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Mechanical Modulation of Glycosaminoglycan and Proteoglycan Production by Valvular Interstitial Cells

by

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ABSTRACT

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Differently loaded regions of the mitral valve contain distinct amounts and proportions of glycosaminoglycans (GAGs) and proteoglycans (PGs); these GAG/PG profiles are altered in abnormal loading conditions such as myxomatous degeneration. However, the role of mechanical stimulation on GAG and PG synthesis by valvular interstitial cells (VICs) is still unclear. This research analyzed first the PGs in differently loaded regions of mitral valve (leaflet and chordae) and then the effects of mechanical strains on GAG and PG synthesis by VICs using an in vitro 3-dimensional tissue-engineering model to develop a deeper understanding of valve mechanobiology.

This original research investigated the specific PGs present in human mitral valves and found that the regions in compression (leaflets) are rich in versican and regions in tension (chordae) are rich in decorin and biglycan; these PGs were also detected in the engineered tissues seeded with VICs. Applying constraint increased the synthesis of decorin, biglycan and 4-sulfated GAGs. Constraint also increased versican secretion but reduced its retention within the engineered tissues. The application of constraint was found to be more influential than the directionality (biaxial vs. uniaxial) of strain. Constrained collagen gels containing leaflet cells retained more decorin and biglycan than did those containing chordal cells. The application of cyclic strains decreased the total GAG synthesis, increased the proportions of 4-sulfated GAGs, and
reduced the proportions of hyaluronan. Synthesis of the PG versican was increased by leaflet cells and decreased by chordal cells in response to cyclic strain. Chordal cells were found to be more responsive to cyclic strains than leaflet cells, which has implications in the dramatic remodeling of myxomatous chordae tendineae. Synthesis of total GAGs, 4-sulfated GAGs and decorin was found to be strain dependent, whereas synthesis of versican and decorin was frequency dependent. In general, VICs within collagen gels synthesize GAG in proportions and amounts close to that of native valve tissue.

This research is the first to show that strains can modulate GAG/PG synthesis by valve cells. These results provide insight into valve mechanobiology and pathology and have implications for understanding the remodeling process of many soft tissues.
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VISHAL GUPTA
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Chapter 1: Introduction

1.1 Specific aims

The objective of this research project was to determine if mechanical loading affects the production of glycosaminoglycans (GAGs) and proteoglycans (PGs) by valvular interstitial cells (VICs) cultured in three-dimensional (3D) collagen scaffolds.

Since myxomatous disease is manifested by an excess of GAGs and PGs, as well as altered tissue mechanics, I utilized a tissue engineering approach to study how mechanical loading of interstitial cells from the leaflets and chordae of mitral valves affects the production of specific GAGs and structural PGs. It was thought that in the leaflets and chordae of diseased mitral valves, abnormally low tensile loading of the tissues stimulates an altered cellular production of GAGs and PGs and hence may be responsible for the changes in their GAG/PG populations.

The overall goal of this project was to apply various stretching protocols to VICs grown from mitral valve leaflets and chordae in order to alter their intrinsic production of GAGs and PGs. This goal was achieved by carrying out the following specific aims:

(i) Analyze different types of PGs present in human normal valve leaflets and chordae tendineae.

(ii) Differentiate between GAGs and PGs produced by VICs from porcine mitral valve leaflet and chordae seeded in collagen gels experiencing different loading (biaxial vs. uniaxial).
(iii) Differentiate between GAGs and PGs secreted by porcine mitral valve leaflet and chordal VICs seeded in collagen gels and subjected to repetitive cyclic tension and relaxation.

(iv) Manipulate the production of GAGs and PGs by porcine mitral valve leaflet and chordal VICs by subjecting the collagen gels to varying cyclic strains and frequencies.

1.2 Extracellular matrix

The extracellular matrix (ECM) of heart valves mainly consists of collagen, elastin, GAGs and PGs. Collagen is the most abundant protein in the majority of connective tissues; it is secreted by cells to provide tensile strength and to serve as an organizational scaffold. The other major fibrillar ECM protein is elastin, which provides elastic recoil. GAGs and PGs are defined in detail in the next two sections. Valvular cells such as VICs bind to components of the ECM via adhesion molecules known as integrins. The integrins transmit mechanical stimuli from the ECM to cytoskeleton and change the gene expression of numerous proteins including ECM molecules [1]. In addition, the synthesis of ECM molecules may be altered in pathological conditions. For example, stenotic bicuspid aortic valves contain disrupted ECM and increased collagen and PGs [2]. Similarly, increased amounts of PGs and GAGs have been found in myxomatous mitral valves [3].
1.3 Glycosaminoglycans

GAGs are long linear carbohydrate chains that are heavily negatively charged due to sulfate and carboxyl groups. GAGs consist of repeating disaccharides that contain N-acetylhexosamine and uronic acid (glucuronic acid or iduronic acid) [4]. Different GAG types include hyaluronan (HA), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS). The GAG disaccharides have different sulfation patterns named after the specific carbon position modified by the addition of a sulfate group. The most common sites of sulfation for CS/DS are the 4 or 6 positions on the hexosamine (i.e., chondroitin 4-sulfate, dermatan 6-sulfate).

Different types of GAGs form complexes with proteins and perform various biological and structural functions in connective tissues. For example, GAGs play an important role in forming protein-protein complexes on the cell surface and also sequester proteins and enzymes within the ECM [5]. Recent advances in chromatographic separation techniques and fluorophore assisted carbohydrate electrophoresis (FACE) make it possible to examine and quantify the different GAG classes [6, 7]. The number of GAG chains, chain lengths and the pattern of sulfation can vary according to different biological needs [8]. For example, the role of DS-protein complexes in the formation and orientation of collagen fibrils has been demonstrated by many investigators [9-11]. HA is unique among GAGs in that it is not synthesized covalently bound to a protein core. HA in solution has an open, random coil structure and entraps large amounts of solvents such as water. With or without aggregation, HA constitutes one of the major structural macromolecule in the ECM, where it can promote cell proliferation and migration [4].
1.4 Proteoglycans

PGs are complex macromolecules that contain a core protein with one or more covalently bound GAG chains. PGs are ubiquitously located throughout the body, both at the cell surface and within the ECM, and there are many different types that perform various functions. For example, basement membrane PGs include perlecan (core protein ~450 kDa, HS/CS GAG chains) and agrin (core protein ~250 kDa, HS GAG chains), which play a role in embryogenesis [12]. The largest hyalactan PGs, which bind to HA and contain a lectin-like region, are versican (core protein ~400 kDa, many CS/DS GAG chains) and aggrecan (core protein ~220 kDa, many CS and KS GAG chains). Decorin, biglycan, lumican and many other small PGs (core protein ~43 kDa with fewer GAG chains), have been shown to play a role in collagen fibrillogenesis. The lowest molecular weight PGs, in particular, may have CS and DS chains with core proteins rich in the amino acid leucine and hence are called “small leucine-rich proteoglycans” (SLRPs).

The main PGs found in heart valves are decorin, biglycan and versican [13]. These PGs play a role in regulating the cellular phenotype and ECM assembly [14]. Decorin and biglycan are small leucine-rich PGs (100-300 kDa) that mediate collagen fibrillogenesis. The arched shape of the leucine rich repeats binds to the triple helix of collagen fibrils and controls collagen fibril diameter [15]. Decorin, so named because it “decorates” collagen fibrils, contains one CS/DS chain while biglycan contain two CS/DS chains (Figure 1.1) [16]. Versican is a very large PG (2,000 kDa) containing 15-20 negatively charged CS chains that branch off the core protein like bristles and aggregate with HA (Figure 1.1) [17]. There are two GAG binding regions in the versican core protein (GAG-α and GAG-β) and four splice variants of versican (V0, V1, V2 and
V3) are possible. The largest splice variant is V0, which contains both GAG-\(\alpha\) and GAG-\(\beta\), whereas the other three variants contain only GAG-\(\beta\) (V1 variant), GAG-\(\alpha\) (V2 variant), or neither region (V3 variant) [18]. The variant V1 is the most common form of versican while the less common V3 has been shown to play a role in elastin fiber formation and in the adhesion and proliferation of smooth muscle cells [19, 20]. The frequent aggregation of versican with HA provides tissue with a hydrated compressive resistance.

![Figure 1.1. Structures of the proteoglycans decorin and versican. Adapted from Kreis and Vale, 1999 [21] (left) and Chang et al., 1983 [17] (right).](image)

### 1.5 Mitral valve

Mammalian hearts contain four valves to help circulating the blood throughout the body. The right heart contains the tricuspid valve and pulmonary valve while the left heart contains the mitral valve and the aortic valve. The mitral valve guides the flow of blood from the left atrium to the left ventricle and helps to maintain the cardiac output. Leonardo da Vinci first drew the anatomy of mitral valve in the 14th Century; Vesalius suggested the term “mitral valve” because it resembled a bishop’s mitre [22]. The porcine mitral valve is anatomically and biochemically very similar to the human mitral
valve [23] and is therefore the most widely used animal model. Other animal models (dog, sheep and bovine) have been used as well [24-26].

Anatomy

The mitral valve is composed of two leaflets (anterior and posterior) and numerous chordae tendineae (Figure 1.2). The chordae tendineae connect the ventricular surfaces of the leaflets to the papillary muscles of the left ventricle and promote proper valve function by preventing the prolapse of the closed valve into the left atrium. Chordae inserting directly into the leaflet free edge are defined as "marginal" and those inserting further underneath the leaflet towards the annulus are "basal" chordae [23].

![Figure 1.2](image.jpg)

**Figure 1.2.** Normal mitral valve structure (left) cut open and (right) closed position (right figure courtesy of Cochran, Kunzelman).

Both the anterior and posterior leaflets are composed of three biochemically and structurally distinctive layers: the atrialis, spongiosa, and fibrosa. The relative thicknesses of each layer vary between the two leaflets and within each leaflet from its attachment edge to its free edge [27]. The fibrosa layer is the thickest in most of the valve leaflet, while the atrialis is the thinnest, elastin layer. The spongiosa layer contains loose
connective tissue extending from the annulus to the free edge and makes up most of the thickness at the leaflet free edge.

The layered structure of the valve leaflet may account for the proper function of the valve system. Cochran et al. mapped the architecture of the collagen fibers in the different layers of the valve and related the resulting arrangement to the mechanical behavior of the mitral valve [28]. They also found that the anterior leaflet bears more load than the posterior leaflet and that regional alterations in the layer thicknesses affect the tensile strength of the leaflets [27]. The variability of the different leaflet layers, and hence the structural constituents within the mitral valve are determined by the specific functional roles of the leaflets and chordae, particularly during valve closure when pressures across the mitral valve may reach 120 mmHg.

Mitral valve closure during systole is maintained by apposition of the anterior and posterior leaflets and the chordal tension produced by the papillary muscles’ contraction. The mitral valve therefore maintains a balance of tensile and compressive loads. The chordae tendineae and the flat, central region of the anterior leaflet bear tensile forces while the posterior leaflet and the free edge of the anterior leaflet experience compressive loads (Figure 1.2) [13].

**Valvular endothelial cells**

The outer surfaces of the leaflet and the chordae are covered with a continuous layer of endothelial cells that are presumed to provide a nonthrombogenic surface. Valvular endothelial cells (VECs) have been shown to express nitric oxide synthase both *in vivo* and *in vitro* conditions; unlike VICs, VECs do not synthesize type I collagen and
chondroitin sulfate GAGs [29]. Aortic VECs were reported to have a different phenotype than other vascular endothelial cells and also show heterogeneity between the atrial and ventricular sides of the leaflets [30]. VECs align perpendicular to the flow direction (dependent on Rho-kinase signaling), unlike aortic endothelial cells, which align parallel to flow (dependent on both Rho-kinase and phosphotidylinositol 3-kinase signaling) [31]. Also, VECs have been reported to express fewer inflammatory genes than do aortic endothelial cells; furthermore, the application of shear stress to VECs has been shown to have an anti-calcification effect [32]. Compared to the ventricular surface VECs, VECs on the aortic side were found to express many different genes and to be more susceptible to calcification [33]. Valve endothelial cells dysfunctions are often associated with valve pathologies such as aortic valve calcification and sclerosis [34].

Valvular interstitial cells

The subendothelial VICs are found in all layers of the valve and are responsible for producing and organizing the ECM proteins. Valves also contain a small number of cardiac muscle cells and smooth muscle cells in the base (attachment edge of leaflet) of the valve, as the base may be slightly vascularized [35]. VICs from all valves show a mixed phenotype, as they have the characteristics of both fibroblasts and smooth muscle cells [36, 37]. The fibroblastic phenotype is marked by elongated cells that contain numerous organelles for matrix synthesis and stain for prolyl-4-hydroxylase. The smooth muscle cell phenotype is denoted by cobblestone cells that stain for smooth muscle α-actin and stress fibers, although there are a variety of other characteristics that have been used to differentiate between these two cell phenotypes. Other sarcomeric proteins and
muscle-specific markers have also been identified in VICs [38]. VICs display this dual phenotype consistently throughout passaging [39, 40]. However, it is still unclear whether these cells are myofibroblast type or if there are two distinct populations of cells within the valve. A recent study by Blevins et al., reported the isolation and characterization of two subpopulations of VICs based on differential adhesion and smooth muscle α-actin expression [41]. Evidences from in vitro studies have shown that VIC originate from epicardially-derived embryonic cells [42], hematopoitic stem cells [43] and bone marrow-derived progenitor cells [44]. In a characterization of the expression of structural (i.e. PGs, collagen) and cell-surface molecules (i.e. CD44, vimentin, myosin) by VICs and mesenchymal stem cells, many similarities between the two cell sources were found [45].

The cell biology of valves has been studied by a growing number of investigators. It has been shown that VICs have contractile properties, which may contribute to controlled tone and valve movement during diastole and the systole [46, 47]. There is also evidence of cell-cell communication junctions and the presence of nerve terminals in the mitral valve, which may further contribute to coordinated valve closure [36, 40, 46, 48, 49]. Recently, it has been demonstrated that mitral VICs have the ability to repair the injured valve leaflet, and that this repair response is mediated by fibroblast growth factor-2 [50]. The valve's response to injury is thought to be associated with an accumulation of VICs and matrix, valvular inflammation, and calcification, which eventually leads to valve dysfunction [51]. Overall, the mitral valve interstitial cells are very dynamic and play a central role in the pathogenesis and remodelling of the valve.
Extracellular matrix composition

As noted previously, both the anterior and posterior leaflets are layered tissues containing a thick collagenous layer on the ventricular side; a thin, predominantly elastic layer on the atrial side; and a “spongiosa” layer in between (Figure 1.3). The spongiosa contains mostly PGs and GAGs [27]. The chordae are strong, thin cords containing a highly aligned collagenous core inside a thin outer sheath of elastic fibers and endothelial cells.

Figure 1.3. Movat stain of the posterior leaflet (F - fibrosa, S - spongiosa, A - atrialis).

On a dry weight basis, the valve leaflets overall are composed of approximately 60% collagen, 10% elastin, 20% GAGs and 10% cells and other components [52, 53]. The mean water content is 87% in the leaflet and 72% in the chordae tendineae [3, 54]. The most commonly found collagen types in the valves are type I (74%), type II (24%) and a small amount of type V [53]. In the mitral valve, collagen provides mechanical and tensile strength; elastin provides extensibility; and GAGs and PGs provide cushioning and absorb shear. All four GAG classes have been found in the mitral valve [54-56]. With the exception of HA, these GAGs are usually found as part of larger macromolecules such as the large, water-binding and HA-binding PG versican and the
small, collagen-binding PGs decorin and biglycan [12]. GAGs mediate collagen fibril to fibril linkages and are responsible for variations in viscoelastic properties of the different types of chordae tendineae [57].

Our laboratory recently differentiated between the distinct classes of GAGs produced by tensile and compressive regions of the mitral valve [13]. The study reported the variations in the chain length (number of disaccharides) of the GAGs: the regions of compression had longer GAG chain lengths than did the regions of tension. In addition, three PGs (decorin, biglycan and versican) were identified in the valve and their relative abundance varied according to the loading regions. Hence, the interpretation of these patterns was that the type of loading the tissue bears dictates the segregation of the GAGs and PGs. In diseased valves such as myxomatous mitral valves, the overproduction of specific GAGs and PGs may be the result of abnormal loading of the tissue.

1.6 Myxomatous mitral valve disease

Mitral valve diseases are widespread in our population and represent significant health care costs. Recent statistics from American Heart Association (January 2006) reported that the prevalence of mitral valve disorders is 1-2% and is equal between men and women. Mitral regurgitation is primarily due to valve prolapse (myxomatous degeneration), ischemic disease, rheumatic fever or infective endocarditis [58, 59]. According to the Framingham Heart Study, up to 2.5% of the United States population is believed to be affected by mitral valve prolapse [60] and it is the most common cause of mitral regurgitation in older patients [61]. This regurgitation is caused by the prolapse of the voluminous myxomatous leaflet cusps into the left atrium during systole as the blood
flows back from the left ventricle to the left atrium. The severity of the mitral regurgitation is usually diagnosed by Doppler echocardiography and defined as mild (1+), moderate (2+), moderately severe (3+) and severe (4+) [22].

There are different opinions regarding the pathogenesis of myxomatous mitral valve disease. The mitral valve is subjected to the highest level of haemodynamic stress in the heart. The diminished healing response of aging valve tissue to mechanical stress may be a factor in the pathogenesis of myxomatous change, or it may result from a variety of possible aetiological factors [62, 63]. Myxomatous degeneration in the mitral valve can be also genetic, as it is often (but not always) associated with Marfan Syndrome, Ehlers-Danlos syndrome and other connective tissue disorders. The chromosomal location of autosomal myxomatous mitral valve prolapse has been previously reported [64] but not all cases of myxomatous mitral valve disease have a clear genetic link. Overall, the precise cause of this condition remains unknown.

The end effects of mitral regurgitation are left ventricular volume overload, reduced cardiac output, left atrial enlargement and pulmonary hypertension [65, 66]. Myxomatous valves are also more likely to develop bacterial endocarditis. Additionally, these left heart dysfunctions may cause left ventricular pump failure and the development of congestive heart failure.

The most common, state-of-the-art treatment for myxomatous degeneration is to repair the myxomatous valve tissue surgically. The posterior leaflet and the chordae tendineae are the most affected regions of the mitral valve. In the most common repair procedure (quadrangular resection and sliding annuloplasty), the ruptured chordae attached to the middle scallop of the posterior leaflet are identified and excised (both
leaflet section and the ruptured chordae). The annulus is then plicated with 2 pledgetted
sutures, the leaflet edges are reapproximated with suture, and an annuloplasty ring is
placed. Mitral valve replacements are also done in selected cases but that procedure is
associated with a higher mortality rate [22].

**Structural and mechanical alterations**

Myxomatous mitral valve disease is characterized by floppy valve leaflets and
elongated or ruptured chordae tendineae (Figure 1.4). Abnormal stress distributions and
transmission of forces to the annulus cause an increase in the size of the mitral annulus as
well as calcification in many patients [66]. The atrial surface of the floppy valve leaflet is
classified by irregularly shaped folds forming an indented surface as opposed to the
smooth surface of the normal valve [66].

![Figure 1.4. Myxomatous mitral valve posterior leaflet and chordae (resected at surgery). (A) atrial surface (B) ventricular surface.](image)

The mechanical properties of the mitral valve chordae and leaflet have also been
reported to be severely affected in myxomatous degeneration. Myxomatous leaflets were
found to be more extensible and less stiff than normal leaflets [67]. The myxomatous
chordae were 50% more extensible than normal chordae, with significantly lower elastic
moduli and failure strengths [68]. Overall, the load bearing capacities of chordae appear to be damaged much more than are those of the leaflet in myxomatous degeneration. Clearly, myxomatous valves are physically and mechanically different from normal mitral valves.

**Biochemical alterations**

Myxomatous mitral valve disease has additional distinctive changes that are apparent histologically and biochemically. Microscopic studies reveal collagen disruption and dissolution, elastin fragmentation and GAG accumulation in myxomatous valve leaflets [63, 66, 69, 70]. The wet weight and water content of the floppy valves is significantly higher than in normal valves. There is also an increase in the elastin concentration in the anterior leaflet of myxomatous valves [53]. Grande-Allen et al. found that myxomatous chordae have same collagen concentration and DNA density as normal chordae, but more water and GAGs [54]. They also differentiated between GAG classes and found abnormally high proportions of 6-sulfated CS/DS and HA in the diseased chordae and leaflet [3]. Another study found that the VICs in myxomatous valves are activated myofibroblasts (mesenchymal type) and that these valves contain elevated levels of proteolytic enzymes [63]. The total number of cells was greater in both myxomatous leaflets and chordae, particularly in the spongiosa, compared to normal valves [54, 63].

It is evident from many reports that although a variety of biochemical changes are manifested in the mitral valve during the development of myxomatous disease, these changes may not be the primary cause of the progression. It has been suggested that
genetic defects in collagen may cause myxomatous degeneration [71]. Certain authors concluded that the collagen dissolution is the primary lesion in the floppy valves [69, 71], whereas others have proposed that the abnormal accumulation of PGs is the basic pathological process [72, 73]. Nasuti et al. suggested that fibrillin, an ECM structural protein (elastic fiber microfibril), plays a role in the development of mitral valve prolapse [74]. The endothelial cells may also affect the pathogenesis of the disease, since these cells regulate metabolic and inflammatory process between blood and the VICs [75]. For example, increased NADPH activity was found in canine myxomatous valves, suggesting increased nitric oxide expression from endothelium [76]. A very recent study reported the upregulation of low-density lipoprotein receptor-related protein 5, involved in chondrogenic and osteoblastic differentiation, in human myxomatous mitral valves [77]. In conclusion, various investigators have different opinions of the disease progression and there is no consensus as to one mechanism of myxomatous degeneration. However, it is evident that GAGs and PGs are obviously abnormally distributed within myxomatous mitral valves along with low tensile tissues loads and hence provide us with the motivation to study the effect of mechanical strains on GAG/PG synthesis by VICs.

1.7 Mechanical loading and cells

It has been known for many decades that mechanical stresses and strains dictate the cellular and ECM organization and remodeling of tissues. Cells generally orient themselves and the ECM they produce in the direction of force [78]. Because cells are connected to the ECM via integrins, mechanical stresses and strains in the ECM result in mechanotransduction of cells and can regulate the expression of ECM genes [1, 79]. To
monitor the response of cells to mechanical stimuli, many types of cells have been subjected to a variety of mechanical strains ranging from 4%-50%. The roles of tension and/or compression on dermal fibroblasts [79, 80], chick embryo fibroblasts [81], vascular smooth muscle cells [82], fetal lung cells [83], mesenchymal cells [84] and cardiac myocytes [85] among other cell lines have been widely studied. As part of this dissertation work, I have reviewed the effect of various types of mechanical stimuli on ECM synthesis by cardiovascular cells (as described in Chapter 2). Most studies involved the application of cyclic strains as opposed to static strains. Also, 2D cultures tended to be used most often even though 3D scaffolds provide more in vivo-like conditions.

**Scaffolds for cell seeding**

Collagen is the most prominent ECM component in the human body and is also a major component of valves. Therefore, using collagen as a scaffold for in vitro studies can provide the seeded cells with a more in vivo like environment. For this reason, collagen has been widely used as a scaffold for cell seeding and for monitoring cell response in a variety of applications. Three dimensional collagen gels have many advantages over other scaffolds, as they are permeable, biocompatible, relevant to the normal environment of the cells, and easy to use and polymerize [86]. Cells within the collagen gel exert tractional forces, migrate preferentially in the direction in which surrounding fibrils are aligned, and compact the gel by excluding water contained within the matrix [87]. The boundary conditions of the gel also play a role in directing the alignment of cells and collagen fibers [88].
Other commonly used scaffolds include collagen sponges, fibrin gels, gelatin sponges, aortic medial elastin and silicone elastomeric membranes. Fibrin gel has been shown to exhibit many advantages as a cardiovascular tissue-engineering scaffold [89], and showed stronger material behavior compared to collagen gel tissue equivalents [90]. VICs seeded within fibrin disk constructs have been shown to remodel fibrin within 3 weeks resulting in engineered tissues with good mechanical properties [91]. Collagen sponge has also been used as a 3-dimensional matrix for cardiac VICs and it was shown that the VICs grown in collagen sponges more closely reproduce the *in vivo* characteristics of valve cells than do these same cells grown in standard monolayer cultures [92]. VICs seeded on top of collagen gels have been shown to secrete the GAGs and PGs found in native valves [93]. Other studies have also found that collagen matrix is a suitable scaffold for VICs [94, 95]. Overall, seeding cells within a collagen matrix is believed to provide a more biologically and anatomically appropriate model than monolayers for studying the regulation of ECM deposition.

