Published in final edited form as:

Cardiovasc Eng Technol. 2015 June ; 6(2): 141–150. doi:10.1007/s13239-015-0214-1.

Heterogeneity of Mitral Leaflet Matrix Composition and Turnover Correlates with Regional Leaflet Strain

Elizabeth H. Stephens, MD, PhD^{1,2}, Patrick S. Connell, BS², Monica M Fahrenholtz, BS², Tomasz A. Timek, MD³, George T. Daughters, MD⁴, Joyce J. Kuo, BS², Aaron M. Patton, BS², Neil B. Ingels, PhD^{4,5}, D. Craig Miller, MD^{4,5}, and K. Jane Grande-Allen, PhD²

¹Dept. of Cardiothoracic Surgery, Columbia University School of Medicine, New York, NY

²Dept. of Bioengineering, Rice University, Houston, TX

³Dept. of Cardiothoracic Surgery, Meijer Heart and Vascular Institute, Spectrum Health, Grand Rapids, Michigan

⁴Dept. of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, CA

⁵Dept. of Cardiovascular Physiology and Biophysics, Research Institute, Palo Alto Medical Foundation, Palo Alto, CA

Abstract

Purpose—To determine how extracellular matrix and contractile valvular cells contribute to the heterogeneous motion and strain across the mitral valve (MV) during the cardiac cycle, regional MV material properties, matrix composition, matrix turnover, and cell phenotype were related to regional leaflet strain.

Methods—Radiopaque markers were implanted into 14 sheep to delineate the septal (SEPT), lateral (LAT), and anterior and posterior commissural leaflets (ANT-C, POST-C).

Videofluoroscopy imaging was used to calculate radial and circumferential strains. Mechanical properties were assessed using uniaxial tensile testing and micropipette aspiration. Matrix composition and cell phenotypes were immunohistochemically evaluated within each leaflet region (basal leaflet (BL), mid-leaflet (ML), and free edge).

Address for correspondence and reprint requests: K. Jane Grande-Allen PhD, Bioengineering, Rice University, PO Box 1892 – MS142, Houston, TX 77251-1892, Fax: 713-348-5877, Phone: 713-348-3704, grande@rice.edu.

Conflict of Interest Declaration: Elizabeth H. Stephens was supported by individual graduate fellowships from the Hertz Foundation and NIH F30HL094019.

Monica M. Fahrenholtz declares that she has no conflict of interest.

Patrick S. Connell was supported by an individual fellowship from the American Heart Association.

Tomasz A. Timek declares that he has no conflict of interest.

George T. Daughters declares that he has no conflict of interest.

Joyce J. Kuo declares that she has no conflict of interest.

Aaron M. Patton declares that he has no conflict of interest.

Neil B. Ingels, Jr. declares that he has no conflict of interest.

D. Craig Miller was supported by NIH R01 HL067025 and R01 HL029589.

K. Jane Grande-Allen has served as a consultant for Edwards Lifesciences.

Human Subjects Declaration: No human subjects studies were carried out by the authors for this article.

Animal Studies Declaration: The animal studies involved in this article were performed according to protocols approved by the Stanford University and Rice University Institutional Animal Care and Use Committees.

Results—SEPT-BL segments were stiffer and stronger than other valve tissues, while LAT segments demonstrated more extensibility and strain. Collagens I and III in SEPT were greater than in LAT, although LAT showed greater collagen turnover (matrix metalloprotease (MMP)-13, lysyl oxidase) and cell activation (smooth muscle alpha-actin, SMaA, and non-muscle myosin, NMM). MMP13, NMM, and SMaA were strongly correlated with each other, as well as with radial and circumferential strains in both SEPT and LAT. SMaA and MMP13 in POST-C ML was greater than ANT-C, corresponding to greater radial strains in POST-C.

Conclusions—This work directly relates leaflet strain, material properties, and matrix turnover, and suggests a role for myofibroblasts in the heterogeneity of leaflet composition and strain. New approaches to MV repair techniques and ring design should preserve this normal coupling between leaflet composition and motion.

Keywords

mitral valve; extracellular matrix; matrix metalloproteases; collagen; leaflet strain

Introduction

Mitral valve (MV) competence is critical to cardiac function. Indeed, mitral regurgitation portends a significantly higher morbidity and mortality to patients with myocardial infarction independent of left ventricular function [1]. MV competence relies on adequate, timely coaptation between the leaflets. For this to be accomplished, the different segments of the MV undergo complex, heterogeneous motion [2,3] culminating in the distal leaflet regions of the various segments forcefully joining in a precise alignment to prevent blood backflow. Recent studies have shown that the degree of coaptation is critical to mitral regurgitation in diseases such as dilated cardiomyopathy [4]. Numerous studies have characterized leaflet motion and found it to be heterogeneous, with the septal leaflet moving substantially further [2] and having a more rapid closure rate than the lateral leaflet [5]. Strains were found to be heterogeneous even within the septal leaflet [6]. The underlying basis for this heterogeneity, however, remains unknown.

