Transitions in Early Embryonic Atrioventricular Valvular Function Correspond With Changes in Cushion Biomechanics That Are Predictable by Tissue Composition

Jonathan T. Butcher, Tim C. McQuinn, David Sedmera, Debi Turner, Roger R. Markwald

Abstract—Endocardial cushions are critical to maintain unidirectional blood flow under constantly increasing hemodynamic forces, but the interrelationship between endocardial cushion structure and the mechanics of atrioventricular junction function is poorly understood. Atrioventricular (AV) canal motions and blood velocities of embryonic chicks at Hamburger and Hamilton (HH) stages 17, 21, and 25 were quantified using ultrasonography. Similar to the embryonic zebrafish heart, the HH17 AV segment functions like a suction pump, with the cushions expanding in a wave during peak myocardial contraction and becoming undetectable during the relaxation phase. By HH25, the AV canal contributes almost nothing to the piston-like propulsion of blood, but the cushions function as stoppers apposing blood flow with near constant thickness. Using a custom built mesomechanical testing system, we quantified the nonlinear pseudoelastic biomechanics of developing AV cushions, and found that both AV cushions increased in effective modulus between HH17 and HH25. Enzymatic digestion of major structural constituent collagens or glycosaminoglycans resulted in distinctly different stress-strain curves suggestive of their individual contributions. Mixture theory using histologically determined volume fractions of cells, collagen, and glycosaminoglycans showed good prediction of cushion material properties regardless of stage and cushion position. These results have important implications in valvular development, as biomechanics may play a larger role in stimulating valvulogenic events than previously thought. (Circ. Res. 2007;100:1503-1511.)

Key Words: chick • development • modeling • ultrasound • flow • aspiration

The development of the atrioventricular (AV) and semilunar valves of the heart from the endocardial cushions occurs concomitantly with a constant barrage of hemodynamic and mechanical forces. Several studies have demonstrated that both blood pressure and velocities increase during morphological development in the heart, implying that the stresses on the endocardial cushions are also increasing.1–3 Early investigations highlighted the motions of cushions in concert with the contracting myocardium, suggesting that they serve a valve-like function before valves form.4 However, a recent study showed that the atrioventricular canal in the tubular early zebrafish heart functions like a suction pump, in contrast to the peristaltic mechanism previously described.5 These observations raise no controversy with respect to current understanding of transitions that occur structurally and molecularly in the myocardium during early tube heart development, but raise major questions with respect to the mechanism through which endocardial cushions function in promoting unidirectional blood flow during the transition from tubular heart to a septated structure. In this study we examined the mechanical properties and myocardial/endocardial cushion mechanical interaction in 3 stages of cardiac development in the chick embryo to better understand how the mechanical properties of the cushions contribute to their functional roles.

Various mutant models demonstrate that genetic defects compromising valve structural maturation result in severe regurgitation, dilatation, and lethality, suggesting that the appropriate maturation of valve matrix is essential for continued function.6,7 These models add weight to the argument that structural properties of AV cushion tissue may be critical determinants of AV cushion function, and that abnormal AV cushion tissue properties (other than mass, which has been well documented) may result in physiological abnormalities in blood flow and cardiac mechanical function before gross morphological disturbances are seen. A link between myocardial elastic and viscous material properties and structural development exists,8,9 and changes in these properties from altered hemodynamic loading argue that mechanical forces do shape embryonic cardiac structural development.1,10–11 The
relationship between mechanics and structure in adult valves has been studied, but not in embryonic cushions. Therefore, connections between cushion structure, hemodynamics, and biomechanics may form a basis for quantifying normal and abnormal embryonic valve function. In this paper we have combined state of the art ultrasonography, optical mapping, and a custom mechanical testing system to quantify and relate these parameters. We found a remarkable fine tuning of the AV cushion biomechanics that coincides with a transition in cardiac pumping mechanisms.

Materials and Methods

In Vivo Kinematics

B-Mode, M-Mode, and Doppler blood velocities were recorded from chick atrioventricular (AV) canals at Hamburger and Hamilton stages 17 through 25 using 55 MHz ultrasound (Vevo660, Visualsonics, Inc; RV708 scanhead) using a custom built environmental chamber (Figure 1). Images and video frames were transferred to image analysis software (ImageJ, NIH) and filter processed for contrast/edge enhancement. B-Mode, M-Mode, and Doppler-generated contours of tissue motions and blood velocities were traced from 6 to 8 different hearts per stage and aligned in the cardiac cycle according to initial cushion opening (supplemental Figure II, available online at http://circres.ahajournals.org). Myocardial contraction, cushion thickness, and blood velocity parameters were then derived from these data streams and compared using ANOVA with P<0.05 denoting significance. Optical recordings of AV canal myocardial activation mapping were obtained as previously described. Additional details are provided in the online supplement.

