparing the expression levels of various contractile markers between interstitial cells and vascular smooth muscle cells, and quantifying contractile and synthetic properties in three-dimensional (3D) collagen gel culture as an in-vitro tissue model.

Materials and methods

Cell isolation and culture

Intact porcine aortic valves with ascending aortas were obtained from a local slaughterhouse (Holifield Farms), and placed in ice-cold Dulbecco's phosphate-buffered saline (PBS; Invitrogen) for shipment. The aortic valve was cut from the ascending aorta above the sinuses, and the leaflets were then excised one-third from the base of the cusp. The leaflets were then serially washed in cold PBS with gradually reduced amounts of antibiotic/antimycotic (ABAM; Invitrogen). The surfaces of the leaflets were then partially

digested with collagenase (600 U/ml; Worthington) for 10 min, after which the endothelium was denuded with gentle scraping. The remaining portions of the leaflets were then digested overnight in fresh collagenase with ABAM. This technique enabled the rapid retrieval of a large number of porcine aortic valve interstitial cells (PAVICs) without concern for migratory phenotype selection (as in the explant method). Porcine aortic smooth muscle cells (PASCs) were isolated from the ascending aorta, above the valve sinuses, but below the carotid artery junctions. The adventitial tissue was removed, and the endothelium removed by collagenase digestion and scraping (as before). The remaining medial layer was minced into small (2-4 mm² sections) and rinsed in gradually colder PBS with gradually reducing concentrations of ABAM, after which the pieces were digested overnight in fresh collagenase.

Both PAVICs and PASCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin/streptomycin (PS; Hyclone) and 1% L-glutamine (LG; Mediatech). Cultures were fed every 48 h, and split 1:3 at confluence. Cultures were used for experiments between passages 5 and 8.

Three-dimensional constructs

Three-dimensional hydrogel constructs were created as described previously (22) using a 2 mg/ml collagen I solution (Coll I, rat tail; BD Biosciences), which forms a fibrillar gel network at neutral pH. A suspension of 1×10^6 cells/ml was created in $5 \times$ DMEM with 10% FBS, 2 mg/ml type I collagen, and enough 0.1 M NaOH to neutralize the solution. Approximately 3 ml of solution was used to make each construct. Constructs were made with either PAVICs or PASCs, and then liberated to compact freely. The medium was changed every 48 h, during which time the gel area was measured. One-way ANOVAs were used to determine significant compaction differences across experimental conditions.

Phenotype analysis

Cell contractile phenotype was determined using antibodies to the cytoskeletal markers α -SMA (Sigma, 1:100), and desmin (Sigma, 1:100). Expression distribution was assessed using confocal microscopy, and expression levels were determined with flow cytometry. It is difficult to distinguish impartially between expression levels using confocal microscopy, and impossible to determine expression pattern (within the cell) by cytometric analysis. Cell phenotype was determined at day 10 for both two-dimensional (2D) and 3D cultures.

For flow cytometry, 2D culture cells were detached from flasks with trypsin at confluency, and centrifuged to form a pellet. Cells from 3D constructs were isolated using collagenase digestion, and the resulting suspension was centrifuged to form a pellet. The pellets were washed by suspending them in PBS, followed by centrifugation to form a pellet. Cells were then fixed in 3.7% paraformaldehyde (Tousimis), and permeabilized with 0.1% Triton X-100 (Sigma). The cells were incubated in blocking buffer (1% neonatal goat serum (NGS; Sigma)) for 1 h, and then washed in PBS before incubation with the primary antibody for 1 h. Cells were washed twice, followed by incubation with a fluorescently conjugated secondary antibody (Anti-rabbit Alexafluor; Molecular Probes, #A-11088; 1:100) for 1 h. Cells incubated with only the secondary antibody served as control. The cells were then washed twice, and approximately 200 μ l of solution was added to a cytometry tube. Flow cytometry was performed to determine the magnitude of phenotype expression using a LSR Flow Cytometer (BD Biosciences). The negative control was used to calibrate the signal, and histograms were collected from four samples from different experimental trials. Population statistics (mean \pm SD) were determined and used for subsequent statistical analysis as described previously (22). A two-way analysis of variance was conducted with a significance level of $p = 0.005$. Pairwise comparisons were then conducted post hoc with $p < 0.005$ considered significant. The increased stringent requirements were added to account conservatively for the comparison of samples of population means instead of direct population comparisons.