Previous studies have explored the differences in material behavior between regions of the septal and lateral leaflets [7,8] and have attributed this heterogeneity to differential thickness of the collagen-rich fibrosa layer across the leaflet anatomy [7,9]. However, these studies performed materials testing on strips of excised leaflets, and did not address *in vivo* strains and hemodynamics, the valve environment, or the commissural leaflets. In addition, these explanations omit the possible contributions of heart valve cells to tissue mechanics, an area of valve biology that warrants further investigation. Given that valve cells are known to be contractile [10] and to produce extracellular matrix in response to mechanical stimulation [11–13], it would be prudent to explore their potential role in the heterogeneity of valve mechanics. All in all, the relationship between the valvular *in vivo* hemodynamic environment, cell behavior, tissue composition, and material behavior has not been fully explored. The interplay between these factors could explain why, in the context of pathophysiology that changes one or more of these factors, coaptation of the leaflet fails. Quantifying how these factors relate to one another in the context of the healthy valve is the first step to a deeper understanding of how this interplay alters valve function. It was the

hypothesis of this study that valvular cell-mediated differences in extracellular matrix composition and turnover underlie the observed heterogeneity in leaflet motion. Therefore, immunohistochemistry (IHC) was performed to characterize valve cell phenotype, matrix composition, and matrix turnover within the different segments of the mitral leaflets. These characteristics were then related to *in vivo* regional leaflet strains and *ex vivo* material behavior.

Methods

All animals received humane care in accordance with the guidelines of the US Department of Health and Human Services (NIH Publ. 85-23, Revised 1985). The use of animals in this study was approved by the Stanford Medical Center Laboratory Research Animal Review Committee.

Animal Protocol

Radiopaque markers were implanted into fourteen sheep delineating the leaflet of four MV segments (Fig. 1): septal (SEPT), lateral (LAT), and anterior and posterior commissures (ANT-C, POST-C). Procedures for marker implantation and biplane video fluoroscopy have been described previously [14]. Strain, defined as the percent change from minimum to maximum dimensions throughout the cardiac cycle, was calculated for radial and circumferential leaflet segment lengths of the different leaflet regions (Fig. 1, basal leaflet (BL) and mid-leaflet (ML) for SEPT; ML for LAT). It was not possible to calculate circumferential leaflet length change for ANT-C and POST-C due to limitations on marker positioning. The hearts were then harvested and stored in formalin.

Materials Testing

For materials testing and micropipette aspiration, lamb hearts were obtained from a local commercial abattoir (Fisher Ham and Meat, Spring, TX). Mechanical analysis was performed as described previously [15]. Circumferential LAT, circumferential mid leaflet SEPT (SEPT-ML), circumferential basal leaflet SEPT (SEPT-BL), circumferential commissure (COMM), radial LAT (LAT-RAD), and radial SEPT (SEPT-RAD) segments were evaluated with a pre-determined uniaxial tensile testing protocol using an EnduraTEC ELF 3220 (Bose, Eden Prairie, MN). In brief, thickness, width and gauge length of the tissue segments were measured prior to testing. Testing was conducted in 37°C phosphate buffered saline. Tissue segments were preconditioned using ten loading-unloading triangle waves, then elongated to failure.

Mechanical data was then converted from load and displacement to stress-strain curves for analysis conducted using MATLAB software (MathWorks, Natick, MA). Gauge length for each test was adjusted according to protocols developed from previously described methods [16]. Maximum tangent modulus was calculated from the slope of the post-transition region of the bilinear stress-strain curve, extensibility was calculated as the x-intercept of the line defined by the maximum tangent modulus, and the ultimate stress and strain were defined as the stress and strain values at the maximum stress obtained throughout failure tensile testing.

Micropipette Aspiration

Sheep hearts for micropipette aspiration were obtained from a local commercial abattoir. A total of 12 mitral valves were obtained. A 5 mm square sample was obtained from each segment of the annulus, corresponding to marker positions 3 (SEPT), 7 (LAT), 5 (ANT-C) and 1 (POST-C). Fresh tissues were then tested by micropipette aspiration as previously described [17] on the atrial side of the leaflet adjacent to the myocardium at the annular border. Experimental images captured during the testing were analyzed using ImageJ software (NIH) to measure the aspiration length and pipette diameter, and pressure data was acquired from a pressure transducer via a National Instruments DAQ system. Data analysis was performed using a finite-strain hyperelastic exponential constitutive model as previously described, and the effective modulus (M) describing the local stiffness of the tissue was derived from the product of the two material constants in the model [18].