Mesomechanical Testing

Pipette aspiration systems have been developed and used for measuring mechanical properties of cells and tissues that are too small to be tested by conventional techniques. We developed a similar system to measure material properties of embryonic cushions (supplemental Figure III). The superior and inferior AV cushions at different stages of development were isolated from avian hearts at HH17, HH21, and HH25, representing early, mid, and late cushion formation stages with myocardial wall intact. Cushions were placed in an isotonic bath supplemented with BSA and other amino acids (RB1 medium, kind gift of S. Kubalak Medical University of South Carolina, Charleston) and positioned to the tip of a smooth tip glass micropipette, and adhered at the central portion of the endocardial surface by a small vacuum pressure not capable of distending the tissue (P=0.1 Pa). Aspirated tissue length was then measured simultaneously with applied vacuum pressure. Increased pressure resulted in incrementally less aspirated length, suggesting a nonlinear material response. Previous computational and experimental studies by Ohashi et al and Aoki et al investigated the ability of pipette aspiration to measure nonlinear finite elasticity of soft tissues. Geometric influences were negligible if the tissue sample was at least 5 times the radius of the pipette in diameter and 4 times the radius of the pipette in thickness, which was the case in all of our tissue samples (supplemental Figure III). They then used strain energy based pseudoelasticity theory to model changes in local tissue mechanics, and found that the principal tissue stress was equivalent to the applied pressure. To apply this theory to embryonic cushions, it was therefore assumed that the cushion material response was homogeneous, isotropic, and nonlinear hyperelastic. Billiar and Sacks postulated a pseudoelasticity-based constitutive model for adult valve segments that incorporated an exponential strain energy formulation. As a prerequisite for pseudoelastic theory, cushions were preconditioned with ~20 loading cycles at low pressure (0<P<1.0 Pa) before quasi-static loading to remove previous strain history. 4 to 10 cushions were tested per anatomical position and stage. The nonlinear cushion loading curve (pressure versus stretch ratio) was then modeled using a similar theory as previously described and curve fit by Newton-Gaussian iteration. Material coefficients and effective modulus were compared between cushion location and developmental stage using ANOVA with P<0.05 considered significant, and the data were also assessed for curve fitting by Normalized Standard Estimation of the Error (NSE). Additional details are provided in the online supplement.

Structural Constituent Modeling

Avian superior atrioventricular cushions were isolated as previously described from HH21 hearts and enzymatically digested by collagenase 2 (Case; Worthington, 300 U/mL) to remove collagen, hyaluronidase (HAD; Sigma, 100 U/mL) to remove glycosaminoglycans (GAGs), or cytochalasin D (CD; Sigma, 1 μmol/L) to remove cellular traction forces by inhibiting actin polymerization. Each treatment was incubated at 37°C 5% CO2 for 6 hours under gentle rocking, with untreated cushions serving as controls. 5 to 10 cushions were used per treatment. Mechanical testing was conducted as before, and the resulting modeling curves compared as before. The collagen, cell, and glycosaminoglycan (GAG) specific loading curves were derived from the individual treatment results by approximating strain energy. Mixture theory was then used to predict material responses based on tissue composition. This assumes the total tissue material response is equivalent to the sum of the individual constituent contributions multiplied by their volume fractions. Serial sections through the AV of 4% paraformaldehyde fixed, paraffin embedded chick embryos from HH17–25 were stained with Movat pentachrome to identify volume fractions of cells, collagen, and glycosaminoglycans using color thresholding. These volume fractions were combined with the derived component material curves using mixture theory to model whole cushion responses, which were compared with the actual loading curves using NSE. Additional details are available in the online supplement.

Results

Transitions in Atrioventricular Function in Vivo

Echocardiography of the embryonic AV canal between HH17 and HH25 shows dramatic changes in cushion motions, indicating important changes in atrioventricular canal mechanics (Figure 1). HH17 cushions were largely acellular as evidenced by histology, a feature which correlated well with the lack of echogenicity in the cardiac jelly (supplemental Movie I). Endocardial and myocardial contours traced from these frames show the early cushions had an almost unmeasurable thickness by ultrasonography during the open filling phase (Figure 2, top), but increased in thickness to coat during peak contraction of the AV myocardium (130 μm thick, 30% contraction at approximately 0.8 cycle fraction). The cushions then “treadmilled” longitudinally down the AV canal. Once the longitudinal tissue contraction wave reached the ventricular segment, the canal opened, the cushions retracted, and blood flow was reinitiated. The cushions exhibited simultaneous changes in thickness with the myocardium through the cardiac cycle at the limits of M-mode resolution. HH17 AV blood flow was biphasic: the first phase coincided with canal opening and carried the majority of the flow energy (Figure 3), whereas the second phase correlated with the acceleration in myocardial contraction preceding cushion coaptation. Maximum blood velocity measured in each phase was approximately 3.0 cm/s.