For confocal microscopy studies, cells were either seeded to confluence onto glass coverslips coated with collagen I (50 μ g/ml), or in constructs. After 10 days of culture, cells were fixed and stained while adhered to the coverslip, or in constructs. Samples were also counterstained with Hoechst stain (Sigma, #33258) to reveal cell nuclei. Five representative pictures were taken of each sample for qualitative analysis.

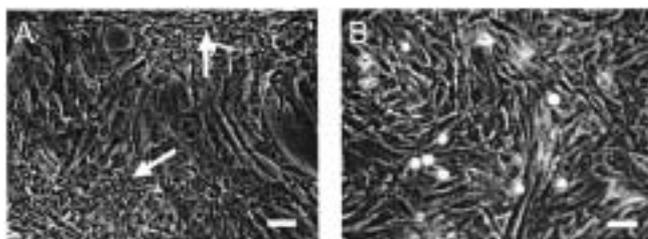


Figure 1: Phase-contrast microscopy images of (A) porcine aortic smooth muscle cells (PASCs) and (B) porcine aortic valve interstitial cells (PAVICs) in monolayer culture. Note the initial stages of classical nodule formation in PASCs (A, arrows). Scale bar = 50 μ m.

Collagen Gel Compaction

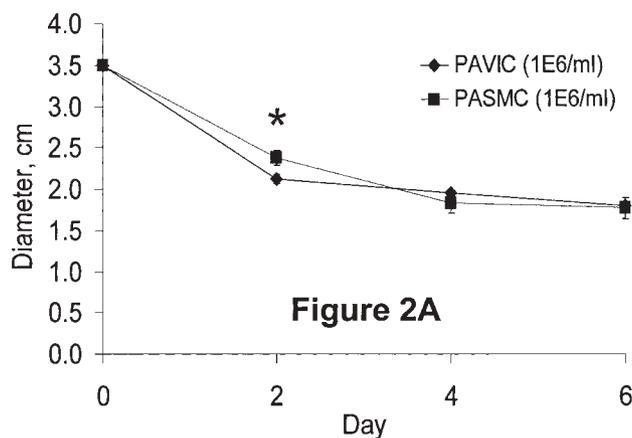


Figure 2A

Construct Cell Number

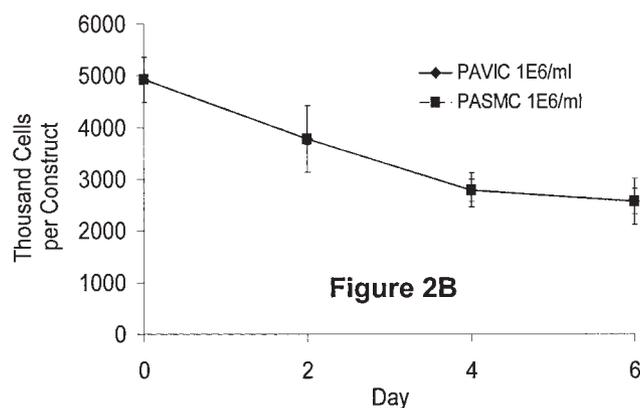


Figure 2B

Construct Cell Density

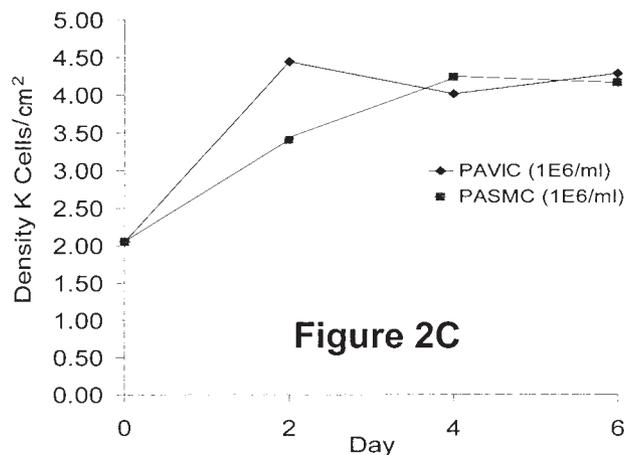


Figure 2C

Figure 2: Construct property comparisons. A) Gel compaction comparison, measured as A/A_0 . B) Construct cell content. C) Construct cell volumetric density. *Statistically significant, $p < 0.05$. ($n = 4$, $n = 2$ for each condition.)