Histology and Immunohistochemistry

For each MV segment a 3-5 mm wide strip was cut from insertion region to free edge (Fig. 1), embedded in paraffin, and sectioned to a thickness of five microns. Each sample was stained with Movat pentachrome as well as IHC to demonstrate the myofibroblast valve cell phenotype, extracellular matrix components, and matrix turnover (Table 1). Staining intensity was quantified on blinded IHC sections using Image J software (NIH, Bethesda, MD) for three regions of the valve, (BL, ML, and free edge (Fig. 2)) and for each histological layer (fibrosa, atrialis, and spongiosa; only spongiosa was quantified in the ML)). Analysis of blinded Movat-stained sections included measurements of leaflet length and distance of proximal muscle insertion relative to total leaflet length using ImageJ Pro software (Media Cybernetics, Bethesda, MD). Semi-quantitative grading was performed to evaluate delineation between leaflet layers using a pre-determined grading rubric ranging from 0 (minimum) to 4 (maximum) as previously described [19].

Statistical Analysis

Multifactorial analysis of variance was performed using SigmaStat (SPSS, Chicago, IL) or R with the level of significance set at 0.05. Tukey's post-hoc tests were applied to examine differences between groups within each level. Data sets that were not normally distributed were rank transformed before analysis. Paired t-tests were used to compare strain between matched leaflet pairs (SEPT/LAT or ANT-C/POST-C).

Correlations between staining intensities of different proteins within individual leaflet layers of specific regions and segments were used to assess protein co-localization as an indication of collagen turnover and/or the presence of the myofibroblast phenotype. Correlations between protein intensities and *in vivo* leaflet strain were analyzed to assess further any relationships between strain and composition. These correlations were calculated using a Pearson rank order test for normally distributed data and Spearman rank order correlation for non-normally distributed data. For correlations between intensities of different proteins, $p = 0.02$ was considered a trend and $p = 0.00625$ was considered significant since 8 proteins were compared. For correlations between intensities of proteins and regional strain, each protein was considered individually, therefore $p = 0.05$ was considered significant, but only highly significant correlations with $p < 0.03$ are reported.

Results

Differences in Leaflet Strain Across Segments

Over the cardiac cycle, the radial strain (percent change in length) of LAT was greater than in SEPT ($p=0.004$, Fig. 3A), as was the ML circumferential strain ($p=0.031$). The radial strain of POST-C was greater than for ANT-C ($p=0.011$). Within SEPT, the radial and circumferential leaflet strain in ML were greater than in BL ($p=0.001$, Fig. 3B).

Differences in Leaflet Structure Across Segments

The total leaflet length differed between leaflets, being greatest in SEPT, followed by LAT, and then ANT-C/POST-C ($p<0.001$, data not shown). Interestingly, delineation between leaflet layers was greatest in SEPT, then in ANT-C/POST-C, and lastly in LAT (Fig. 4A, $p=0.011$). The length of the muscle insertion into the leaflet region relative to total leaflet length was highest in ANT-C (32%), much less in LAT (13%) and POST-C (10%), and minimal in SEPT (2%, $p<0.001$). Analysis of Movat-stained sections revealed further structural differences between the leaflets. In the ML region of LAT where the chordae tendineae insert, the leaflet fibrosa contained collagen, PGs, and other matrix components, and the chordae tendineae insertions were apparent as branches coming off the fibrosa. In contrast, in the commissural leaflets, the collagen-rich chordae tendinae often appeared as continuous with the fibrosa. Therefore, the leaflet fibrosa of the commissures contained proportionately more collagen than did the LAT leaflet (Fig. 4B).

Compositional Heterogeneity Between Leaflet Segment Pairs

The expression of Col I in SEPT was greater than in LAT across all leaflet regions (Fig. 5a; $p<0.025$ for ML, BL; $p=0.06$ (trend) for free edge), as was Col III ($p<0.005$). In contrast, the expression of collagen-degrading enzyme MMP13 was greater in LAT than in SEPT (Fig. 5b; $p<0.005$ for BL, ML). LOX, an enzyme involved in collagen and elastin crosslinking, was similarly greater in LAT compared to SEPT ML (Fig. 5c; $p<0.008$). The proteoglycan DCN, which mediates collagen fibrillogenesis, was greater in LAT BL than in SEPT BL ($p<0.007$; data not shown). Two indicators of valve cell activation, SMaA and NMM, were greater in LAT than in SEPT (both BL and ML, $p<0.0002$ for SMaA, $p<0.0001$ for NMM (not shown)).

These patterns of expression for collagen, collagen turnover enzymes, and markers of cell activation paralleled those of radial and circumferential strains. For example, MMP13 abundance was correlated with circumferential strain in LAT ML ($r=0.733$, $p=0.020$) and radial strain in SEPT BL ($r=0.99$, $p=0.001$). SMaA expression in the atrialis layer was correlated with circumferential strain in SEPT-BL (0.935, $p<0.001$) and with radial strain in SEPT-ML ($r=0.700$, $p=0.03$). In LAT ML, NMM expression was correlated with both radial and circumferential strain ($r=0.857$, $p=0.006$ and $r=0.821$, $p=0.0145$, respectively). Furthermore, in SEPT, the abundance of NMM, SMaA, and MMP13 was greater in the ML fibrosa than in the BL fibrosa ($p=0.037$), which was consistent with greater strains in SEPT ML compared to BL (Fig. 3b).