HH21 endocardial and myocardial AV contours (Figure 2, middle panels and supplemental Movie II) showed persistent thickness throughout the cardiac cycle, ranging from 140 to 260 μm, rather than longitudinal treadmilling. The cushions rocked caudally such that their coaptation occurred at a point (or line in 3D) that translated along the AV canal. Also unlike HH17, changes in tissue thickness were approximately 0.20
cycle (72 degrees) out of phase with the contraction of the myocardium. Peak cushion thickness occurred within the range of peak AV myocardial contraction (again 30%), but minimum thickness occurred while the myocardium is rapidly contracting. Blood flow at this stage was also markedly different. Though still biphasic, the initial phase had much slower blood velocity than the second, which now carried most of the flow energy. AV canal flow pathways were still mostly open during the cardiac cycle (Figure 3).

At HH25, as can be seen in Figure 2, bottom panels (and supplemental Movie III), there was no longer any longitudinal tissue motion through the AV canal and the cushions coapted along their entire length simultaneously. The cushions came together and parted rapidly, and the principle flow was a single atrially propelled jet of blood. Cushion thickness varied in phase with the myocardial contraction, becoming thinnest (260 μm) at peak myocardial relaxation and thickest (450 μm) at peak contraction (now only 16%). Average peak blood velocity during this phase was 17.1 cm/s (Figure 3). Our reported AV Doppler velocity profiles compare well with previously published studies.23,24

Comparison of tissue motions and blood flow from each stage suggested that the flow regulation mechanics of the AV canal were tied directly to the material properties of the cushions (Figure 4). A significant inverse linear correlation is apparent when comparing the tissue wave speed and peak blood velocity between embryos over stages 17 to 25 (R=0.899). The nearly acellular gelatinous AV cushions of HH17 heart undulated as a wave with myocardial contraction. The tissue wave speed was approximately 8 mm/s, slower than the peak blood flow. Myocardial tissue wave velocity was diminished at HH21 (≈6 mm/s), whereas peak blood flow velocity was increased (≈11 cm/s). There was persistent though changing thickness of the cushions throughout the cardiac cycle during compression and stretching. At HH25, there was almost no tissue wave (≈2 mm/s) yet the peak blood velocity was much higher (≈20 cm/s). The cushions at this stage change less in thickness over the cardiac cycle than the other stages, suggesting that they were more rigid. These findings highlight changes of AV canal cushion mechanics as being critical to the normal function of the AV canal in regulating unidirectional blood flow.

Alterations in Atrioventricular Conduction Patterns

The propagation of the myocardial depolarization signal changes significantly between HH17 and HH25. As shown in Figure 5, depolarization at HH17 was activated at the superior aspect of the primitive atrial segment (⋆) and progressed in a pseudo-linear manner as indicated by the isochrone lines through the ventricular and outflow segments, compatible with peristaltic/suction pumping. At HH21 the conduction pattern began to transition to a binodal configuration. Depolarization progressed radially through the atrium to the atrioventricular canal, followed by earliest ventricular activation of a portion of the superior ventricle near the inner curvature. By HH25, depolarization of the atria was followed by ventricular activation that was earliest at a zone clearly distant from the AV canal and which then spread independently through the right and left ventricular myocardial tissues (⋆), supporting function as independent piston-like pumps. To determine the velocity of conduction propagation through the atrioventricular canal at these different stages, the length of the canal was measured using the B-Mode ultrasound images, this length being between the initial and final cushion extremes. This length was then divided by the conduction delay (approximately 49, 40, and 46 ms, respectively), which resulted in HH17, HH21, HH25 conduction velocities being 20.1, 18.4, and 10.3 mm/s, respectively. These results show that AV conduction velocity is slowing concomitant with the mesenchymal growth of the cushions, as has been previously shown.25
Nonlinear Pseudoelastic Material Modeling of Developing AV Valve Cushions

To quantify changes in AV cushion material properties during these stages, a mesomechanical test system was developed to apply tensile tests to these small tissues while limiting gripping artifacts. The stress-strain loading curves of isolated valve cushions all showed a monotonically increasing nonlinear mechanical response. As evidenced by low NSE values, our data were modeled well by pseudoelasticity theory, but there were distinct differences between developmental stage (Figure 6 and supplemental Table I). Generally, both AV cushions were extremely pliable at HH17 ($E_{\text{gap}} \approx 0.15$).
Pa) but became successively more rigid at HH21 (E_{eff} = 0.85 Pa) and HH25 (E_{eff} = 3.6 Pa). Effective moduli were statistically significant between stages (P<0.05), but not between cushions. Statistical differences between linear and nonlinear coefficients (supplemental Table I) suggested that the mechanical response of HH17 inferior and superior AV cushions were different, but similar at HH21 and HH25.