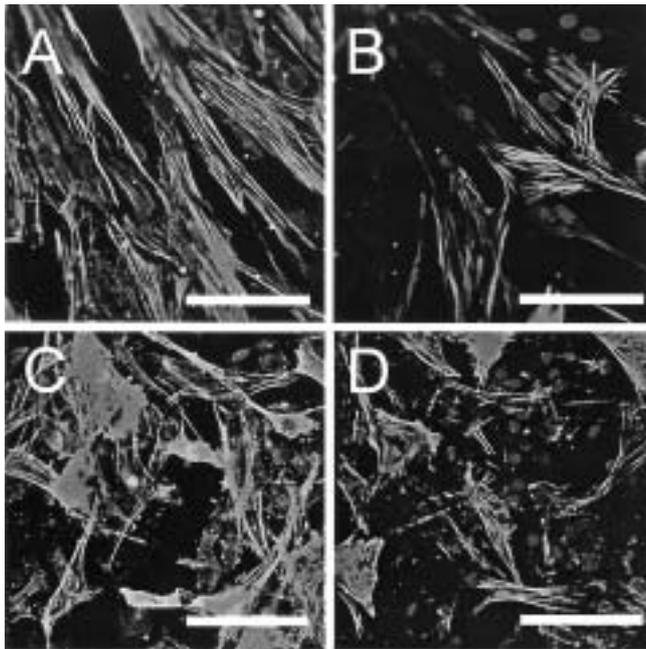


Figure 3: Confocal microscopy images of α -smooth muscle actin expression. A,B) Porcine aortic smooth muscle cells (PASCs). C,D) Porcine aortic valve interstitial cells (PAVICs). A,C): 2D culture. B,D): 3D culture. Scale bar = 50 μ m.

Extracellular matrix synthesis

Total soluble protein and sulfated glycosaminoglycan (GAG) content was measured at days 2 and 6. Protein content was assessed using the BCA Total Protein Assay Kit (Pierce; Cat. No. 23225), and sulfated GAG content was determined using the Blyscan sGAG Kit (Biocolor; Cat. No. B1000). Constructs were washed thoroughly with room temperature PBS to remove all residual media, lyophilized with a vacuum centrifuge (Jouan RC 10.10), and dissolved in a Tris-HCl-buffered Proteinase-K (Sigma) solution overnight. The BCA and Blyscan protocols were followed for sample analysis, and data were obtained using an ultraviolet spectrophotometer (Biotech Powerwave 340). The Hoechst DNA assay was used to determine construct cell number to normalize protein and GAG content (22). An analysis of variance followed by *t*-tests were used as before, with $p < 0.05$ considered significant.

