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GENE EXPRESSION AND COLLAGEN FIBER MICROMECHANICAL INTERACTIONS OF THE SEMILUNAR HEART VALVE INTERSTITIAL CELL

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Abstract

The semilunar (aortic and pulmonary) heart valves function under dramatically different hemodynamic environments, and have been shown to exhibit differences in mechanical properties, extracellular matrix (ECM) structure, and valve interstitial cell (VIC) biosynthetic activity. However, the relationship between VIC function and the unique micromechanical environment in each semilunar heart valve remains unclear. In the present study, we quantitatively compared porcine semilunar mRNA expression of primary ECM constituents, and layer- and valve-specific VIC-collagen mechanical interactions under increasing transvalvular pressure (TVP). Results indicated that the aortic valve (AV) had a higher fibrillar collagen mRNA expression level compared to the pulmonary valve (PV). We further noted that VICs exhibited larger deformations with increasing TVP in the collagen rich fibrosa layer, with substantially smaller changes in the spongiosa and ventricularis layers. While the VIC-collagen micro-mechanical coupling varied considerably between the semilunar valves, we observed that the VIC deformations in the fibrosa layer were similar at each valve's respective peak TVP. This result suggests that each semilunar heart valve's collagen fiber microstructure is organized to induce a consistent VIC deformation under *its respective diastolic TVP*. Collectively, our results are consistent with higher collagen biosynthetic demands for the AV compared to the PV, and that the valvular collagen microenvironment may play a significant role in regulating VIC function.

Keywords

Valve morphology; extracellular matrix; microstructure; cellular deformations; mechanobiology; heart valve remodeling; tissue engineered heart valve

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INTRODUCTION

The aortic (AV) and pulmonary (PV) heart valves, termed “semilunar” due to their leaflet shape, have the basic function to prevent retrograde blood flow back into the ventricles during diastole. Valve mechanical function spans multiple length scales from organ-scale hemodynamic phenomena to underlying tissue and cellular components (21). Both the AV and PV experience significant in-plane tension resulting from transvalvular pressure (TVP) during diastole (21); approximately 90 mmHg and 15 mmHg for the AV and PV, respectively. As a result of this difference, the AV and PV extracellular matrix (ECM) organization and concomitant mechanical properties differ considerably (1, 6, 11). Normal tissue morphology and function rely upon the constituent cell population's ability to detect forces appropriate to their correct tissue context (8). However, it is not known whether the AV and PV valvular interstitial cell (VIC)-tissue mechanical coupling and VIC function for each valve are also distinct.

The semilunar valves consist primarily of three histologically distinct fibrosa, spongiosa, and ventricularis layers, except in the coaptation region that is a single collagenous layer (18). The fibrosa is oriented towards the associated artery and is composed mainly of a dense, highly aligned network of Type I collagen fibers. For the AV the fibrosa layer dominates the mechanical response compared to the ventricularis layer (25). The ventricularis faces the ventricle and is composed of primarily of elastin and a more loosely structured collagen fiber network. The spongiosa, located between the fibrosa and ventricularis, is rich in glycosaminoglycans, and has been observed to have collagen fibers transverse it that interconnect the fibrosa and ventricularis layers (25). Structural adaptations to applied stress, especially in the collagen fiber network, are also distinct for each valve (11). In the unloaded configuration, the AV leaflet demonstrates substantial regional variations in collagen fiber alignment as compared to the relatively uniform PV (11). As loading commences, the AV collagen fibers straighten more than observed for the PV (11). These differences in valvular tissue structure also are apparent in the mechanical behavior under biaxial load at aortic physiological membrane tension (60 N/m)(6), where the PV is more compliant in the radial direction compared to the AV, while both valves respond similarly in the circumferential direction (6).

Clinical interest for the PV has focused on auto transplantation into the aortic position as a replacement for the AV, especially for the pediatric population (12). This results in a six-fold increase in the PV autograft TVP, with explants from 2-10 weeks having a higher VIC biosynthetic activity compared to explants from 3-6 years (19). These results suggest that the PV autograft can adapt to the increased loading at the cellular level, possibly until a new homeostatic state is achieved although such information is lacking. Moreover, the development of engineered tissue approaches to congenital PV and AV reconstruction require a detailed knowledge of semi-lunar heart valve mechanics and the underlying mechanobiology.