**Cell stretching devices**

A great variety of cell-loading devices have been developed over the past two decades, ranging from the commercially available Flexcell systems to independently designed stretching systems. Different devices have been used to apply equibiaxial, uniaxial or biaxial mechanical loading to cells seeded within or on a scaffold. These systems may be simple, hand-manipulated mechanical devices or have complex computer control [1, 78, 80, 96, 97]. Recently, a novel MEMS device was developed to apply biaxial mechanical stimulation to a single cell [98].
GAG and PG synthesis and gene expression have been studied in mechanically loaded culture systems although not previously for VICs. In chondrocytes grown on bovine medial elastin membranes under 10% tensile strain for 8 hours, overall GAG synthesis was elevated while collagen synthesis was depressed [99]. In vascular smooth muscle cells grown on silicone membranes exposed to 4% tensile strain for 12 hours, mRNAs for several PGs including versican were increased, but mRNA for collagen-binding decorin was decreased [82]. Fetal lung cells grown in a gelatin sponge exposed to 5% tensile strain for 48 hours showed an increase in GAG synthesis but no change in the relative proportions of HA, total chondroitin/dermatan sulfates, and heparan sulfate [83]. In a study of mesenchymal cells embedded in an agarose matrix and subjected to cyclic compression for 3 days, overall GAG synthesis was increased with cyclic frequency and loading duration [84]. These and other studies of GAG/PG responses to loading have used $[^{35}S]$sulfate and $[^3H]$glucosamine incorporation to study the rates of synthesis, Western and Northern analyses to examine the PG core proteins and mRNAs, and either HPLC or cellulose acetate electrophoresis [100] to identify different GAG classes [101]. Studies describing the role of mechanical strains or stressed on VICs are very limited as described in the following section.

*Valve mechanobiology*

The mechanobiology of valves and valve cells is a fairly new area of research with only a few publications. It has been shown that VICs seeded within a polymeric mesh will synthesize more ECM if they are grown within a pulsatile flow bioreactor [102]. VICs have been shown to be mechanoresponsive to tensile strains in 2D cell
culture [103], and to pressure, shear forces and circumferential stretch in organ culture [104, 105], resulting in altered ECM synthesis. VICs also showed altered expression of general pro-inflammatory genes (VCAM-1 upregulation and osteopontin downregulation) in response to elevated pressure in 2D culture [106]. However, the effect of varying magnitudes of strain and frequencies, such as those found in vivo, have not been investigated in the context of VICs in 3D cultures. Only one study on VICs examined the effect of strain magnitudes on collagen synthesis (increased with strain magnitude), but that was in 2D culture [103]. This paucity of research on VICs is notable because investigations on other cardiovascular cell types (described in detail in chapter 2) have shown that ECM synthesis is clearly influenced by strain and frequency. Co-culture of VECs with VICs in 3D culture under flow conditions reduced the expression of α-smooth-muscle actin by VICs and differentiated them to a more quiescent phenotype, which is closer to native conditions [107]. This study suggests that VECs communicate with VICs to properly transmit the mechanical stimulus to VICs and to regulate their phenotype and matrix synthesis.

The exact mechanistic pathways of the effect of mechanical stimulus on ECM synthesis are still unclear, particularly in heart valves. However, it is well known that integrins bind to ECM (i.e., fibronectin, other glycoproteins, and matrix proteins) outside the cell and inside the cell to intracellular proteins that constitute the cytoskeleton (i.e., actin) via numerous intracellular anchor proteins, which recruit focal adhesion kinase (FAK) and thereby initiate a number of potential signaling pathways [1, 108]. Focal adhesions are thus comprised of integrin clusters, cytoskeletal proteins, and FAK. In 2D culture, cells adhere through integrins binding to the serum proteins that adsorb to the
tissue culture plastic. It has been shown that in 3D collagen gels, the focal adhesions of fibroblasts are smaller and fewer in number than those present in the 2D monolayer cultures [109], but the cells likely also bind to fibronectin and matrix in these gels as well. Application of mechanical stretch initiates conformational changes in integrins, focal adhesion assembly, and FAK activation, ultimately resulting in reorientation of the actin cytoskeleton to reposition the cell in the direction of strain. An additional consequence of FAK activation is the activation of Ras, which triggers the mitogen-activated protein kinase (MAPK) pathway. There are also other possible pathways, but MAPK has been shown to be heavily involved in mechanotransduction. The MAPK pathway activates many transcription factors such as activating protein-1 (AP-1) or nuclear factor kappa B (NF-κB), which bind to the promoter sequence within the nucleus and regulate gene expression. The resulting altered gene expression could be the synthesis of ECM or many other proteins.

In many pathological conditions of the cardiovascular system (i.e., hypertension, atherosclerosis, and myxomatous mitral valve disease) significantly altered profiles of GAGs and PGs have been found to accompany alterations in mechanical strains within the tissue, which has spurred investigations into the effects of mechanical strains on GAG and PG synthesis [3, 110]. Therefore, I aimed to fill the gap in the literature between valve mechanobiology and pathology. As the starting point, I first reviewed the literature on the effect of various mechanical strains on ECM synthesis by cardiovascular cells (as described in Chapter 2).
1.8 Summary

The anatomy and pathophysiology of the mitral valve is well studied. However, there is only limited literature available about the cell biology of VICs and biochemical characterizations of the valve. There are also many studies investigating the effects of mechanical stress and strain on different types of cells seeded within or on a variety of scaffolds. However, only a few of these mechanical loading studies were actually performed on VICs. In myxomatous mitral valves, it has been widely reported that the GAGs are abnormal. However, the role of the PGs, which contain the GAGs and perform many biological and structural functions in the valve, has not previously been studied in myxomatous mitral valve disease. Overall, there is a need for more investigation of the relationship between the biochemical makeup, structure, and mechanics of the valve to better understand valve biology and pathology.

This dissertation contains a rigorous investigation into the mechanical regulation of GAG and PG synthesis by mitral VICs. A comprehensive literature search to survey the effects of strain types on various cardiovascular cells was performed and is explained in Chapter 2. Chapter 3 describes the analysis of different PGs present in the different regions of the mitral valve. I analyzed GAGs and PGs synthesized by VICs seeded within collagen gels under constrained or unconstrained condition as described in Chapter 4, which is followed by Chapter 5 describing the effect of cyclic strains on GAG/PG secretion into the culture medium. Chapter 6 consists of the investigation of variations of strain and frequency on the VIC-seeded collagen gels. Finally, the overall contribution of this work are discussed and future directions are suggested in Chapter 7.
Chapter 2: Effects of static and cyclic loading in regulating extracellular matrix synthesis by cardiovascular cells

2.1 Introduction

Mechanical stimulation is an important modulator of cell function and plays a critical role during tissue development and repair. Mechanical stimuli are transmitted to cells via the ECM, which provides an adhesive surface for cells and structural organization to tissue. Cells sensing mechanical strains will then reciprocate by remodeling their surrounding ECM. The role of mechanical stimuli was described first in bone remodeling and is now being actively investigated for many tissue types. Cells within the cardiovascular tissues have been shown to respond to mechanical stimuli by modulating the synthesis of almost all major components of the ECM, including collagen, elastin, PGs, GAGs, matrix metalloproteinases (MMPs), glycoproteins, and various soluble proteins such as growth factors. This review was performed to understand the mechanical regulation of ECM by cardiovascular cells, which was used as a basis of comparison for the VICs’ response to the various types of mechanical strains to be applied in this research.

The major cell types found in cardiovascular tissues include cardiac fibroblasts, cardiomyocytes, endothelial cells and smooth muscle cells (SMCs); all of these cells interact dynamically with the ECM in response to mechanical strains during development and disease [111, 112]. Fibroblasts are the major cell type in cardiac muscle, representing two thirds of cardiac cells in number, and are mainly responsible for cardiac matrix production [113]. The other cell type in the cardiac muscle is cardiomyocytes, which
primarily have a contractile role [114]. Blood vessels, in contrast, predominantly contain
SMCs and fibroblasts in concentric layers. Heart valves contain valvular interstitial cells
(VICs), which are myofibroblasts and have the characteristics of both fibroblasts and
SMCs [37]. All these cardiovascular tissues are lined with endothelial cells, which act as
a semi-permeable barrier between the tissues and body fluids.

These various types of cardiovascular cells experience complex mechanical
strains, which are either mainly static or cyclic in nature with myriad amplitudes and
frequencies. Because distinctive strain patterns differentially affect ECM synthesis
according to the structural and functional needs of the tissues, it is important to
understand the role of various types of mechanical stimulation on these cells. This review
was focused on the modulation of ECM synthesis by cardiovascular cells in response to
mechanical stresses and strains, with a particular emphasis on the effects of static vs.
cyclic mechanical strains. VICs were not covered in this review, as there is a scarcity of
data on the mechanical loading of these cells. However, the known literature about VIC
mechanobiology is described in Chapter 1.

2.2 Extracellular matrix and non-matrix proteins relevant to mechanical loading of
cardiovascular cells

Each ECM component fulfills a different structural or functional need in connective
tissues but has also been shown to influence cell growth and migration. Collagen is the
most abundant protein in cardiovascular tissues; it is secreted by cells to provide tensile
strength and serve as an organizational scaffold. The other major fibrillar ECM protein is
elastin, which provides elastic recoil and is therefore an essential component of arteries.
PGs consist of one or more GAG chains attached to a core protein; PGs and GAGs serve diverse biological functions, including as acting as "space fillers," within cardiovascular tissues [12]. The GAG hyaluronan and the large PG versican, in particular, sequester large volumes of water and provide resistance to repeated compressive loading, while the small leucine rich PGs such as decorin and biglycan have been shown to contribute to collagen fibrillogenesis [9, 115]. In many pathological conditions of the cardiovascular system (i.e., hypertension, atherosclerosis, and myxomatous mitral valve disease) significantly altered profiles of GAGs and PGs have been found to accompany alterations in mechanical strains within the tissue, which has spurred investigations into the effects of mechanical strains on GAG and PG synthesis [3, 110]. PGs also influence cell proliferation, migration, and phenotype and their synthesis is in turn regulated by growth factors and mechanical strains [14]. Tissue homeostasis is maintained by the synthesis of new matrix by cells and the degradation of matrix by MMPs and other proteases. Mechanical stimuli normally vary physiologically, but increased strains resulting from stenting, hypertension, or atherosclerosis may lead to enhanced matrix degradation and remodeling such as by MMPs [116]. The gelatinases MMP-2 and MMP-9 are the easiest to study using zymography and hence are the most commonly characterized MMPs in these reports. Other proteases such as MMP-1, -8 and -14, which have collagenase activity, have also been investigated. In addition to synthesizing ECM proteins and proteases, cells also secrete a variety of signaling and adhesion molecules including growth factors. Of these non-matrix mediators, transforming growth factor (TGF-β), which can influence proliferation, differentiation, and many other cell functions, and fibronectin, a glycoprotein involved in cell-matrix attachment and thus cell growth and
migration, have been the most widely studied with respect to mechanical loading [117-119]. The mechanical modulation of cardiovascular cells' secretion of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), endothelin-1 and angiotensin-II has also been investigated since these proteins mediate cell growth, migration and signaling [120, 121]. The ECM itself directs the gene expression of many transcribed proteins [122] by changing the physical and chemical environment of the cells and the cytoskeleton association with mRNA through transmembrane receptors.

2.3 In vivo mechanical strains experienced by cardiovascular tissues and relation to ECM remodeling

Cells in the cardiovascular system are exposed to a complex variety of shear, tensile, and compressive strains. Vascular endothelial cells constantly experience shear strains from blood flow, whereas pulsatile pressures result in both tensile and compressive strains on the subendothelial cells within vascular and cardiac tissues. The nonhomogeneous and multiaxial strains experienced by the cardiac wall cells are about 10% in magnitude on average [123, 124]. All cardiovascular cells experience these strains with every heartbeat, i.e., at pulsatile frequencies close to 1 Hz.

Alterations in these physiological mechanical loads may cause compensatory remodeling of the ECM, a common process in cardiovascular pathologies such as hypertension, cardiac hypertrophy, myocardial infarction, myxomatous heart valve disease, and atherosclerosis [112, 125]. In hypertension, the arterial mechanical strains reportedly increase by 15% with a corresponding increase in collagen [126]. Cardiac
hypertrophy is also associated with excess collagen deposition (fibrosis) due to mechanical overload. Atherosclerosis, which develops in part due to reduced and disturbed shear stresses, is characterized by accumulation of collagen, GAGs and cholesterol [110]. Myxomatous mitral heart valves, which are enlarged and flail and thus are speculated to be under reduced tissue tension, have an overabundance of GAGs [3]. To understand these pathologies in more depth, many investigators have studied the effects of mechanical strains on ECM synthesis in vitro to clarify the relationships between tissue microstructure, cell mechanotransduction, and loading conditions using either commercial devices such as the Flexcell system [127] or custom built devices to apply mechanical strains in two-dimensional (2D) [128-131] or three-dimensional (3D) culture [132-134].

Mechanical strains regulate ECM synthesis through cell-matrix interactions, cytoskeletal rearrangements, and by opening stretch-activated ion channels, thereby activating membrane-bound enzymes or releasing growth factors in an autocrine or paracrine manner [79, 118]. The transmembrane receptor integrins, which connect the ECM to the cytoskeleton, have been shown to play key roles in transducing mechanical signals to the cell interior [1]. The subject of cell mechanotransduction pathways has been widely reviewed [108, 120, 121, 135, 136]. Mechanical stimuli also affect other aspects of cell phenotype such as their orientation, growth and differentiation [135]; the overall effects of mechanical strains on these characteristics of vascular smooth muscle cells (SMCs) and cardiac fibroblasts have been reviewed elsewhere [135, 137].
2.4 Effect of various types of mechanical strains on ECM synthesis by cardiovascular cells

Cardiac fibroblasts

Cardiac fibroblasts experience high mechanical loads and remodel the ventricular and atrial ECM during development, growth, and pathogenesis. These cells are always under cyclic strains whose magnitude and frequency vary with heart rate and pressure load. Cardiac chamber walls contain both cardiomyocytes and fibroblasts with the latter serving as scaffolding primarily through maintaining a network of collagen fibers. Correspondingly, cyclic strains applied to cardiac fibroblasts have been found to modulate collagen synthesis and to cause the secretion of various growth factors into the ECM. When these cells were subjected to cyclic loading, their collagen I gene expression increased up to 4-fold compared to no loading conditions [138-141]. This response was similar whether the cells were grown on untreated elastomeric membranes or membranes coated with collagen, fibronectin or laminin [141]; collagen type III mRNA expression by these same cells, however, did not change appreciably. Cyclic stretching has been shown to accelerate the degradation of procollagen, but not to the same extent that new procollagen is expressed, resulting in a net increase [138]. In this same study, the exogenous addition of TGF-β to these stretching conditions further increased procollagen mRNA expression by 4.3 fold. In a different study, the cyclic-strain-induced increase in collagen synthesis was accompanied by increased secretion of TGF-β [139]. TGF-β can enhance collagen expression in a direct or indirect manner, often via the renin-angiotensin system, which is highly relevant to collagenous scarring in the myocardium. There has been only one direct comparison of the effect of static and cyclic strains on
collagen synthesis by cardiac fibroblasts [142]. In this study, 5% cyclic strain (0.33 Hz) induced a 70% increase in the ratio of collagen type III to type I, whereas 5% static strain increased the ratio by only 5% compared to nonstretched controls [142].

One additional report investigated the effect of static strains alone on ECM synthesis. Cardiac fibroblasts subjected to uniaxial static strains showed a significant increase in collagen I, collagen III, and fibronectin expression with 10% strain, while 20% strain decreased collagen III and fibronectin expression when compared to nonstretched controls [143]. Similarly, when tensile equibiaxial strains were applied, cells subjected to 3% strain expressed more collagen III and fibronectin but at 6% strain the mRNA levels for collagen III decreased as compared to nonstretched controls [143]. In contrast, compressive equibiaxial strains of either 3% or 6% caused decreased expression of collagen III and fibronectin, suggesting that tensile strains might be the primary signal for stretch-induced matrix synthesis by cardiac fibroblasts. In the same study, TGF-β activity was increased at both 10% or 20% uniaxial strain and 6% biaxial strain (either tensile or compressive); 3% biaxial strain had no effect on TGF-β activity.

In general, mechanical strains increase the synthesis of collagen and release of growth factors by cardiac fibroblasts, which can cause myocardial hypertrophy [144]. This regulation of collagen synthesis is mainly mediated by TGF-β and MAP kinase pathways [139, 140]. However, there is a paucity of information regarding cardiac fibroblasts’ synthesis of other ECM components and proteases in response to mechanical strains.
Cardiomyocytes

The cardiomyocytes are mainly involved with muscle contraction [114] yet they also produce various ECM molecules and release growth factors. Physiologically, cardiomyocytes are exposed to cyclic loading but can also experience hemodynamic (pressure) overload manifested as an increase in baseline static stretch. This hemodynamic overload causes cardiac hypertrophy (increase in cell size), which can significantly affect cell structure and function [121]. Therefore, the effects of both cyclic and static strain are relevant to these cells. Interestingly, much less attention has been given to cardiomyocytes than to other cardiovascular cells; only three studies have investigated the effect of mechanical strains on their synthesis of extracellular proteins relevant to ECM and tissue remodeling, likely because chemical (altered blood chemistry) and electrical (heart contraction) stimulations are more relevant to cardiac muscle. Regardless, mechanical stimulation has been shown to be important in hypertrophy. In two in vitro models of load-induced hypertrophy, static uniaxial strains of 20% were applied to cardiomyocytes, resulting in significant increases in the secretion of endothelin-1 and angiotensin-II [145, 146], both growth-promoting factors whose release would activate phosphorylation cascades, initiate cell growth, and subsequently cause hypertrophy [146]. A third study reported that 20% cyclic stretch caused an increase in MMP-2 and MMP-14 expression via the angiotensin II-JAK-STAT1 pathway; the JAK/STAT pathway has been demonstrated to be involved in cell-specific MMP expression [147]. Although mechanotransduction pathways may initiate signal transduction through many possible mechanisms, growth factor secretion results in the release of second messengers in stretch induced cardiac hypertrophy [120, 121, 148]. At
this time, no study has explored how other ECM constituents such as collagen, elastin, and PGs are synthesized by cardiomyocytes in mechanical stretch-induced hypertrophic conditions, presumably because cardiac fibroblasts bear the majority of this function.

**Endothelial cells**

All cardiovascular tissues are lined with a layer of endothelial cells, which experience both pulsatile pressures (normal tensile stresses) and shear stresses imposed by the flowing blood in the vasculature [149]. Shear stresses are transmitted to the ECs through the glycocalyx (a thin layer of glycoproteins and PGs surrounding the plasma membrane), stretch-activated ion channels, or integrin binding among other possible pathways [150]. Because cyclic strains and shear stresses on ECs regulate vascular architecture, tone, and remodeling, their effects on the synthesis of various ECM molecules have been widely investigated. ECs mainly synthesize GAGs, PGs, MMPs, fibronectin and a variety of growth factors such as PDGF and FGF. These ECM components and growth factors are critical to anticoagulation, anti-atherogenicity, and growth of the underlying SMCs.

The range of shear stresses on ECs, which normally vary with the flow rate of blood during diastole and systole, can affect the synthesis of various proteins. Shear stresses equivalent to laminar flow (1 dyne/cm²) decreased PG secretion whereas high shear stresses (5 to 40 dynes/cm²) increased GAG and PG synthesis compared to static no-flow conditions. These results were shown both in non-pulsatile conditions for durations of 24 hrs or less [151-153] and in pulsatile conditions of 72 hrs duration [154]. These ranges of high shear stresses were similar to those of veins (~5 dynes/cm²) and
arteries (~23 dynes/cm²) [154], which indicates that high shear stresses are necessary for normal synthesis of GAGs and PGs and to maintain homeostasis within vascular tissue. When pulsatile shear stresses were applied bidirectionally, as compared to static or unidirectionally, ECs increased MMP-9 expression, which has been shown to promote atherosclerosis [155].

Cyclic strains have also been shown to modulate ECM synthesis by ECs. High strains (4.9-12.5%) increased total protein synthesis and decreased fibronectin secretion into the medium [129]; the authors speculated that these high strains possibly caused cell injury. In other studies, cyclic strains of 10-24% decreased collagen and non-collagenous protein, but increased PDGF-B expression [156, 157]. The expression of MMP-2 and membrane type I MMP, partially mediated by p38 and ERK-dependent pathways, was found to increase with cyclic strains in a magnitude and time-dependent manner [158, 159]. When aortic ECs were co-cultured with SMCs in tubular constructs, the cells decreased collagen and GAG deposition after 15 days under pulsatile shear stress conditions [160]. This study demonstrated the significance of cell-cell interactions as the presence of ECs caused SMCs to express more of a contractile phenotype compared to a synthetic phenotype. Similar to growth factors, the cytoskeletal proteins are major intermediates in transmitting the strains through integrins and regulating gene expression; Davies et al. have comprehensively reviewed hemodynamic mechanotransduction within ECs [149]. Hemodynamics is also important in the development of pathogeneses such as atherosclerotic lesions. The heparan sulfate GAGs that are synthesized by ECs, located in their glycocalyx and underlying basement membrane, have anticoagulation activity;
hence, the ECs lining vessels are subject to high shear stresses are each surrounded by a thick glycocalyx for improved anti-atherogenicity [161].

There has been only one study to directly compare the effects of shear and cyclic strains on ECs. This study found that shear stress increased PDGF-B and bFGF expression, whereas cyclic strains had no effect [162]. These results indicate the importance of fluid shear in regulating EC secretion of growth factors, which then regulate mitogenic activity and govern the vascular structural response during atherosclerosis.

Overall, investigators have shown that low shear stresses and cyclic strains decrease the synthesis of ECM molecules by ECs. Static strains, which are less relevant to ECs, have not been investigated in this context. Cyclic strains on ECs decrease matrix building and cell-matrix binding proteins (collagen, fibronectin) and increase matrix degrading proteins (MMPs), consequently reducing net ECM. In contrast, high shear stresses variably regulate ECM expression and synthesis and maintain arterial wall tone and architecture.

**Vascular smooth muscle cells**

Smooth muscle cells, located in the medial layer of the vasculature, are responsible for vessel contractility and remodeling during growth and pathogenesis. *In vivo*, SMCs mainly experience cyclic tensile strains due to pressure forces of the blood and compression due to thinning of the vessel wall during inflation. Shear stresses experienced by the ECs in the blood vessels can also be transferred to SMCs but the magnitudes of these transferred stresses are low compared to the tensile stresses [149]. As
SMCs are the predominant cell type in blood vessels, there are abundant studies on the mechanical regulation of ECM synthesis by SMCs.

Cyclic strains tend to increase collagen and elastin synthesis by vascular SMCs but these responses are sensitive to the strain magnitude, frequency and duration [133, 163-165]. In a study of very high strains (25%) at 0.05 Hz, a significant increase in total protein and collagen synthesis could be seen only after 5 days of stretching [166]. The authors attribute this delay to the extremely high strain, which might have damaged the cells; the frequency may have also been too low to provide proper mechanical stimulus. Another study showed the dependence of total protein and collagen synthesis on strain magnitude; synthesis was increased between 5% and 10% strain, but then was unchanged between 10% and 20% strain, compared to nonstretched controls [167]. In a different investigation using 4% cyclic strain, collagen I expression increased to a maximum at 12 hrs, then remained constant up to 48 hrs [82]. Although the majority of these reports noted that strain increased overall protein synthesis, there is one exception. Kulik et al. reported a slight decrease in protein and collagen synthesis when pulmonary arterial SMCs were subjected to cyclic stretch using strains of 10 or 20% and frequencies of 0.33-0.5 Hz [131]. Possible explanations for these unexpected findings included the loss of cell surface receptors, a lack of cell adhesion molecules, or insufficient stretching time (3 or 6 hrs only). The culture environment is also highly relevant. For example, the addition of serum did not cause any increase in total protein or collagen synthesis by cyclically stretched aortic SMCs, whereas in serum-free medium the total protein and collagen synthesis doubled over stationary controls [168]. When seeded in 3D tubular collagen scaffolds and cyclically stretched (2.5-10%, 0.5 Hz), SMCs demonstrate no
change in collagen synthesis, but elastin synthesis increased enormously [132]. In another 3D study, when SMCs were seeded together with ECs on a polyglycolic acid (PGA) scaffold, collagen content increased under pulsatile conditions (5% radial distension, 2.75 Hz) compared to non-pulsatile conditions [169]. These variations in elastin and collagen contents alter tissue material properties, emphasizing the critical role of mechanical strain in tissue remodeling and tissue-engineering applications.