Compared to ANT-C, POST-C demonstrated greater expression of Col I and Col III in BL and ML (each $p < 0.0005$), SMaA in ML ($p < 0.008$), and MMP13 in ML ($p < 0.003$). The greater expression of SMaA and MMP13 in POST-C vs. ANT-C was consistent with the greater radial strain in POST-C ($p = 0.011$).

Compositional Heterogeneity Across All Mitral Valve Segments

To understand the heterogeneity of collagen expression and remodeling enzymes throughout the mitral valve leaflets, all segments were compared. Expression of Col I and Col III in both ML and BL were greatest in POST-C, followed by SEPT, then ANT-C, and lastly LAT (overall $p < 0.001$ for each of ML and BL; Fig. 5a). MMP13 expression was greatest in POST-C, followed by ANT-C, then LAT, and lastly SEPT (each region overall $p < 0.007$; Fig. 6). Similarly, expression of the crosslinking enzyme LOX (Fig. 5c) was greater in the commissural leaflets than in the SEPT and LAT, both closer to the annulus (BL, overall $p = 0.022$) and in the ML (overall $p < 0.001$). In contrast, expression of DCN in the BL was greatest in LAT, followed by ANT-C/POST-C, and then SEPT (overall $p = 0.019$).

With regards to cell activation, expression of SMaA in BL and ML was greatest in POST-C, followed by ANT-C and LAT, and then lowest in SEPT (overall $p < 0.001$ for each of BL and ML; Fig. 5b). In BL, NMM was greatest in LAT, followed by POST-C/ANT-C, and then was again lowest in SEPT (overall $p = 0.016$; data not shown).

For proteins related to elastic fibers, expression of elastin in ML was greatest in ANT-C/POST-C, followed by SEPT and LAT (Fig. 5c, overall p -value: $p < 0.001$). Similarly, expression of MMP9, an elastin degrading enzyme, in ML was greatest in ANT-C/POST-C, followed by SEPT and LAT (overall $p = 0.032$; data not shown).

Correlation Between Proteins Within Leaflet Regions

Within specific leaflet regions and layers, strong correlations were found between markers for cell activation and enzymes involved in matrix turnover, suggesting the presence of myofibroblasts. For example, MMP13 and SMaA were correlated in the ML across all layers (0.764, $p < 0.00001$), as well as in the BL fibrosa (0.810, $p < 0.00001$) and free edge (0.656, $p = 0.003$). MMP9 was correlated with both SMaA and NMM in the BL fibrosa (0.576, $p < 0.0022$ and 0.578, $p < 0.0014$, respectively).

Material Properties of Leaflet Regions

SEPT-BL regions (circumferential) were stiffer (larger maximum tangent modulus) and stronger (larger ultimate stress) than LAT, SEPT-ML, LAT-RAD, and COMM regions as shown by tensile testing (Fig. 7a, $p < 0.05$). This finding was confirmed by micropipette aspiration, where SEPT-BL regions were stiffer than SEPT-ML, LAT, and ANT-C regions (Fig. 6, $p < 0.05$). In addition, LAT-RAD regions were found to be more extensible than SEPT-RAD and LAT regions by tensile testing (Fig. 7b, $p < 0.05$). There was no difference in ultimate strain between the different segments.

Discussion

This research provides the first *in vivo* evidence that leaflet strains are directly related to local leaflet material properties, matrix composition, matrix turnover enzyme levels, and valve cell activation, and moreover that these relationships are heterogeneously patterned across the valve leaflets. *Ex vivo* material properties are consistent with the *in vivo* strain patterns, with regions of low strain associated with stiffer and stronger tissues (SEPT-BL) and areas of high strain associated with high extensibility (LAT and SEPT-RAD). Although collagen content in SEPT was greater than LAT, collagen remodeling enzymes were expressed at higher levels in LAT. The greater abundance of these enzymes suggest increased collagen turnover, which would be consistent with greater strains in that leaflet. The presence of activated valve cells paralleled these greater strains and increased turnover enzyme presence in LAT. Similarly, collagen turnover enzyme levels and valve cell activation in POST-C was greater than ANT-C, again suggesting greater collagen turnover, which is consistent with greater strain in POST-C. Comparison of all four leaflets revealed that the commissures had the highest collagen content, as well as the highest collagen and elastic fiber remodeling enzyme levels and valve cell activation. Strong correlations between cell activation and matrix turnover markers suggest the role of myofibroblasts in the observed compositional heterogeneity.