VICs maintain the ECM structure through protein synthesis and enzymatic degradation (28), and are phenotypically plastic in that they are quiescent under homeostatic conditions and active in periods of growth and disease (20). Yet, relatively little is known about how organ level mechanical stimuli result in alterations in semilunar valve biosynthetic activity. Both in our and other laboratories cyclic mechanical stimuli during *in-vitro* culture has been utilized in valvular tissues to elicit alterations in biosynthetic activity (10, 15). These results suggest a link between the VIC phenotype- biosynthetic activity and cellular loading resulting in the specific valve *in-vivo* hemodynamic environment. These studies were directed in part towards understanding how changes in TVP induce concomitant changes in structural and mechanical properties of the AV and PV *in-vivo*. Along these lines, we have determined that

porcine AV VICs undergo larger deformations in the fibrosa layer compared to the spongiosa and ventricularis layers (9).

The above results clearly suggest specific VIC-ECM interactions that may be also unique for each semi-lunar valve, including layer-specific adaptations. To gain insight into this question, we undertook the present study to quantitatively link the semilunar valve VIC biosynthetic activity at the genetic level to differences in VIC location (valve type, layer), deformation level, and quantitative mechanical interactions with the collagen fiber network with TVP. VIC mRNA expression level of ECM constituents and phenotypic markers was measured with qRT-PCR from both semilunar valves. Changes in VIC shape were quantified using semilunar valves exposed to increasing TVP, and compared to differences in valve ECM layer structure and collagen fiber structure. Note for the PV we utilized both physiologic (~15 mmHg) and hyper-physiologic (~90 mmHg) TVP levels in order to compare both its physiological and autograft environments.

METHODS

Histomorphometry

Porcine hearts (from animals 10 months old, and approximately 250 lbs) were obtained within 10 minutes of death from a local USDA approved abattoir (Thoma Meat Market, Saxtonburg, PA). AV and PV leaflets were dissected on site, with the surrounding myocardium trimmed from the leaflets. A central 2 mm strip in the radial direction was removed and fixed in 10% neutral buffered formalin (VWR) for histology. The remaining leaflet was frozen in liquid N₂ and stored at -80°C prior to homogenization for mRNA analysis.

The resulting formalin fixed leaflet tissue was processed as previously described (7, 14). Briefly, Movat's modified pentachrome staining was used to visualize fibrous collagen (yellow), proteoglycan and GAGs (blue), and elastic fibers (black), on 5 µm sections on the radial-transverse surface (Fig. 1). Leaflet thickness, as well as the percent total thickness of fibrosa, spongiosa, and ventricularis layers were evaluated in 3 regions per leaflet for n=7 AV and n=7 PV leaflets using ImageJ software. Leaflet layers were identified by distinct color boundaries whereby the fibrosa layer contained yellow and blue, the spongiosa layer contained blue with no black or yellow, and the ventricularis layer contained only black. Statistical significance of differences between AV and PV dimensions was determined by independent-samples *t*-test with *p*<0.05, with values reported as mean ± standard error.

Real-time quantitative PCR

Porcine AV and PV leaflets were obtained as above and frozen in liquid N₂ then stored at -80°C prior to homogenization and total RNA isolation using Trizol as previously described (5). Samples were then treated with DNase using a DNA-free kit (Invitrogen). RNA was quantified, and 0.5 µg was used for cDNA synthesis with the Superscript RT-PCR kit according to manufacturer's instructions. 1µL cDNA was used for real-time PCR by Taqman gene expression assays and the StepOnePlus system (Invitrogen). Taqman assays used were porcine *GAPDH* (Ss03373286_u1), *Col1a1* (Ss03373340_m1), *Col3a1* (Ss03375691_g1), *Hsp47* (Ss03373335_g1), *matrix gla protein (MGP)* (Ss03394091_m1) and *biglycan* (Ss03375454_u1). An additional custom assay was designed for porcine *periostin* (forward 5'-GGCAAACAGCTCAGGGTCTTC-3'; reverse 5'-TTCCTCTAACCATGCATGAATTTTC-3'; probe 6FAM-TGTATCGTACTGCTGTCTG-MGBNFQ). The identity of the amplified *periostin* DNA fragment was confirmed by sequencing. Taqman assays were used at 1X and custom assays were performed using 900 nM forward and reverse primers with 250 nM probe. Amplification conditions were step 1

95°C 20s; step 2 95°C 1s; step 3 60°C 20s, with steps 2 and 3 repeated for 40 cycles. All amplifications were performed in triplicate for each sample and data were normalized to *GAPDH* levels. Relative expression values also were determined based on normalization to *beta-actin* (Ss03376563_uH) expression, and no significant differences were observed for expression values normalized to either *GAPDH* or *beta-actin*. Data were analyzed using the comparative $\Delta\Delta C_t$ method according to manufacturer's instructions (Invitrogen). Expression values for each leaflet were reported relative to the AV from the same animal, set at 1.0. A total of 15 AV and 15 PV leaflets from 5 individual AV and PV were evaluated. Statistical significance for each marker of interest between AV and PV was determined by a paired samples t-test with $p < 0.05$ (PASW Statistics, V. 18). Values are reported as mean $\pm$ one standard error.