Cyclic mechanical strains generally increase the GAG and PG synthesis by cardiovascular cells but the increases are often specific to certain types of GAGs. For example, cyclic stretching of aortic SMCs in 2D culture tripled the synthesis of the GAGs hyaluronan and chondroitin 6-sulfate over stationary cultures, but did not affect the synthesis of chondroitin 4-sulfate and dermanan sulfate [163]. Similarly, cyclic stretching increased expression of the PGs versican, biglycan, and perlecan, but decreased the expression of the PG decorin [82]. These two reports are consistent as versican contains abundant chondroitin 6-sulfate and aggregates with hyaluronan, whereas decorin is associated with dermanan sulfate. SMC expression of the heparan sulfate PG syndecan-4, a cell-adhesion molecule colocalized with integrins in focal adhesions, was increased after 1 hr of biaxial cyclic stretching (10%, 1 Hz) but then decreased over 24 hrs [170]. The expression of syndecan-4 did not change when strain was lowered from 10% to 3%. Mechanical stretch also caused syndecan-4 shedding from the cell surface, which served to promote cell motility via decreased focal adhesions.

MMP synthesis by SMCs has also been modulated with the application of cyclic strains, but different types of MMPs are regulated in distinct ways. One study found a significant downregulation of MMP-1 (collagenase) with 4% cyclic stretching [171]
whereas another study reported no changes in the expression of MMP-2 or MMP-9 (both gelatinases) [172]. Two other studies, however, did find an increase in MMP-2 with cyclic strains ranging from 10% to 16% [165, 173]. In a tubular collagen construct, which provides more of an in vivo-like 3D environment, the application of 10% cyclic stretch to the embedded vascular SMCs increased MMP-2 levels more than 5-fold compared to nonstretched controls [134].

Cyclic stretching has almost uniformly been found to increase SMC synthesis of growth factors and signaling molecules. The expression and secretion of TGF-β, PDGF, and VEGF increase substantially with cyclic stretching [165, 171, 174-176]. Cyclic strain-induced secretion of TGF-β and FGF-2 into the culture medium was found to be time and strain dependent [130, 177]. These growth factors regulate SMC growth, differentiation and gene expression in an autocrine or paracrine manner. For example, secreted TGF-β promotes L-proline transport and hence collagen synthesis and cell growth during arterial remodeling in hypertension [177].

Although the role of static stretching on ECM synthesis has received less attention than cyclic stretching, several reports have described its effect on SMCs. Static stretch has been reported to increase SMC synthesis of tropoelastin, the precursor of elastin [178]. Three studies in particular have directly compared the effects of static and cyclic strains [116, 179, 180]. In the first study, cyclic strain (10%, 0.86 Hz), compared to 10% static strain, caused a 3-fold increase in total protein and collagen synthesis [179]. Moreover, artificially generating an increase in intracellular cAMP levels (by adding theophylline, inhibitor of cAMP degrading enzyme) inhibited the collagen synthesis in cyclically stretched cultures only, suggesting that cAMP was involved in the response to
cyclic stimuli but not static stimuli. cAMP mediates protein synthesis by SMCs; its levels decrease during hypertension and are inversely related to increased collagen synthesis [181]. In the second study, a 50-fold increase in MMP-2 mRNA was found after 24 hours of 5% static stretch but no change was found in the absence of stretch or in cyclic stretch (1 Hz) [116]. Additionally, secretion of MMP-2 and MMP-9 increased with static stretch, but decreased with cyclic stretch. The stretching protocol of the third study simulated an arterial balloon injury to explain the tight regulation of syndecan-4 expression and cell migration during the injury process [180]; the high (30%) static strains rapidly increased syndecan-4 expression compared to 10% cyclic strains but subsequent application of 5% cyclic strains downregulated the expression. All three of these studies clearly demonstrated that static strains promote ECM degradation and synthesis of adhesion molecules whereas cyclic strains enhance SMC synthesis of fibrillar ECM proteins.

Alternative mechanical loading conditions such as centrifugal force and shear stresses have also been used to investigate ECM synthesis by SMCs. Although centrifugal forces are less physiologically relevant to SMCs than the methods discussed above, they serve as a simple tool to apply circumferential stresses, as found in hypertension. Centrifugal forces applied to vascular SMCs increased total GAG synthesis, predominantly affecting heparan sulfate (found in the glycocalyx and basement membrane) but minimally affecting hyaluronan (which can be present in either the pericellular matrix or in the ECM) [182, 183]. These are the same trends found with the application of static strains, which show some mechanical equivalence to centrifugal forces. Shear stresses have also been investigated because they are transferred from ECs to SMCs in vivo. Shear stresses (10-20 dynes/cm²) applied to SMCs decreased MMP-2
activation and significantly inhibited cell migration; SMC migration from the media is a characteristic finding in intimal hyperplasia [184, 185].

Overall, cyclic strains applied to SMCs increase ECM building proteins and signaling molecules whereas static strains and shear stresses upregulate ECM degrading proteases. ECM synthesis also depends on strain magnitude and durations. The results were also found to be sensitive to cyclic frequency and the culture conditions such as 2D vs. 3D or biaxial vs. uniaxial strain.

2.5 Summary and future directions

Cardiovascular cells show selective responses to different types of strains (Table 2.1). In general, the literature reviewed here reports that strains enhance the synthesis of most ECM components in cell culture. Although cells tend to respond rapidly to strains in a magnitude dependent manner, there are no overwhelming trends regarding time dependence. Cyclic strains are the obvious choice to apply mechanical stimuli to cardiovascular cells, which experience cyclic stresses under pulsatile blood flow. However, in certain circumstances such as when failing hearts are supported by continuous-flow left ventricular assist devices (LVADs) [186], the cardiac cells will experience predominantly static tensile stresses. The growing use of these continuous-flow LVADs mandates understanding the effect of static strains on ECM synthesis. From the limited investigations regarding static strains published to date, the comparative effects of static vs. cyclic strains appear to vary for different ECM macromolecules. Cyclic strains tend to affect collagen and elastin synthesis more profoundly than do static strains whereas the reverse is true for MMP-2 synthesis. Given that MMP expression
increases in heart failure and that many patients are put on LVAD support [187], the potential for alterations in MMP expression warrants continued in vivo and in vitro research to understand the effect of various loading conditions on ECM synthesis and degradation. Furthermore, although SMCs and collagen were the focus of most of these reports, changes in ECM components such as GAGs, PGs and MMPs are prominent in many cardiovascular pathologies, so the effects of strains on their synthesis by SMCs or all cell types should be given more emphasis in the future.

Table 2.1 Overview of ECM modulation studies for various cardiovascular cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ECM protein changes with strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECM protein changes with strain</td>
</tr>
<tr>
<td></td>
<td>Static</td>
</tr>
<tr>
<td>Cardiac fibroblasts</td>
<td>↑ Collagen [142, 143]</td>
</tr>
<tr>
<td></td>
<td>↑↓ Others [143]</td>
</tr>
<tr>
<td></td>
<td>↑↑ Others [143]</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>↑↑ Others [145,146]</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>↑ GAGs &amp; PGs [151-153]</td>
</tr>
<tr>
<td></td>
<td>↑↑ Others [162]</td>
</tr>
<tr>
<td></td>
<td>↑↑ GAGs &amp; PGs [154,160]</td>
</tr>
<tr>
<td></td>
<td>↑↑ Others [157,127]</td>
</tr>
<tr>
<td>VSMC</td>
<td>↓ Collagen [179]</td>
</tr>
<tr>
<td></td>
<td>↑↑ Elastin [178]</td>
</tr>
<tr>
<td></td>
<td>↑↑ GAGs &amp; PGs [180,182,183]</td>
</tr>
<tr>
<td></td>
<td>↓ MMPs [114,184,185]</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

"Others" include total protein, growth factors and signaling molecules. Double arrow = 100%, single arrow = 60-99%, combined arrow = 40-60%, contradictory studies, or different responses to various strain magnitudes.
Many culture conditions have been used to apply mechanical strains to cardiovascular cells. 2D cell culture is appealing due to easy handling, maintenance, and manipulation of the mechanical strains. In addition, tissue-engineering techniques have recently been used to characterize vascular cell phenotype and modulation of ECM synthesis [86, 188]. The cell shape and pericellular environment in 3D cultures is more like that found in native tissues and cells may utilize cell-matrix interactions (i.e., focal adhesions) and mechanotransduction signaling differently than when they are in 2D cultures, although admittedly mechanical strains can be more complex to apply in 3D [189]. In the future, 3D cultures may become more widely used to characterize cellular responses to mechanical strains.

The effect of cyclic strains on ECM synthesis appears to be specific to the different cell types in cardiovascular tissues. For example, collagen synthesis increased with strain in SMCs and cardiac fibroblasts, whereas the body of responses in ECs is less uniform. Another cardiovascular cell type is heart valve interstitial cells, which (as described in Chapter 1) resemble myofibroblasts [37]. Heart valve cells’ responses to mechanical strains have only recently been explored in 2D and 3D culture systems [95, 106] although no study has focused exclusively on ECM modulation. Porcine aortic valve interstitial cells produced more protein and GAGs than did porcine aortic SMCs in 3D culture [95]; clearly, valve cells require further investigation as they are responsible for valvular remodeling and disease. Among all the reports of cardiovascular cells, inconsistencies regarding the matrix production elicited by mechanical stimuli may be due to different breeds, species, and ages of animals from which the cells were derived. Furthermore, variations in ECM gene expression with stretch may be due to the effects of
pure tensile or compressive strains imposed by different stretch devices. In addition to the stretch applied to the cultured cells, their metabolic state, culture conditions, and accumulation of secretory products likely also affect their synthesis of matrix components and matrix mediators.

While the role of integrins in mechanotransduction is well known [149], other membrane-bound proteins such as the ADAMs (a disintegrin and a metalloproteinase domain) also support integrin-mediated cell adhesion because they can cleave ECM proteins by their metalloproteinase domains [190]. Recently, a new mechanism of mechanotransduction was proposed in which mechanical stimulation encourages growth factor shedding into the ECM [191]. Because ADAMs have the ability to shed many cell-adhesion molecules and cell-surface proteins including cytokines and growth factors, they may be key regulators in mechanotransduction signaling pathways and their synthesis by cardiovascular cells certainly merits further investigation. The relevant intracellular signaling pathways, however, may differ for various types of strains. Numerous mechanotransduction pathways (most involving MAP kinase) for specific cell types have been proposed [1, 108, 120, 121, 125, 135, 136, 149, 150], but only a few reports have indicated which pathways are specifically activated by static stresses. The application of static strains to cardiac fibroblasts, for example, activated G protein subunits [192] and the ERK2 or JNK1 pathways [193], whereas release of endothelin-1 from cardiomyocytes under static strains activated MAP kinases and Raf-1 in stretch-induced cardiac hypertrophy [146]. It has yet to be determined which particular pathway is required for regulation of the synthesis of each of the ECM molecules under the diversity of mechanical stimuli experienced in vivo. Overall, determining the precise role
of static and cyclic mechanical stimulations on the regulated synthesis of various cardiovascular matrix macromolecules should remain an intriguing area of research for many years to come.
Chapter 3: Proteoglycan measurement in human mitral valve regions

3.1 Introduction

The mitral valve is one of the most complex connective tissue structures in the entire body. It consists of two leaflets and numerous chordae tendineae. These chordae have a highly aligned collagenous core and a thin outer sheath of elastic fibers and endothelial cells. Both leaflets are laminated tissues containing a heavily collagenous layer on the ventricular side; a predominantly elastic layer on the atrial side; and an inner spongiosa layer containing abundant proteoglycans (PGs) and hyaluronan (HA) (Figure 1.3). The relative thicknesses of these layers vary between the two leaflets and also within each leaflet from its attachment edge to its free edge [27]. The variability of the different leaflet layers, and hence the structural constituents within the mitral valve, are determined by the specific functional roles of the leaflets and chordae. The closed valve, in particular, maintains a balance of tensile and compressive loads, in which the chordae and the flat central region of the anterior leaflet are in tension, whereas the free edge of the anterior leaflet and most of the posterior leaflet are in appositional compression. Accordingly, the most collagenous components of the mitral apparatus are the chordae and the portion of the anterior leaflet between the annulus and the upper appositional border [27]. In the posterior leaflet and in the free edge of the anterior leaflet, the collagenenous layer is relatively thinner, whereas the PG rich spongiosa is substantially thicker. The wide diversity of glycosaminoglycans (GAGs) and their parent PGs exert considerable yet variable control over the physical properties of the extracellular matrix [8]. Over the past 50 years, exhaustive biochemical analyses have been conducted on the
GAGs of heart valves of pigs [194], cows [195-201], rodents [202, 203], and humans [55, 56, 204-206]. Despite this wealth of literature, there has been only one group that attempted to link these GAG profiles with the presence of a particular PG [9]. In general, there is scant information regarding the influence of PGs on the mechanical properties and function of the valve tissues.

This study was designed with the objective to determine if the fine structure and PGs present in mitral valve tissues are dependent on the predominant type of tissue loading. For this purpose, mitral valves from normal human autopsy subjects were cut into regions of compressive and tensile loading, and their PG characteristics were measured using western blotting.

### 3.2 Materials and methods

Normal mitral valves (n=35) were obtained at autopsy from persons who died of noncardiac-related causes and were harvested after less than 24 h in cold storage. Our laboratory has determined that valves kept at 4°C retain their matrix-based mechanical properties for at least 5 days [207]. We therefore believe that the matrix in these cadaveric valves was not measurably degraded during the postmortem period. All chemicals were obtained from either Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA), except for glycerol, guanidine HCl, and proteinase-K (Invitrogen, Carlsbad, CA); hyaluronidase SD, chondroitinases ABC and ACII, and 2-B-1 antibody for versican (Seikagaku America, Falmouth, MA); Q-Sepharose Fast Flow beads and peroxidase-linked secondary antibodies (Amersham, Uppsala, Sweden); Triton X-100 (Roche, Indianapolis, IA); and 2-aminoacridone HCl (Molecular Probes, Eugene, OR).
Monosaccharide electrophoresis running buffer and the preformed monosaccharide gels were purchased from Glyko (Novato, CA). The polyclonal antibodies LF136 for decorin and LF51 for biglycan were generously provided by Larry Fisher at the National Institute of Dental and Craniofacial Research, NIH.

**Sample Preparation**

Mitral valve samples were prepared by first removing the chordae then sectioning the valve into anterior and posterior leaflets (Figure 1.2). The anterior leaflets were sectioned into the central region and the free edge. The chordae were trimmed from the leaflets at their point of attachment, and divided into four groups: anterior basal, anterior marginal, posterior basal, and posterior marginal. The wet weights of the leaflet samples and chordal samples (chordae were pooled into the four groups) were determined. The water content was calculated after lyophilizing the samples for 16 h and then weighing again to obtain the dry weight. The dried samples were stored in microcentrifuge tubes at 20°C prior to analysis.

**Proteoglycan analysis**

Five fresh mitral valves were dissected into regional samples as described, lyophilized overnight, and reweighed to obtain dry weight. Each sample was minced with fine scissors in a 2-ml centrifuge tube and then agitated in extraction buffer overnight at 4°C (4 M guanidine HCl, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.05 M ammonium acetate, 0.01 M ethylenediamine tetraacetic acid (EDTA), 0.1 M 6-aminohexanoic acid, 0.08% benzamidine HCl, 10 mM N-ethyl
maleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 ml per 25 mg tissue dry weight). After extraction, the samples were centrifuged (13,000 rpm) and the supernatant dialyzed four times against 7 M urea buffer (containing 2 mM EDTA, 0.05 M Tris, 0.5% Triton X-100, pH 7.5) to remove the guanidine. After dialysis, volumes of extract solutions containing equivalent proportions of starting dry mass were mixed with Q-Sepharose beads, and the beads were rinsed with 40 column volumes of 7 M urea buffer containing 0.25 M NaCl. The bound purified PGs were eluted with 7 M urea buffer containing 3 M NaCl. Equivalent aliquots of the purified PG samples were mixed with water to a final volume of 300 ml and precipitated by adding 1 ml 95% ethanol/1.3% potassium acetate and incubating at 20°C for 2 h. The precipitate was suspended in 20 ml of enzyme digest solution (containing 2.5 mU/ml chondroitinase ABC, 0.01% bovine serum albumin (BSA), 0.05 M Tris, 3 mM Na acetate, 8 mM 6-aminohexanoic acid, 0.42 mM benzamidine HCl, and 0.08 mM PMSF) and incubated at 37°C for 3 hour. Samples were then mixed with an equivalent volume of sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and run on a 4%-12% SDS-PAGE gel at constant 180 V. The gel was transferred to a 0.2-mm nitrocellulose membrane at 100 V (1 h for decorin and biglycan, 3 h for versican). The membrane was blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 and 2% BSA overnight at 4°C, then treated with primary antisera to decorin, biglycan, or versican (1:6000 dilution in TBS/Tween containing 2% fetal bovine serum) overnight at 4°C. After four washes in TBS/Tween, the membrane was treated with horseradish peroxidase-linked secondary antibodies (1:20,000 dilution in 2% BSA) for 2 h at room temperature, then washed six times more. Proteins were detected using
chemiluminescent exposure (using ECL detection kit from Amersham) to radiographic film (Kodak, Rochester, NY). PG bands were identified by comparison with positive controls and quantified using densitometry. Each PG band (decorin, biglycan, or versican) was normalized by the content of the corresponding band from the anterior leaflet free edge sample run on the same gel. The complete process of PG isolation, purification and detection is shown in the flow chart (Figure 3.1).

*Optimization of proteoglycan detection*

As noted previously in Chapter 1, Section 1.4, there are three major kinds of PGs found in the mitral valve: decorin, biglycan and versican. Mitral valve leaflets and chordae contain different kinds of PGs in variable amounts. The isolation and purification of these PGs were optimized for detection on a SDS-PAGE gel and western blot membrane. Purification optimization was determined by comparing different Q-sepharose bead (0.25, 0.5 and 0.75 ml) and elution (6 column) volumes. Appropriate precipitation volumes were considered to be optimized for specific PGs when a detectable (but not saturated) band could be visualized on western blot membrane. Procedures for SDS-PAGE and western blotting are explained in the previous section.

*Statistical analysis*

Band intensities of PGs from different regions of mitral valve were compared using analysis of variance (ANOVA). These samples were run on the same gel and were taken from equivalent amounts of starting material.
Figure 3.1 Flow chart for the detection of proteoglycans.
The band intensities from all valve regions were normalized to band intensity of the anterior leaflet free edge region for each valve separately. The rationale for this normalization method was that all regions from the same valve were run on the same gel and that this approach would eliminate the gel to gel variability. The anterior leaflet free edge was chosen for this normalization since it was consistently available for each valve received from autopsy and there was always sufficient quantity available for PG analysis, unlike some of the other leaflet regions.

3.3 Results

Proteoglycan detection optimization

First, the amount of Q-Sepharose beads and the elution volume were optimized to purify the PGs. As shown in the SDS-PAGE gel (Figure 3.2), higher volumes of beads caused the PG band intensity to increase in the elution fractions. Also, there was no loss of the PGs in the washing step (lanes 3, 8 and 13). There were no bands of PGs in the fourth elution for each bead volume, so there was no need to collect the 4th elution. The enzyme control (chondroitinase ABC with protease inhibitor) was also run in the 2nd lane so it could be identified in each lane.

It was found that 0.75 mL of beads per 1 mL of the guanidine-extracted sample (25 mg of dry tissue weight) were required to bind all the PGs, and 4.5 mL (3 elutions with 2 column volumes each) of 3 M NaCl in 7M urea buffer were required to elute the bound PGs. All these optimization studies were performed on PGs extracted from porcine mitral valves. Different precipitation volumes were also optimized to determine the detectable amount of the PGs on western blot film. It was found that the following
approximate quantities of original dry mass should be taken: 3-4 mg for SDS-PAGE; 4 mg for western blots of versican; 1 mg for western blots of decorin; and 1.5 mg for western blots of biglycan (Table 3.1).

<table>
<thead>
<tr>
<th>Lanes: S EC W E₁ E₂ E₃ E₄</th>
<th>0.25 mL beads</th>
<th>0.50 mL beads</th>
<th>0.75 mL beads</th>
</tr>
</thead>
</table>

Versican

V₀

V₁

decorin/ biglycan

**Figure 3.2.** SDS-PAGE gel for purification optimization (S-standard, EC-enzyme control, W-washing, E₁₋₄ – elution fractions; amount of Q-sepharose beads taken 0.25 mL, 0.5 mL and 0.75 mL).

*Proteoglycans present in human normal and myxomatous mitral valve*

When the intact PGs were run on an SDS-PAGE gel, it was found that versican was so large that it barely entered the stacking gel, biglycan was present as a smear from 200-300 kDa, and decorin was a smear from 100-200 kDa (Figure 3.3A). After treatment with chondroitinase ABC to remove the GAG chains, the core proteins for decorin and biglycan both appeared about 43 kDa, with decorin having a characteristic doublet appearance (Figure 3.3B).
Table 3.1. Optimization of PG detection via SDS-PAGE and western blotting.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDS-PAGE</th>
<th>Western Immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Versican</td>
</tr>
<tr>
<td>Valve Tissue</td>
<td>3-4 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td>(original dry mass)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen gel</td>
<td>3-4 mg</td>
<td>6 mg</td>
</tr>
<tr>
<td>(original dry mass)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td>6 mL</td>
</tr>
<tr>
<td>(original volume)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The gels were always run with equivalent amounts of starting material as measured by dry weight. Also, tissue regions from the same valve were always run together in order to normalize the band intensities to the anterior leaflet free edge [13]. The relative band intensities had significant differences between leaflet regions, with decorin being most abundant in the center of the anterior leaflet (a region under tension), and biglycan being more abundant in the center of the anterior leaflet and in the chordae tendineae (regions in tension).

An ANOVA found a statistical difference between valve regions for the decorin and biglycan (both $p<0.001$). In contrast, versican was present in greater quantities in the posterior leaflet and free edge of the anterior leaflet (compressive regions) with lower quantities in the chordae tendineae and center of the anterior leaflet (tensile regions), although this trend was not a significant difference, likely due to the small sample size. The core protein of versican is usually present in two isoforms: V0, which is the complete
version, and V1, which lacks the alpha region (Figure 3.3C). V1 is usually more abundant in the tissues.

Figure 3.3. Detection of proteoglycans in normal mitral valves. (A) SDS-PAGE gel, (B) western blots for decorin and biglycan, (C) western blot for versican.
Figure 3.4. Relative abundances of the proteoglycans decorin, biglycan, and versican in different regions of the mitral valve, normalized to the specific proteoglycan content in the free edge of the anterior leaflet (mean ratios ± SD, n=5, see text for explanation). Compressive regions = posterior leaflet and free edge of the anterior leaflet. Tensile regions = chordae tendineae and center of the anterior leaflet. (b) Representative western blots of the proteoglycans in these same leaflet regions.
The decorin and biglycan samples were analyzed eight times (2 analysis, 2 lanes, 2 precipitation) to establish intraobserver variability as 3.9\% for band quantitation, 5.5\% for lane-lane repeatability from the same precipitated volume, and 9.7\% for repeatability in separately precipitated samples from the same extraction volume. There were no statistical differences between data from different gel runs, precipitations, and GelPro analyses (paired t-tests, two-tailed p<0.05). Due to the large volume of sample required to visualize the versican bands, the versican samples were not run in duplicate.

3.4 Discussion

This study showed that the components of the mitral valve apparatus that experience predominantly tensile loads, such as the central portion of the anterior leaflet and the chordae, contain relatively less water and more decorin and biglycan. Although the division of the mitral valve into regions that experience tensile and compressive loading certainly simplifies its very complex loading regime, a rationale for this segregation is provided by the valve's mechanical and microstructural heterogeneity. For example, the elastic moduli of the center region and of the chordae tendineae are higher than those of the anterior leaflet free edge and the posterior leaflet [208-210]. In addition, both the central portion of the anterior leaflet (adjacent to the annulus) [28] and the chordae [211] contain collagen fibers that are highly oriented in the predominant loading direction, whereas the posterior leaflet and the free edge of the anterior leaflet have less collagen fiber alignment [212] and a thicker, GAG-rich spongiosa [27].

The hypothesized differential abundances of these PGs in tensile versus compressive loading regions of the mitral valve was partially confirmed by our
immunoblotting of valve extracts. It was evident that versican, decorin, and biglycan were present in some quantity throughout the valves, which agreed with the earlier results showing that all regions had a wide distribution of GAGs (Figure 3.4). There were significant regional differences in the relative amounts of decorin and biglycan. Although there were not significant differences between regions for versican, a larger sample size may have been able to detect a greater abundance of versican in the compressive loading regions.