Results

Two-dimensional culture

PAVICs grew in a similar manner to PASCs, with similar growth rates and morphological appearance (Fig. 1). Smooth muscle cells tended to be slightly larger than interstitial cells, and formed directional patterns in 2D culture. Interstitial cells showed less pattern formation, and were somewhat less likely to

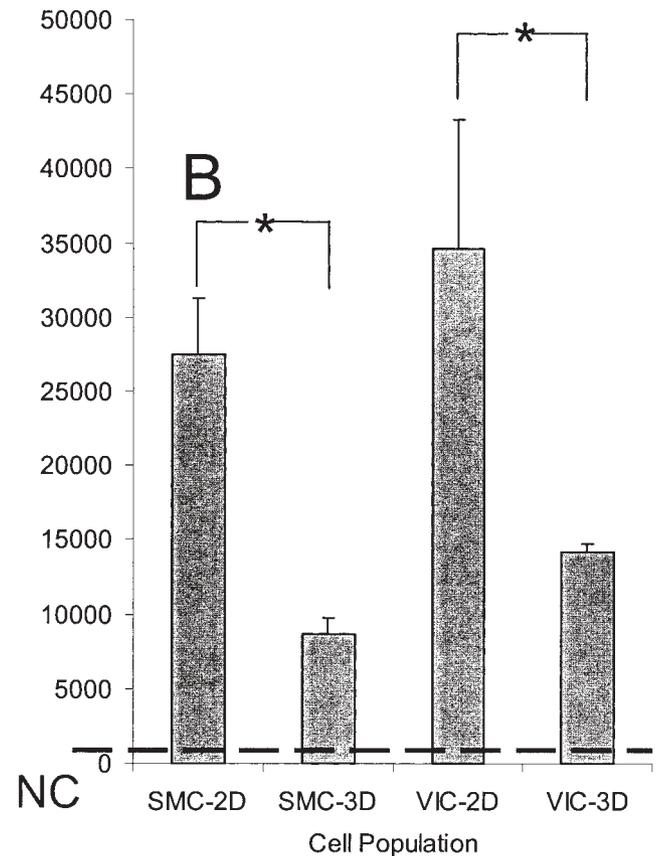
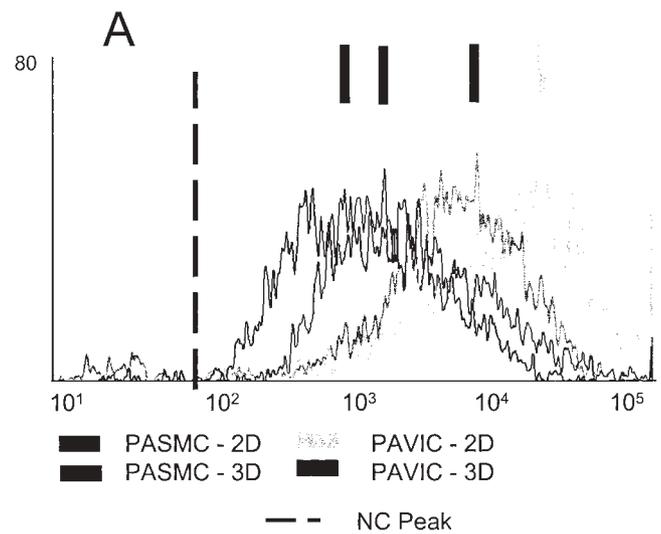


Figure 4: Flow cytometry analysis of α -smooth muscle actin expression. A) Representative population distributions. Lines indicate mean population values. B) Population means comparisons ($n = 4$, $n = 2$ for each condition). *Statistically significant, $p < 0.005$. NC: Negative control expression.

form nodules common to super-confluent smooth muscle cells in flask culture.