Valve tissue preparation and imaging

All AV and PV tissue sections were acquired from a previous study by Joyce et al., as described in (11). In brief, pairs of AVs and PVs were pressurized and fixed at 0,1,2,4,10,20,60, and 90 mmHg in aqueous glutaraldehyde solution. For each pressure level and valve, the belly region of one of the three leaflets (Fig. 1) was paraffin embedded, and two 5 μm sections were taken and stained with Movats Pentachrome to distinguish VIC nuclei and the three leaflet layers: Fibrosa, spongiosa, and ventricularis (9, 11). Additional 5 μm sections were stained with Picrosirius red and imaged under polarized light microscopy, as previously described, to visualize the natural birefringence of collagen fibers by the periodic bands that correspond to collagen fiber crimp (11). Additional sections were taken at 30 μm for 3D analysis at pressures of 0 and 90 mmHg for the AV to represent both the free float and physiological TVP, respectively, and 0, 20, and 90 mmHg for the PV to represent free float, physiological TVP, and hyper-physiological TVP (in the case of the Ross procedure), respectively.

Each section was deparaffinized, permeabilized with Triton-X for 20 minutes at 1:1000 concentration, stained with Sytox green nucleic acid stain (Invitrogen) for 1:2000 for 15 minutes, and gelvatol mounted with a coverslip for multi-photon microscopy (MPM) (29) with an Olympus FV1000 MPM system equipped with a Chameleon ultra diode-pumped laser operated with Olympus fluoview software. MPM was used to simultaneously image collagen and VIC nuclei via second-harmonic generation (SHG – filter set at 400 ± 100 nm) and two-photon excited fluorescence (TPEF – filter set at 525 ± 50 nm), respectively. Each sample was imaged with a 25x XL Plan N objective (N.A. of 1.05) forming a 500 by 500 μm field of view with a 0.5 μm /pixel resolution to a subsurface z-axis depth of 25-30 μm with a 1 μm step size resulting in 25-30 images composing a stack. An excitation wavelength of 830 nm was used for all samples with a laser transmissivity of 7%. Image stacks were reconstructed into 3D projections for a localized volume (Fig. 4-a) using VOXX (Indiana University Medical Center, Indianapolis, IN, USA), a freeware voxel-based volume-rendering program.

Determination of nuclear orientation and deformation

In the present study we measured VIC nuclear orientation and deformation states between the valves as a function of layer and TVP. Orientation information was acquired from the MPM image stacks, which were imported into a custom Matlab program (The Math-Works, Natick, MA) to acquire the 3D point cloud of each nucleus, from which we performed principle component analysis (PCA) on the segmented VIC nuclei to acquire the 3D nuclear orientation (Figs. 5a-b) (24). On average, ~300 VICs were counted per sample. To distinguish the layers in the MPM images, we utilize the collagen content derived from the SHG signal. Specifically, we found that the spongiosa layer had minimal collagen fiber content as compared to the fibrosa and ventricularis layers. The fibrosa layer was easily

distinguished due to its high collagen content, direction, and thicker size. This observation was confirmed to match the Movat's Pentachrome stain.

We have shown that 2D analysis of VIC nuclear aspect ratio (NAR) is a sufficient and robust measure of total VIC deformation (9, 21). Thus, in the present study we performed additional 2D analysis of VIC deformation on the AV and PV as a function of spatial location and TVP to extend our previous studies on the AV (9). Briefly, using Movat's pentachrome stained sections in the transverse/circumferential plane {Ikhumetse, 2006 #29589; Merryman, 2007 #21716}, a panoramic image was created from one to five overlapping images in order to span the complete thickness of the leaflet (including fibrosa, spongiosa, and ventricularis). This process was repeated for approximately five different locations across the section. The nuclei were identified, spatial locations recorded, and nuclear aspect ratio (NAR, ratio of the longest to shortest axial lengths) determined. The NAR has been shown to be an accurate index of VIC deformation, with NAR determined in the transmural-circumferential plane (Fig. 1). Approximately 60 VICs were counted per panoramic image across five locations of each leaflet, resulting in 300 VIC counted per pressure level. Statistical analysis for each TVP was determined using an independent-samples *t*-test at $p < 0.05$ (PASW Statistics, V. 18). Values are reported as mean $\pm$ standard error.

Relation of VIC deformation to tissue stress level

In the present study we sought to clarify the observed changes in NAR to estimated local tissue stress levels. While a complete computational biomechanical simulation of the semilunar valve response to TVP is beyond the scope of the present study, we approximated the average tissue and fibrosa layer Cauchy stresses, σ , using the Laplace relation $\sigma = \frac{1}{2}PR/t$, P is the TVP, R is the current radius of curvature, and t is the current thickness. We assumed that the deformed semilunar heart valves shape could be approximated using spherical geometry with a radius of curvature of 10 mm that remained approximately constant with increasing TVP. For the thickness we utilized both the fibrosa and total thickness of the AV and PV in the deformed configuration to bracket the estimated stress range.

