THESIS

# CONNECTING STRUCTURAL CHANGES TO CELL TRANSFORMATION PATTERNS IN THE CANINE DEGENERATIVE MITRAL VALVE

Submitted by

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ABSTRACT

CONNECTING STRUCTURAL CHANGES TO CELL TRANSFORMATION PATTERNS IN THE CANINE DEGENERATIVE MITRAL VALVE

Degenerative mitral valve disease (DMVD) is a significant problem in the canine population and also affects humans. Recent studies have provided insight into molecular and cellular mechanisms that likely contribute to disease progression. Better understanding of the cellular processes that mediate the degenerative process could lead to treatments that prevent or slow this degeneration benefiting both canine and human patients. Structural changes to degenerative valves such as nodules, leaflet thickening, increased opacity, loss of elasticity and loss of valve architecture have been well documented. Abnormal cell transformation patterns such as the transformation of valvular interstitial cells to activated myofibroblasts have been characterized in degenerative mitral valve tissue, as well as other irregular cell behavior such as the overproduction of glycosaminoglycan and matrix remodeling factors that have become hallmarks of the disease. Despite these important discoveries, much remains unknown about cell signaling in degenerative mitral valve disease and how cell activity changes a normal valve to the diseased phenotype. An overarching hypothesis of this study is that investigating signaling mechanisms active in degenerative valves could provide insight into cellular processes mediating the disease. A specific hypothesis that emerged from initial results is that endothelial to mesenchymal transition (EndMT), a process important in valvulogenesis, could be active in degenerative mitral valves.

The first goal of this study was to compare protein abundance in degenerative and normal mitral valves to determine if there exists previously unidentified signaling molecules that could be initiating or perpetuating the cellular transformations and abnormalities present in DMVD. The second goal was to investigate these proteins using immunohistochemistry to characterize their activity in the tissue matrix and show evidence of their contribution to structural changes of the valve. The first goal was accomplished by doing a targeted microarray analysis of signaling proteins comparing their relative abundance in normal and degenerative mitral valves. This analysis yielded an increased abundance of signaling proteins that have been associated with EndMT. The second goal was accomplished by immunohistochemistry to determine the spatial distribution of selected proteins from the microarray analysis with markers of endothelial cells and mesenchymal cells (activated myofibroblasts).

Targeted microarray analysis of signaling proteins revealed increased abundance of 18 proteins including the growth factor HB-EGF, its partner molecule ADAM17, and the cell adhesion molecule integrin β3, all possible mediators of EndMT (Chapter 4). Immunohistochemistry studies demonstrated the presence of cells positive for the endothelial marker CD31 within the valve interstitum. These CD31 positive cells co-localized with areas of myofibroblast transformation in degenerative valves identified by positive staining for α-smooth muscle actin (αSMA). Expression of signaling proteins including HB-EGF and ADAM17 also co-localized to these areas (Chapter 5). In conclusion, these results support active EndMT in canine degenerative mitral valves. EndMT could be contributing to the formation of high cellular density myofibroblast transformation which has been postulated to mediate mitral valve degeneration.

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| | |
|---|---|
| αSMA | Alpha smooth muscle actin |
| ECM | Extracellular matrix |
| DMVD | Degenerative mitral valve disease |
| MMP | Matrix metalloproteinases |
| TGFβ | Transforming growth factor beta |
| ERK | Extracellular signal regulated kinase |
| ADAM 17 | A disintegrin and metalloproteinase, also known as TACE |
| HB-EGF | Heparin binding epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| CD31 | A marker of endothelial cells, also known as PECAM-1 |
| BMP | Bone morphogenic protein |