Three-dimensional culture

Both smooth muscle and interstitial cells compacted the polymerized type I collagen gel over the six-day period (Fig. 2A). Very slight differences in gel compaction were observed at day 2 ($p < 0.05$), but no differences in compaction were recorded at any other time point analyzed ($p > 0.05$). The discs generally maintained a circular profile during compaction, indicating homogeneous contractile properties and/or cell distribution. The constructs generally decreased in DNA content over the culture period, indicative of a reduction in cell number, but there was no difference between cell types (Fig. 2B). Both the compaction and cell number of each construct stabilized at six days, and the constructs appeared to maintain a specific cell density of approximately 4.2×10^6 cells per cm^3 (Fig. 2C). This may have been due to specific cell-mediated requirements, or limitations of nutrient diffusion. Cell viability could not be assessed directly on constructs used for these specific studies, but similarly made constructs were used in live-dead assays. No difference was observed between cell type, and constructs demonstrated consistently a viability of over 70% (data not shown).

Cell marker expression

Cell phenotype was determined through expression of cytoskeletal markers as previously described. Confocal microscopy images of α -SMA expression for the two cell types in the two different culture conditions are shown in Figure 3. Both cell types expressed α -SMA in both 2D and 3D culture, but to varying degrees. Flow cytometry results highlighting these differences are shown in Figure 4. No differences were observed between the cell types ($p > 0.005$), but differences were observed between culture conditions ($p < 0.005$). A reduction in α -SMA expression occurred in 3D cultures for both cell types, but almost all the cells were still positive as compared to negative controls. The cytometry histograms indicated broad expression peaks, which indicated that these cells expressed α -SMA in an inhomogeneous manner. There was a subpopulation of cells that did not express the marker at all, suggesting caution when interpreting mean expression levels. It is therefore important to conduct replications to ensure improved accuracy. Desmin expression levels as determined by confocal microscopy and flow cytometry are shown in Figure 5. Clearly, smooth muscle cells expressed desmin to a greater degree than interstitial cells, regardless of culture conditions, in contrast to the α -SMA expression.

Protein content

Constructs containing either interstitial or smooth

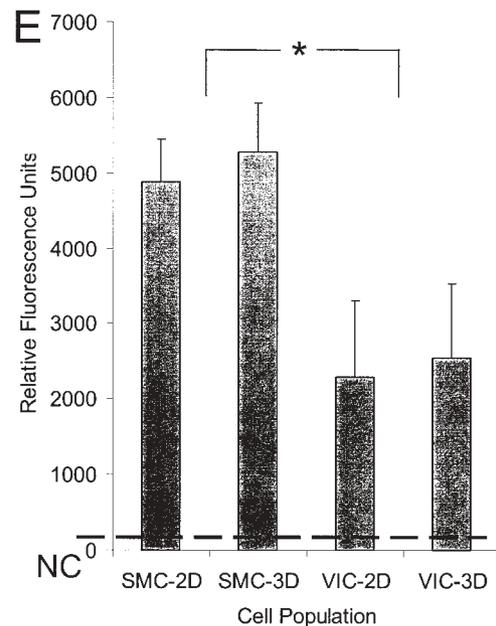
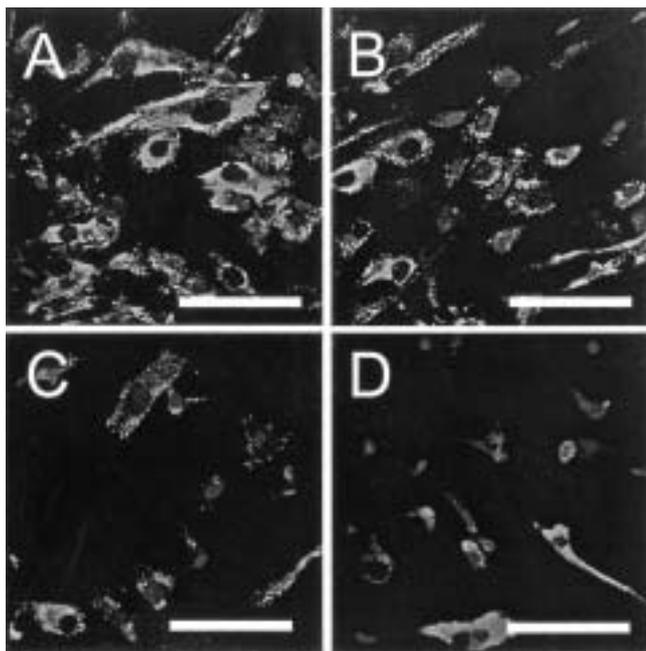