RESULTS

Structural differences between the AV and PV leaflets

In both leaflets the collagen-rich fibrosa, proteoglycan-rich spongiosa, and elastin-rich ventricularis layers are clearly evident (Fig. 1). The overall AV and PV leaflet thickness in addition to relative thickness of fibrosa, spongiosa and ventricularis layers were quantified morphometrically (Table 1). Overall, the AV leaflets are confirmed to be significantly thicker than the PV leaflets. Comparison of the average thicknesses for the fibrosa layer confirmed that the AV fibrosa layer is twice as thick as the PV leaflet fibrosa layer, as previously reported (11). However, the PV leaflet is not simply a scaled down version of the AV leaflet. The relative thickness of the fibrosa layer of the AV is greater than that of the PV, while the relative thickness of the spongiosa layer of the PV is greater than that of the AV (Table 1). The relative thickness of the ventricularis layer of the AV and PV leaflets is approximately the same for both valves. Hence, on a per layer basis it is confirmed that the overall thickness of the AV leaflet is not only higher than the PV leaflet but the relative layer proportions are also distinct with the fibrosa being thicker for the AV and the spongiosa being thicker for the PV.

Differential expression of ECM genes in AV and PV

The mRNA expression levels of genes corresponding to ECM constituents present in all the leaflet layers of the porcine AV and PV were evaluated. Expression of the major load

bearing fibrillar collagens *Col1a1* and *Col3a1* was determined, in addition to the expression of the molecular chaperone *Hsp47*, which is an indicator of collagen biosynthesis (13, 27). *Biglycan* was assessed as an indicator of proteoglycan gene expression, based on its expression in all layers, with filamentous strands in the spongiosa layer (13). Previous studies have indicated differential expression of *MGP* that serves a protective role in calcification, with higher expression in porcine VIC isolated from PV relative to AV (4). Expression of *periostin*, demonstrated to have a role in collagen fibrillogenesis and aortic valve disease, also was assessed (23). *Col1a1*, *Col3a1*, *Hsp47*, and *Biglycan* expression levels are significantly increased in the AV versus PV (Fig. 2a). *Periostin* is expressed at similar levels in AV and PV (Fig. 2b), as previously reported (13, 23). The higher level of *MGP* expression in AV compared to the PV is distinct from what is observed in cultured VIC (Fig. 2b).

Differences in VIC deformations as a function of valve layer

From visual inspection of circumferential-transverse sections stained with Movat's Pentachrome stain (Fig. 3) both the AV and PV total thickness decreases as a function of pressure. At 0 mmHg, undulations present in the fibrosa layer represented the crimp pattern of the collagen fibers under low tension; the VICs appeared to have a similar circular shape throughout the three layers. As the pressure increased to 90 mmHg, the undulations were no longer present in the fibrosa layer as the collagen fibers were fully loaded (For visualization of this crimp under Picrosirius red stain, see (11) Fig. 7a-b). At 90 mmHg, the VICs in the fibrosa layer are mechanically compacted and elongated with an ellipsoidal shape between organized collagen fibers while the VICs in the spongiosa and ventricularis layer still are fairly circular.

A localized 3D reconstruction of the AV collagen fibril organization and VIC nuclei as a function of the layer and physiological pressure from MPM imaging of a 30 μm circumferential transverse section (Fig. 4) confirmed visual observations of layer dependent VIC deformations with pressure from 5 μm circumferential transverse sections stained with Movat's Pentachrome (Fig. 3). At 0 mmHg for the fibrosa layer, the collagen fibers are undulated and oriented in the circumferential direction. At 0 mmHg, the VIC nuclei exist above, below and between the collagen fibers, conforming to the fibers' natural undulations, and are ellipsoidal in shape. Although collagen is present in the spongiosa and ventricularis layer, the collagen fibers are not organized into large fibrous bundles with a distinct orientation. Compared to the fibrosa layer, the spongiosa and ventricularis layer have large areas without collagen, which may contain other ECM constituents. At 90 mmHg, the collagen fibers are straightened with a decrease in crimp period and amplitude. The space between the fibers is decreased. The VIC nuclei are decreased in sphericity, and are mechanically compacted between the fibers while elongated in the space along the fibers in the circumferential direction. VICs have a higher degree of compaction in the circumferential-transverse plane compared to the circumferential-radial plane, indicating that the spacing between the collagen fibers is smaller in the circumferential-transverse plane. At 90 mmHg, the VIC nuclei in the spongiosa and ventricularis layer do not deform along the circumferential direction. The VICs in the spongiosa and ventricularis layer may not deform with pressure because of the lack of oriented collagen fibers to mechanically compact nearby VICs. Thus, VIC deformation mainly occurs in the fibrosa layer likely due to mechanical compaction by nearby collagen fibers.