The difference in relative PG composition of the compressive as compared to tensile loading regions of the valve reflects the structural and functional differences of these regions. The long GAG chains from the PG versican extend away from the core protein to minimize electrostatic interactions. The resulting large hydrodynamic volumes are ideal to respond to variable compressive loads and to withstand high pulsatile forces [4]. Versican would thus allow the loosely layered appositional surfaces of the mitral valve to reversibly buffer the considerable impact and shearing deformations that occur during valve opening and closing. The core protein of versican also contains a HA-binding region [213, 214] that anchors the PGs on long strands of hyaluronan and thereby retains them in the tissue. The combined versican-HA aggregate has been suggested to perform a lubrication and antiadhesive role in tissues by preventing ligand-receptor interactions between cells and matrix [215, 216]. This lubrication could also aid in viscous dissipation for the impact forces during valve closure [201]. In contrast, decorin and biglycan were present in the more solid tensile loading structures of the valve. Decorin in particular is abundant in connective tissues with high concentrations of type I collagen [217], in which it stabilizes and orients collagen fibrils [9, 218]. Decorin
therefore may play a role in the overall strength of high load-bearing tissues. Biglycan, which typically does not colocalize with decorin, also contributes to collagen fibril diameter and tissue strength [219].

In conclusion, these data on PGs from human mitral valves indicate that the regions in compression are rich in versican and regions in tension are rich in decorin and biglycan. The compositional patterns of mitral valve PGs thus provide new insight into the roles of these molecules in load-bearing tissues.
Chapter 4: Synthesis of glycosaminoglycans and proteoglycans in differently loaded regions of collagen gels seeded with valvular interstitial cells

4.1 Introduction

It has been known for many decades that mechanical stresses and strains dictate the cellular and ECM organization and remodeling of tissues. Because cells are connected to the ECM via integrins, mechanical stresses and strains in the ECM result in cell mechanotransduction and can regulate the expression of ECM genes [1, 79]. In heart valves, the VICs experience uniaxial, biaxial or multiaxial strains in vivo; uniaxial and biaxial strains are relatively straightforward to investigate within a controlled in vitro study. Using 2D models, the application of uniaxial vs. biaxial strains was shown to induce distinct responses by fibroblasts [143, 220]. Recently, the response of valve cells to pressure and shear forces has been characterized in organ culture [104, 221], but there has been only one study published to date that has examined the effect of mechanical strains in other forms of valvular cell culture [106].

PGs, which are composed of a core protein and various glycosaminoglycan (GAG) chains [5], are one of the major ECM components in soft tissues, where they perform many biological and structural functions [4, 8]. The chondroitin/dermatan sulfate PGs decorin, biglycan and versican are the most commonly found PGs in heart valves [13]. Decorin and biglycan are small PGs that are known to aid in collagen fibril formation [10, 11], whereas the large PG versican aggregates with the GAG hyaluronan, together accumulating large volumes of water that provide compressive resistance to the
tissues [4]. Previous research on mitral valves has shown that valve regions in tension (chordae and center of anterior leaflet) contain abundant quantities of GAG dermatan 4-sulfate, and PGs decorin and biglycan, while regions experiencing compression (posterior leaflet and free edge of anterior leaflet) contain more of the GAGs hyaluronan and chondroitin 6-sulfate, and PG versican [13]. Furthermore, mitral valves with myxomatous degeneration, which are subjected to altered tissue loads, contain more GAGs and PGs than normal [3].

Many different types of scaffolds and cell environments have been used to investigate the effects of mechanical stress and strain conditions on various cell types. 3D collagen gels are a popular choice for scaffolds, as they are easy to use and polymerize, permeable, biocompatible, and relevant to the normal environment of the cells [86]. Overall, seeding cells within a collagen matrix is believed to provide a more biologically and anatomically appropriate model for studying the regulation of ECM deposition than growing cells in monolayers [188, 222-224]. Indeed, VICs seeded on top of or within collagen gels have been shown to retain their native phenotype and secrete GAGs and PGs that are normally present in heart valves [92, 93, 95]. To our knowledge, there has not been any 3D model that has studied the effects of different strain types on GAG and PG synthesis by VICs seeded within engineered tissues. Therefore, the purpose of this study was to determine the effect of different loading conditions on GAG and PG production, which is relevant to heart valves as well as other tissues. To investigate this effect, VICs from mitral valve leaflets and chordae were cultured within 3D collagen gels and subjected to regionally varying uniaxial or biaxial static tension.
4.2 Materials and methods

Cell culture

Porcine mitral valves from an abattoir were used to develop primary cultures of VICs. Valve tissues were first extensively washed with sterile phosphate buffered saline (PBS) in a laminar flow hood. To loosen endothelial cells, the tissues were soaked in 15-20 mL of serum-free Dulbecco’s Modified Eagles Medium (DMEM, Mediatech, Herndon, VA), containing 2 mg/mL collagenase type II (Worthington, Lakewood, NJ), within an incubated shaker for 20 minutes (140 rpm, 37°C). The endothelial cells were then removed by wiping all valve tissue surfaces with a sterile cotton swab. All chordae tendineae were removed from the leaflet using sterile scissors, and the annular attachment edge was trimmed away from valve leaflets.

The leaflets and chordae were then separately finely minced and dissociated with 10-20 mL of serum-free DMEM containing 1 mg/mL collagenase type III and 0.1 mg/mL hyaluronidase (both from Worthington) for 4 hours in an incubated shaker (140 rpm, 37°C). Each resulting cell suspension was filtered through a sterile 70 µm cell strainer (BD Falcon, San Jose, CA) to remove undissolved debris and the resulting cell pellet was resuspended in DMEM:F12 (Mediatech) medium (1:1, containing low glucose with HEPES) with 10% bovine growth serum (BGS, HyClone, Logan, UT) and 1% antibiotic-antimycotic solution (Mediatech). The culture was then incubated in a humidified atmosphere of 95% air/ 5% CO₂ at 37°C with changes of medium every 48 hours. The cells were split in a 1:3 ratio when they became 90-95% confluent. Three separate primary leaflet and chordal cell cultures of passage 5-10 were used to prepare the collagen gels.
Mold preparation

A cross-shaped mold was designed to hold the biaxially stretched collagen gels. The dimensions of the cross (60 mm x 20 mm x 7 mm) were chosen to provide sufficient amounts of collagen gel for PG and GAG analysis. To generate the mold, a counter mold with a cross protruding from the surface was designed using ProEngineer CAD/CAM software (Parametric Technology Corp., Needham, MA). A circular rim with a diameter (88 mm ID) was designed to create a mold that would fit into the petri dish (Figure 4.1a). Rapid prototyping technology (Laser Reproductions, Columbus, OH) was used to prepare the counter mold and the circular rim. Silicone rubber (Silicones Inc., High Point, NC) was used to make the final mold. The liquid rubber was mixed with the activator in 10:1 ratio until the mixture became homogenous and was then poured within the rim set on top of the counter mold. After curing (10-12 hours), the mold was detached from the counter mold and rim. The mold was then placed within the glass petri dish and autoclaved before use (Figure 4.1b).

Holders for static and dynamic loading of the collagen gel were prepared using stainless steel tubing (0.050 inch OD for static loading and 0.0625 inch OD for dynamic loading). On the inner edge of the holders, 2 layers of the polyester mesh (Sefar America Inc., Los Angeles, CA) were sewn together with nylon thread to provide an anchor for the collagen gel (Figure 4.2). The final holders (polyester mesh sewn to the stainless steel tubes) were sterilized by soaking in 70% ethanol and fixed into the autoclaved silicone mold for static loading. Several molds were prepared without anchors to test the collagen gels in an unconstrained environment (Figure 4.3).
Figure 4.1. (a) Cross-shaped counter mold and circular rim, (b) Silicone rubber mold in glass petri dish.

Figure 4.2. 3D constrained collagen gel (5 days old) in a cross-shaped silicone rubber mold. (A) mesh, (B) uniaxial and (C) biaxial region.
Cell seeding in collagen gels

To mimic the 3D aspect of the cells’ native tissue environment, the VICs were seeded within collagen gels. These collagen gels were prepared using 8 parts rat-tail collagen type I in 0.02 M acetic acid, 1 part 10X DMEM and 1 part cells suspended in 1X DMEM using a protocol adapted from Eastwood et al [225]. Briefly, rat tail tendon collagen (BD Biosciences, Bedford, MA) at 2.28 mg/mL was brought to physiological pH by the addition of 5 M NaOH and kept over ice to delay the gel formation. The volume of the manufacturer’s collagen type I was calculated using the following formula:

\[
\text{Volume of rat tail collagen type I} = \frac{\text{volume of collagen solution} \times \text{desired collagen concentration}}{\text{initial collagen concentration}}
\]

One million cells per mL of gel, suspended in 1X DMEM, were added to the solution and immediately poured into the mold (with or without anchors). A number of constrained (8 leaflet, 6 chordal) and unconstrained (4 leaflet, 3 chordal) collagen gels
were prepared for direct comparison. The holders anchored the gel to prevent complete
gel contraction. After the collagen gel had set in the incubator (1-2 hours), 2-3 mL of
regular DMEM:F12 medium with serum was added. As the cells contracted the gel,
tension was developed in the anchored gels. Every other day the conditioned medium
from the collagen gels was collected and replaced with fresh medium. All of the medium
collected during 7-day period for 3D culture was later combined for the PG analysis.
After 7 days, the collagen gels were harvested and three distinct regions of the collagen
gels were identified as biaxial, uniaxial and mesh, and separated accordingly for analysis
(Figure. 4.2). The mesh region was included in the analyses because the cells within this
region likely experienced uniformly low strains, in contrast to the uniaxial and biaxial
regions, where cells experienced greater magnitude strains due to the collagen gel
contraction. Furthermore, the conditioned medium contained PGs secreted by cells from
all regions, therefore all three regions of the collagen gel were analyzed to provide
compete correspondence to the medium analysis.

*Image analysis for contraction assessment*

At the time of each medium change, an image of the collagen gel was taken by a
digital camera and analyzed using Image-Pro software (Media Cybernetics, Silver Spring,
MD). The area of the collagen gel was measured and the percentage contraction was
calculated relative to the original mold size. Only the contraction of the area could be
measured, as the depth of the collagen gel could not be assessed.
**Histology**

One additional collagen gel was prepared and grown for 7 days under constrained conditions. The gel was fixed with 10% formalin while still constrained and divided into uniaxial and biaxial regions. These samples were paraffin embedded and sectioned en face (10 μm sections). The sections were stained with hematoxylin to demonstrate the cell nuclear orientation.

**Glycosaminoglycan analysis**

Fluorophore-assisted carbohydrate electrophoresis (FACE) is a simple, sensitive, and rapid technique for analyzing a wide range of carbohydrate structures [54, 226] in which GAG disaccharides are labeled with a fluorescent tag and then separated electrophoretically on a carbohydrate gel. The overall process is described in Figure 4.4.

For FACE, the collagen gel and medium samples were treated with proteinase-K (EMD Pharmaceutical, Durham, NC) to degrade proteins and large protein aggregates and to liberate the GAG chains into solution. The collagen gels were weighed to measure the wet weight and then lyophilized overnight. The gels were weighed again to calculate the dry weight and percentage hydration and rehydrated with 500 μL of 100 mM ammonium acetate. The gel was minced slightly and a 100 μL aliquot of proteinase-K solution (10 mg/mL) was added and kept at 60°C for 2 hours. Similarly, appropriate medium aliquots were digested with proteinase-K (50 μg per mL of the conditioned medium) for 2 hours at 60°C. All the samples were well mixed and heated for 30 minutes at 80°C to denature the proteinase-K at the end of the digestion. At this point, an aliquot of the resulting sample solutions was taken for a fluorescent Hoechst assay to quantify
the DNA, estimate the number of cells in the collagen gel, and normalize the data [227].

The medium samples were diluted with ultrapure water in a 1:1 ratio and treated with
10% Triton-X (final concentration 0.5% Triton-X). Ion exchange purification, with Q-
sepharose beads, was performed to remove the glucose from the medium samples. 0.25
mL of 50% Q-sepharose bead slurry per mL of the original volume of the medium
samples was added and beads were washed with 40 column volumes of 7 M urea buffer
(pH 7.5). Bound GAGs were eluted with 4 column volumes of 3 M NaCl in 7 M urea and
pooled together. The purified GAGs were precipitated by addition of ethanol and vacuum
dried.

Each specimen was then analyzed using FACE analysis by digestion with highly
GAG-specific enzymes (Seikagaku, USA) to cleave the GAG chains into disaccharides
(Table 4.1). All samples were fluorotagged with 40 μL of 0.0125 M 2-aminoacridone
(AMAC, Molecular Probes, Eugene, Oregon) in 85% DMSO/15% acetic acid, and 40 μL
of 1.25 M sodium cyanoborohydride in ultrapure water, and then incubated for 16 hours
at 37°C. After fluorotagging, 20 μL of glycerol was added to each sample. 5 μL of each
sample was electrophoresed for 75-85 minutes at a constant 500 V on a monosaccharide
gel.

Table 4.1. Enzymes that cleave specific GAGs (Seikagaku America, Falmouth, MA)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate GAG(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chondroitinase ABC</td>
<td>unsulfated chondroitin chondroitin and dermatan sulfate</td>
</tr>
<tr>
<td>chondroitinase AC-II</td>
<td>unsulfated chondroitin chondroitin sulfate hyaluronan</td>
</tr>
<tr>
<td>hyaluronidase SD</td>
<td>hyaluronan</td>
</tr>
</tbody>
</table>
Valve tissue, collagen gel or medium sample

1) lyophilization
2) proteinase-K digestion
3) GAG specific enzyme treatment

Purified GAGs

Fluorotagging with AMAC

Labeled GAGs

1) addition of maltotriose
2) electrophoresis
3) image analysis

FACE analysis

Specific GAG concentrations in the sample

Figure 4.4. Flow chart for the analysis of glycosaminoglycans.
The gels were imaged with a Kodak Gel Logic 100 imaging system (Kodak, Eastman, MA) and then analyzed using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). Specific GAGs were identified by corresponding bands in a disaccharide standard lane. Because the fluorescent tag gives the same molar fluorescence for each saccharide derivative, the resulting GAG quantities could be determined from a single standard curve. Known picomolar quantities of maltotriose were incorporated into the FACE samples prior to running the gel to calibrate the integrated optical density of the bands. When appropriate, the total volume of the medium, the experimental time point, and the original gel dry weights were used to normalize the GAG concentration. An example of a FACE gel is shown in Figure 4.5.

![FACE gel](image)

**Figure 4.5.** FACE gel to separate GAGs in collagen gel samples (ML-mitral leaflet, MCh-mitral chordae, ACII-chondroitinase ACII, CABC- chondroitinase ACII + chondroitinase ABC).
Proteoglycan analysis

Core proteins of the PGs were identified using western blotting techniques. PGs were extracted from the collagen gel samples using 4 M guanidine HCl with protease inhibitors overnight at 4°C [13], then dialyzed into 7 M urea (pH 7.5). Dialyzed gel samples and medium samples were then purified by ion-exchange. Equivalent amounts of purified PGs were ethanol-precipitated from solution and digested with chondroitinase ABC to remove the GAG chains from the core proteins. The samples were vacuum dried, dissolved in SDS buffer at 100°C, separated on a 4-12% SDS-polyacrylamide gel, and then blotted for western analyses. The nitrocellulose membrane was stained with antibodies specific for PGs (anti-decorin LF-122 and anti-biglycan LF-104, courtesy of Larry Fisher, NIH; anti-versican 2-B-1, Seikagaku) [217, 228]. Intensities of various bands on western blot films were scanned and analyzed using Gel-Pro Analyzer software. To reduce the variability in the magnitudes of PGs secreted by different gels, the band intensities from all constrained regions were normalized to the corresponding PG band intensity from the unconstrained gels grown from the same cell type.

DNA and collagen measurement

Biochemical assays for DNA [227] and collagen were used to monitor the cell and collagen density within collagen gels at 4 time points throughout the culture duration. Collagen content within collagen gels was measured to assess any variability in collagen in the different regions over time in order to identify or rule out that factor as a basis for PG differences between regions. The starting DNA and collagen concentrations were calculated from the amounts of cells and type I collagen solution used to prepare the
collagen gels. These concentrations were then measured after initial gelation (4 hours) and initial contraction (16 hours) in constrained and unconstrained collagen gels with duplicate gels for each combination of 2 constraint conditions, 2 cell types, and 4 and 16 hour time points. The final time point was at the end of 7 days, when DNA and collagen contents were measured in different regions (biaxial, uniaxial and mesh). For each cell type, 7 constrained and 4 unconstrained collagen gels were analyzed for DNA, and 2 constrained and 1 unconstrained collagen gels were analyzed for collagen. For the DNA and collagen assays, similar aliquots (0.5-1.0 mg dry weight) of collagen gel samples were solubilized in either proteinase-K or pepsin, respectively. The Hoechst DNA assay was performed on aliquots of the proteinase-K digested collagen gel samples [227, 229]. The Sircol sirius red assay for collagen (Biocolor, Ireland, UK) was performed on collagen gel samples dissolved in pepsin (1 mg/mL in 0.5 M acetic acid, keeping the ratio of pepsin to sample wet weight as 1:10) overnight at room temperature. The cell and collagen densities in different regions were determined by normalizing to the dry weight of those portions of the collagen gels.

The same DNA and collagen analysis was applied to a small group (n = 3 leaflet and 5 chordal) of identically sized and shaped collagen gels cyclically stretched for 7 days at 10% strain and 1.16 Hz; the analysis of PGs and GAGs from these gels and the explanation of the cyclic stretching mechanism is described in Chapter 5.

**Data analysis**

Differences in the specific GAG classes and PGs were compared between different loading regions (biaxial, uniaxial or mesh) of the collagen gel and between cells
grown from different regions of the valves (leaflet vs. chordae). Since the PG band intensity data consisted of normalized ratios (each constrained region was normalized to the unconstrained region data from the same cell group run on the same western blot gel to reduce gel-gel variability), these ratios served as the basis for the statistical analysis. GAG quantities were either normalized to collagen gel dry weight or volume of medium. GAGs present in the control volume of fresh medium were subtracted from the GAG quantities in the collected medium samples. Proportions of various GAG classes were then determined by normalizing the quantity in each class to the total GAG quantity as measured by FACE. The ratio of 4-sulfated to 6-sulfated GAGs, which is indicative of compression vs. tensile load patterns, was also determined for every sample [13]. One-way and two-way analysis of variance (ANOVA) was used to compare group data using SigmaStat statistical software (SPSS Inc., Chicago, IL). In the two-way ANOVA, factor one was the cell type (leaflet and chordae) and factor two was the different loading regions (biaxial, uniaxial, mesh and unconstrained). A post-hoc Tukey test was used for pairwise comparisons and significance was accepted at p<0.05.

4.3 Results

Collagen gel holder study

Different kinds of mesh holders were used to anchor the collagen gel in the cross-shaped silicone rubber molds. The polyester mesh and the stainless steel tubing used are biocompatible. It was previously established in our lab (in consultation with Dr. Mark Eastwood of the University of Westminster, London, UK) that mesh holders work best for holding the 2D sheet like structures. Therefore, two different mesh sizes were
compared. It was found that the fine mesh worked much better than the coarse pore size mesh. Collagen gels attached to the big pore size mesh holders ripped off from the inner edge of the mesh (Figure 4.6) while the fine mesh was able to hold the gel well even after 15 days. Therefore, all further experiments were conducted using the fine pore size polyester mesh.

![Image of mesh holders](image)

**Figure 4.6.** Coarse (above) and fine (below) mesh holders for collagen gels.

**Water content in different regions of collagen gels**

Percentage water content was calculated in different regions of collagen gels from the measured wet and dry weights (Figure 4.7). The water content was not significantly different between collagen gels seeded with leaflet and chordal cells. The mean water content in different regions of collagen gels (leaflet and chordal groups combined) ranged from 89.2±3.4% (baxial region) to 92.4±1.9% (unconstrained gels) with a statistically significant difference (p<0.05) only for biaxial vs. unconstrained gels.
Figure 4.7. Water percentage in different regions of collagen gels.

Cell orientation

The histological images of the biaxial and uniaxial regions (Figure 4.8) showed that the cell orientation was unidirectional in the uniaxial region and random in the biaxial region.

Figure 4.8. Cell orientation in biaxial and uniaxial regions of constrained collagen gels.
Optimization of GAG analysis from conditioned medium

The optimization of the GAG measurement in the conditioned medium was performed by Sherket Peterson, an undergraduate student in our laboratory, who was mentored in conjunction with this research project during the summer of 2004. The conditioned medium samples contain a high amount of glucose, which hinders visibility of the GAG bands. An ion-exchange purification step using Q-sepharose beads was performed and then ethanol precipitation was used to remove all the glucose from the medium samples. A variety of volumes of Q-sepharose bead slurry were tested. When 1.0 ml of 50% Q-sepharose bead slurry was used, the samples contained little to no chondroitin 4-sulfate, but when 0.6 ml of slurry was used, there was a significant amount of all GAGs (Figure 4.9).

Figure 4.9. FACE gel of GAGs isolated from conditioned medium samples. Image courtesy of Sherket Peterson.
These results led us to believe that 0.6 ml per 2.5 mL of the conditioned medium was the optimal slurry volume. Different dilutions of the samples (25%, 50% and 100%, no dilution) with AMAC buffer were used to find out the detectable concentration of the sample. The GAG bands from diluted samples (25% and 50%) were easily detectable and were not saturated in the shorter image exposures.

**Total GAGs secreted in collagen gels and conditioned medium**

GAGs produced by the cells were either retained within the collagen gels or secreted into the surrounding medium. Uniaxially strained regions of constrained collagen gels tended to contain the least total GAGs (chordae 5.96±2.92, leaflet 5.59±2.18 nmol/mg dry weight), independent of the cell source, compared to other regions (chordae biaxial 7.77±2.09, chordae mesh 7.61±2.91, leaflet biaxial 7.92±1.99, leaflet mesh 8.20±3.11 nmol/mg dry weight), although this trend was not statistically significant (Figure 4.10). Unconstrained collagen gels seeded with leaflet cells contained slightly fewer GAGs (chordae 7.16±3.01, leaflet 4.65±1.71 nmol/mg dry weight) than did constrained gels. Even when leaflet and chordal samples were grouped together, the difference between biaxial and uniaxial regions was not statistically significant. The medium samples from the unconstrained gels, when both cell types were grouped, contained slightly less GAGs (p=0.091) than found in the medium from the constrained gels (Figure 4.11). No significant differences in total GAGs were found, however, between collagen gels seeded with chordal cells (constrained 6.13±2.93, unconstrained 3.77±1.45 nmol/mL of the medium) and leaflet cells (constrained 6.16±2.33,
unconstrained 3.79±1.22 nmol/mL of the medium). The repeatability of the Gel-Pro analysis of FACE gels was found to be within 7%.

**Figure 4.10.** Total GAGs in biaxial, uniaxial, and mesh regions of constrained collagen gels and in unconstrained collagen gels. Data are mean ± standard deviation.

**Total GAGs in the Medium**

**Figure 4.11.** Total GAGs secreted into the medium. Data are mean ± standard deviation.
Relation of total GAGs produced to collagen gel contraction

For constrained collagen gels, the total GAGs secreted into the collagen gel and the surrounding medium were significantly but weakly correlated to the percentage gel contraction. The constrained collagen gels that contracted less contained fewer GAGs in all three regions of the gel as shown in Figure 4.12 ($r^2=0.25$, $p<0.001$). The mean contraction for constrained collagen gels was 33.5±6.2% while for unconstrained gels it was 84.0±11.1%.

**Constrained collagen gels**

![Graph showing the relation between total GAGs and % contraction](image)

$$y = 0.1065x + 2.8938$$

$$r = 0.51, \ p<0.001$$

**Figure 4.12.** Relation of total GAGs produced and the percentage contraction of collagen gels.

GAG proportions in collagen gels

VICs within collagen gels produced both sulfated and unsulfated GAGs. The 4-sulfated (4-S) GAGs alone accounted for 67-75% of total GAGs in various regions of constrained collagen gels (Figure 4.13). In contrast, the unconstrained collagen gels, compared to constrained gels, contained fewer 4-S GAGs ($p<0.01$ vs. chordal biaxial...
region) and more 6-sulfated (6-S) GAGs (p<0.01 vs. all regions of leaflet gels, p<0.05 vs. all regions of chordal gels). When the leaflet and chordal samples were grouped together, there was a significantly greater proportion of 4-S in each constrained region vs. unconstrained (p<0.05) and the unconstrained gels contained a greater proportion of 6-S than each region of the constrained gels (p<0.001). The only difference between biaxial vs. uniaxial regions was found for 6-S GAGs in chordal gels (p<0.05).