Figure 5: Confocal and cytometric analysis of desmin expression. A-D) Confocal microscopy; original magnification, $\times 40$. E) Cytometric analysis of sample distribution means. A,B) Porcine aortic smooth muscle cells (PASCs). C,D) Porcine aortic valve interstitial cells (PAVICs). A,C): 2D culture. B,D): 3D culture ($n = 4$, $n = 2$ for each condition). *Statistically significant, $p < 0.005$. NC: Negative control expression. Scale bar = $50 \mu\text{m}$.

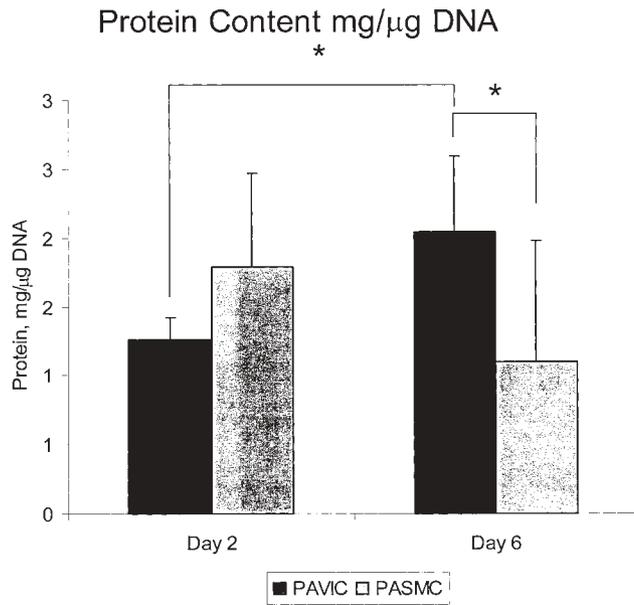


Figure 6: Total soluble protein content of constructs normalized to DNA content ($n = 6, n = 2$ for each condition). *Statistically significant, $p < 0.05$.

muscle cells were assayed for protein content as described previously (Fig. 6). During the course of construct compaction, between days 2 and 6, there was a slight (but not significant, $p > 0.05$) decrease in protein content in smooth muscle cell constructs, but a dramatic increase in protein content over the same period in interstitial cell constructs ($p < 0.05$). Differences in protein content between cell types at day 2 were insignificant ($p > 0.05$), but by day 6 the interstitial cell constructs contained more protein than did the smooth muscle constructs ($p < 0.05$). Taken together, these results suggested that the balance of protein synthesis and degradation was shifted towards synthesis in the interstitial cells, this being in contrast to the degradation observed in smooth muscle cells in type I collagen gels. There may have been some variation in initial protein content because of the differences that might exist between the cellular protein levels.

Glycosaminoglycan content

The sulfated GAG content of interstitial and smooth muscle cell constructs is shown in Figure 7. There were fewer GAGs per DNA in the interstitial constructs at day 2 compared to the smooth muscle constructs ($p < 0.05$). By day 6, however, there was no difference in content between the two cell types ($p > 0.05$). The smooth muscle constructs did not increase in GAG content over the six-day period ($p > 0.05$), whereas the interstitial cells did ($p < 0.05$). Differences in GAG content at day 2 may indicate differences in cellular GAG content between PAVICs and PASCs. These results