To confirm the visual observation that VICs are oriented along the circumferential direction in the fibrosa layer, VIC 3D nuclear orientation was measured from MPM of 30 μm circumferential transverse sections at physiological TVP for both the AV and PV. Overall, the preferred nuclear direction is along the circumferential direction in the circumferential-radial plane, as evidenced by the centroid of θ and Φ at approximately $9.7 \pm 2.8^\circ$ and

$5.3 \pm 4.1^\circ$ for all measured nuclei for both the AV and PV (Figs. 5a,b). For θ (Fig. 5c) there is an increase in alignment for the nuclei in the fibrosa layer of the PV while the nuclear alignment of the AV remained relatively constant. In the spongiosa and ventricularis layer, for θ there is a decrease in overall alignment with load for both the AV and PV. For Φ (Fig. 5d) there is a substantial increase in alignment for both the AV and PV in the fibrosa layer, but a decrease or relatively constant alignment in the spongiosa and ventricularis layers. For the fibrosa layer of the PV, the majority of cellular realignment for both the θ and Φ directions and the majority of fiber realignment occurred by 20 mmHg, the PV physiological TVP (Table 2). Thus, VIC nuclear orientation in the fibrosa layer correlates with collagen fiber alignment. Although VIC nuclear deformation correlated with collagen fiber alignment, this is not the only factor contributing to VIC deformation. The nuclei normalized orientation index (NOI) for θ (Table 2) in the PV fibrosa layer continues to increase alignment along the circumferential direction in the circumferential-radial plane after physiological TVP at 20 mmHg, although the NOI remained constant after 20 mmHg. Further, the nuclei NOI is higher than the collagen fiber NOI. This indicates that after the majority of collagen fibers align, the nuclei still undergo further deformation, and are actually more aligned than the fibers. This confirms visual observations (Fig. 3-4) that the mechanism of VIC deformation is mechanical compaction, not just collagen fiber organization.

Differences in VIC deformation between the semilunar valves

As VICs are oriented along the circumferential direction in the circumferential-radial plane, we were able to simplify the NAR deformation analysis to 2D in evaluating valve-specific differences. The NAR was found to be both layer- and valve-dependent as a function of TVP (Fig. 6). At 0 mmHg the VIC NAR is between 2.0-2.5 (i.e. moderately ellipsoidal as compared to a spherical value of 1.0). The AV and PV fibrosa layer NAR did not change from 0 to 2 mmHg, but from 2 mmHg to 4 mmHg NAR in both valves experienced a similar rapid increase. At 4 mmHg the PV NAR continues to increase at a more gradual rate, exceeding the rate of increase of the AV NAR. By 60 mmHg, the AV NAR achieved a plateau while the PV NAR continued to gradually increase. Interestingly, under diastolic loading, the AV fibrosa NAR (90 mmHg diastolic TVP) was similar to the PV fibrosa NAR (15 mmHg diastolic TVP) at approximately six. The AV and PV spongiosa and ventricularis layers combined have a similar modest but significant increase in NAR from 0 to 10 mmHg, and then remain constant from 20 to 90 mmHg reaching a NAR of 2.47 ± 0.07 and 2.67 ± 0.08 , respectively (not statistically different). There are minimal differences between the trends of the separated spongiosa and ventricularis layers compared to the combined spongiosa and ventricularis (data not shown). At a TVP level of 90 mmHg, mimicking the pressure that the PV experiences in the Ross procedure, the PV fibrosa reached a NAR of 9.67 ± 0.61 that is significantly greater than the AV fibrosa NAR of 5.95 ± 0.27 . This difference in VIC deformation in the fibrosa layer is also visually confirmed (Fig. 3). Hence, the AV and PV have complex nonlinear layer-specific VIC NAR as a function of pressure.

VIC mechanical coupling to collagen fibers in the fibrosa layer for both the AV and PV was further examined by comparing VIC deformations to collagen fiber straightening (Fig. 7). As the TVP increased, the observable straight collagen fiber area increased. The increase in observable straight collagen fiber area is more rapid for the AV compared to the PV, and the range of observable straight collagen fiber area is larger for the AV compared to the PV (Fig. 7-a). The NAR of the VICS in the fibrosa layer of the AV and PV also correlate with observable straight collagen fiber area as a function of TVP (Fig. 7-b). From 0% to 70% observable straight collagen fiber area there is relatively little change in VIC NAR. Above 70% observable straight collagen fiber area, corresponding to approximately 4 mmHg, there is a rapid increase in NAR for the PV compared to the AV. Further, at pressures above 4

mmHg the PV has a higher NAR and a lower observable straight collagen fiber area compared to the AV. At 90 mmHg both the AV and PV have approximately 93% observable straight collagen fiber area, but the PV reached a NAR of 9.67 and the AV reached a NAR of 5.95. This valve-specific correlation between VIC deformation and observable collagen fiber straightening indicated that VIC deformation is specific to the unique mechanical interactions within each valve's collagen fiber network.