Relations Between Strain, Material Properties, Matrix Composition, and Myofibroblasts

Overall, this work suggests that there is a relationship between leaflet strains during the cardiac cycle, material properties of the tissue, levels of matrix turnover proteins, and valve cell activation towards the myofibroblast phenotype, as demonstrated through analysis of different MV leaflets and leaflet regions. This work validates previous *ex vivo* aortic valve studies showing that leaflet strain leads to altered leaflet composition, such as increased collagen content and SMaA expression [20] and altered MMP expression [21]. It is also consistent with *in vitro* studies that have demonstrated altered matrix synthesis [11–13] and increased SMaA [11] by valve cells exposed to different strains or residing in valves experiencing different transvalvular pressures. Additionally, leaflet regions that demonstrated greater stiffness and strength (such as SEPT-BL) were also found to have increased collagen content and LOX content than weaker tissues. These stiffer regions were also associated with lower *in vivo* strains.

The myofibroblast cell phenotype, as demonstrated by SMaA and NMM expression, may be a factor in the observed heterogeneity in composition, since myofibroblasts can sense and respond to their mechanical environment [22]. These cells influence the leaflet composition, as they synthesize extracellular matrix and express abundant MMPs [23]. Myofibroblasts are also contractile [10]; although this contractility does not occur at the time scale of the valve opening and closing, it does affect leaflet stiffness *in vitro* [24], and could affect strain. Taken together, these results suggest that the normal MV continually alters levels of matrix remodeling enzymes in response to its mechanical environment, and that these processes may be mediated in part by cells demonstrating the myofibroblast phenotype.

Characterization of Commissural Leaflets

This work also provided novel information regarding the structure and dynamic matrix composition of the commissural leaflets. For decades, there has been scant research on the composition of these leaflets; the one exception was a description of their collagen fiber alignment [25]. In an extensive study of MV histology from 1956, it was noted that the “junctional” tissues of the normal MV are “anatomically and histologically not different from the rest of the valvular tissue” [26]. In contrast, this study demonstrated that the commissures have significantly different structure and composition compared to the other MV segments. As a result, ANT-C and POST-C defy easy classification as resembling either SEPT or LAT, or even as resembling each other. In terms of collagen content, delineation of leaflet layers, and extent of muscle insertion, ANT-C resembled LAT and POST-C resembled SEPT. Those results agree with the material property findings obtained through micropipette aspiration, in which ANT-C was significantly less stiff than SEPT-BL tissues whereas POST-C was not. In terms of the abundance of collagen remodeling enzymes and markers of cell activation, however, ANT-C more closely resembled SEPT (lower MMP13 and SMaA), and POST-C more resembled LAT. Significant differences in protein expression between ANT-C and POST-C (Col I, Col III, SMaA, and MMP13) suggest inherent differences between the two commissures. It is also noteworthy that expression of MMP13, LOX, elastin, and SMaA was greater in the commissures than in SEPT/LAT, which suggests that the commissural leaflets undergo more remodeling than do the leaflets. This finding could reflect differences between ANT-C/POST-C and SEPT/LAT in leaflet strain; in the future, it will be important to assess *in vivo* leaflet strains in a manner that permits direct comparison between ANT-C/POST-C and SEPT/LAT.

Heterogeneity in Valve Composition and Structure

Significant heterogeneity in composition between the four MV leaflets reflects their differences in structure as well as function. For instance, the SEPT leaflet showed greater abundance of Col I and III compared to LAT, likely due to the dominance of the fibrosa layer, but less evidence of collagen remodeling enzymes than in LAT. The amount of cardiac muscle present atop the proximal leaflet has not been previously reported, even though the general nature of the muscular and fibrous portions of the mitral annulus has been widely observed. Compared to SEPT, muscle was found to extend much further into the leaflets of LAT, ANT-C, and POST-C. This muscle tissue would likely impart significant contractility to these regions, and is consistent with greater changes in leaflet lengths across the cardiac cycle in LAT compared to SEPT. It is interesting to note that SEPT had significantly greater delineation of leaflet layers compared to LAT. Since a layered leaflet structure decreases the resistance to bending [9], this greater delineation of SEPT may functionally allow the SEPT leaflet to bend further compared to LAT, as previously reported [2]. Bending of SEPT is also likely aided by the thinness of the BL within the center “clear zone” of the anterior leaflet.

Implications for Mitral Valve Disease and Treatment

The relationships between leaflet strain, material properties, and composition emphasizes the need to restore normal leaflet strain in valve repair procedures, such as through judicious

design of annuloplasty rings. Restoring normal strain patterns may also affect the abundance of myofibroblasts, which are a probable link between strain and matrix composition, and are considered to be highly relevant to the progression of mitral valve disease [23]. Furthermore, these results provide a more complete picture of the complexity of MV composition and structure, including the commissural leaflets, which may be relevant to the design of a tissue-engineered mitral valve and in commissural repair procedures. It may be insightful to incorporate the commissural leaflets into future finite element models of the mitral valve. Future studies are warranted to determine causative mechanisms of the relationship between *in vivo* strain, cell phenotype and matrix composition. It will also be important to investigate other aspects of the *in vivo* mechanical activity of the valve, such as flexure, shear, and coaptation, on regional leaflet composition and structure.