![Diagram of GAG proportions in biaxial, uniaxial, and mesh regions of constrained collagen gels and in unconstrained collagen gels. Data are mean ± standard deviation. Because many comparisons of different regions were significantly different when the leaflet and chordae factor was disregarded (and vice versa), see text for further details of the two-way ANOVA results. *p<0.05 vs. unconstrained, †p<0.05 chordae vs. leaflet, ‡p<0.05 vs. biaxial.](image)

**Figure 4.13.** GAG proportions in biaxial, uniaxial, and mesh regions of constrained collagen gels and in unconstrained collagen gels. Data are mean ± standard deviation. Because many comparisons of different regions were significantly different when the leaflet and chordae factor was disregarded (and vice versa), see text for further details of the two-way ANOVA results. *p<0.05 vs. unconstrained, †p<0.05 chordae vs. leaflet, ‡p<0.05 vs. biaxial.

There was also a significant difference for 6-S GAGs between the collagen gels seeded with chordal cells vs. leaflet cells for uniaxial (p<0.03) and mesh regions (p<0.02). The level of significance increased for chordal vs. biaxial differences when all the regions were grouped together (p<0.01). The unsulfated (0-S) and disulfated (4,6-S) GAGs were present in very small proportions (1-7%) in all the gels. The 4,6-S GAG class
was different for chordal vs. leaflet gels in the mesh region (p<0.05) and for all the regions grouped together (p<0.03). HA was also present in low proportions (6-12%) with no significant difference between regions or cell type. All the samples were run in duplicate on separate FACE gels to check the repeatability of specific GAG proportions, which was found to be within 16% of their value.

**GAG proportions in conditioned medium**

Some of the GAGs produced by the VICs within the 3D collagen gels were secreted into the surrounding medium (Figure 4.14). The GAG quantities in the conditioned medium samples were analyzed after subtracting the GAGs in the fresh medium control (abundant 0-S and 4-S with small quantities of 6-S).

![Pie charts showing GAG proportions](image)

**Figure 4.14.** GAG proportions in the surrounding medium of constrained and unconstrained collagen gels. Data are mean ± standard deviation.
Interestingly, no di- or tri-sulfated GAGs were found secreted into any of the medium samples. The constrained gel medium contained slightly more HA (p=0.084) and significantly less 4-S (p<0.05, leaflet and chordal gels grouped together) than did the unconstrained gel medium, whereas 0-S and 6-S GAGs were present in nearly the same quantities in all the medium samples. The proportions of HA secreted into the medium (26-52%) were much higher than that retained within collagen gels (6-12%).

**Ratio of 4-sulfate to 6-sulfate**

Because 4-S and 6-S GAGs tend to be found on distinct PGs that are predominantly located in tensile and compressive loading regions, respectively, the ratio of 4-S to 6-S can serve as an indicator of the type of loading experienced by a tissue. The unconstrained collagen gels demonstrated a far lower ratio of 4-S to 6-S (chordae 2.03±0.27, leaflet 2.85±0.90) than found in constrained gels for both leaflet (biaxial 5.21±0.75, uniaxial 5.71±2.08, mesh 6.33±1.58) and chordal cells (biaxial 8.30±5.41, uniaxial 4.32±2.30, mesh 4.31±1.15) as shown in Figure 4.15. This comparison was significant for constrained biaxial region vs. unconstrained for chordal gels alone (p<0.05) and for chordal and leaflet gels grouped together (p<0.02). No significant differences were observed between the regions of the constrained collagen gels. The collagen gels seeded with leaflet cells (constrained or unconstrained) generally showed higher 4-S to 6-S ratios, with the exception of biaxial region, than gels seeded with chordal cells. There was no significant difference in 4-S to 6-S GAG ratios in the culture medium collected from constrained (chordae 1.25±0.29, leaflet 1.83±0.66) and unconstrained gels (chordae 1.65±0.41, leaflet 2.26±0.74, Figure 4.16).
Figure 4.15. 4-S/6-S ratio of GAGs in biaxial, uniaxial, and mesh regions of constrained collagen gels and in unconstrained collagen gels. Data are mean ± standard deviation. *p<0.05 vs. unconstrained, †p<0.02 vs. unconstrained (leaflet and chordae grouped).

Figure 4.16. 4-S/6-S ratio of GAGs in the medium. Data are mean ± standard deviation.
Proteoglycan measurement in the collagen gels

This work involved the measurement of three PGs, decorin, biglycan and versican, which have been previously found in heart valve tissues [13]. All three of these PGs were present in the 3D cultures of either leaflet or chordal cells (Figure 4.17). PGs from all 3 regions from the same collagen gel and from the unconstrained gel were run on the same western blot and the resulting band intensities of the constrained regions were normalized to the unconstrained PG band intensity. Although this data normalization greatly reduced the gel-to-gel variability, some variability in the band intensity ratios remained. The content of decorin was generally higher in all regions of the constrained collagen gels relative to unconstrained gels for both leaflet and chordal cells, but this trend reached statistical significance only for the leaflet uniaxial region (Figure 4.18a, p<0.01). In addition, there was greater relative content of decorin in the leaflet uniaxial region compared to the same region of chordal gels (p<0.02). When leaflet and chordal gels were grouped together, all three regions contained significantly more decorin than was found in the unconstrained conditions (p<0.05).

Biglycan content was also higher in the constrained collagen gels compared to unconstrained gels seeded with either leaflet cells (biaxial, uniaxial and mesh vs. unconstrained, p<0.01) or chordal cells (biaxial vs. unconstrained, p<0.03, Figure 4.18b). Within constrained leaflet gels, the biaxial region contained more biglycan than the uniaxial and mesh regions (p<0.001). There was a significant difference in biglycan content between leaflet and chordal gels, when all three regions were grouped together (p<0.01); this difference was also significant for the biaxial region alone (p<0.02). When
leaflet and chordal gels were analyzed together, all constrained regions contained significantly more biglycan than found in the unconstrained gels (p<0.001).

The large molecular weight PG versican was demonstrated in collagen gels seeded with either leaflet or chordal cells (Figure 4.18c). Overall, the unconstrained collagen gels retained more versican than constrained gels, but only the biaxial region was significantly lower (p<0.04 for leaflet gels, p<0.01 for all gels).

![Image of western blotting results](image)

**Figure 4.17.** Representative band intensities from western blotting of different PGs.

**Proteoglycan measurements in the culture medium**

PGs synthesized by VICs within collagen gels were also secreted into the surrounding medium (Figure 4.19). All of the medium collected during 7-day period for 3D culture was combined for PG analysis. Although decorin and biglycan were not different in the conditioned medium from constrained collagen gels, as compared to unconstrained collagen gels, versican was significantly more abundant (p<0.01 for each cell type, p<0.001 for both cell types combined).
Figure 4.18. Proteoglycan synthesis in differently loaded regions of collagen gel (n=8 for ML and n=6 for MCh). (a) Decorin, *p<0.01 vs. unconstrained. (b) Biglycan, †p<0.03 vs. unconstrained, †p<0.03 vs. biaxial. (c) Versican, ‡p<0.04 vs. unconstrained. *p<0.05 vs.
unconstrained, when leaflet and chordal data were grouped together. MCh- mitral chordae, ML-mitral leaflet. Data are mean ± standard deviation. Due to gel-to-gel variations, the band intensities for each western blot was normalized to unconstrained condition so the “mean” was 1 and “standard deviation” was 0 for unconstrained conditions.

Figure 4.19. Proteoglycan synthesis in the conditioned medium (n=8 for ML and n=6 for MCh). (a) Decorin, (b) Biglycan, (c) Versican, *p<0.01 vs. unconstrained. Data are mean ± standard deviation. The band intensities from western blots were normalized to unconstrained condition so the “mean” was 1 and “standard deviation” was 0 for unconstrained conditions.
Time-dependency of DNA and collagen contents within collagen gels

An analysis of the consistency of cell density showed that there was no change from the original DNA concentrations at 4 and 16 hours but a 55-80% decrease after 7 days (Figure 4.20a). Though there was no cell loss during collagen gel preparation, the cell number reduced over time for all types of loading (unconstrained, static, and cyclic). The collagen present in the gels showed slight variations but no significant differences over the 7 day period (Figure 4.20b). After 7 days of incubation under static or cyclic strains, there were no significant differences between collagen or DNA content between the constrained and unconstrained collagen gels or between leaflet and chordal cells.

Cell and collagen density in differently loaded regions of the collagen gels

The cell density in unconstrained collagen gels was less than in the constrained collagen gels (Figure 4.21a). The biaxial region from the gels seeded with leaflet cells had significantly higher cell density compared to the unconstrained gels (p<0.04) and even the leaflet mesh region compared to unconstrained suggested a trend of higher cellularity (p=0.088). Within the constrained gels, the leaflet cell density was higher than chordal cell density for the biaxial region alone (p<0.04) and for all regions grouped together (p<0.02). Neither constraint, directional loading, nor cell source had any significant effect on collagen density (Figure 4.21b).
Figure 4.20. Time-dependency of (a) DNA (n=2 for starting, 4 hrs and 16 hrs; n=7 constrained and 4 unconstrained for 7 days; n=3 leafluft and 5 chordal for 7 days cyclic) and (b) collagen contents (n=2 for 4 hrs and 16 hrs; n=2 constrained and 1 unconstrained for 7 days; n=3 leafluft and 5 chordal for 7 days cyclic) within collagen gel. ----- indicates calculated initial collagen content (25.5 mg). Data are mean ± standard deviation.
Figure 4.21. DNA and collagen densities in differently loaded regions of collagen gel after 7 days. (a) DNA (n=7 constrained and 4 unconstrained), *p<0.04 vs. unconstrained, (b) Collagen (n=2 constrained and 1 unconstrained). Data are mean ± standard deviation.

4.4 Discussion

In this study, I characterized the specific GAG and PG production by VICs within a 3D scaffold exposed to static tensile strain. I found that these VICs produced and
retained abundant chondroitin/dermatan 4- and 6-sulfate within their scaffold, and
secreted abundant hyaluronan, 6-sulfated and unsulfated chondroitin/dermatan into the
culture medium, in total GAG magnitudes close to those found in native heart valve
tissues. I also found that these VICs produce decorin, biglycan and versican, which are
both retained within the scaffold and secreted into the culture medium. The effect of cell
type (leaflet VICs vs. chordal VICs) was only significant for the abundance of biglycan
and 6-S and 4,6-S GAGs. Overall, the presence or absence of tensile constraint, as
opposed to region-specific loading, was the most significant factor determining the VICs’
production of different GAGs and PGs.

The profile of GAG classes and PGs secreted into the culture medium was
distinctly different from those retained within the scaffold. This finding appears to be
very reasonable given that collagen gels are porous 3D structures, which allow the
GAGs/PGs produced by VICs to leach into the surrounding medium. HA was more
abundantly secreted into the surrounding medium than retained within the gel because
these molecules do not covalently bind to a proteoglycan core protein and are therefore
more likely to leach out. In contrast, proportions of 4-S GAGs (and correspondingly the
4-S/6-S ratio) were higher in the collagen gels than in the surrounding medium, which is
likely due to the close association of C/D 4-S containing PGs with collagen fibrils [4].
The amount of decorin and biglycan retained in the collagen gels increased with the
application of static strains, whereas versican decreased under the same conditions.
Secretion of versican, however, increased in the conditioned medium when the gels were
constrained. This distinctive versican result may be a consequence of its greater
abundance in compressive tissues, i.e., this large PG may have less utility in tissues
subjected to static tension and hence is released into the medium [13]. In contrast, VICs largely retained decorin and biglycan within the engineered tissues under static tensile strains, which is logical given that these two PGs are largely found in numerous tissues that bear tension [13]. All of these GAGs/PGs were newly synthesized by the VICs, and hence their relative abundance in the constrained gels was possibly due to a proportionally higher GAG/PG synthesis in those gels as opposed to loss of GAGs/PGs from the unconstrained gels.

There were only a few differences in GAG/PG synthesis between VICs isolated from leaflet and chordal tissues of the valve. The application of any type of static strain increased GAG retention in collagen gels seeded with leaflet VICs, but not chordal VICs. Strain also increased the secretion of GAGs into the medium of collagen gels, but it did so equally for leaflet and chordal VICs. It is interesting to note that the most unique profile of GAG classes was found in the collagen gels seeded with chordal cells and subjected to uniaxial strain. Chordae tendineae in the mitral valve normally experience uniaxial tension and undergo dramatic remodeling (both mechanically and biochemically) in pathological conditions [3, 54]. VICs from chordae may be particularly responsive to mechanical strains. Constrained collagen gels containing leaflet cells retained more decorin and biglycan than did those containing chordal cells. This interesting difference between leaflet and chordal cells may be due to a more dramatic difference between their in vivo conditions and our 3D model, or it may be due to inherent phenotypic differences between the cell types. Leaflet cells normally experience a combination of compression or tensile strains in vivo, whereas in our 3D model cells experienced either tensile or no strains. Also, the cells have somewhat different
phenotypes; our lab has found that mitral chordal cells have approximately 50% higher levels of smooth muscle α-actin expression than leaflet cells [230]. Smooth muscle α-actin, which is reportedly expressed at low levels in normal human VICs, has been associated with a more contractile phenotype [46, 231, 232]. This study is the first to report on characteristics of the chordal cell phenotype. The proportion of 6-S GAGs (as well as the degree of hydration) was higher in the unconstrained gels whereas the proportion of 4-S GAGs was higher in the constrained gels, suggesting a role for tension in the production of specific proteoglycans, as previously found for tensile and compressive regions of heart valves [13].

The effect of constraint itself was more influential than the directionality of the constrained conditions. The only difference between regions experiencing biaxial and uniaxial loading was found for the PG biglycan, which was most abundant in the biaxial region. Distinctive collagen fibril and cell alignment may have contributed to the differences in constrained and unconstrained collagen gels. Other possible factors that are intrinsically associated with unconstrained collagen gels are cells demonstrating decreased actin stress fibers and downregulated cyclin and ERK pathways resulting in increased cell apoptosis compared to cells in constrained matrices [109, 233]. Fringer et al. also mentioned the possible role of focal adhesions in 3D gel compaction [109], despite the observations that these adhesions are smaller than those in monolayer cultures. Also, the biaxial region may not experience consistent strain patterns across its entire area. To monitor the consistency of strain pattern and cell alignment, many different sections of biaxial region could be analyzed in the future using a more comprehensive histological analysis or by tracking the motion of visible beads embedded
within collagen gels. Even though our study investigated static as opposed to cyclic tension, this resulting differential abundance of distinctly sulfated GAGs and PGs between the constrained and unconstrained groups also has correlations in “floppy” myxomatous mitral valves, which are subjected to reduced tensile loading (or no tensile loading in the case of ruptured chordae) and contain elevated concentrations of chondroitin 6-sulfate. Static loading is also relevant to the aortic valve in hearts supported by left ventricular assist devices and has been shown by Warnock et al. to modulate the expression of inflammatory genes in porcine aortic VICs [106]. There was also a proportional relationship between the degree of contraction and total GAG production; the constrained collagen gels that demonstrated relatively lower contraction produced fewer total GAGs (which were predominantly 6-S, data not shown). In both the unconstrained gels and in the constrained gels with reduced contraction, the cells would have experienced low or no tensile stretch, which would affect the production of specific GAGs.

Most differences in GAGs between the biaxial and uniaxial regions were not significant within chordal or leaflet groups, but many comparisons attained significance when the chordal and leaflet groups were combined. The proportions of different GAG classes were similar between the uniaxial, biaxial, and mesh regions, but total GAGs were lowest in the uniaxial regions. This total GAG trend may be analogous to that found in native chordae, which are subjected only to uniaxial stretch and which contain lower total GAG concentrations than found in the biaxially stretched leaflets. The reason why biaxial and mesh regions showed the same trend may be due to the absence of one predominant direction of loading in both regions. The rationale for measuring the
GAGs/PG in the mesh region was based on two factors. First, in the mesh region the collagen gel is embedded within and outside the polyester mesh; hence, the cells within this region experience low and likely almost uniform strain patterns as opposed to the adjacent region, where cells experience pure uniaxial strains. Because this is a distinctive loading region, the mesh region was compared to the uniaxial and biaxial regions. Second, GAGs were secreted into the medium by cells from all regions, requiring us to therefore measure GAGs from all three regions of the collagen gel.

Overall, many of the GAG/PG trends within our 3D collagen gels were very similar to those found in native valve tissues, which has promising implications for tissue engineering. The water content of these collagen gels ranges from 80-95%, which is comparable to that of native leaflet and chordae tendineae (76% for chordae and 86% for leaflet) [13]. The concentration of total GAGs produced by the VICS seeded within collagen gels were approximately ½ and ¼ of the concentration of total GAGs found in native chordae and leaflet tissue, respectively [3, 13]. The predominant GAG class in the cell-seeded collagen gels was chondroitin/dermatan 4-sulfate, which is also the most common GAG in native chordae. Compared with native chordae and leaflet, however, these collagen gels had greater proportions of 4-sulfated GAGs and lower proportions of hyaluronan and 6-sulfated GAGs. These differences in total GAG concentration and the proportions of selected GAG classes retained within these gels may be due to the constant tensile stretch experienced by the constrained cells, resulting in the production of 4-sulfated GAGs, which are presumed to be part of the collagen-binding PGs decorin and biglycan [13]. The predominant PGs in the constrained collagen gels were decorin and biglycan, which are also the most common PGs in native chordae [13]. Similarly,
versican was more abundant, and decorin and biglycan were less abundant, in unconstrained collagen gels as been found in the compressive regions (posterior leaflet and free edge of anterior leaflet) of the mitral valve. Given that GAGs and PGs are diverse molecules that serve different biological roles in tissues, it appears reasonable that their synthesis by VICs is not uniformly regulated by mechanical strains. Investigations on smooth muscle cells (SMCs) have shown that mechanical strains cause variable effects on the synthesis of distinct GAGs and PGs on application of mechanical strains [82, 163]. For example, increased synthesis of versican and decreased synthesis of decorin was observed by SMCs under mechanical strains [82]. This study therefore provides additional guidance for constructing engineered tissues in which a tensile environment will induce specific GAG or PG production.

Our results and methods provide new information yet are quite consistent with previous findings that mechanical stretching of cells regulates ECM production [1, 79]. Berry et al. reported that the collagen production by two types of fibroblasts was dependent on strain profile (biaxial vs. uniaxial) and cell type [220]. Lee et al. compared the effect of uniaxial and equibiaxial strains and found an increase in collagen and fibronectin for both uniaxial and biaxial strains of low magnitude [143]. However, both of these studies used separate 2D systems to apply uniaxial or biaxial strains as opposed to our 3D cross-shaped model, in which various strains could be experienced by valve cells within a single engineered tissue. 3D cultures have also been shown to provide more in vivo like conditions to characterize cell phenotype and the modulation of ECM synthesis [86]. Because collagen is the most prominent ECM component of heart valves, collagen was selected as a scaffold in order to provide the seeded VICs with a more in
*vivo* like environment; VICs seeded within 3D collagen scaffolds have been shown to maintain their phenotype and synthesize abundant ECM [92, 95].

The application of mechanical strains also enhanced cell retention within collagen gels, as shown by the lower cell retention within the unconstrained collagen gels. Within the constrained gels alone, higher gel contraction was found together with higher cell density (data not shown). Additionally, in a previous study [234], it was reported that higher GAG synthesis with increased gel contraction. This trend is distinct from the comparison of constrained vs. unconstrained gels because in unconstrained gels, I found increased contraction with lower cell density. As reported previously for fibroblasts seeded within 3D collagen gels (especially unanchored gels) [109, 235], the reduced cell number in our engineered tissues suggests that many of these cells underwent apoptosis or migrated to the surrounding medium or culture dish between the 16 hour and 7 day time points. Although the final cell density in the leaflet VIC-seeded gels was higher than those seeded with chordal cells, the possible cause of this difference may be because the chordal VICs do not regularly experience biaxial strains (chordal VICs experience uniaxial strains *in vivo*). When the collagen gels were subjected to cyclic strains as opposed to static strains, however, the final cell concentration increased slightly, suggesting that cyclic tension is an anti-apoptotic signal for VICs. This improvement was greatest in the chordal VIC-seeded gels, possibly because those cells experience such regular cyclic strains *in vivo*. The other time dependent study was performed on collagen, which remained the same as original for both static and cyclic strains at the end of 7 days but exhibited a dip in the 4 hour samples. It is speculated that the decrease at this time point may have been due to loss of some collagen before gelation.
There were a small number of limitations to this study. To begin, the large amounts of cells and collagen gels required to analyze PGs limited our sample size. This restriction was particularly the case for versican, which required substantial masses of engineered tissues to be able to detect its 450 kDa core protein by western blotting. Other possible methods that could have been used to characterize the PGs are chromatography and immunohistochemistry, but these methods are also semi-quantitative. It was difficult to demonstrate differences between uniaxial, biaxial, and mesh regions of the collagen gels; this comparison rarely approached statistical significance until the leaflet and chordal samples were grouped together. This difficulty may be due to the small size of the uniaxial regions; different aspect ratios of the cross shaped mold would have produced longer uniaxial sections. The 16% variability between duplicate FACE gel runs of the same sample may have also contributed to the high standard deviations in the data. The fact that all regions shared the same culture medium may also have dampened or mixed the effects of any soluble autocrine or paracrine feedback based on the regionally specific type of stretch. Separate molds for the application of biaxial or uniaxial strains may have also provided greater magnitude differences but potentially at the loss of some experimental control. Another limitation is that the gels were subjected to static stretch only, although static loading is relevant in selected circumstances; cyclic stretch would be more appropriate to analyze the strain response of VICs as these cells continuously experience cyclic strains in vivo. Finally, these results address only one aspect of myxomatous mitral valve degeneration: the relationship between tensile stretch and the production of specific GAGs. Although these are grossly observable characteristics of myxomatous mitral valves, the initial cause of the disease and the temporal progression
of the complex myxomatous remodeling remain uncertain. This relationship between tension and GAGs, however, does offer direction for tissue engineering efforts.

In conclusion, the production of specific GAGs and PGs by VICs in 3D cultures was shown to be more sensitive to the presence or absence of mechanical constraints than to VIC type or to the directionality of loading. Total GAG concentrations were greater in constrained gels, but the balance of distinctly sulfated GAGs was altered. Synthesis of the collagen-binding PGs decorin and biglycan was greater in constrained collagen gels, whereas the large hydrated PG versican was more abundant in unconstrained 3D cultures. This study improves our understanding of the role of mechanical strains on GAG and PG synthesis in the remodeling of the mitral valve.
Chapter 5: Reversible synthesis of glycosaminoglycans and proteoglycans by cyclically stretched valvular cells in 3D culture

5.1 Introduction

The organization and remodeling of ECM is partially controlled by mechanical stresses and strains. GAGs and PGs are essential ECM components in mitral valve tissues and have been shown to be segregated according to the type of loading that distinct regions (leaflet or chordae) experience [13]. GAGs and PGs regulate and perform a variety of biological and biophysical functions during the growth, remodeling and pathogenesis of tissues [5, 12]. GAGs consist of linear chains of disaccharides that bind to the core protein of PGs. The abundance of particular GAGs and PGs can vary according to different biological needs of the tissues [8]. For example, the dermatan sulfate (mostly 4-sulfated) PGs decorin and biglycan regulate the formation and orientation of collagen fibrils and hence tissue tensile strength [10, 11], whereas the GAG HA, which does not covalently bind to a core protein, entraps large amounts of water to create a swelling force [4]. Correspondingly, previous research on mitral valves has shown that dermatan 4-sulfate and small leucine-rich PGs are prominent in valve regions experiencing cyclic tension (chordae), while GAGs HA and chondroitin 6-sulfate and the PG versican are abundantly present in regions of cyclic compression (such as the leaflet free edges) [13]. Furthermore, in diseased conditions such as myxomatous degeneration, valves are subjected to altered tissue stress [236] and contain more GAGs and PGs than normal [3, 229]. Hence, leaflet and chordal regions of the mitral valve experience distinct
cyclic strains and during pathological conditions the magnitude of these strains changes, as do the amounts of GAGs and PGs.