NAR of both valves are closer in value at equivalent stresses compared to equivalent TVP regardless of which stress metric was utilized, but are still surprisingly distinct for each valve. Specifically, the AV is under three to six times higher stress than the PV at their respective diastolic TVP assuming that either the fibrosa (Fig 8-b) or the entire thickness (Fig 8-a) bore the load. Thus, the VIC NAR is also a unique function of the local tissue stress within each valve.

DISCUSSION

ECM gene expression and the organization of the AV and PV

The present study demonstrated clear structural and functional differences, from the macro to the micro scale, between the semilunar valves. At the macro scale, the ECM stratification is valve specific, with the AV having a greater total and fibrosa layer (both absolute and proportional) thickness compared to the PV (Fig. 1) (11). The fibrosa layer is predominately composed of dense, type I fibrillar collagen that provides the large structural resilience required by the valve to resist the TVP. Thus, the finding that the AV has a thicker fibrosa layer (both in terms of absolute measurement and relative to the total thickness) was not surprising as its diastolic TVP that is six times greater than the PV. Moreover, we estimated larger tissue stresses in the AV (either total or by the fibrosa layer only, Fig. 8). While based on a simple Laplace law approximation, this finding suggests that the thicker AV fibrosa does not fully compensate for the increased AV TVP; the AV fibrosa tissue is actually under effectively higher stresses compared to the PV.

These findings are consistent with the observed higher baseline collagen gene expression in the AV compared to the PV (Fig. 2), as well as with our *in-vitro* study on isolated VIC culture from all four heart valves (16) where the VICs from valves under the highest TVP (mitral and aortic valves) demonstrated the highest biosynthetic levels. Further, in a recent study that examined collagen hydrothermal stability of heart valve tissues, it was found that collagen in heart valves subjected to higher TVP had lower molecular stability and cross-linking (3). The molecular stability of the collagen in the AV also decreased compared to that of the PV from transition from fetal to neonatal when the TVP in the AV increases due to closure of the foramen ovale and ductus arteriosus (2). Aldous et al. speculated from these findings that a relationship exists between the turnover rate of collagen and mechanical loading whereby there is a faster rate of damage accumulation and collagen turnover in the valves under higher TVP as a mechanism of resistance to biomechanical fatigue loading. While intriguing, these findings are at present correlative and further studies needed to establish specific mechanisms, such as normalization for any differences in total collagen protein levels and to clarify how mRNA changes correspond to specific changes in protein levels in valvular tissues.

VIC ECM layer specific deformation response

Cellular deformation has been shown to occur as a function of organ level load for both native tissues and engineered tissue scaffolds (9, 22, 24). Critical to understanding the mechanobiological response of VICs is how the collagen fiber network locally deforms VICs with application of organ level loads. We note further that VIC-collagen

micromechanical interactions were investigated under increasing TVP to simulate diastolic loading because it is during this phase the leaflets undergo the highest strains. In contrast, during opening/closing the leaflets are submitted primarily to flexure, wherein the leaflets undergo large deflections but small strains (approximately $\pm 7\%$ strain (17)).

In the present study, we observed that VICs in the collagen-rich fibrosa layer significantly deform at higher pressures compared to the VICs in the spongiosa-ventricular layer that deform minimally (Fig. 3-4,6). Visual inspection of 3D image stacks (Fig. 4) and quantification of VIC orientation and deformation (Fig 5-6 and Table 2) suggests that VICs in the spongiosa-ventricularis layer deform minimally because of a lack of large organized collagen fibers. Hence, the VIC-ECM coupling in the fibrosa and spongiosa-ventricularis layers is completely different and may have implications on VIC function in each layer. The present study, taken together with our previous findings (6, 11), clearly indicates that the collagen fiber network organization of each semilunar valve is distinct.