Study Limitations

One of the limitations in this study is the inherent variability of IHC, which was quantified to be 13.7% within batches and 16.5% between batches. Averaging of duplicate sections and batch staining methods were performed to limit this variability. With respect to radiopaque marker placement, it was not possible to compare ANT-C and POST-C radial leaflet strain to that of LAT and SEPT, or to measure commissural circumferential leaflet lengths. The use of uniaxial testing to assess the material properties of the different tissue regions simplifies the analysis by estimating the characteristics of interest of each tissue section in an orientation-dependent manner. It would be worthwhile to follow up to this work with a more comprehensive mechanical analysis conducted using biaxial testing, in which the circumferential and radial behavior could be controlled and measured at the same time [27-29]. Additionally, slight anatomic differences between the ovine and human MV could affect translation of these data to human patients. For example, the fibrous mitral annulus ring in humans appears to be more well defined than in ovine hearts, which could impact the motion of the annulus and the resulting strains on leaflets in healthy valves. In addition, the fibrosa layer of the ovine valve is reported to have more dense collagen than that in humans, which would also impact strains [30].

In conclusion, these observations are the first to show a direct relation between *in vivo* valve strain, collagen turnover, and cell activation, as well as *ex vivo* material behavior. Myofibroblast-like valve cells, which demonstrate contractile and synthetic capabilities, may contribute to both the observed compositional heterogeneity as well as the heterogeneity in leaflet strain important for proper leaflet coaptation. In the future, the myofibroblast may be an important therapeutic target for diseases in which leaflet motion and composition are altered. Furthermore, it will be important for valve repair techniques and annuloplasty ring design to preserve this heterogeneity in leaflet strain and thereby attempt to prevent any further deterioration in leaflet composition that accompanies these disease processes.

Acknowledgments

The authors thank all the members of the Grande-Allen lab, especially Indrajit Nandi. The authors also appreciate the statistical expertise of Dr. Scott Baggett and use of the micropipette aspiration equipment from Dr. Robert Raphael.

Sources of Funding: Funding for this project came in part from graduate fellowships from the Hertz Foundation (EHS), National Institutes of Health (F30HL094019, EHS) and American Heart Association (PSC) and National Institutes of Health grants R01HL067025 and R01HL029589 (DCM).

References

1. Grigioni F, Enriquez-Sarano M, Zehr KJ, Bailey KR, Tajik AJ. Ischemic mitral regurgitation: long-term outcome and prognostic implications with quantitative Doppler assessment. *Circulation*. 2001; 103:1759–64. [PubMed: 11282907]
2. Karlsson MO, Glasson JR, Bolger AF, Daughters GT, Komeda M, Foppiano LE, et al. Mitral valve opening in the ovine heart. *Am J Physiol*. 1998; 274:H552–H563. [PubMed: 9486259]
3. Tsakiris AG, Von Bernuth G, Rastelli GC, Bourgeois MJ, Titus JL, Wood EH. Size and motion of the mitral valve annulus in anesthetized intact dogs. *J Appl Physiol*. 1971; 30:611–8. [PubMed: 5576071]
4. Chaput M, Handschumacher MD, Tournoux F, Hua L, Guerrero JL, Vlahakes GJ, et al. Mitral leaflet adaptation to ventricular remodeling: occurrence and adequacy in patients with functional mitral regurgitation. *Circulation*. 2008; 118:845–52. [PubMed: 18678770]
5. Tsakiris AG, Gordon DA, Mathieu Y, Irving L. Motion of both mitral valve leaflets: a cinerentgenographic study in intact dogs. *J Appl Physiol*. 1975; 39:359–66. [PubMed: 1176402]
6. Sacks, MS.; Enomoto, Y.; Graybill, JR.; Merryman, WD.; Zeeshan, A.; Yoganathan, AP., et al. *Ann Thorac Surg*. Vol. 82. Elsevier; 2006. In-Vivo Dynamic Deformation of the Mitral Valve Anterior Leaflet; p. 1369-77.
7. Stephens EH, de Jonge N, McNeill MP, Durst Ca, Grande-Allen KJ. Age-related changes in material behavior of porcine mitral and aortic valves and correlation to matrix composition. *Tissue Eng Part A*. 2010; 16:867–78. [PubMed: 19814589]
8. Kunzelman KS, Cochran RP. Stress/strain characteristics of porcine mitral valve tissue: parallel versus perpendicular collagen orientation. *J Card Surg*. 1992; 7:71–8. [PubMed: 1554980]
9. Kunzelman KS, Cochran RP, Murphree SS, Ring WS, Verrier ED, Eberhart RC. Differential collagen distribution in the mitral valve and its influence on biomechanical behaviour. *J Heart Valve Dis*. 1993; 2:236–44. [PubMed: 8261162]
10. Messier RH, Bass BL, Aly HM, Jones JL, Domkowski PW, Wallace RB, et al. Dual structural and functional phenotypes of the porcine aortic valve interstitial population: characteristics of the leaflet myofibroblast. *J Surg Res*. 1994; 57:1–21. [PubMed: 8041124]
11. Merryman WD, Youn I, Lukoff HD, Krueger PM, Guilak F, Hopkins RA, et al. Correlation between heart valve interstitial cell stiffness and transvalvular pressure: implications for collagen biosynthesis. *Am J Physiol Hear Circ Physiol*. 2006; 290:H224–H231.
12. Gupta V, Werdenberg JA, Mendez JS, Jane Grande-Allen K. Influence of strain on proteoglycan synthesis by valvular interstitial cells in three-dimensional culture. *Acta Biomater*. 2008; 4:88–96. [PubMed: 17928282]
13. Ku CH, Johnson PH, Batten P, Sarathchandra P, Chambers RC, Taylor PM, et al. Collagen synthesis by mesenchymal stem cells and aortic valve interstitial cells in response to mechanical stretch. *Cardiovasc Res*. 2006; 71:548–56. [PubMed: 16740254]
14. Timek TA, Dagum P, Lai DT, Liang D, Daughters GT, Tibayan F, et al. Tachycardia-induced cardiomyopathy in the ovine heart: Mitral annular dynamic three-dimensional geometry. *J Thorac Cardiovasc Surg*. 2003; 125:315–24. [PubMed: 12579100]
15. Gheewala N, Schwarz KA, Grande-Allen KJ. Organ Culture of Porcine Mitral Valves as a Novel Experimental Paradigm. *Cardiovasc Eng Technol*. 2013; 4:139–50.
16. Carew EO, Vesely I. A new method of estimating gauge length for porcine aortic valve test specimens. *J Biomech*. 2003; 36:1039–42. [PubMed: 12757813]
17. Krishnamurthy VK, Guilak F, Narmoneva DA, Hinton RB. Regional structure-function relationships in mouse aortic valve tissue. *J Biomech*. 2011; 44:77–83. [PubMed: 20863504]
18. Zhao, R.; Sider, KL.; Simmons, CA. *Acta Biomater*. Vol. 7. Acta Materialia Inc.; 2011. Measurement of layer-specific mechanical properties in multilayered biomaterials by micropipette aspiration; p. 1220-7.