Composite Modeling Predicts Cushion Material Properties
Specific enzymatic digestion treatments were applied to HH21 AV cushions to ascertain the contribution of collagen, GAGs, and cells to the material properties. As shown in Figure 7A and 7B, hyaluronidase digestion resulted in a very nonlinear rigid cushion, whereas collagenase digestion resulted in an extremely fragile, more linear elastic tissue. Cytochalasin D treatment of cushions resulted in minimal differences compared with control tissues, and only at large stretch ratios (also supplemental Table I) and without significant differences in effective modulus, suggesting that cell traction forces are not significant contributors to the material properties of the AV cushions at these stages of development.

Histological staining of AV valve cushions at HH17 through HH25 shows dramatic changes in cushion morphology and composition (supplemental Figure IV). It is important to note that from a biomechanical perspective, the structural composition of the material is the driving factor in the material properties of the tissue rather than the size of the tissue or absolute amounts of the constituents. HH17 AV cushions are comprised mostly of GAGs (hyaluronan), but some invading cells are present. Between HH21 and HH25, AV cushions increase in collagen and cell proportion, and image analysis shows that collagen and cell content approach 30% each of the total cushion volume (Figure 8A and 8B).

Interestingly, the derived individual component loading curves (Figure 7C) were similar in trend to the stage specific cushion loading curves, suggesting that cushion structure may regulate tissue biomechanics. To this end, composite modeling of the cushions at HH17 and HH25 was achieved by combining the component strain energies for GAGs, collagen, and cell traction fractions determined from HH21 cushions with the HH17 and HH25 histologically determined volume fractions. Because CD treatments resulted in little differences in material response and cushion mesenchymal cells were surrounded by collagen fibers at HH21 and HH25, we postulated that the collagen volume fractions at these stages were better represented mechanically by adding to it the cell volume fraction. The component fractions are shown in supplemental Table II, and the resulting curves are shown in Figure 8C and 8D. The mixture model generally predicted the measured material response to tensile stress at HH17 and HH25 for both superior and inferior cushions. The low NSE values for these curves indicate that these curves are still relatively accurate given the number of data points and small number of empirical coefficients. These results show that good prediction of the mechanical properties of valvular cushions were possible by composite modeling of the component volume fractions.

Discussion
Embryonic heart development is characterized by dramatic growth and change over a relatively short amount of time, all the while generating unidirectional blood flow. Prior models of endocardial cushion function and their contribution to flow regulation have not accounted for cushion material properties and have been biomechanically oversimplified. The results of
the current study link hemodynamic function with myocardial activation and cushion mechanics to show the spectrum of atrioventricular flow regulation during the transition from a single AV lumen to separate right and left atrioventricular connections. Our studies show a defined progression in AV canal mechanics necessary for the transition in cardiac function from a suction-like pump at HH17 to a piston-like pump at HH25. The relatively weak HH17 cushions are propelled in a treadmill-like fashion by the contracting myocardium, which occurs in a quasi-linear fashion along the looped heart tube. During migration, mesenchymal cells secrete collagen (HH21), rendering the cushions more rigid and assuming rocking mechanics, rather than a wave-like mechanism of apposition. Slowing of AV myocardial conduction and increases in AV blood flow velocities are also noted. By HH25, the cushion mechanics resembled rigid structures meeting along a broad zone of apposition—a probable prelude to successful AV cushion fusion. When fully populated with mesenchyme, cushions contained much more collagen, and exhibited increases in thickness and rigidity. The AV myocardial conduction velocity was further decreased, whereas the AV blood flow velocity was further increased. The increases of blood flow velocity observed, and the transition from passive early filling dominance to atrial

Figure 5. Developmental changes in heart activation. Top row, Maximum intensity projection confocal images from rhodamine-phalloidin stained hearts (method by Germroth et al). Scale bar 500 μm. Second row, Optically recorded action potentials from different heart compartments (raw data). The atrioventricular delay in these examples is 122, 131, and 115 ms, respectively. Time scale 100 ms. Note that the upstroke velocity is slower at the AV region, and at the latest studied stage it is impossible to obtain signal from it (only contraction-related motion is present). Below are shown actual images (80×80 pixels) from the CCD camera. Activation maps in the bottom show separately the atrial and ventricular isochrones in 2 ms intervals. Dark stippling denotes the AV region, light gray marks the contractile part of the conotruncus.