# Chapter 1

## Introduction

Degenerative mitral valve disease (DMVD) is a significant health concern for many dogs. It often presents in older, smaller dogs, and its rate of progression can vary. Studying cellular changes apparent in degenerative valves increases our understanding of this common disease in both human and canine patients. Several patterns consistently seen in diseased valves have been identified, including overabundance of GAG, aggregation of αSMA positive cells, breakdown of collagen and elastin organization and dissolution of the normal stratification of the valve cells. Some patterns such as serotonin signaling have been implicated as causes of these degenerative changes, but overall cellular signaling in mitral valve cells is complex and still under investigation. To gain a more complete understanding of the disease, the present study began with a protein array which sought to identify proteins whose distributions varied in the diseased state and therefore recognize potential signaling cascades that contribute to degeneration. After studying the results of this initial array and comparing the significant proteins to previous studies and hypotheses about cellular change in DMVD, the hypothesis that endothelial to mesenchymal cell transformation, or EndMT, was occurring was formulated. EndMT is a specialized from of the boarder cellular mechanism of epithelial-to-mesenchymal transformation (EMT) which plays a prominent role during development and has an emerging role in a variety of diseases including cancer, inflammation, and degenerative diseases. This hypothesis could explain the increased cell density and modification of cell type found in areas of active disease and would also concur with theories that developmental processes are being re-activated in other degenerative valve disease. To test this hypothesis a marker of endothelial

cells, the protein CD31, also known as PECAM-1, was targeted with immunohistochemistry techniques to identify the location of endothelial cells in valve tissue. In conjunction with this marker, other significant proteins from the array were studied to determine if they co-localized with each other and with αSMA, which would indicate they play a role in the cellular transformation processes. Overall, determining the location in diseased tissue of these molecules of interest and comparing this information to healthy tissue will lead to a better understanding of mitral valve disease.

# Chapter 2

## Literature Review

Degenerative mitral valve disease (DMVD), also known as myxomatous mitral valve disease, is the most common heart disease observed in dogs.[1] Estimates place the number of dogs affected in the United States between 3 and 7 percent of the population, correlating to 2.3 to 5.3 million individuals. This prevalence is about ten times higher than that of DMVD in humans[1], where it is still a major health concern, as it the most common cause of mitral regurgitation[2]. Because of similarities in its presentation and progression, research on canine mitral valve disease is also relevant to humans patients with the same disease[3-4]. DMVD is age related, progressive and yields a thickened, less elastic, nodular phenotype with an impaired ability to close properly that leads to regurgitation and heart remodeling. Without surgical intervention to repair or replace the valve, eventually the increased workload placed on the left ventricle by regurgitating flow overcomes the heart's ability to compensate, leading to heart failure. While the primary causes of DMVD are incompletely understood, in some dog breeds it appears to have a genetic component[5], it correlates positively with age[6] and negatively with size[7], and it is associated with other conditions such as hypertension[8]. Many cellular mechanisms present in the disease progression have been identified, but none yet seem to explain all aspects of the degenerative and phenotypic changes that exist in diseased valve tissues. To understand the causes of DMVD, it is important to investigate the changes and patterns occurring at the cellular level.

*Tissue structure and morphology*

Healthy mitral valve tissue is composed of three principal layers, the atrialis, the spongiosa, and the fibrosa which each contribute to the valve's ability to function properly. The atrialis, the layer facing the left atrium when the valve is closed, contains radially oriented elastin fibers which allow the valve to stretch as it opens and closes and encounters the high pressures of the left ventricle[9]. The fibrosa, which faces the left ventricle when the valve is closed, contains collagen fibers which provide structural strength to the valve leaflet when it is closed during ventricular systole[10]. The spongiosa exists between the atrialis and fibrosa, and is primarily composed of hydrated proteoglycan (PG) and glycosaminoglycan (GAG) which are thought to lubricate and provide support for the atrialis and fibrosa layers as they move with respect to each other during valve flexure.[11]

Mitral valves exhibiting a degenerative (diseased) phenotype have some very distinct changes in their cellular structure. Diseased valves are commonly known to show an increase in both GAG and αSMA, and a loss of proper valve architecture where the layers of atrialis, spongiosa, and fibrosa become intermixed[12](as in Figure 2.1). GAG content in degenerative valves can be increased by 30% to 150% and may contribute to mechanical weakness of the valve[13]. Interestingly, a study by Tamura et al. found that "the presence of excessive amounts of proteoglycans may interfere with the normal assembly of collagen and elastic fibers"[14]. This interference may explain the increased weakness and decreased distensibility of degenerative valves. Collagen fiber disorganization and disruption, recognized by King et al. (1982) is considered to be a principle characteristic of the disease contributing to valve dysfunction.