19. Stephens EH, Nguyen TC, Itoh A, Ingels NB, Miller DC, Grande-Allen KJ. The effects of mitral regurgitation alone are sufficient for leaflet remodeling. *Circulation*. 2008; 118:S243–9. [PubMed: 18824762]
20. Balachandran K, Konduri S, Sucosky P, Jo H, Yoganathan AP. An ex vivo study of the biological properties of porcine aortic valves in response to circumferential cyclic stretch. *Ann Biomed Eng*. 2006; 34:1655–65. [PubMed: 17031600]
21. Balachandran K, Sucosky P, Jo H, Yoganathan AP. Elevated cyclic stretch alters matrix remodeling in aortic valve cusps: implications for degenerative aortic valve disease. *Am J Physiol Heart Circ Physiol*. 2009; 296:H756–64. [PubMed: 19151254]
22. Wang J, Chen H, Seth A, McCulloch CA. Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. *Am J Physiol Heart Circ Physiol*. 2003; 285:H1871–H1881. [PubMed: 12842814]
23. Rabkin E, Aikawa M, Stone JR, Fukumoto Y, Libby P, Schoen FJ. Activated Interstitial Myofibroblasts Express Catabolic Enzymes and Mediate Matrix Remodeling in Myxomatous Heart Valves. *Circulation*. 2001; 104:2525–32. [PubMed: 11714645]
24. Merryman WD, Shadow Huang HY, Schoen FJ, Sacks MS. The effects of cellular contraction on aortic valve leaflet flexural stiffness. *J Biomech*. 2006; 39:88–96. [PubMed: 16271591]
25. Cochran RP, Kunzelman KS, Chuong CJ, Sacks MS, Eberhart RC. Nondestructive analysis of mitral valve collagen fiber orientation. *ASAIO Trans*. 1991; 37:M447–8. [PubMed: 1751231]
26. Chiechi MA, Lees WM, Thompson R. Functional anatomy of the normal mitral valve. *J Thorac Surg*. 1956; 32:378–98. [PubMed: 13358125]
27. Grashow JS, Yoganathan AP, Sacks MS. Biaxial stress-stretch behavior of the mitral valve anterior leaflet at physiologic strain rates. *Ann Biomed Eng*. 2006; 34:315–25. [PubMed: 16450193]
28. Grashow JS, Sacks MS, Liao J, Yoganathan AP. Planar biaxial creep and stress relaxation of the mitral valve anterior leaflet. *Ann Biomed Eng*. 2006; 34:1509–18. [PubMed: 17016761]
29. Liao J, Yang L, Grashow J, Sacks MS. The relation between collagen fibril kinematics and mechanical properties in the mitral valve anterior leaflet. *J Biomech Eng*. 2007; 129:78–87. [PubMed: 17227101]
30. Walmsley R. Anatomy of human mitral valve in adult cadaver and comparative anatomy of the valve. *Br Hear J*. 1978; 40:351–66.