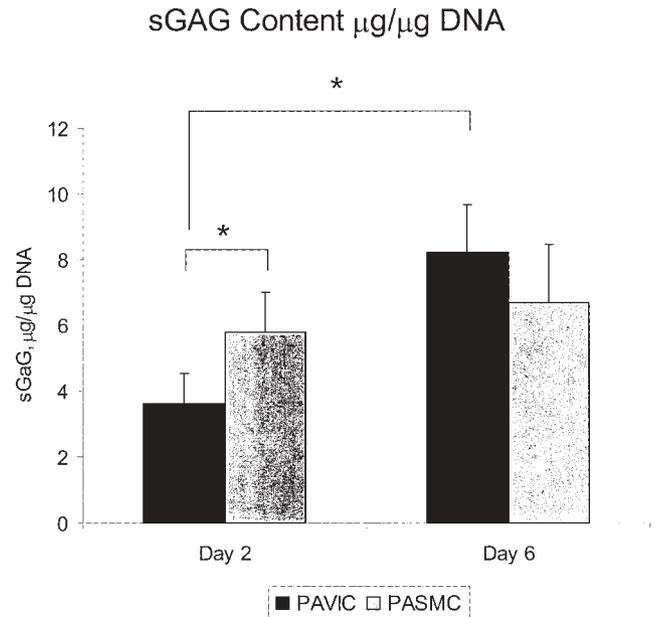


Figure 7: Total soluble sulfated glycosaminoglycan content of constructs normalized to DNA content ($n = 6, n = 2$ for each condition). *Statistically significant, $p < 0.05$.

indicate that the balance between synthesis and degradation of GAGs for interstitial cells is shifted towards synthesis, whereas for smooth muscle cells, such a shift was not observed.

Discussion

The mechanical environment of the aortic valve is highly complex and demanding, and bioprosthetic valve substitutes eventually fail under the loads imposed. These non-living valves lack a viable cell component that can remodel and repair the tissue matrix as it develops microscale defects under stress, without these growing to macro tissue failure. Tissue engineering approaches to replacing a diseased aortic valve incorporate living cells and relevant matrix components to create a viable valve that, in theory, can grow and remodel with the patient. This of course would be particularly beneficial for pediatric patients. While this technology shows promise, little is known about the biological functions that these devices are intended to replace. Certainly, adequate tissue mechanical properties are fundamentally critical to the success of these devices, but many biological functions may need to be present for long-term implant success. Understanding these functions will certainly become important in the future.

The results of the present studies demonstrated some similarities and some differences between the phenotypes of aortic valve interstitial cells and aortic smooth muscle cells. Both interstitial cell- and smooth

muscle cell-populated constructs compacted to similar degrees over the culture period, and both cell types expressed similar levels of α -SMA. The latter is a structural protein which is present in smooth muscle, and its expression is correlated with gel compaction in other cell types (23), indicating a contractile phenotype. A significant reduction in α -SMA expression was measured in 3D gel culture, highlighting the role that matrix interaction plays in modulating cell phenotype (24). Desmin expression was different between the two cell types, with expression by aortic smooth muscle cells greater than that of interstitial cells. Desmin is an intermediate filament that is involved in supplementing contractile function (25). Based on the results of the present study, desmin expression is not correlated directly to gel compaction, but rather to cell type. The increased expression of desmin may be related to differences in the synthetic properties observed, and this is discussed later. Several reports have been made of interstitial cell expression of fibroblast markers in situ, with limited expression of α -SMA (26,27). Others have shown that cell phenotype can be modulated through culture conditions. α -SMA expression can be enhanced through mechanical or biochemical stimulation, and matrix composition is also involved in this process (22,24). Rabkin et al. demonstrated that the long-term culture of tissue-engineered valvular substitutes modulated the expression of α -SMA to a degree similar to that seen in vivo, but that the expression levels of these molecules in vitro were initially different (28). It is therefore probable that culture conditions and time in culture are also modulators of marker expression. It is also important to note the relatively broad expression peaks of the different markers, indicating a relatively wide range of expression between individual cells within these groups. There were no observations of multiple positive expression peaks in any experiment, suggesting that the expression range is associated with a single cell type, and not multiple cell types. Many factors may be associated with the range of expression between individual cells; hence, an assessment must be made of the distribution of expression levels among cells in any particular sample.