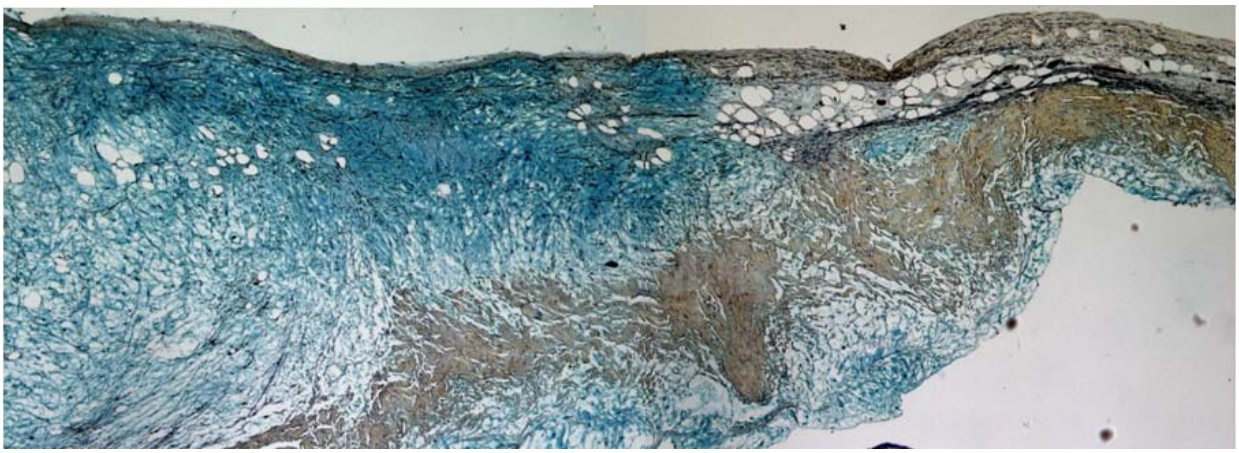

**Figure 2.1 Photomicrograph of a Movat Stained Degenerative Canine Mitral Valve**
Figure 2.1 is a photomicrograph of a cross section of a degenerative valve stained with the Movat pentachrome procedure. To the far right (proximal end), normal valve layers are visible. The elastin fibers are stained black, collagen is stained yellow, and GAG is stained blue. As the valve thickens towards its distal end (left side), GAG becomes much more prominent and both collagen and elastin fibers appear less organized, demonstrating the loss of valve architecture characteristic of the disease.

In addition to this elastin and collagen dysregulation, valvular interstitial cells begin to express catabolic enzymes, such as matrix metalloproteinases (MMPs), in the diseased state which degrade the extracellular matrix[12]. This causes matrix remodeling which changes the proper stratification of the layers, leading to abnormalities in the valve architecture. These weakened valve leaflets do not close properly or are forced open when they should be closed during ventricular systole, resulting in a regurgitant jet of blood flow back into the atrium. This backwards blood flow forces the heart to work harder in order to obtain the same amount of forward flow out the aorta to the body.

*Disease initiation and progression and the roles of serotonin, αSMA, and TGFβ*

As the heart generates more pressure to fulfill the body's need for oxygenated blood, the already compromised valve is subjected to this increased pressure. Accordingly, valve tissue will sense this abnormal stress and strain and increase ECM remodeling. Studies show that valve cells exposed to increased strain in culture respond by escalating expression of αSMA, serotonin, MMPs, and GAG synthetic enzymes.[15-16] This indicates that mechanoreceptors on valve cells are sensitive to cyclic tensile strain and respond by altering protein synthesis in a pattern consistent with MMVD. Besides causing diseased valves to degenerate further, this mechanism of progression may be responsible for the initiation of valve disease in hypertensive individuals, as high blood pressure has been shown to be associated with an increased risk of DMVD in humans.[8]

However, initiating mechanisms and causes of the disease are still not fully understood. Hypertension and age related tissue weakening are accepted contributing factors but do not account for the total prevalence of DMVD in dogs and humans, especially in younger animals

and certain breeds. Serotonin signaling may be an important mechanism involved in DMVD, as Disatian et al. found that diseased canine and human mitral valves exhibited increased serotonin synthetic enzymes and decreased transporters necessary for serotonin breakdown.[17] This study also shows increased TGFβ receptors and phosphorylated ERK ½ which may be activated by the serotonin mechanism. These molecules are involved in activating transcription factors which influence the cell to undergo phenotype transformation, proliferation, and stimulate matrix remodeling. TGFβ expression in canine mitral valves has been examined in depth by Aupperle et al. and seems to be a contributing factor to disease phenotype proteins such as αSMA.[18] While the serotonin hypothesis (Figure2.2) for the initiation of DMVD is very promising, the causes and downstream effects of this signaling still need more investigation. Overall, the initiation and progression of DMVD is a multifaceted and complicated biochemical process. There does not seem to be one clear answer as to the cause, but investigation of cellular signaling has contributed to our knowledge of what diseased cells express and how they change from the healthy state.