Figure 6. Pseudoelastic modeling of embryonic cushions. Superior (A) and Inferior (B) cushions were tested using the mesomechanical aspiration device, and the resulting data were modeled using pseudoelasticity theory (solid line). The averaged α and C values for an entire data set (4 to 7 cushions) was used to determine the goodness of fit of the data (shown in supplemental Table I). C, Effective moduli of cushions according to M = αC. Each stage is significantly different (P<0.05) from the others, but no differences were observed between anatomical position.
contraction dominance, correspond to the atrioventricular canal flow channels becoming more restrictive in relation to the net volume of blood crossing the AV junction as the junction remodels.

A seminal study by Forouhar et al determined that the early zebrafish heart functions as a suction pump through several criteria, most notably that (1) maximum cushion thickness occurs at peak myocardial contraction, (2) blood velocity exceeds that of tissue velocity, and (3) wave propagation is initiated by a single myocardial source. Our HH17 data are consistent with these criteria, suggesting that this stage chick heart may also behave like a suction pump, in contrast to the peristaltic mechanism previously suggested. A thermodynamic characterization of piston pumping is a volume change–driven propulsion of fluid, with negligible contribution to pumping by the orifice except to throttle the outlet flow. Our data at HH25 is again consistent with this notion, and therefore appears that the HH21 AV may be a transitional stage between 2 pumping styles.

Our data show that material properties of embryonic prevalvular tissues change in concert with changes in their observed mechanics. Using the enzymatic digestions and Cytochalasin D treatments, the individual contributions of collagen, GAGs, and cell traction forces were approximated. Collagen contributed to the majority of the cushion tissue nonlinear rigidity, as has been shown for numerous soft tissues. GAG (mostly hyaluronan in these tissues) material properties were found to be extremely weak, extensible, and mostly linear. Previous studies measuring the material properties of cartilage, which also contains glycosaminoglycans, showed that hydration and swelling also contribute to biomechanical response. GAGs are complex, highly coiled chains that exhibit entropic elasticity: they resist uncoiling from stretching to maintain disorder. The mechanical contribution of GAGs in valve cushions is likely attributable to a combination of entropic elasticity and altered extracellular matrix hydration properties.

We used mixture theory to combine the stress-strain behavior attributable to individual components and found that material properties could be reasonably predicted across developmental stage and anatomical position. Similar formulations were applied to predict material behavior in the aorta as a function of collagen, elastin, and cell contractions, and could predict functional consequences of alterations in component configurations. Deviations in the model at large strains of HH25 AV cushions may be caused by unmodeled interaction effects between constituents or from additional cell/matrix components not considered. This is most clearly demonstrated by the apparent negative strain energy induced by HAD treatment in Figure 7C, resulting in a more rigid tissue but thermodynamically impossible. We believe that the HAD effect is the result of the removal of an important but as yet unknown interaction effect between GAGs and collagen. Indeed, it is likely that all of the assumptions posed in this model will need to be revisited in more complex experimental and computational models, but nevertheless these results demonstrate the utility of this simplified model for predicting material response to changes in matrix.

Hemodynamics may be an important factor in cushion tissue strengthening required for embryonic development to progress. Temporal increases in blood pressure and blood flow velocity must be supported by changes in cushion tissue structure to ensure proper structural integrity. The increasing cushion tissue elastic rigidity with developmental stage reported here may help to maintain morphological integrity under increasing mechanical stress, as has been postulated in adult valves. The nonlinear nature of the stress-strain curve suggests the tissue is pliable under lighter loads but has an innate resistance to deformation by increased loads.
suggests that cushions can preserve their structure under a wide range of hemodynamic forces, and in that way inhibit regurgitation under temporary variations from ideal hemodynamic conditions.

Appropriate transitions in AV cushion properties are likely to include signaling through the endocardial cells that line the lumenal surface of the cushions. It is already known that these cells are unique in their ability to undergo EMT, but they may also play a role in regulating post-EMT cushion morphogenesis through integration of hemodynamic stimuli and signaling of underlying mesenchyme. Vascular endothelial cells are the primary mechanosensitive agents in normal vascular function. Germane to this discussion, it was recently shown that adult valvular endothelial cells possess mechanosensitive functions not shared by vascular endothelial cells, and can stimulate valvular interstitial cells in coculture in response to shear stress. This raises the question whether similar mechano sensitivity mechanisms are factors in normal and abnormal cushion and valvular development. Several studies have shown that obstructing or altering blood flow patterns results in defective heart development. One recent study demonstrated changes in embryonic endocardial expression of mechanosensitive genes in vivo by altered hemodynamic loading. 