General VIC-collagen micromechanical interactions

Our findings suggest that VIC deformation within the fibrosa layer is driven by changes in the local (as opposed to larger tissue level) collagen fiber structure as a function of TVP (Fig. 7). VICs were found to preferentially align along the circumferential direction, which is the same as the preferred direction of collagen fibers. At a cell-matrix level, VICs are interconnected to the collagen fibers by integrins. It has been shown that blocking of the $\alpha_2\beta_1$ integrin or preventing actin polymerization resulted in a decrease in VIC orientation in the circumferential direction (26). Although demonstrating that integrins are a critical component of VIC-collagen interaction under small loads, under physiological loads we have found that VICs in the fibrosa deform due to mechanical compaction of surrounding collagen fibers (Fig. 7, Table 2). Thus, specific organ level deformations do not directly translate to cellular level deformation but are highly modulated by the local collagen fiber structure.

Valve specific differences in VIC-collagen micromechanical interactions

The substantial differences in the highly nonlinear fibrosa AV NAR compared to the PV as a function of stress confirms that the VIC-collagen micromechanical interactions are fundamentally different between the two valves (Fig. 8). At 90 mmHg the PV had approximately a two times greater NAR than the AV. Thus, in pulmonary hypertension or the Ross procedure, where the PV is under an increased pressure, the PV VICs undergoes a greater deformation than the AV VICs. This increase in cellular deformation may have implications to the PV VIC biosynthetic state, and subsequent leaflet remodeling. The most surprising finding is that AV VIC and PV VIC in the fibrosa have similar NARs at their respective diastolic TVP (Fig. 5c, Table 2). Thus the valve collagen fiber network may be organized to induce a nearly constant NAR at peak diastolic TVP. This finding may indicate that the semilunar valves adjust the fiber network organization in response to TVP and this may be an important feature in maintaining semilunar valve homeostasis and disease. Moreover, we speculate that this is a common feature to all heart valves; which will require additional studies of the other heart valves to elucidate.

Summary and implications

Collectively, our results indicate an interrelationship between ECM organization, VIC gene expression, and deformation with increasing TVP that is highly valve specific. At the tissue level, the AV has both a thicker fibrosa layer that is also subjected to greater stresses than the PV. At the gene level, this is consistent with higher levels of *Hsp47*, *Col1a1* and *Col3a1* mRNA expression in the AV than the PV. At the cell level, VICs undergo substantially larger deformations in the fibrosa layer compared to the spongiosa and ventricularis layers.

VIC deformation in the fibrosa layer also appears to be due to mechanical compaction and elongation between local collagen fibers, whereas in the spongiosa and ventricularis layer there are not large organized collagen fibers and hence may not be able to induce large cellular deformations. Most interestingly, at their respective diastolic TVP for each valve VICs have a similar magnitude of deformation, even though there is a 6-fold difference in pressure and an estimated 3-6 fold difference in stress. Further, at 90 mmHg the PV VIC undergoes a much larger deformation than the AV VIC, which can be reached in pulmonary hypertension or the Ross procedure. Hence, different levels of VIC deformation may lead to modification of ECM gene expression and architecture, as seen with age, disease, or repair (1, 2, 7, 19, 27). This information sets the foundation for a more detailed study on VIC tissue formation and remodeling as a function of hemodynamic loading and VIC-collagen micromechanical coupling.

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Figure 1.

Histological assessment of porcine AV and PV leaflet stratification (inset: partially polarized image of an AV leaflet where C and R represent circumferential and radial directions, respectively). AV and PV leaflets were sectioned along the radial-transverse plane (line labelled as 1) and examined using Movat's pentachrome stain. Elastin fibers predominant in the ventricularis (V) layer are black, proteoglycans predominant in the spongiosa layer (S) are blue, and collagen fibers predominant in the fibrosa (F) are yellow. Relative thickness of the valve ECM layers is indicated by brackets. The AV had a thicker fibrosa layer than the PV. This is consistent with the higher fibrillar collagen gene expression in the AV compared to the PV.

Figure 2.

(A) Differential ECM constituent gene expression in porcine AV and PV as determined by qRT-PCR showing that the AV has a significantly higher baseline expression of fibrillar collagen (*Col1a1*, *Col3a1*, and *Hsp47*) and the proteoglycan *Biglycan* than the PV. (B) Differential gene expression of VIC phenotypic markers was detected by qRT-PCR showing that AV has a significantly higher baseline expression of MGP but expression levels of periostin are not significantly different (* $p < 0.05$).

Figure 3.

Histological assessment of circumferential-transverse sections (see Fig. 1 line label 2) using Movat's pentachrome stain of porcine AV and PV leaflet tissue stratification at 0 and 90 mmHg TVP. At 90 mmHg the PV decreases in thickness proportionally more than the AV, and the VICs deformed more in the fibrosa layer compared to the spongiosa and ventricularis layer in both valve tissues.

Figure 4.