These observations suggest that mechanical strains may regulate GAG and PG synthesis by VICs, which have been characterized as myofibroblasts [37]. There are several studies that have shown that various mechanical strains such as centrifugal forces [182, 183], shear forces [154, 161] and cyclic tensile strains [82, 163, 170] can modulate GAG and PG synthesis; cyclic mechanical strains are the most relevant to the in vivo loading of valve cells. In addition, an elegant investigation of fibroblasts seeded on collagen gels subjected to repeated cycles of static stretch and relaxation showed that collagen XII synthesis was increased by tensile strains in a repeatable and reversible manner [81]. A combination of cyclic stretch and relaxation conditions in repeated cycles, therefore, offers a precise method to study strain-induced ECM production by VICs. The response of valve cells to pressure and shear forces has been characterized in organ culture [104, 221], but only two studies published to date have examined the effect of cyclic mechanical strains in 2D valvular cell culture [103, 106]; these latter investigations, however, did not measure GAGs and PGs. Determining how GAG and PG synthesis responds to mechanical strains is important since these ECM molecules are altered extensively during myxomatous valve degeneration. Recently, it was shown that static strains induce the synthesis of specific GAGs by VICs in 3D cultures [237]. This study performed a cyclic strain-based analysis in which the 3D cultures were stretched in a custom designed bioreactor. Many different types of scaffolds and cell environments have been used to investigate the effects of mechanical stress and strain conditions on various cell types. In this investigation, VICs were seeded within a collagen matrix since
that approach was believed to provide a more biologically and anatomically appropriate model for studying the regulation of ECM deposition than growing VICs in monolayers [78, 86]. Indeed, VICs seeded within collagen gels have been shown to retain their native phenotype and secrete GAGs and PGs that are normally present in heart valves [92, 93, 95]. Therefore, the purpose of this study was to determine if the GAG and PG production by mitral VICs cultured in 3D collagen gels is inducible by cyclic stretch, reversibly regulated and dependent upon cell loading \textit{in vivo} (leaflet vs. chordae).

5.2 Materials and methods

Cell culture

Primary cultures of VICs were dissociated from porcine mitral valve leaflets and chordae, harvested from porcine hearts obtained from an abattoir. Valves were first washed extensively with sterile phosphate buffered saline and then placed in serum-free Dulbecco’s modified Eagle medium (DMEM, Mediatech, Herndon, VA), containing 2 mg/mL collagenase type II (Worthington, Lakewood, NJ), within an incubated shaker for 20 min (140 rpm, 37°C). Afterwards, the loosened endothelial cells were removed by rubbing the valve surface with a cotton swab and the chordae tendineae were separated from the leaflet. The leaflet and chordae were minced and digested in serum-free DMEM containing 1 mg/mL collagenase type III and 0.1 mg/mL hyaluronidase (both from Worthington) for 4 h in an incubated shaker (140 rpm, 37°C). The resulting cell suspension was filtered through a sterile 70 μm cell strainer (BD Falcon, San Jose, CA) and cultured in DMEM:F12 (1:1, containing low glucose with HEPES, Mediatech) with 10% bovine growth serum (BGS, HyClone, Logan, UT) and 1% antibiotic-antimycotic
solution (Mediatech). The culture was incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C with changes of medium every 48 hours. The cells were split in a 1:3 ratio when they became 90% confluent. Separate leaflet and chordal cell cultures between passages 5-9 were used to prepare the collagen gels.

Device design and assembly

To apply cyclic strains with different amplitude and frequency to the VICs seeded within collagen gels, a prototype bioreactor was developed in our lab. The conceptual cyclic stretching mechanism and initial design of the bioreactor was done by Mr. Brian Lawrence, an engineering co-op student working with Dr. Grande-Allen. The expert suggestions by Mr. Mike Allen and Dr. Fazle Hussain were also extremely helpful in the design and setup of the device.

The interchangeable cam traces have a variable radius of curvature with sinusoidal flanges that stretch the cell-seeded collagen gel four times with each cam rotation (Figure 5.1). The cam radii \( R_{outer} \) and \( R_{inner} \), initial collagen gel construct length \( L_0 \), and percent strain \( \epsilon \) are related by equation

\[
R_{outer} = R_{inner} + \frac{L_0 \epsilon}{4} \left( 1 + \sin \left( 4\theta - \frac{\pi}{2} \right) \right)
\]

where \( \theta \), in radians, is the angle around the perimeter of the cam.

Since the original design, several revisions have been performed. Different parts of the bioreactor and the complete assembly in working condition are shown in Figures 5.2-5.4. The first revision was to pour the gel into a cross-shaped mold as opposed to an open circle mold. The purpose of this change was primarily to minimize the amount of
collagen/cell solution required to create a gel, and secondarily to add some distance between the main body of the gel and the gel holders in order to minimize stress concentrations caused by edge effects. Next, the CAD/CAM software Pro Engineer was used to design and modify the bioreactor. Notable recent modifications to make the bioreactor robust and to improve its function include replacing the beads of the old design with the roller bearings. The cam perimeter and holes in the lid, through which the wires are inserted, were lubricated with grease to provide frictionless motion.

![Cam Shape for 20% Stretch](image1)

![Sinusoidal Cam Radius, 20% Stretch](image2)

**Figure 5.1.** Cam trace shape for 20% stretch. In this example, $L_0=60$ mm and $R_{\text{inner}}=80$ mm.

A design challenge was encountered when the rapid prototyping Somos resin (photopolymer) material started to warp slightly in the incubator ($37^\circ$C, 5% CO$_2$ and 100% humidity), which caused the steel wires to stop moving through the lid holes. To prevent this warping of the lid holes, stainless steel tubing was inserted into the lid holes to provide an interface between the steel wires of the gel holder and the polymer material,
resulting in smooth motion of the steel wires even in the incubator. An example of the collagen gel prepared within the bioreactor is shown in Figure 5.5.

Figure 5.2. Parts of the bioreactor assembly (A) rectangular block with the roller bearing, (B) 24 V DC gearhead motor, (C) aluminum box, (D) cam, and (E) lid.
Figure 5.3. CAD drawings showing assembly of the device with lid, cam and box. Top view (left) and side view (right), arrow indicates the direction of rotation of the cam.

Figure 5.4. The complete device assembly connected to the power supply.
Cell seeding in collagen gels

To mimic the 3D aspect of the cells’ native tissue environment, the VICs were seeded within collagen gels as previously described. After the collagen gel was poured, incubated, and began to contract (1-2 hours), 2-3 mL of regular DMEM:F12 medium with serum was added. Twenty-four hours after pouring the gel, the “pre-conditioning” culture medium was removed and stored (-20°C) and the culture was replenished with fresh medium. The collagen gel was then subjected to 10% strain at 1.167 Hz for 24 hours, then no cyclic stretch (relaxation) for 24 hours; this cycle of stretching and relaxation was repeated 2 more times. Conditioned media samples were removed and stored after each stretching or relaxation period, for a total of 7 days, before the collagen gels were collected for biochemical analysis. Four collagen gels were prepared from each cell type (leaflet or chordal VICs).
Image analysis for contraction assessment

At the time of each medium change, the area of the collagen gel was digitally measured using Image-Pro software (Media Cybernetics, Silver Spring, MD) and the percentage contraction was calculated relative to the original mold size [234].

Glycosaminoglycan analysis

Fluorophore-assisted carbohydrate electrophoresis (FACE) was used to analyze the different GAG classes (chondroitin/dermatan sulfates and HA) [234]. Briefly, intact collagen gels were weighed to measure the wet weight, lyophilized overnight, and then re-weighed to calculate percentage hydration. Samples were then dissociated with proteinase-K (EMD Pharmaceutical, Durham, NC); conditioned medium aliquots were similarly digested with proteinase-K. The medium samples (in addition to a control volume of fresh medium) were then subjected to ion exchange purification to remove the glucose from the medium samples and isolate the GAG chains. Each medium and gel sample was analyzed using FACE after digestion with chondroitinase ABC and ACII (Associates of Cape Cod, Falmouth, MA) to cleave the GAG chains into disaccharides exactly as described previously [13, 234]. FACE gels were imaged with a Kodak Gel Logic 100 imaging system (Kodak, Eastman, MA) and analyzed using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). Specific GAGs were identified by corresponding bands in a disaccharide standard lane and quantified by comparison to a fluorescence standard curve. Total GAGs were calculated as the sum of various classes of GAGs.
Proteoglycan measurement

To identify the core proteins of the PGs, gel samples were extracted using 4 mol/L guanidine HCl with protease inhibitors overnight at 4°C using previously reported techniques [13, 238]. The extracted samples were dialyzed into 7 mol/L urea (pH 7.5). Dialyzed gel samples and conditioned medium samples were then purified by ion exchange. The purified PGs were ethanol-precipitated from solution and digested with chondroitinase ABC to remove the GAG chains from the core proteins. The samples were vacuum dried, dissolved in SDS buffer at 100°C, separated on a 4-12% SDS-polyacrylamide gel, and then either visualized by Coomassie blue staining or blotted for western analyses. The nitrocellulose membrane was blotted with antibodies for specific PGs (anti-decorin LF-122 and anti-biglycan LF-104, courtesy of Larry Fisher, NIH; antiversican 2-B-1, Associates of Cape Cod) [217, 228].

DNA and collagen analysis

Cell and collagen density within the cell-seeded collagen gels were monitored by the Hoechst dye DNA assay [227] and a Sirius red collagen assay, respectively. The DNA assay was performed on aliquots of the proteinase-K digested collagen gel samples, as prepared for GAG analysis [54]. The Sircol sirius red assay (Biocolor, Ireland, UK) for collagen was performed on collagen gel samples dissolved in pepsin (1 mg/mL in 0.5 mol/L acetic acid, keeping the ratio of pepsin to sample wet weight as 1:10) overnight at room temperature.
Data analysis

Differences in specific GAG classes were compared between each 24-hour period of stretching and relaxation for leaflet and chordal cells. Total GAGs present in the conditioned medium were calculated by subtracting GAGs present in the fresh medium. The proportions of specific GAGs (relative to total GAGs) were also determined for each day. Proteoglycan band intensities on days 2-7 were normalized to the band intensity of the same PG for the same gel from day 1 (preconditioning). This normalizing was performed to eliminate blot-to-blot variability. Band intensities on each electrophoresis gel and western blot were analyzed in duplicate and then averaged for each gel. Data was expressed as mean ± standard deviation. Two-way ANOVAs were performed to compare leaflet vs. chordal groups (factor 1) and the different 24-hour periods (factor two). When a significant effect of the time factor was found, post-hoc Tukey tests were used to examine period-to-period differences. Paired t-tests were performed to compare GAGs secreted during sequential stretching and relaxation periods. The level of significance was chosen as \( \alpha = 0.05 \).

5.3 Results

Collagen gel contraction

Gel contraction increased over the seven-day culture period. The collagen gels contracted faster during the first 2-3 days and more slowly afterwards. The maximum contraction at the end of 7 days was found to be 50%. The total percentage that a given gel contracted was slightly dependent on cell lines but the overall trend was the same (Fig. 5.6). The gels contracted more during relaxation periods as compared to stretching.
periods; this response was repeated with each cycle of stretching and relaxation. There was no significant difference in total percentage contraction between gels seeded with leaflet cells and chordal cells.

**Collagen gel contraction**

![Collagen gel contraction graph](image)

*Figure 5.6.* Representative contraction profile of collagen gel seeded with mitral chordal cells over 7 day period. PC- preconditioning, S- stretching, R- relaxation.

**Total GAGs secreted into conditioned medium**

GAGs produced by the cells were either retained within the collagen gels or secreted into the surrounding medium. In our analysis only GAGs secreted into the medium over each 24-hour period of stretching or relaxation were measured. Culture medium from both leaflet and chordal cell-seeded collagen gels showed significant changes in total GAGs with time (p<0.02), which specifically tended to show an up-regulation of total GAGs secreted during periods of stretching compared to periods of relaxation (p<0.02, chordal days 4 S and 6 S vs. day 7 R, Fig. 5.7). Although chordal cells demonstrated this pattern starting with the first stretch-relax periods, leaflet cells showed this repetitive response only for the last 2 cycles of stretching and relaxation. A
significant difference between stretching and relaxation was found for chordal cells when the total GAGs released during each cycle were averaged together (p<0.004, Fig. 5.8). A similar trend was found for leaflet cells when only the last 2 cycles of stretching and relaxation were considered (p<0.004). The reproducibility of the analysis of bands on FACE gels was found to be within 7%.

**Figure 5.7.** Total GAGs secreted into the medium of collagen gels seeded with cells isolated from regions of (a) mitral leaflet and (b) mitral chordae. Data represented as mean ± standard deviation. *p<0.02 vs. Day 7 R.

**Figure 5.8.** Total GAGs secreted into the medium combining cycles of stretching and relaxation (a) leaflet VICs (2 cycles) (b) chordal VICs (3 cycles). Data represented as mean ± standard deviation.
Specific GAG classes secreted into the medium

The most common GAG classes found in heart valves are HA and unsulfated, 4-sulfated and 6-sulfated chondroitin/dermatan (0-S, 4-S and 6-S) [3]. All four of these GAGs were found in the medium of collagen gels; HA and 4-S GAGs were the most prominent GAGs secreted into the medium whereas 0-S and 6-S were present in lesser amounts. HA, 0-S, 4-S, and 6-S were all significantly different between culture periods when both leaflets and chordal data were grouped together (p<0.05). All four GAG classes tended to be upregulated during stretching periods compared to relaxation periods for chordal cells (Fig. 5.9), with significant differences found for HA (p<0.05, day 6 S vs. days 3, 5 and 7 R), 6-S (p<0.03, day 7 R vs. days 2, 4 and 6 S) and 4-S (p<0.02, day 4 S vs. day 7 R). In general, the various GAG classes secreted by leaflet cells were also slightly upregulated with stretching (compared to relaxation) but only after 1 stretch-relax cycle (Fig. 5.10). When GAGs secreted with each stretch-relax cycle were averaged (3 cycles for chordal and the latter 2 cycles for leaflet), the differences between stretching and relaxation periods were particularly evident. In collagen gels seeded with chordal cells, all GAG classes except 0-S were more abundant in stretching periods (p<0.008), whereas in gels seeded with leaflet cells only HA (p<0.04) and 4-S (p<0.03) were more abundant. When all VICs (leaflet and chordae) were grouped together for combined ANOVA, HA (p<0.004), 6-S (p<0.01) and 4-S (p<0.01) were all upregulated during stretching periods.
Figure 5.9. Various GAG classes secreted into the medium of collagen gels seeded with mitral chordal cells. Data represented as mean ± standard deviation. *p<0.05 vs. Day 6 S, †p<0.03 vs. Day 7 R, ‡p<0.02 vs. Day 7 R, §p<0.05 vs. Day 7 R, b<0.05 vs. Day 2 S, when leaflet and chordal data were grouped together.

Figure 5.10. Various GAG classes secreted into the medium of collagen gels seeded with mitral leaflet cells. Data represented as mean ± standard deviation. *p<0.05 vs. Day 7 R, †p<0.03 vs. Day 6 S, ‡p<0.05 vs. Day 7 R, b<0.05 vs. Day 2 S, when leaflet and chordal data were grouped together.
There were also general differences found between the medium from chordal cells and leaflet cells. Compared to chordal cells, leaflet cells secreted more HA during relaxation periods (p<0.02, averaged cycles) and less 6-S during stretching periods (p<0.001, averaged cycles). Overall, secretion of 6-S GAGs by leaflet cells was higher than for chordal cells even when all stretching and relaxation cycles were averaged together (p<0.001).

**Proportions of various GAG classes**

Proportions of GAG classes relative to total GAGs secreted into the medium for each cycle were different for leaflet and chordal cells (Fig. 5.11). Although there appears to be an up-regulation of total GAGs produced during stretching compared to relaxation (Fig. 5.7), the actual composition of GAGs secreted into the medium demonstrated some notable trends. The proportions of HA and 6-S decreased (both p<0.04) and 4-S increased (p<0.05) over nearly the entire culture duration irrespective of stretching or relaxation for collagen gels seeded with leaflet cells. For collagen gels seeded with chordal cells, a decrease in proportions of 6-S (p<0.05) over the culture duration was the only significant trend; the proportions of all other GAGs remained steady. Compared to chordal cells, leaflet cells secreted higher proportions of HA (p<0.002) and 4-S (p<0.03), and lower proportions of 6-S (p<0.001) when all stretching and relaxation cycles were averaged together. The leaflet cells' secretion of 0-S was negligible in many samples. For those two samples that did contain an appreciable amount of 0-S (data shown in Figure 5.11a), there was an overall effect of culture day on the proportion of 0-S (p<0.05) and the proportion of 0-S increased over time.
Figure 5.11. Proportions of various GAGs in the medium of collagen gels seeded with cells isolated from (a) leaflet, *p<0.04 vs. Day 2, †p<0.04 vs. Day 2, ‡p<0.05 vs. Day 2 and (b) chordae, *p<0.04 vs. Day 2, †p<0.05 vs. Day 3 R. Data represented as mean ± standard deviation.
Proteoglycans secreted into the medium

Proteoglycans are another major component of ECM that are secreted by valve cells. Decorin, biglycan and versican, three PGs previously found in heart valve tissues were analyzed [13]. All three PGs were present in the conditioned medium of collagen gels seeded with either leaflet or chordal cells. Biglycan secreted by chordal cells showed a trend of increasing with each stretching cycle compared to relaxation (Fig. 5.12), although these differences did not reach statistical significance (p=0.077). Similar to GAG synthesis, leaflet cells also showed, albeit weakly, delayed responsiveness for biglycan synthesis (stretch-relax pattern apparent after 1 cycle only). Neither decorin nor versican secretion showed any specific trend for either leaflet or chordal cells.

ECM and cells in collagen gels after stretching protocol

The identities and abundances of GAG classes, PGs, collagen and DNA within the collagen gels were measured and normalized to dry gel weight at the end of 7-day stretching and relaxation protocol. Higher amounts and proportions (65-75%) of 4-S GAGs were found in the collagen gels compared to conditioned medium; the proportions of 4-S GAGs were higher in the gels seeded with chordal cells compared to leaflet cells. PG contents in the collagen gel again did not show any specific trends (similar to the PGs in the medium). The collagen content remained almost the same after the 7-day period, compared to initial amounts used for collagen gel preparation, for both chordal and leaflet collagen gels. However, DNA content in collagen gels was reduced to nearly 50% at the end of 7 days for both leaflet (from 308±30 μg to 140±26 μg) and chordal cells (from 392±8 μg to 180±76 μg).
Figure 5.12. Proteoglycans present in the medium of collagen gels seeded with leaflet cells (a) decorin (b) biglycan (c) versican and chordal cells (d) decorin (e) biglycan (f) versican. PG band intensities on western blots were normalized to preconditioning (Day 1 PC). Data represented as mean ± standard deviation.

5.4 Discussion

In this study, a novel custom-designed bioreactor was used to perform the first characterization of GAG and PG secretion by VICs subjected to cyclic strains. It was found that VICs from both leaflet and chordae secrete abundant GAGs (in similar total magnitudes) into the culture medium of collagen gels, confirming our previous static tension model [13]. Repetitive stretching and relaxation periods demonstrated that these
cells respond reversibly to mechanical strains. Mitral chordal VICs showed stretching-induced upregulation of total GAGs secreted into the medium followed by a relaxation-induced decrease in GAGs. Mitral leaflet VICs showed the same trends as chordal VICs but it was only apparent after at least one cycle. This same delay of upregulation with stretching was also observed for the secretion of the PGs decorin and biglycan. This interesting difference between leaflet and chordal VICs may be due to a more dramatic dissimilarity between their in vivo conditions and our 3D model, or it may be due to inherent phenotypic differences between the cell types. Chordae tendineae in the mitral valve normally experience uniaxial tension and undergo dramatic remodeling (both mechanically and biochemically) in pathological conditions [3, 54]. Also, research in our lab has shown that mitral chordal cells have approximately 50% higher levels of smooth muscle α-actin expression than leaflet cells [230]. Based on findings in this study, it is proposed that VICs from chordae may be particularly responsive to mechanical strains.

The various GAG classes were also reversibly modulated by cyclic stretch. The most abundant GAG class found in the medium was 4-S; this GAG is associated with the PGs decorin and biglycan and is the most abundant GAG in native chordae, which experience tension during the cyclic closure of the valve [3, 54]. All GAG types (except 0-S) had increased secretion during stretching although this trend was only apparent in the leaflet 3D cultures after a delay (similar to total GAGs). In contrast to the trends displayed by the GAG amounts, the proportions of various GAG classes did not show specific upregulation and downregulation patterns during stretching and relaxation periods. This trend of upregulation of GAGs during stretching but finding no change in GAG composition is supported by reports in other cell lines, such as endothelial cells.
subjected to shear stresses [154]. Our finding of a statistically significant increase in the amount of 6-S GAGs during stretching is supported by the similar findings of Leung et al., who tested arterial smooth muscle cells in 3D collagen constructs [163]. Because 4-S and 6-S GAGs tend to be found on different PGs that are predominantly located in tensile and compressive loading regions, respectively, the relative abundance of 4-S and 6-S is an indicator of the type of loading experienced by a tissue. For leaflet cells, the proportions of HA and 6-S decreased and whereas 4-S increased during the 7 day period irrespective of stretching and relaxation. Also, the proportions of 6-S decreased each day for chordal cells. Therefore, the ratio of 4-S to 6-S for both leaflet and chordal cells increased with each day regardless of cyclic stretching or relaxation conditions. Interestingly, this ratio was much higher for leaflet cells than chordal cells as more 4-S GAGs were secreted into the medium compared to 6-S GAGs. It is speculated that application of cyclic tensile strains caused the leaflet cells to reverse their in vivo GAG synthesis pattern (which is normally more 6-S and less 4-S) [13]. The other possibility is that less 4-S was retained in the collagen gels seeded with leaflet cells, since more 4-S was found in the collagen gels seeded with chordal cells than leaflet cells (data not shown); a higher proportion of 4-S may be released into the medium during relaxation periods because it is not needed in the gel. This explanation would account for 4-S being present in lesser amounts in the medium and in higher amounts in the chordal gels, since chordal tissues (which experience high cyclic tension in vivo) also have an abundance of 4-S GAGs [3]. This study therefore provides guidance for constructing engineered tissues in which a tensile environment will induce specific GAG or PG production. It has also provided an in vitro confirmation that leaflet VICs will alter their proportional secretion
of GAG classes when exposed to cyclic tension. This trend would support the remodeling of the myxomatous mitral valve leaflets post surgical repair (when normal leaflet loading is restored) as well as the fibrotic-type remodeling found in mitral valves in heart failure (when their tensile loading increases).

This is the first study to apply cyclic strains to valve cells to modulate GAG and PG synthesis, yet our results are quite consistent with previous investigations of other cell types [79]. In a time-dependent study, cyclic strains applied to SMCs induced GAG/PG synthesis within 24 hours [82], which supports our selection of 24-hour periods of stretching and relaxation. That same study reported that cyclic strain increased the abundance of chondroitin/dermatan sulfate GAGs and the PGs versican and biglycan in the medium and cell layer but decreased decorin synthesis; clearly all GAGs and PGs are not mechanically regulated in the same manner, which befits their diverse biological roles. Biaxial cyclic strains applied to SMCs cultured on 2D elastic membranes also increased the expression of syndecan-4, a cell surface PG [180]. Collagen XII secretion by fibroblasts has been shown to accumulate in the medium of statically stretched collagen gels after 24 hours; whereas static relaxation for another 24 hours decreased secretion [81]. This response was repeatable for up to 3 cycles of stretching and relaxation, which has excellent agreement with our findings. Hence, this study demonstrated that the valve cells (especially chordal) are quickly responsive to cyclic mechanical strains and that response is reversible and repeatable.

Limitations to this study include the large standard deviations in the data, which can be attributed to the variability that arises in the analysis of bands on FACE gels and western blots. Also, the significant loss of cells during the culture duration may have
caused slight variability in ECM secretion between different collagen gels; however, these cell results are comparable to other reports of cell-seeded collagen gels [235]. It appears that the sample size that was able to detect differences in GAGs is not sufficient to detect changes in PGs, which may be due to the less quantitative methodologies of blot analysis. The levels of all PGs increased during stretching and relaxation periods compared to preconditioning; this increase limited our ability to detect changes in versican secretion since the magnitude of the versican levels, even after normalizing to day 1, were highly variable between gels. In addition, the time dependent secretion of GAGs and PGs was measured in the medium only, as opposed to those retained within the collagen gel during each 24 hours period. Future investigations on the time-dependent ECM synthesis and turnover by VICs within collagen gels may provide more mechanistic information. Finally, our 3D model used the same biaxial construct format for both chordal and leaflet cells even though these cells experience different strain patterns in vivo. However, our rationale was that providing the same collagen gel design conditions (gel volume, medium volume, cell number) for both leaflet and chordal cells would permit the most direct comparison of the effect of mechanical strains.