These and the results of this study point to endocardial cells as excellent candidates for the mechanosensitive signaling in normal morphological and physiologic cushion morphogenesis.

How endothelial signaling corresponds with myocardial contractile function is currently unknown, but many of the signals implicated in EMT, such as bone morphogenetic proteins, transforming growth factor beta, and vascular endothelial growth factor, require coordination between the myocardium and endocardium. The role of hemodynamics in EMT is still controversial. One study suggests that disruption of myocardial contraction (and likely flow) is sufficient to inhibit EMT. The fact that EMT can be initiated in vitro through the expression or inhibition of a variety of signals (reviewed by Person et al) without hemodynamics does not rule out a necessary role for hemodynamic processes in vivo.

Our article therefore adds to the conclusion that biomechanics play an important signaling role in valvulogenesis. There are many examples where genetic deficiencies result in altered biomechanical properties, which over time lead to early valvular degeneration, as in some palliated congenital heart defects as well as functional defects like bicuspid aortic valve, myxomatous valves, and mitral valves in patients with Marfan syndrome. It may be that biomechanical abnormalities arising from a number of genetic causes may contribute to the development of abnormal morphological phenotypes such as common AV canal as well as normal...
structures that subsequently become dysfunctional prenatally. New biomechanical tools and analytical techniques such as those presented in this study will help to characterize more subtle phenotypes with greater predictive power and, ultimately, clinical potential.

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**Disclosures**

None.

**References**

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Supplemental Materials and Methods

Echocardiography – 48 hour incubated chick eggs were cracked into hexagonal weigh boats placed inside a humidified 150 mm diameter culture dish, and the embryos were further incubated at 38°C until they reached the appropriate stage. Dishes containing embryos from HH17, HH21, and HH25 were placed onto a 3D controlled stage with temperature and humidity maintained by a recirculating heated water bath, heat lamp, and a tent of clear plastic wrap (Saran wrap). Recordings were made at steady state temperatures between 37 and 38 degrees centigrade. Data were recorded for analysis from embryos exhibiting heart rates within previously published norms and without evidence of arrhythmias or atrioventricular regurgitation. It is important to note that bradycardia, irregular rhythms, and significant levels of atrioventricular regurgitation were only seen in hypothermic or stressed embryos. 55 MHz high frequency ultrasound (Vevo660, Visualsonics, Inc.) with an oscillating scan head (RMV708, Visualsonics, Inc.) was used to image the embryonic atrioventricular canal and corresponding cushion motions. 2D B-mode video frames were acquired at 55 fps (at approximately 30 µm axial x 70 µm lateral resolution) of the AV canal from the side of the embryos, resulting in long axis planar views of the myocardium and cushions at each side (Supp. Movies 1-3, and Supp. Fig. 1). Additionally, M-mode images of cushion and myocardial motions were obtained by rotating the scan head to obtain short axis views of the cushion coaptation region (Fig. 1). Finally, Doppler blood flow measurements were taken through the AV canal. Both M-mode and Doppler data were acquired simultaneously under B-mode ultrasound guidance to ensure accurate probe positioning. Because of the variability of embryo rotation and the time required to acquire the aforementioned signals, it was not possible to generate all three data streams for every embryo at each stage.
Image Analysis – B-Mode video frames were transferred to image analysis software (ImageJ, NIH) and processed for contrast/edge enhancement using the 3D Hybrid Median Filter including the center pixel. Doppler waveforms were processed via Fourier fast transform using a bandpass filter. Myocardial and cushion contours were traced over each frame of a cardiac cycle and rendered graphically in a spreadsheet. M-Mode and Doppler-generated contours (Y vs. time and Velocity vs. time respectively) were also digitized through ImageJ (NIH). M-Mode and Doppler data were then registered within the cardiac cycle by aligning the opening of the cushions with the initial spike in blood flow (Supp. Fig. 2). Myocardial contraction and cushion thickness were quantified by subtraction of the appropriate data streams, e.g. superior cushion thickness determined by subtracting the superior cushion Y data from the superior myocardial Y data. 6-8 embryos were analyzed per stage.