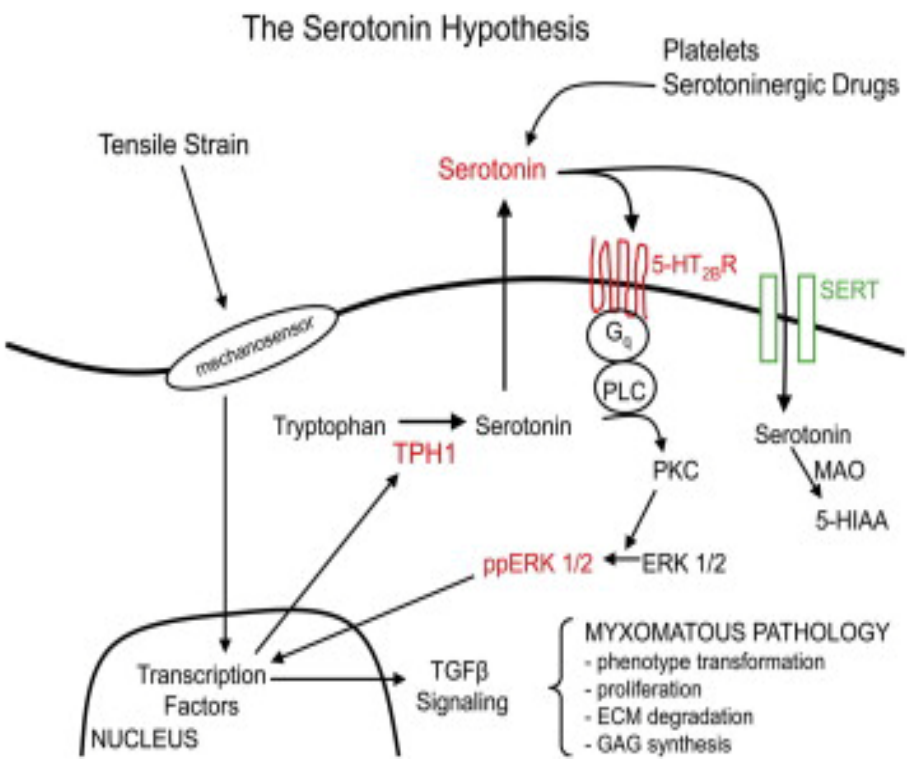


**Figure 2.2 The Serotonin Hypothesis**

Figure 2.2 illustrates the serotonin hypothesis of DMVD initiation. Principle molecules involved in this process include the serotonin transporter, SERT, the serotonin receptor 5-$HT_{2B}R$, and the serotonin degrading enzyme MAO. Studies of these molecules indicate that serotonin signaling is upregulated in DMVD. This figure was borrowed from Orton 2012.[19]

Previous studies have identified patterns of interstitial cell phenotype transformation[20], changes in cell density[21], and changes in protein expression in degenerative valve tissue[22]. One example of this is the change from a normal or quiescent fibroblastic type interstitial cell to an "activated myofibroblast" which expresses catabolic enzymes such as MMPs implicated in the pathological remodeling process present in diseased valves[21]. The activated myofibroblast cell is identified by the expression of αSMA, which increases dramatically in diseased valves and positively correlates with severity[23]. Besides simply increasing however, the distribution of this cytoskeletal protein also varies. Instead of only appearing in a thin layer of the atrialis which occurs in normal valves, αSMA positive cells are present throughout the atrialis and into the spongiosa and seem to form clusters[21] .This αSMA expression pattern in degenerative valves usually correlates with distinct nodules and is considered a good identifier of areas of active cellular change in valve tissue. (Figure 2.3)