In conclusion, it was shown that GAG secretion by VICs seeded within 3D collagen gels is dependent on the presence or absence of cyclic strains. GAG secretion was significantly upregulated during cyclic stretch and downregulated during relaxation. The proportional composition of distinct GAG classes remained fairly consistent for chordal cells but tended to change for leaflet cells, which reflects the leaflet VICs’ adaptation to high cyclic tensile strains. It was also shown that the chordal cells respond to cyclic strains more rapidly than leaflet cells, which is an exciting finding given that
chordal regions undergo most dramatic changes during myxomatous degeneration. Overall, this study improves our understanding of the role of mechanical strains on GAG synthesis in the remodeling of mitral valve as well as other native and engineered tissues. This study also provides valuable information for tissue engineering research and applications in which mechanical strains can be used to regulate ECM synthesis and organization.
Chapter 6: Effect of cyclic mechanical strain and frequency on glycosaminoglycan and proteoglycan synthesis by heart valve cells

6.1 Introduction

The application of mechanical strains has been shown to induce remodeling of the ECM in many tissues, including heart valves [79, 239]. Heart valves are maintained in vivo under a range of strain magnitudes and frequencies during opening and closing of the valve and during relaxed or stressed conditions of the heart, such as exercise or pathological conditions. Correspondingly, the VICs within heart valves have been shown to be mechanoresponsive to tensile strains in 2D and 3D cell cultures [103, 234] and to pressure and shear forces in organ culture [104, 221], resulting in altered ECM synthesis. However, the effect of varying magnitudes of strain and frequencies, such as those found in vivo, have not been investigated in the context of VICs in 3D cultures. Only one study on VICs examined the effect of strain magnitudes on ECM synthesis, but that was in 2D culture [103]. This paucity of research on VICs is notable because investigations on other cardiovascular cell types have shown that ECM synthesis is clearly influenced by strain and frequency. For example, cardiac fibroblasts subjected to 10% or 20% uniaxial strain and 3% or 6% equibiaxial strain expressed more collagen III mRNA at lower strains and less at higher strains compared to unstretched controls [143]. SMCs subjected to 10% or 20% strain synthesized more collagen than those subjected to 5% strain [167], although another study on SMCs did not find any significant effect of strain magnitude on collagen synthesis [132]. Secretion of transforming growth factor-β [177] and fibroblast growth factor-2 [130] by SMCs was found to be proportional to both strain and frequency. The
release of heparan sulfate GAGs and fibronectin by endothelial cells was decreased when shear rates [151] and biaxial strains [129], respectively, were elevated. Therefore, strain and frequency demonstrably but variably affect ECM synthesis in different cardiovascular cell types and their effects on VICs should be investigated in order to improve our understanding of valve mechanobiology.

GAGs and PGs are integral ECM components of valve tissues and play an important role in defining the material and structural behaviour of valves. PGs consist of GAG chains (linear molecules of repeating disaccharides) attached to a core protein; these molecules perform many biological functions in tissues [5]. The sulfation pattern of GAG chains, meaning whether they are 4-sulfated or 6-sulfated, characteristically varies according to the biological and biomechanical needs of the tissue [8]. Dermatan sulfate PGs such as decorin and biglycan, which contain mostly 4-sulfated GAGs, regulate collagen fibril diameter and fibrous tissue organization [10, 11]. In contrast, the GAG HA, which does not bind to a protein core but tends to aggregate with large PGs such as versican (mainly containing 6-sulfated GAGs), entraps large amounts of aqueous solvent to provide compression resistance [4]. Previous studies of mitral valves from our laboratory showed that 4-sulfated GAGs, decorin, and biglycan were abundant in tensile loading regions such as chordae tendineae, while regions experiencing compression such as the leaflet free edges contained more hyaluronan, 6-sulfated GAGs, and versican [13]. In pathological conditions such as myxomatous mitral valve disease, the valve tissues experience altered tissue loading along with an overabundance of GAGs and PGs [3, 54].

Static mechanical strains have recently been applied to study GAG synthesis by VICs grown in 3D cell cultures [234]. Although VICs have been investigated under
cyclic straining conditions (in 2D culture), no study has examined the effect of strain and frequency on GAG and PG synthesis by VICs [103, 106]. Determining how GAG and PG synthesis responds to different strains and frequencies is important since the patterns of strain and distribution of GAGs and PGs are evidently altered during myxomatous valve degeneration. In this study, VICs seeded within 3D collagen gels were stretched at different strains and frequencies in a custom designed bioreactor. A 3D collagen matrix was chosen, as opposed to a 2D cell culture approach, in order to provide a more biologically and anatomically appropriate model, since 3D is closer to in vivo conditions [86]. Furthermore, previous studies have shown that VICs seeded on top of or within 3D collagen scaffolds retain their native phenotype and secrete GAGs and PGs comparable to those found in vivo [92, 93, 95, 240]. Therefore, the purpose of this study was to determine if the GAG and PG synthesis by VICs isolated from distinct regions of the mitral valve is regulated by different cyclic strain magnitudes and frequencies.

6.2 Materials and methods

Cell culture and seeding in collagen gels

Porcine mitral valves were obtained from an abattoir and VICs were isolated using a previously described protocol [234]. Briefly, the tissues were first soaked in serum-free Dulbecco’s Modified Eagles Medium (DMEM, Mediatech, Herndon, VA), containing 2 mg/mL collagenase type II (Worthington, Lakewood, NJ), within an incubated shaker for 20 minutes (140 rpm, 37°C). The endothelial cells were then brushed from the valve surface using cotton swabs and the chordae tendineae were dissected from the leaflet. The leaflets and chordae were separately minced and
dissociated with serum-free DMEM containing 1 mg/mL collagenase type III and 0.1 mg/mL hyaluronidase (both from Worthington) for 4 hours in an incubated shaker. Leaflet and chordal VICs were cultured separately in DMEM:F12 medium (1:1, containing low glucose with HEPES, Mediatech) with 10% bovine growth serum (BGS, HyClone, Logan, UT) and 1% antibiotic-antimycotic solution (Mediatech). Similar primary leaflet and chordal cell cultures (passages 6-7) were used to prepare all the collagen gels.

VICs were seeded within collagen scaffolds to provide them with an in vivo like 3D environment. The collagen gels were prepared using a protocol from Eastwood et al. [225]. Briefly, 8 parts rat-tail collagen type I at 2.28 mg/mL (BD Biosciences, Bedford, MA) in 0.02 M acetic acid, 1 part 10X DMEM and 1 part cells suspended in 1X DMEM (1 million cells per mL of gel) were mixed together and brought to physiologic pH using 5M NaOH dropwise. The resulting gel solution was immediately poured into the mold (with or without anchors) within the cyclic stretching device (Figure 6.1).

**Stretching device**

A stretching device was developed in our laboratory to apply cyclic mechanical strains to collagen gels seeded with VICs (Figure 6.1). The device components were built at a rapid prototyping company (Laser Reproductions, Columbus, OH) and in the machine shop at Rice University. The main components of the device consisted of an aluminum base, stretching cam and culture lid. The collagen gel anchors were connected to roller bearings, which were displaced by the stretching cam.
Figure 6.1. Images of stretching device and collagen gels. (a) Cyclic stretching device used to apply biaxial mechanical strains to cell-seeded collagen gels; (b) constrained collagen gel, L0-initial collagen gel length; (c) unconstrained collagen gel.

The stretching cam was shaped as a 4-sinusoid waveform superimposed around the circumference of a circle and caused the collagen gel to be stretched four times with each cam rotation. The cam radii $R_{outer}$ and $R_{inner}$, angle around the perimeter of the cam
\( \theta \), initial collagen gel construct length \( L_0 \), and percentage strain \( \varepsilon \) were related by the equation

\[
R_{\text{outer}} = R_{\text{inner}} + \frac{L_0 \varepsilon}{4} \left( 1 + \sin \left( 4\theta - \frac{\pi}{2} \right) \right)
\]

The cam rotation was performed using a 24 V DC gearhead motor (Barber Colman, Rockford, IL) and controlled by an external power supply (Jameco Electronics, Belmont, CA). The whole bioreactor assembly, except for the power supply, was maintained within the incubator.

A cross-shaped silicon rubber mold that fit snugly within the culture lid was designed as described previously [234]. The collagen gels within the stretching device were incubated at 37°C and 5% CO\(_2\) for 24 hours to let the collagen gel attach firmly to the anchors. After 24 hours, the medium from the collagen gels was collected for GAG/PG analysis and replaced with fresh medium. The collagen gels were then subjected to cyclic mechanical strains at 2, 5 or 10% strain and 0.83, 1.16, 1.5 Hz for 48 hours. These frequencies represent the number of times each gel anchor was displaced per second. Two molds each were also prepared to expose the collagen gels to conditions of static stretch and no stretch (without anchors, Figure 6.1c).

**Glycosaminoglycan analysis**

Various GAG classes (chondroitin/dermatan sulfates and HA) were analyzed using fluorophore-assisted carbohydrate electrophoresis (FACE) [226, 234]. Briefly, collagen gels were lyophilized overnight, weighed and then digested with proteinase-K (EMD Pharmaceutical, Durham, NC). The conditioned medium aliquots were also
digested with proteinase and then subjected to ion exchange chromatography to remove the glucose from the medium samples and to isolate the GAG chains. Conditioned medium and collagen gel samples were then analyzed using FACE after digestion with chondroitinases ABC and ACII (Associates of Cape Cod, Falmouth, MA) to cleave the GAG chains into disaccharides as described previously [13, 234]. The fluorescent disaccharide bands within the FACE gels were imaged with a Kodak Gel Logic 100 imaging system (Kodak, Eastman, MA) and analyzed using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). GAG classes were identified by correspondence to bands in a disaccharide standard lane and quantified by comparison to a fluorescence standard curve. Total GAGs were calculated as the sum of the different classes of GAG disaccharides. The ratios of 4-sulfated (4-S) to 6-sulfated (6-S) GAGs and sulfated GAGs (4-S and 6-S) to unsulfated GAGs (HA) were also calculated.

**DNA measurements**

The DNA assay was performed on aliquots of the proteinase-K digested collagen gel samples [54]. Total cells retained within the collagen gels after the stretching protocol were measured using the Hoechst dye DNA assay [227].

**Proteoglycan analysis**

PGs within the collagen gel samples were extracted using 4 mol/L guanidine HCl with protease inhibitors overnight at 4°C as described previously [13]. The extracted samples were dialyzed into 7 mol/L urea (pH 7.5). Dialyzed collagen gel samples and conditioned medium samples were then purified by ion exchange chromatography. The
purified PGs were precipitated from solution using 95% ethanol (containing 1.3% potassium acetate) and digested with chondroitinase ABC to remove the GAG chains from the core proteins. The samples were vacuum dried, dissolved in SDS buffer at 100°C, separated on a 4-12% SDS-polyacrylamide gel, and blotted for western analyses. The nitrocellulose membrane was blotted with antibodies for the specific PGs (anti-decorin LF-122 and anti-biglycan LF-104, courtesy of Larry Fisher, NIH; anti-versican 2-B-1, Associates of Cape Cod) [217, 228]. The band intensities were analyzed using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) and normalized to band intensity of PGs from unconstrained collagen gels run on the same western blot.

**Data analysis**

The cell-seeded collagen gels subjected to various frequencies (0.83, 1.16 and 1.5 Hz) were grouped together for strain dependency analysis. Similarly, gels subjected to different strains (2, 5 and 10%) were grouped together for frequency dependency analysis. The absolute amounts of GAGs secreted into the conditioned medium were analyzed after accounting for the GAG contents of fresh medium and multiplying by total medium volume collected during the culture period. The proportions of specific GAGs synthesized by leaflet and chordal cells were determined by normalizing to total GAG abundance for each sample. The samples for PG analysis were run on electrophoresis gels in duplicate and their band intensities were averaged. Data were expressed as mean ± standard deviation. A two-way analysis of variance (ANOVA) was performed to analyze the effect of cell type (factor one: leaflet and chordal) and strain (factor two: no strain, static, 2%, 5% and 10%) or frequency (factor two: no strain, static, 0.83 Hz, 1.16 Hz and
1.5 Hz). Post-hoc Tukey tests were used for pair-wise comparisons and the level of significance was chosen as 0.05.

6.3 Results

**DNA content within collagen gels**

For each type of cell considered separately, there was no significant difference in the final DNA content (at the end of the stretching protocol) between the cyclic stretching conditions as compared to static or no strain conditions. However, by combining the leaflet and chordal data, DNA content was found to be significantly affected by strain condition \((p<0.05)\) and specifically lower for the 10% cyclic strain than for 5% cyclic strain \((p<0.04)\). Otherwise, there was no strain or frequency effect on cellularity (Figure 6.2).

**Glycosaminoglycans synthesized within collagen gels**

Leaflet and chordal cells synthesized various GAG classes that were retained within the collagen gels. Total GAGs, calculated as the sum of the different GAG disaccharides, were significantly affected by the strain conditions for the leaflet group \((p<0.001)\), the chordal group \((p<0.001)\), and for both groups combined \((p<0.001)\). Total GAGs were lower in the collagen gels subjected to cyclic strains than in gels subjected to static strain or no strain (Table 6.1). Significantly fewer GAGs were synthesized by leaflet cells exposed to all cyclic strain regimens than the gels exposed to static strain \((p<0.03)\) but only the 5% cyclic \((p<0.04)\) and 10% cyclic strains \((p<0.02)\) were significantly lower than in the no strain case. Similarly, for chordal cells, GAGs
synthesized under 5% and 10% cyclic strains (but not 2%) were significantly lower than the static strain (p<0.002) and no strain gels (p<0.05).

![DNA dependence on strain](image1)

*Figure 6.2.* Strain and frequency dependency of DNA content within collagen gels seeded with leaflet or chordal cells. Data are mean ± standard deviation. *p*<0.05 vs. 10%, when leaflet and chordal data were grouped together.
The effect of frequency was only significant for chordal cells alone (overall effect \( p < 0.01 \), static vs. 1.16 Hz, \( p < 0.04 \)) or for leaflet and chordal cells grouped together (overall effect \( p < 0.01 \), static vs. 0.83, 1.16, or 1.5 Hz, \( p < 0.02 \)). When the cyclic strain groups were compared to each other, the decrease in total GAGs was more dependent on the magnitude of strain than on the frequency. For chordal cells alone and for all cells grouped together, application of higher strains decreased GAGs synthesis (\( p < 0.04 \)).

Both unsulfated (HA) and sulfated GAGs (4- and 6-sulfated chondroitin/dermatan sulfate) were synthesized by valve cells within the collagen gels (Figure 6.3). Within the leaflet group, the chordal group, and/or both groups combined, the presence of cyclic strain, regardless of strain magnitude or frequency, significantly affected the proportions of HA, 4-S, and 6-S retained within the gels (\( p < 0.05 \)). In the collagen gels seeded with leaflet cells, cyclic strains of 2% caused significantly higher proportions of HA (\( p < 0.04 \)) and lower proportions of 4-S (\( p < 0.03 \)) compared to the static strain condition. Collagen gels seeded with chordal cells retained higher proportions of 4-S at 10% strain than in the no strain condition (\( p < 0.02 \)). A strain magnitude of 10% increased the proportions of 4-S compared to 2% strain when leaflet and chordal cells grouped together (\( p < 0.05 \)). Compared to the unconstrained collagen gels seeded with chordal cells, the highest strain magnitudes (\( p < 0.05 \)) and lowest frequency (\( p < 0.03 \)) of cyclic strain reduced the proportion of 6-S. Collagen gels seeded with leaflet cells contained significantly higher proportions of HA and lower proportions of 4-S than gels seeded with chordal cells for all strain (\( p < 0.01 \)) and frequency variations (\( p < 0.02 \)). In contrast, the proportions of 6-S were much more uniform between gels seeded with chordal cells vs. leaflet cells, only
showing greater proportions for chordal cells at 2% strain (p<0.002) and under the static and no strain conditions (p<0.02).

The ratio of 4-S to 6-S GAGs and the ratio of sulfated GAGs (sum of 4-S and 6-S) to unsulfated GAGs (HA) were calculated to gather more information about the mechanical behavior of the cell-seeded collagen gels during the different strain regimens, since 6-S GAGs and HA have been previously linked with low tension/compressive loading regions of valves whereas 4-S GAGs were more characteristic of high tensile loading regions [13]. On average, the ratio of 4-S to 6-S was higher in collagen gels seeded with leaflet cells than in gels seeded with chordal cells (p<0.05, Table 6.1). In contrast, the ratio of sulfated GAGs to HA was higher for chordal cells than for leaflet cells for the 2% (p<0.004) and 10% strain conditions (p<0.02) and for all frequency variations (p<0.04). Furthermore, the cyclically stretched collagen gels seeded with chordal cells showed much greater sulfated GAG to HA ratios than did the static or no strain gels (p<0.03, for 1.16 Hz).

Glycosaminoglycans secreted into culture medium

Besides being retained within the collagen gels, GAGs synthesized by the valve leaflet and chordal cells were also secreted into the surrounding culture medium. On average, more total GAGs were secreted into the medium by leaflet cells than by chordal cells (p<0.04). When exposed to increasing strain magnitudes, GAG secretion was progressively decreased and increased for leaflet and chordal cells, respectively. However, the frequency variations had no effect on total GAG secretion. Cyclic strains of only 2% applied to collagen gels seeded with leaflet cells caused a substantially greater
secretion of total GAGs into the medium compared to higher strains (p<0.02) and the no
strain condition (p<0.05, Table 6.2). With respect to the proportions of specific GAGs, in
general the only difference in HA proportions between groups was that leaflet cells
secreted more HA than did chordal cells (p<0.02), particularly at 10% strain (p<0.03,
Figure 6.4). There were no significant factors affecting the proportions of 4-S GAGs
secreted into the medium, although the proportions of 6-S GAGs showed many
differences. Collagen gels seeded with chordal cells secreted more 6-S when subjected to
a cyclic frequency of 0.83 Hz (compared to static, p<0.001). When the cell types were
grouped together, more 6-S was secreted at both 0.83 Hz (p<0.002) and 1.5 Hz (p<0.05).
6-S was also abundant in gels seeded with chordal cells than leaflet cells at 5% strain
(p<0.04), 1.16 and 1.5 Hz (p<0.02), and under static strain (p<0.04).

The ratio of secreted 4-S to 6-S GAGs was significantly affected by strain and
frequency conditions when leaflet and chordal data were analyzed together (p<0.05) and
by frequency conditions for the chordal group alone (p<0.01). Specifically, the 4-S/6-S
ratio secreted by chordal cells was highest at the lowest cyclic frequency (p<0.05, Table
6.2). Overall, the 4-S/6-S secreted GAG ratio tended to be greater in the cyclically
strained gels compared to static strain and no strain conditions, for 5% strain (p<0.04)
and 0.83 Hz (p<0.03). Although the secreted S-GAG to HA ratios were quite consistent
and far lower than those corresponding to the GAGs retained within the collagen gels, on
average the chordal cells produced greater secreted S-GAG/HA ratios than did the leaflet
cells (p<0.01).
Figure 6.3. Specific GAG proportions retained within collagen gels seeded with (a) leaflet cells and (b) chordal cells. Data are mean percentage ± standard deviation. *p<0.05 vs. chordae, †p<0.04 vs. 2%, ‡p<0.05 vs. unconstrained. *p<0.05 vs. 2%, ‡p<0.05 vs. unconstrained, when leaflet and chordal data were grouped together.
Table 6.1. Strain and frequency dependence of total GAGs (nmol/mg dry weight), ratio of 4-S to 6-S, and ratio of S-GAGs to HA calculated from amount of total GAGs retained within collagen gels seeded with leaflet or chordal cells.

<table>
<thead>
<tr>
<th></th>
<th>No strain</th>
<th>Static</th>
<th>Cyclic 2%</th>
<th>Cyclic 5%</th>
<th>Cyclic 10%</th>
<th>Cyclic 0.83 Hz</th>
<th>Cyclic 1.16 Hz</th>
<th>Cyclic 1.5 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total GAGs</strong></td>
<td>12.0±0.1</td>
<td>13.7±2.7&lt;sup&gt;x&lt;/sup&gt;</td>
<td>8.3±1.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.7±1.7&lt;sup&gt;ix&lt;/sup&gt;</td>
<td>5.8±1.2&lt;sup&gt;ix&lt;/sup&gt;</td>
<td>7.6±1.6&lt;sup&gt;y&lt;/sup&gt;</td>
<td>7.3±3.2&lt;sup&gt;y&lt;/sup&gt;</td>
<td>6.6±1.4&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Leaflet</strong></td>
<td>5.2±0.8</td>
<td>6.7±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8±2.3</td>
<td>6.6±1.8</td>
<td>5.4±0.5</td>
<td>6.4±2.2</td>
<td>5.6±2.2</td>
<td>6.4±0.7</td>
</tr>
<tr>
<td><strong>S-GAGs/HA</strong></td>
<td>6.9±0.4</td>
<td>7.9±1.1</td>
<td>3.2±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7±4.2</td>
<td>5.9±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0±1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0±1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total GAGs</strong></td>
<td>12.5±0.5</td>
<td>15.3±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.0±2.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7.5±2.2&lt;sup&gt;ix&lt;/sup&gt;</td>
<td>4.1±0.6&lt;sup&gt;ix&lt;/sup&gt;</td>
<td>7.5±4.6&lt;sup&gt;y&lt;/sup&gt;</td>
<td>7.1±3.0&lt;sup&gt;y&lt;/sup&gt;</td>
<td>8.0±3.8&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Chordae</strong></td>
<td>3.2±0.1</td>
<td>3.8±0.2</td>
<td>4.1±0.6</td>
<td>6.2±0.7</td>
<td>6.0±1.0</td>
<td>6.0±1.0</td>
<td>4.9±0.6</td>
<td>5.8±1.5</td>
</tr>
<tr>
<td><strong>S-GAGs/HA</strong></td>
<td>7.1±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7±1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.2±35.1</td>
<td>29.0±12.8</td>
<td>40.2±10.4</td>
<td>34.9±19.3</td>
<td>48.6±23.0</td>
<td>37.8±27.4</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation. *p<0.03 vs. static, **p<0.04 vs. no strain, *p<0.002 vs. 5% or 10%, †p<0.009 vs. chordae, ‡p<0.05 vs. chordae, †p<0.02 vs. chordae, ‡p<0.004 vs. 1.16 rpm. *p<0.05 vs. 2%, †p<0.05 vs. static, when leaflet and chordal data were grouped together.
Table 6.2. Strain and frequency dependence of total GAGs (nmol), ratio of 4-S to 6-S, and ratio of S-GAGs to HA calculated from amounts of GAGs secreted into the medium of collagen gels seeded with leaflet or chordal cells.

<table>
<thead>
<tr>
<th></th>
<th>No strain</th>
<th>Static</th>
<th>Cyclic 2%</th>
<th>Cyclic 5%</th>
<th>Cyclic 10%</th>
<th>Cyclic 0.83 Hz</th>
<th>Cyclic 1.16 Hz</th>
<th>Cyclic 1.5 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total GAGs</strong></td>
<td>92.4±7.7*</td>
<td>125.2±10.6</td>
<td>174.5±55.4†</td>
<td>127.6±44.6</td>
<td>85.3±14.5*</td>
<td>92.8±16.8</td>
<td>166.1±81.3</td>
<td>123.6±32.0</td>
</tr>
<tr>
<td><strong>Leaflet</strong></td>
<td>3.2±0.1y</td>
<td>3.1±0.1y</td>
<td>3.9±0.3</td>
<td>4.6±0.3†</td>
<td>3.4±0.5</td>
<td>4.1±0.9</td>
<td>3.7±0.5</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>S-GAGs/HA</td>
<td>0.7±0.2</td>
<td>0.9±0.5</td>
<td>1.0±0.3</td>
<td>1.0±0.2</td>
<td>0.7±0.2</td>
<td>0.8±0.3</td>
<td>0.8±0.1</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td><strong>Total GAGs</strong></td>
<td>65.2±19.5</td>
<td>83.6±17.5</td>
<td>84.9±23.3</td>
<td>91.2±13.1</td>
<td>138.1±24.2</td>
<td>123.6±36.5</td>
<td>95.1±30.3</td>
<td>105.5±11.6</td>
</tr>
<tr>
<td><strong>Chordae</strong></td>
<td>3.0±0.3y</td>
<td>2.6±0.0y</td>
<td>3.5±0.8</td>
<td>3.7±1.4†</td>
<td>3.6±0.4</td>
<td>4.5±0.7</td>
<td>3.0±0.3†</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>S-GAGs/HA</td>
<td>1.0±0.5</td>
<td>1.6±0.4</td>
<td>1.1±0.2</td>
<td>1.4±0.4</td>
<td>1.3±0.7</td>
<td>1.1±0.4</td>
<td>1.3±0.6</td>
<td>1.4±0.4</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation. *p<0.05 vs. 2%, †p<0.001 vs. chordae, ‡p<0.05 vs. 0.83 Hz. §p<0.05 vs. static, yp<0.05 vs. 0.88 Hz, when leaflet and chordal data were grouped together.
(a) Unconstrained | Cyclic 2% | Cyclic 5% | Cyclic 10%

Static

- HA | 49.5±1.5
- 6.5 | 27.2±0.5
- 4.5 | 19.0±0.5

Cyclic 0.83 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

Cyclic 1.16 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

Cyclic 1.5 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

(b) Unconstrained | Cyclic 2% | Cyclic 5% | Cyclic 10%

Static

- HA | 49.5±1.5
- 6.5 | 27.2±0.5
- 4.5 | 19.0±0.5

Cyclic 0.83 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

Cyclic 1.16 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

Cyclic 1.5 Hz

- HA | 40.8±1.0
- 6.5 | 37.2±0.3
- 4.5 | 31.0±1.0

Figure 6.4. Specific GAG proportions secreted into culture medium from collagen gels seeded with (a) leaflet cells (b) chordal cells. Data are mean ± standard deviation. *p<0.04 vs. chordae, †p<0.001 vs. static, ‡p<0.05 vs. static, when leaflet and chordal data were grouped together.