Atrioventricular Canal Myocardial Activation Mapping - Freshly isolated chick embryonic hearts at stages 17, 21, and 24 (N ≥ 6 per stage) were stained with voltage-sensitive dye Di-4-ANEPPS and imaged with high-speed CCD camera at 35 C with 20 μm cytochalasin D to control motion artefacts. In most cases, separate recordings of atria and ventricles were necessary because of different focal plane. Computer-generated isochronal activation maps were manually superimposed on high-resolution images of the preparation; for illustration purposes, typical atrial and ventricular waveforms from individual pixels are shown to demonstrate the AV delay.

Mesomechanical Testing System – The modified aspiration system presented in this study consists of a micropipette (inner diameters between 80 and 300 μm have been used) attached to silicone tubing, which in turn is connected to a fluid reservoir (Supp. Fig. 3A). A micromanipulator positions the micropipette tip to the endocardial surface of the excised cushion that is in a bath of RB1 medium. Different vacuum pressures are
obtained by adjusting the height of a column of water initially equilibrated between the
tissue bath and fluid reservoir, and calibrated with an angled micromanometer (Supp.
Fig. 3A, Inset). The tissue bath is placed on a dissection stereomicroscope attached to
a high resolution camera (Zeiss) that records the displacement of the tissue (Supp. Fig.
3B).

Material Modeling – Most micropipette aspiration experiments have been conducted on
single cells using an infinite halfspace configuration with linear elastic material properties
(stress being related to strain by a constant – elastic modulus)\(^1\). Preliminary aspiration
experiments of the AV cushions indicated that they were inherently nonlinear in
response (Supp Fig. 4). Therefore, nonlinear finite elasticity models were employed.
Aoki et al performed computational modeling of hypothetical tissues using strain energy
based finite strain theory\(^2\). They determined that the contributions of tissue geometry to
the material response is negligible (a constant) if the tissue thickness is 4x the pipette
radius and 3x the pipette radius. Also, the thickness of the pipette relative to the radius
results in a single constant (approximately = 1.0 at ratios between 1.5 and 2). Their
finite element analysis determined that the principal tissue stress was equivalent to the
applied pressure. These studies were validated experimentally by measuring local
elastic moduli of atherosclerotic arteries in rabbits\(^3\). A recent paper by Ohashi et al
extended these studies with nonlinear strain energy based finite elasticity theory, namely
pseudoelasticity. They used a polynomial formulation, and measured local elastic
moduli two different ways: pipette aspiration under biaxial tension and pressure-diameter
relationships. They found that the data from the pipette aspiration technique agreed well
with the other methods, supporting its use for measurement of nonlinear finite
deformations.
Therefore strain energy based nonlinear finite pseudoelastic theory was employed to model the material response. This theory states that the loading portion of the stress/strain response can be modeled according to changes in strain energy density, which takes a polynomial or exponential form depending on the tissue and loading conditions. Billiar and Sacks developed a constitutive theory for adult valves that involved an exponential pseudoelasticity theory of the form:

$$ S = \frac{\delta W}{\delta E}; W = \frac{C}{2} \exp[\alpha E^2-1] $$

Where $S$ is the Piola/Kirchoff stress, $W$ strain energy, $E$ finite strain, and $C$ and $\alpha$ are material coefficients that represent the nonlinear and linear portions of the stress-strain curve (Supp. Fig. 3C, 3D). As per the Aoki et al results, the applied pressure was equivalent to the Lagrangian stress $T$, which was then related to strain energy through $T = \lambda S$. An effective modulus $M$ was then determined as the product of the linear and nonlinear coefficients, or

$$ M = \alpha C. $$

Composite modeling of cushions – The changes in material properties with treatments were then used as a basis for composite modeling of the developing cushion. The collagen, cell, and glycosaminoglycan (GAG) specific loading curves were extracted from the individual treatment results by appropriating the strain energy according to the following relationships.

$$ S_{Coll} = S_{HAD} - S_{Ctrl} - S_{CD} $$

$$ S_{Cells} = S_{Ctrl} - S_{CD} $$

$$ S_{HA} = S_{Case} $$

Based on the extreme extensibility and fragility of the collagenase-digested cushions, it was assumed that cell contributions were negligible for the HA case.
Serial sections of the atrioventricular and proximal outflow tract cushions were stained with Movat’s Pentachrome to identify the relative amounts of cells, collagen, and glycosaminoglycans at the different stages of development. The relative contributions changed little across the length (cranial-caudal axis) of the cushions (data not shown) so only the central thickest portion of each cushion was analyzed. Images of sections were taken and color thresholds created in NIH Image were used to determine the volume fractions of each contributor. For composite modeling, mixture theory was used to combine the strain energies of the different components (cells, collagen, GAGs) in proportion to their ratios in the different stages and cushion locations, namely:

\[ S_{\text{TOT}} = \sum \phi_i S_i \]

The composite lagrangian stress strain (T vs. \( \lambda \)) model as determined by the volume fractions was then compared to the cushion data from all stages to ascertain its predictive capability.