(A) Cross-section of an AV leaflet in the circumferential-transverse (C-T) plane (see Fig. 1 line label 2) showing the fibrosa (F), spongiosa (S), and ventricularis (V) layers as visualized with second harmonic generation microscopy. The white boxes indicate the regions used for the 3D reconstruction in (B), and C, R, and T represent circumferential, radial and transverse directions, respectively. (B) Multi-photon microscopy images used to simultaneously image AV collagen (red) and cell nuclei (green) for a 60x80x20 μm localized volume, via second harmonic generation and two-photon excited fluorescence, respectively. The VIC nuclei (green) can be seen to undergo large deformations only in the fibrosa layer only under 90 mmHg, whereas in the spongiosa and ventricularis layers deformations remain modest.

Figure 5.

(A) A schematic showing the coordinate system used to define the 3D VIC nuclei geometry, with principal axial directions e_1 - e_2 - e_3 , ordered by decreasing magnitude. e_1 was thus used to define the nuclei 3D orientation using the spherical angles θ and Φ . The resulting nuclei angle probability distributions for θ (B) and Φ (C) as a function of TVP in the F-fibrosa layer and S+V - spongiosa and ventricularis layers. Results clearly demonstrated that the majority of the nuclei were oriented along the circumferential axis in the circumferential-radial plane. In addition, VIC nuclei aligned increased with TVP for the fibrosa layer for AV and PV; whereas the PV VIC orientations did not measurably change above 20 mmHg.

Figure 6.

(A) Combined results for the AV and PV NAR as a function of TVP and layer, with F = fibrosa, and S+V = the combined spongiosa and ventricularis layers. Consistent with Fig. 5, VICs deformed significantly more in F compared to S+V. The vertical arrows denote approximate diastolic TVP of the AV and PV, while the horizontal dashed line highlights their respective NAR, and highlights the finding that the nuclei in both AV and PV experience very similar deformations at their respective TVPs.

Figure 7.

(A) Observable straight collagen fiber area expressed as percent total measured area as a function of TVP for both the AV and PV (values for the AV taken from {Joyce, 2009 #22468}). The AV experienced both initially more rapid and also larger total changes compared to the PV. In order to highlight the relation between collagen crimp and VIC deformation, the NAR of the AV and PV fibrosa layer was assessed as a function of both observable straight collagen fiber area from percent total area and TVP in (B). From 0 to 70% observable straight collagen fiber area there was minimal VIC deformation, whereas above 70% observable straight collagen fiber area there was a rapid increase in NAR, with the PV having a higher rate of increase in NAR compared to the AV.

Figure 8.

NAR of the AV and PV fibrosa (F) layer as a function of Cauchy stress, both the total (A) and fibrosa (B) thickness values. At diastolic TVP the AV was under approximately three times greater stress than the PV; yet their respective NAR values are similar. This suggests that the VICs in the AV may be more stress-shielded as a result of the AV fibrosa layer more robust collagen structure.

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Table 1

Morphometry of AV and PV stratification. Layer thickness was measured from Movat's Pentachrome images. Calculations of % relative thickness were made from layer thickness/total thickness. Values are reported as mean $\pm$ standard error of mean (n=7). Statistical significance of differences between AV and PV dimensions was determined by an independent student's *t*-test.

	Total (um) [*]	V (um) [*]	S (um)	F (um) [*]	V/Total (%)	S/Total (%) [*]	F/Total (%) [*]
AV	446 $\pm$ 24	111 $\pm$ 9	140 $\pm$ 16	194 $\pm$ 13	25 $\pm$ 1	30 $\pm$ 2	45 $\pm$ 3
PV	282 $\pm$ 17	68.2 $\pm$ 5.0	118 $\pm$ 13	96 $\pm$ 6	25 $\pm$ 1	41 $\pm$ 3	35 $\pm$ 2

V represents ventricularis, S represents spongiosa, and F represents fibrosa.

^{*} p<0.05

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Table 2

3D VIC nuclear orientation for the semilunar leaflet layers. The normalized orientation index (NOI) for θ represents nuclei alignment, and the NOI for small angle light scattering technique (SALS) represents the ECM fiber alignment. AV and PV represent aortic valve and pulmonary valve, respectively. F, S, and V represent fibrosa, spongiosa, and ventricularis, respectively. Values are reported as mean $\pm$ SEM.

	Nuclei - θ NOI (%)						SALS NOI (%)	
	AV			PV			AV	PV
TVP	F+S+V	F	S+V	F+S+V	F	S+V	F+S+V	
0	75.6	75.6	73.3	66.7	71.1	57.8	50.8 $\pm$ 0.9	36.3 $\pm$ 2.4
20	NA			57.8	75.6	13.3	62.4 $\pm$ 1.5	59.4 $\pm$ 2.1
90	57.8	73.3	33.3	46.7	80.0	24.4	62.0 $\pm$ 1.3	60.3 $\pm$ 1.1