Fig. 1.

Circles indicate the location of the implanted radiopaque markers. Dark gray rectangles indicate tissue section location (sections did not include markers or their sutures). Two tissue sections were analyzed and the results averaged for the septal (SEPT) and lateral (LAT) segments, respectively; one tissue section was used for each of the commissures (ANT-C, POST-C). BL=basal leaflet, ML=mid-leaflet. Double-headed arrows indicate leaflet segment length measurements; rad=radial, circ=circumferential. For SEPT, BL rad + ML rad=total rad. Due to limitations regarding marker placement, in the case of ANT-C and POST-C, total rad was calculated as the average of the two measurements

Fig. 2.

Locations of leaflet regions and histological layers. Red line indicates measurement for calculating proportion of muscle insertion into leaflet. Scale bar represents 2 mm

Fig. 3.

A) Comparison of LAT vs. SEPT radial and circumferential percent contraction. *p 0.031 vs. SEPT for a given direction (radial or circumferential). B) Comparison of BL and ML radial and circumferential percent contraction within SEPT. *p 0.001 vs. BL for a given direction (radial or circumferential). For all bar graphs error bars indicate the standard error of the mean

Fig. 4.

A) Degree of delineation between layers. $*=p<0.001$. B) The two left sections represent examples of commissure sections in which the collagen core of the chordae tendinae is continuous with the fibrosa of the leaflet resulting in a particularly collagen-rich fibrosa in this section of the leaflet. That pattern contrasts with the chordal origins in LAT (far right), in which chordae has branched off of the existing fibrosa. Red arrows in left two images point to chordae continuous with leaflet fibrosa; red arrow in right image points to chordae branching off fibrosa. Scale bars represent 2 mm

Fig. 5.

A) Relative amounts of Col I and III in leaflet sections. Overall Col I and Col III each $p < 0.001$. * $p < 0.008$ vs. LAT and ^ $p < 0.011$ vs. ANT-C for a given protein (Col I or Col III). B) Relative amounts of MMP13 and SMaA in leaflet sections. Overall MMP13 and SMaA each $p < 0.001$. * $p < 0.001$ vs. SEPT-ML and SEPT-BL and ^ $p < 0.015$ vs. POST-C for a given protein (MMP13 or SMaA). C) Relative amounts of elastin and LOX in leaflet sections. Overall elastin and LOX $p < 0.001$. * $p < 0.001$ vs. SEPT-ML for a given protein (elastin or LOX)

Fig. 6. Comparison of Effective Modulus of leaflet sections tested using micropipette aspiration. Overall $p < 0.05$. * $p < 0.05$ vs. SEPT-ML, ANT-C, and LAT.

Fig. 7.

A) Comparison of maximum tangent modulus and ultimate stress in leaflet sections based on uniaxial tensile testing. Overall maximum tangent modulus and ultimate stress $p < 0.05$.

* $p < 0.05$ vs. SEPT-BL for given material property (maximum tangent modulus or ultimate stress). B) Comparison of extensibility and ultimate strain in leaflet sections based on uniaxial tensile testing. Overall extensibility $p < 0.05$. [^] $p < 0.05$ vs. LAT-RAD for extensibility

Table 1
Panel of antibodies used in immunohistochemistry

Protein	Function
<u>Collagens</u>	
Collagen I (Col I) ^a	Predominant collagen in valve, provides tensile strength
Collagen III (Col III) ^a	Reticular collagen
<u>Collagen Turnover Proteins</u>	
Matrix Metalloproteinase (MMP)-13 ^b	Collagen degradation
Lysyl oxidase (LOX) ^d	Involved in collagen and elastin crosslinking
<u>Elastic Fiber-Related Proteins</u>	
Elastin ^e	Predominant component of elastic fibers
Matrix Metalloproteinase (MMP)-9 ^c	Elastin degradation
<u>Proteoglycans (PG) and Glycosaminoglycans (GAG)</u>	
Decorin (DCN) ^a	PG involved in collagen fibrillogenesis
<u>Valve Cell Activation</u>	
Smooth muscle alpha-actin (SMaA) ^g	Indicates an “activated” myofibroblast-like phenotype
Non-muscle Myosin Heavy Chain (NMM) ^h	Indicates an “activated” myofibroblast-like phenotype

^a gift of Dr. Larry Fisher, NIH (Bethesda, MD);

^b Chemicon (Temecula, CA);

^c Assay Designs (Ann Arbor, MI);

^d Imgenex (San Diego, CA);

^e Abcam (Cambridge, MA);

^f Associates of Cape Cod (Falmouth, MA);

^g Dakocytomation (Denmark);

^h Covance (Berkeley, CA).