Proteoglycans synthesized within collagen gels

All three PGs of interest (decorin, biglycan and versican) were found within collagen gels subjected to the different stretching protocols. Synthesis of decorin, biglycan and versican was not found to be significantly affected by strain magnitude or frequency despite some trends that appeared promising (Figure 6.5). However, when the
leaflet and chordal gels were grouped together, decorin synthesis was significantly greater in collagen gels subjected to 1.5 Hz than in gels subjected to 0.83 or 1.16 Hz (p<0.04). Versican synthesis by leaflet cells was significantly greater than chordal cells for the lowest and highest strains (p<0.04) and frequencies (p<0.006).

**Figure 6.5.** Western blot detection of various PGs synthesized by valve cells. PGs synthesized at various strains are shown in (a) decorin, (b) biglycan and (c) versican, *p*<0.04 vs. chordae; and PGs synthesized at various frequencies are shown in (d) decorin, *p*<0.05 vs. 1.5 Hz, when leaflet and chordal data were grouped together. (e) biglycan and (f) versican, *p*<0.005 vs. chordae. Data are mean ± standard deviation.

**Proteoglycans secreted into culture medium**

Similar to GAG secretion, PGs were also secreted into the surrounding medium (Figure 6.6). There were only two effects of strain or frequency on decorin secretion. At 5% cyclic strain, the secretion of decorin by gels seeded with chordal cells was lower
than that of chordal gels strained 2% (p<0.01), and lower than the leaflet gels strained 5% (p<0.01).

Figure 6.6. PGs secreted into culture medium surrounding the collagen gels seeded with leaflet and chordal cells. PGs secreted at various strains are shown in (a) decorin, \(^*\)p<0.01 vs. chordae, \(^+\)p<0.05 vs. 5% (b) biglycan and (c) versican, \(^*\)p<0.04 vs. chordae; and PGs secreted at various frequencies are shown in (d) decorin, (e) biglycan and (f) versican, \(^*\)p<0.001 vs. chordae, \(^+\)p<0.005 vs. 1.5 Hz, \(^+\)p<0.03 vs. 0.83 Hz, \(^*\)p<0.05 vs. 1.5 Hz when leaflet and chordal data were grouped together. Data are mean ± standard deviation.

Collagen gels seeded with leaflet cells and subjected to 0.83 or 1.5 Hz strain secreted more versican than gels stretching at 1.16 Hz and in the static strain condition (p<0.03). As with the PGs retained within the collagen gel, versican secretion by leaflet cells was greater than secretion by chordal cells for all applicable strains (p<0.04) and frequencies of 0.83 and 1.5 Hz (p<0.001). However, the secretion of biglycan was not affected by either strain or frequency.
6.4 Discussion

In this study, a custom built stretching device was used to show that the synthesis of specific GAGs and PGs by VICs is strain and frequency dependent. Total GAGs synthesized within collagen gels decreased under cyclic strain; this decrease was strain magnitude dependent with the least GAGs produced at 10% cyclic strain. Endothelial cells have likewise been shown to decrease GAG synthesis with increasing strain magnitudes [129]. Not all GAGs and PGs showed this trend; the synthesis of 4-sulfated GAGs increased with strain magnitude and the secretion of decorin into the culture medium was lowest at the medium strain magnitude (5%). That strain magnitudes variably affect GAGs and PGs has been previously found for collagen synthesized by other cardiovascular cell types [132, 143, 167]. DNA content was also somewhat affected by strain magnitudes, being significantly lower for 10% strain as compared to 5%. It was speculated that the high strains might have been damaging to the cells [109, 235]. Frequency also showed an influence on GAG/PG synthesis, with the retention of decorin from chordal cells and the retention/secretion of versican from leaflet cells tending to be lower at the medium frequency tested (1.16 Hz). The secretion of these characteristic PGs was different at the higher and lower frequencies, 0.83 or 1.5 Hz (~1 Hz is the normal frequency experienced by valve cells). Interestingly, these frequency effects were noted for tissue-relevant pairings of PGs and cell types, i.e., the PG decorin is prevalent in chordae, whereas the PG versican is more abundant in leaflets.

Cyclic strains were applied in this study because it was believed that these would elicit ECM synthesis patterns that more closely resembled the in vivo behavior of the specific cell types. Overall, the application of cyclic mechanical strains to the valve cell-
seeded collagen gels reduced the synthesis of GAGs, although the proportions of 4-sulfated GAGs and total sulfated GAGs tended to increase whereas the 6-sulfated GAGs and the unsulfated GAG HA tended to decrease. This trend was previously reported for SMCs, which synthesized more sulfated GAGs and less HA when exposed to centrifugal mechanical forces [182]. In contrast, other studies have reported an increase in versican (more associated with 6-sulfated GAGs and HA) and decrease in decorin (associated with 4-sulfated GAGs) retained within the cell layer as well as secreted into the medium upon the application of cyclic strains on SMCs [82]; these differences may be due to the specific magnitudes of strain and frequency applied in the various investigations.

Leaflet and chordal tissues experience distinctive strain patterns in vivo and in our 3D model the cells derived from these tissues showed different trends with cyclic strain. GAGs that were secreted into the medium by chordal cells (as opposed to those being retained within the engineered tissues) increased with cyclic strain in a magnitude dependent manner, whereas the GAGs secreted by the leaflet cells showed the opposite trend. In general, the chordal cells tended to produce greater proportions of 6-sulfated GAGs (static strain, no strain) and 4-sulfated GAGs (cyclic strain) than the leaflet cells, which produced greater proportions of HA. Compared to the profile of GAGs produced by the cells under static strain conditions [234], the application of cyclic strains to the cell-seeded collagen gels brought the profile of GAGs synthesized by valve cells closer to what is observed in vivo. This recapitulation of in vivo behavior was also demonstrated by the synthesis of the PG versican, which was upregulated by cyclically strained leaflet VICS. Versican and its associated 6-sulfated GAGs were decreased by chordal VICS.
under cyclic strain, which again match the GAG and PG patterns found in native mitral valve tissues under continuous cyclic strains [13].

Because this study was the first to examine the variable effect of strain and frequency on VICs’ synthesis of ECM, our study parameters were guided by investigations of other cardiovascular cell types. Our selection of 48 hour periods of stretching was justified by previous studies showing that the application of mechanical strains causes changes in PG and collagen synthesis within a few hours [82, 156]. Since other cardiovascular cell types have been examined using a wide range of strains and frequencies [237], our selection of the frequency regimens was chosen based on heart rate (72 beats per minute), which the VICs normally experience in vivo, although this frequency certainly varies depending on health and activity. Furthermore, most cardiovascular cells experience strains at 10% [123], which was the maximum strain could be applied to cell-seeded collagen gels. As noted above, the results obtained in this investigation are quite consistent with previous investigations of other cell types [82, 129, 143, 182].

These results also contribute to the growing field of heart valve mechanobiology, in which the experimental mechanical stimulation of heart valves and VICs has proven highly relevant to our understanding of the pathological remodeling of valves and to the improvement of tissue engineered heart valves. Our findings that strains modulate GAG and PG synthesis by VICs are supported by recent studies showing that mechanically strained VICs regulate their expression of other extracellular matrix (collagen) as well as of inflammatory genes, which may play a role in the development and prevention of valve pathologies [103, 106]. In the area of tissue engineering, it has been widely shown
that tissue engineered heart valves that are cyclically mechanically stimulated during their *in vitro* incubation and maturation will have improved matrix production, greater cell density, more compact matrix organization, and greater mechanical strength [241]. As GAGs and PGs are also structurally and biologically important ECM components, the regulation of these molecules by applying various cyclic strain regimens will aid in the development of a tissue engineered heart valves that more closely recapitulates the native valve.

This study had a number of limitations, the first being that only a narrow range of strains and frequencies could be applied due to the delicacy of the engineered tissue and to device constraints. Although a wider range of strain and frequency conditions were pilot tested, cell-seeded collagen gel tended to tear at high strains and frequencies such as 20% and 2 Hz. The minimum frequency that could consistently be applied at all strains was 0.83 Hz because the cam for 10% strain could not overcome the motor torque to rotate at very low frequencies. In addition, since each collagen gel required 14 million cells that took several passages to grow, only one collagen gel could be prepared for each strain and frequency combination. To overcome this limitation, collagen gels subjected to various frequencies (0.83, 1.16 and 1.5 Hz) were grouped together for strain dependency analysis and visa versa. Finally, it might be speculated that the cross-shape of the collagen gel would impose different strain patterns throughout the engineered tissue (biaxial in the center and uniaxial along the arm of the gel, Figure 6.1b) that would influence the local production of GAGs or PGs. In previous studies, however, it was found that the biaxial vs. uniaxial loading in these gels did not cause substantial changes
in their GAG and PG synthesis; stretch alone was the primary factor influencing the synthesis of these matrix components [234].

Overall, it was found that strain magnitudes and frequencies affected the synthesis of specific GAGs and PGs by VICs seeded within 3D collagen gels. Cyclically straining the VICs grown from mitral valve leaflets and chordae caused the upregulation of specific GAGs that are usually in abundance in these distinctive tissue regions. The controlled synthesis of specific GAGs and PGs in mechanically loaded collagen gels will be important in tissue engineering applications as these ECM molecules have diverse biological and biomechanical functionalities that are desirable to recapitulate in engineered tissues [242, 243]. This study also contributes to the growing body of knowledge about heart valve mechanobiology in normal and pathological conditions.
Chapter 7: Discussion, conclusions and future directions

7.1 PGs present in normal mitral valve

In this research, I investigated the PGs present in the mitral valve as well as the effect of various types of mechanical strains on GAG and PG synthesis by VICs. I found that three PGs (decorin, biglycan and versican) were present in the mitral valve leaflet and chordal regions. When I isolated VICs from these valve regions and seeded them in 3D collagen gels, all 3 PGs found in the mitral valve were also synthesized within these tissue surrogates. I then applied static and cyclic mechanical stimulus at various strains and frequencies; again, all 3 PGs were detected during various strain conditions but were present in different amounts and proportions. Various GAGs (present in native mitral valve) were also synthesized by VICs in 3D cultures subjected to various mechanical strains. Similar to PGs, GAGs were synthesized in different amounts and proportions during diverse strain conditions.

I found that the small PGs decorin and biglycan were more abundant in tensile loading regions (chordae tendineae and center of anterior leaflet) than in the compressive loading regions (posterior leaflet and free edge of anterior leaflet). Since the posterior leaflet and chordae tendineae undergo dramatic biochemical and mechanical changes during myxomatous disease, the presence of specific PGs in the myxomatous valves needs to be investigated in the future. Overall, the compositional patterns of mitral valve PGs provide new insight into the roles of these molecules in load-bearing tissues. Because studies reported the presence of chondroitin/dermatan sulfate GAGs associated with the above-mentioned PGs within the mitral valve [13], VICs' synthesis of these
GAG types was analyzed. However, I observed one unidentified band on the FACE gels, which may be another class of GAG such as heparan sulfate. Similarly, western blots showed several other unidentified bands, which may be other isoforms of versican that are synthesized by VICs, since some lower molecular weight bands (possibly V2 and V3) other than the known measured bands (V0 and V1) can be observed. In the future, the presence of additional PGs, such as perlecan and syndecans, should be investigated. This is the first time specific PGs were detected in the native valve tissues. The detection of these PGs in differently loaded valve regions strengthens the available body of knowledge about valve ECM structure and tissue loading, which is also relevant to pathological conditions.

7.2 Effect of constraint conditions on GAG and PG synthesis by VICs

Throughout this research project, the effect of strain itself was more influential than the directionality of the constrained conditions. These investigations were the first to apply static and cyclic strains to VICs in 3D culture and compare them to unconstrained conditions (no mechanical strain). The amount of decorin and biglycan retained in the collagen gels increased with the application of static strains, whereas versican decreased under the same conditions. The proportion of 6-S GAGs (as well as the degree of hydration) was higher in the unconstrained gels whereas the proportion of 4-S GAGs was higher in the constrained gels, as previously found for compressive and tensile regions of heart valves [13]. Distinctive collagen fibril and cell alignment may have contributed to the differences in constrained and unconstrained collagen gels. Other possible factors that are intrinsically associated with unconstrained collagen gels are cells demonstrating
decreased actin stress fibers and downregulated cyclin and ERK pathways resulting in increased cell apoptosis compared to cells in constrained matrices [109, 233]. Fringer et al., also suggested the possible role of focal adhesions in 3D gel compaction [109], despite the observations that these adhesions are smaller than those in monolayer cultures.

In this unique 3D cross-shaped model, VICs could experience either uniaxial or biaxial strains within a single engineered tissue. Total GAGs and the proportions of different GAG classes were similar between the uniaxial, biaxial, and mesh regions. The only difference between regions experiencing biaxial and uniaxial loading was found for the PG biglycan, which was most abundant in the biaxial region. The resulting differential abundance of distinctly sulfated GAGs and PGs between the constrained and unconstrained groups has correlations in "floppy" myxomatous mitral valves, which are subjected to reduced tensile loading (or no tensile loading in the case of ruptured chordae) and contain elevated concentrations of chondroitin 6-sulfate. Static loading is also relevant to the aortic valve in hearts supported by left ventricular assist devices and has been shown by Warnock et al. to modulate the expression of inflammatory genes in porcine aortic VICs [106].

After static strains, cyclic strains were applied because it was believed that these conditions would elicit ECM synthesis patterns that more closely resembled the in vivo behavior of the specific cell types. In these cyclic studies, I did not separate the differently loaded regions of collagen gels (biaxial vs. uniaxial) to analyze ECM components because I did not find substantial differences in these regions under static conditions. The application of cyclic mechanical strains to the valve cell-seeded collagen
gels reduced the retention of total GAGs within collagen gels, although the proportions of 4-sulfated GAGs and total sulfated GAGs tended to increase whereas the 6-sulfated GAGs and the unsulfated GAG HA tended to decrease. The decrease in GAG synthesis was strain magnitude dependent with the least GAGs produced at 10% cyclic strain. Endothelial cells have likewise been shown to decrease GAG synthesis with increasing strain magnitudes [129]. Not all GAGs and PGs showed this trend; the synthesis of 4-sulfated GAGs increased with strain magnitude and the secretion of decorin into the culture medium was lowest at the medium strain magnitude (5%). That strain magnitudes variably affect GAGs and PGs has been previously found for collagen synthesized by other cardiovascular cell types [132, 143, 167]. DNA content was also somewhat affected by strain magnitudes, being significantly lower for 10% strain as compared to 5%. I speculate that the high strains might have been damaging to the cells [109, 235].

Frequency also showed an influence on GAG/PG synthesis, with the retention of decorin from chordal cells and the secretion of versican from leaflet cells tending to be lower at the medium frequency tested (1.16 Hz). The secretion of these characteristic PGs was different at the higher and lower frequencies, 0.83 or 1.5 Hz (~1 Hz is the normal frequency experienced by valve cells). Interestingly, these frequency effects were noted for tissue-relevant pairings of PGs and cell types, i.e., the PG decorin is prevalent in chordae and decorin synthesized by chordal cells was the most susceptible to frequency, whereas the PG versican is more abundant in leaflets and here again the leaflet cells’ synthesis of versican was the most frequency dependent. The effects of strain and frequency data presented in this research provide an insight into the mechanobiology of VICs for a range of physiological conditions. In the future, it would be useful to integrate
a load cell into the bioreactor to measure the exact magnitude of tensile loads experienced by the VICs within the 3D collagen gels.

7.3 GAGs and PGs present in collagen gels vs. conditioned medium

Collagen gels are porous 3D structures, which allow the GAGs/PGs produced by VICs to leach into the surrounding medium. The profile of GAG classes and PGs secreted into the culture medium was distinctly different from those retained within the scaffold. HA was more abundantly secreted into the surrounding medium than retained within the gel because these molecules do not covalently bind to a proteoglycan core protein and are therefore more likely to leach out. In contrast, proportions of 4-S GAGs (and correspondingly the 4-S/6-S ratio) were higher in the collagen gels than in the surrounding medium, which is likely due to the close association of C/D 4-S containing PGs with collagen fibrils [4]. The amount of decorin and biglycan retained in the collagen gels increased with the application of static strains, whereas versican decreased under the same conditions. Secretion of all three PGs, however, increased in the conditioned medium when the gels were constrained, although the only significant increase was found for versican. This distinctive versican result may be a consequence of its greater abundance in compressive tissues, i.e., this large PG may have less utility in tissues subjected to static tension and hence is released into the medium [13]. In contrast, VICs largely retained decorin and biglycan within the engineered tissues under static tensile strains, which is logical given that these two PGs are largely found in numerous tissues that bear tension [13].
I also investigated the reversibility and reproducibility of GAG/PG secretion during stretching and relaxation cycles. I have shown that GAG secretion by VICs in the culture medium is dependent on the presence or absence of cyclic strains. GAG secretion was significantly upregulated during cyclic stretch and downregulated during relaxation. This study showed that the VICs’ response to cyclic stretch in my 3D model was reversible. In the future, the cyclic stretch results could be extended to provide a complete picture of the reversible synthesis of GAGs/PGs by measuring these components within collagen gels after each stretching and relaxation cycle. This information, along with strain and frequency data, should also be further extended to analyze the secretion of GAGs and PGs by VICs isolated from human normal and myxomatous mitral valves to complement the porcine-cell based research described here. The synthesis of GAGs and PGs from myxomatous VICs could in theory be reversed to be more like normal VICs, and if so then this approach will provide an in vitro model to simulate the myxomatous disease condition. In the future, specific enzymes for the synthesis of HA (which is abundantly found in diseased conditions) such as the HA synthases and matrix degrading enzymes such as MMPs can also be measured within normal and diseased tissues and their expression by VICs could potentially be modulated by the application of static or cyclic strains. Another future research possibility could be to use HA synthase overexpressing cells and subject them to static and cyclic strains in order to understand the role of GAGs such as HA in defining the tissue microstructure and mechanical behavior; these type of investigations could pinpoint whether HA and other GAGs are the cause or the effects of altered mechanics. The role of HA in tissue engineering applications has great promise [242].
7.4 GAGs and PGs synthesized by leaflet vs. chordal cells

There were many interesting differences in GAG/PG synthesis between VICs isolated from leaflet and from chordal tissues of the mitral valve. It is interesting to note that the most unique profile of GAG classes was found in the collagen gels seeded with chordal cells and subjected to uniaxial strain. Chordae tendineae in the mitral valve normally experience uniaxial tension and undergo dramatic remodeling (both mechanically and biochemically) in pathological conditions [3, 54]. This research has shown that VICs from chordae are particularly responsive to mechanical strains, which was also confirmed in the cyclic strain analysis in which the reversible pattern GAG/PG secretion into the medium showed a delayed response by leaflet VICs (Chapter 5). Interestingly, the proportional composition of distinct GAG classes remained fairly consistent for chordal cells but tended to change for leaflet cells, which reflects the leaflet VICs' adaptation to high cyclic tensile strains. Constrained collagen gels containing leaflet cells retained more decorin and biglycan than did those containing chordal cells. GAGs that were secreted into the medium by chordal cells (as opposed to those being retained within the engineered tissues) increased with cyclic strain in a magnitude dependent manner, whereas the GAGs secreted by the leaflet cells showed the opposite trend. In general, the chordal cells tended to produce greater proportions of 6-sulfated GAGs (static strain, no strain) and 4-sulfated GAGs (cyclic strain) than the leaflet cells, which produced greater proportions of HA. Another difference between leaflet and chordal cells was demonstrated by the comparison of their responses to static vs. cyclic strains. Compared to the profile of GAGs produced by the cells under static strain conditions [234], the application of cyclic strains to the cell-seeded collagen gels brought
the profile of GAGs synthesized by valve cells closer to what is observed in vivo. This recapitulation of in vivo behavior was also demonstrated by the synthesis of the PG versican, which was upregulated by cyclically strained leaflet VICs. In contrast, versican and its associated 6-sulfated GAGs were decreased by chordal VICs under cyclic strain, which again match the GAG and PG patterns found in native mitral valve tissues under continuous cyclic strains [13].

The notable differences between leaflet and chordal cells described above may be due to a more dramatic difference between their in vivo conditions and our 3D model, or it may be due to inherent phenotypic differences between the cell types. Leaflet cells normally experience a combination of compression or tensile strains in vivo, whereas in my 3D model cells experienced either tensile or no strains. Also, the cells have somewhat different phenotypes; our lab has found that mitral chordal cells have approximately 50% higher levels of smooth muscle α-actin expression than leaflet cells [230]. Smooth muscle α-actin, which is reportedly expressed at low levels in normal human VICs, has been associated with a more contractile phenotype [46, 231, 232]. This research is the first to report on characteristics of the chordal cell phenotype.

7.5 Comparison of in vitro cultures with normal valve

Overall, many of the GAG and PG trends within 3D collagen gels were very similar to those found in native valve tissues, which has promising implications for tissue engineering. The water content of these collagen gels ranges from 80-95%, which is comparable to that of native leaflet and chordae tendineae (76% for chordae and 86% for leaflet) [13]. The concentration of total GAGs produced by the VICs seeded within
collagen gels were approximately ½ and ¼ of the concentration of total GAGs found in native chordae and leaflet tissue, respectively [3, 13]. The predominant GAG class in the cell-seeded collagen gels was chondroitin/dermatan 4-sulfate, which is also the most common GAG in native chordae. The predominant PGs in the constrained collagen gels, decorin and biglycan, are also the most common PGs in native chordae [13]. Similarly, versican was more abundant, and decorin and biglycan were less abundant, in unconstrained collagen gels as been found in the compressive regions (posterior leaflet and free edge of anterior leaflet) of the mitral valve. Given that GAGs and PGs are diverse molecules that serve different biological roles in tissues, it appears reasonable that their synthesis by VICs is not uniformly regulated by mechanical strains. Investigations on SMCs have shown that the application of mechanical strains causes variable effects on the synthesis of distinct GAGs and PGs [82, 163]. For example, increased synthesis of versican and decreased synthesis of decorin was observed by SMCs under mechanical strains [82]. This research therefore provides additional guidance for constructing engineered tissues in which a tensile environment will induce specific GAG or PG production.

These results also contribute to the growing field of heart valve mechanobiology, in which the experimental mechanical stimulation of heart valves and VICs has proven highly relevant to our understanding of the pathological remodeling of valves and to the improvement of tissue engineered heart valves. This is the first research to investigate GAGs/PGs in the context of VIC mechanobiology. My findings that tensile strains modulate GAG and PG synthesis by VICs are supported by recent studies showing that mechanically strained VICs regulate their expression of other extracellular matrix
(collagen) as well as of inflammatory genes, which may play a role in the development and prevention of valve pathologies [103, 106]. It may be that if compressive strains were applied to VICS, they would respond by synthesizing more HA and 6-S GAGs and PG versican. Although no single area of the heart valve experiences both tensile and compressive loading, it may be of interest to observe what VICS would do if these modes of loading were alternated in vitro. In the area of tissue engineering, it has been widely shown that tissue engineered heart valves that are cyclically mechanically stimulated during their in vitro incubation and maturation will have improved matrix production, greater cell density, more compact matrix organization, and greater mechanical strength [241]. For example, specific GAGs such as C/D 4-S and PG decorin/biglycan play a role in collagen fibrillogenesis and provide tensile mechanical strength to the tissue. Similarly, HA and 6-S GAGs associated with PG versican provide compression resistance to the tissue. Specific GAG and PG production can be controlled by mechanical strains to obtain particular structural and biological properties of tissue surrogate.

Future research possibilities that could be pursued to build on the data described here include the addition of selected growth factors and cofactors such as TGF-β and vitamin C in an effort to find out how these influence GAG and PG synthesis by VICS. As GAGs and PGs are structurally and biologically critical components of the ECM, the regulation of these molecules through various cyclic strain regimens will aid in the development of a tissue engineered heart valves that more closely recapitulates the native valve. In addition, mechanical stimulation can be combined with chemical stimulation to learn more about the intracellular pathways involved in the regulation of GAG and PG
expression and synthesis by VICs. In the future, a more comprehensive base of knowledge of the regulation of GAG and PG synthesis by VICs may possibly lead to novel drug therapies that can be used to treat heart valve diseases without requiring surgical intervention.
References


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