References

**Supplemental Data Legends**

Supplemental Movie 1 – HH17 cushion motion video. 55 fps, Scale increment is 100 µm.

Supplemental Movie 2 – HH21 cushions motion video. 55 fps, Scale increment is 100 µm.

Supplemental Movie 3 – HH25 cushions motion video. 55 fps, Scale increment is 100 µm.

Supplemental Fig. 1 – Cushion and myocardial contours of HH17 (top panels), HH21 (middle) and HH25 (bottom) AV canal at intervals of the cardiac cycle, highlighting differences in cushion apposition and thickness. HH17 cushions treadmill across the myocardium and are thickest during peak contraction. HH21 cushions have a persistent thickness and block blood flow by rocking cranially to caudally along the AV canal. HH25 cushions have an almost constant thickness and appose far more rigidly than during the other two stages.

Supplemental Fig. 2 – Composites of M-Mode tracings from 6 different hearts at each stage. Solid lines represent the mean cushion and myocardial positions with error bars denoting standard deviations.

Supplemental Fig. 3 – Design, calibration, and modeling of the mesomechanical test system used in these studies. (A) A smoothed micropipette is placed on the endocardial surface of the excised cushion and a cylindrical portion of tissue is aspirated inside by applying a small vacuum pressure by removing a small volume of bath (B). This pressure was linearly related to the volume of bath removed (Inset). Nonlinear cushion response was modeled by pseudoelasticity theory by the formula $W = C \exp(\alpha E^2 - 1)$, where $C$ varies the nonlinear portion of the curve (C) and $\alpha$ the linear portion.

Supplemental Fig. 4 – Raw data from the mesomechanical testing system. L/a denotes aspirated length (L) divided by the pipette radius (a). Change in height of water column measured from an angled manometer.

Supplemental Fig. 5 – Movat’s Pentachrome staining of serially sectioned AV cushions used to create volume fractions of cells (red) collagens (orange/yellow) and glycosaminoglycans (blue/green). Changes in cushion size and architecture are clearly apparent between HH17, HH21, and HH25.

Supplemental Table 1 – Material modeling coefficients and goodness of fit parameters for HH17, HH21, and HH25 cushions. At HH17, * denotes significant difference between cushions (P<0.05). With enzymatic treatments, * denotes significantly different from control, # from HAD, and $ from Case conditions (P<0.05).

Supplemental Table 2 – Average volume fractions for cells, collagen, and GAGs for each stage and anatomical position of AV cushions. NSE values indicate goodness of fit of the data to the non-linear curve.
Cushion Function Changes over Development

HH17

HH21

Supp. Movies 1-3

HH25
Supplemental Fig. 2
Supplemental Fig. 3
Raw Cushion Response Data

Inferior AVC Response Curves

Superior AVC Response Curves

Supplemental Fig. 4
Superior Cushion

HH17  HH21  HH25

Inferior Cushion

HH17  HH21  HH25

Supp.
Fig. 5
### HH17 Cushion Material Modeling Constants

<table>
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<tr>
<td>AVCI</td>
<td>0.052 +/- 0.01</td>
<td>3.23 +/- 3.77</td>
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<tr>
<td>AVCS</td>
<td>0.028 +/- 0.01*</td>
<td>5.773 +/- 5.12</td>
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### HH21 Cushion Material Modeling Constants

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<tr>
<td>AVCI</td>
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<td>0.162 +/- 0.054</td>
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### HH25 Cushion Material Modeling Constants

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<tr>
<td>AVCI</td>
<td>0.296 +/- 0.11</td>
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<td>AVCS</td>
<td>0.230 +/- 0.10</td>
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### Effects of Enzymatic Treatment on Cushion Material Properties (HH21 AVC)

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<td>Control</td>
<td>0.016 +/- 0.02</td>
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<td>Hyaluronidase</td>
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<tr>
<td>Collagenase</td>
<td>0.014 +/- 0.002#</td>
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<tr>
<td>Cytochalasin D</td>
<td>0.017 +/- 0.004#$</td>
<td>57.637 +/- 13.41#$</td>
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### HH17 Structural Composite Volume Fractions

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### HH21 Structural Composite Volume Fractions

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### HH25 Structural Composite Volume Fractions

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<td>OFTP</td>
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Supplemental Table 2