The marked differences in protein and GAG content indicate an ability of the valvular interstitial cell to synthesize matrix in 3D culture, apart from biochemical or mechanical stimulation. This was not evident for smooth muscle cells in the present investigation. It is possible that the increased expression of desmin in conjunction with α -SMA may commit the smooth muscle cell to a more singularly contractile phenotype, and thus limit its synthetic capabilities. It should be understood that a combination of synthesis and degradation is likely to occur within these cells, and content measures can only indicate the 'net' results of this bal-

ance. As the gel constructs were created with cells and a collagen solution, limited conclusions can be drawn about the initial protein content. There might also be more protein associated with the larger smooth muscle cells, and changes in protein content may provide a framework for further studies related to these effects.

The limited initial GAG content, followed by dramatic increases in GAG levels in PAVIC constructs that were not apparent in PASMIC constructs, demonstrates the different GAG synthetic capacity of PAVICs in 3D cultures. GAGs are an important component of valve leaflets, and particularly of the spongiosa layer, which is primarily responsible for lubrication between the two load-bearing layers (ventricularis and fibrosa) and for reducing internal shear between them (29). A successful living valve substitute would need the ability to synthesize GAGs in order to maintain this important function.

Taken together, these results indicate that valvular interstitial cells possess the ability to contract tissue and synthesize matrix components in collagen gels. Normal smooth muscle cells have a limited capacity to synthesize matrix, and dedifferentiated smooth muscle cells have a limited ability to control matrix synthesis, as well as a reduced contractile ability. This raises concern about the use of vascular smooth muscle for tissue-engineered valvular substitutes. Such concern must be balanced, however, with the ease of obtaining smooth muscle cells, for example from a peripheral vessel, compared to interstitial cells, for which there is no readily available source. Further investigations are required to elucidate more fully the differences between these two cell types.

Acknowledgements

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Meeting discussion

DR. IVAN VESELY (USA): Besides the dimension of your two groups, 2D versus 3D, the other factor is the presence and absence of tension. You have tension on the 2D plates, and because you have a free-formed

contraction you have almost an absence of tension. I know that you are working on tubular grafts where there is some tension in a 3D environment. Have you looked at this pattern of expression in the constrained or 3D structures with tension in them?

DR. JONATHAN T. BUTCHER (Atlanta, GA, USA): We have only carried out a form of cursory Movats immunohistochemistry with the tubular constructs, and they do have a geometric constraint that allows the development of some tissue fiber anisotropy. We think that will be useful in progressing towards a tissue-engineered leaflet model for use in other studies, but we have not yet progressed much in that area.

DR. PATRICIA TAYLOR (United Kingdom): When you were growing the cells in the two dimensions, did you coat the flasks with anything, or were the tissue culture flasks uncoated?

DR. BUTCHER: No, we did not coat the tissue culture flasks.

DR. TAYLOR: That is another difference in the 3D environment, where the cells are surrounded by collagen.

DR. BUTCHER: Correct, but these cells grow over sev-

eral days, and it is unclear what they are producing - we have seen that they do several different things.

DR. PETER ZILLA (South Africa): Smooth muscle cells, when cultured, are secretory cells and look very similar to fibroblasts or myofibroblasts, with very low levels of actin. They are also very slow in changing to the resting type, which is packed with actin. Do you think that 10 days was long enough for them to accumulate the actin content of a true contractile smooth muscle cell, or did you consider longer 3D culture times?

DR. BUTCHER: We picked the 10-day point based on our other studies with vascular smooth muscle cells. When using different mechanical stimuli, we showed that the smooth muscle cells in these constructs behave differently. To keep everything the same, we chose the 10-day time point, but we could extend it to see if there are any changes in expression.

DR. ZILLA: A very old method such as transmission electron microscopy is wonderful for smooth muscle cells, because you see instantly when they change to the resting state.