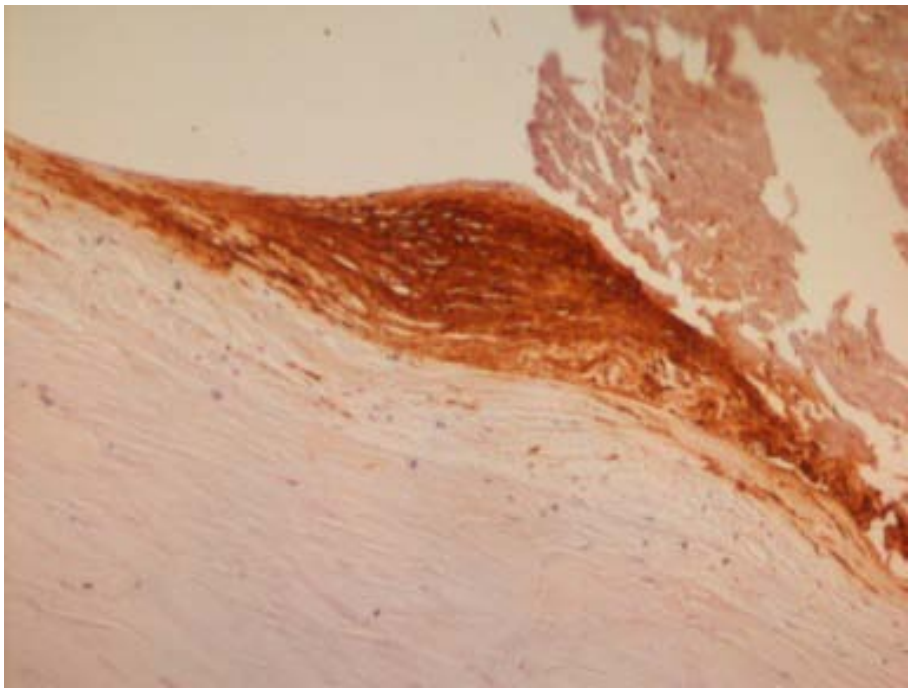

**Figure 2.3 αSMA Immunohistochemistry Stained Canine Degenerative Mitral Valve**
The dark brown staining on this photomicrograph of a valve with DMVD indicates the presence of αSMA (alpha smooth muscle actin). Clearly, the positive cells form a nodule in the atrialis layer, which is characteristic of the disease.

As mentioned previously, TGFβ, a cytokine involved in ECM regulation, plays a significant role in DMVD. In cultured valvular interstitial cells TGFβ-1 contributed to myofibroblast transformation, linking it to the expression of αSMA in valve cells[24]. Besides this role, TGFβ has other widespread effects in the body that contribute to many diverse outcomes, including ECM management, inhibiting cell proliferation, immune function, and mesenchymal cell transformation. Examples of processes where TGFβ signaling is important would be in wound healing, fibrosis, cancer progression or suppression, and embryonic development. In these processes TGFβ often functions by causing cells to undergo epithelial to mesenchymal cell transition, or EMT[25]. This mechanism involves two TGFβ receptors, I and II, which transmit the signal initiated by TGFβ binding and activate canonical and non-canonical pathways. The canonical pathway activates the molecules Smad 2/3 which bind Smad 4 and this complex enters the nucleus and acts as a transcription factor, changing the activity of the cell[25]. The non-canonical pathway uses a variety of signaling cascades, including Erk, JNK, and MAP kinases which play important roles in the initiation of EMT[26] .

*Developmental pathways*

EMT is a programmed cellular process which involves the transformation of organized epithelial cells into motile mesenchymal cells by the removal of anchoring proteins and the restructuring of the actin cytoskeleton[25]. EndMT is a variation of this process wherein endothelial cells transform. Of particular interest is TGFβ's role in contributing to EMT in cardiac valve development, along with BMP and Notch signaling[27]. The BMP ligands are members of the TGFβ superfamily of signaling molecules. BMP signaling is the first step in the commencement of EMT in the developing heart valve, followed by Notch signaling. Notch is a single transmembrane

receptor that when bound to its ligand exposes an active site which a disintegrin and metalloproteinase (ADAM17 or ADAM10) will cleave and the remaining portion will be endocytosed, migrate to the nucleus, and cause its effects, including stimulating multiple genes that are present in EMT as well as production of αSMA[27]. The effects of BMP, Notch, and continued TGFβ signaling support the cellular changes taking place by engaging in "cross talk" as the endothelial cells begin to form the mesenchymal cells of the developing heart valve[28].

Both BMP and TGFβ are involved in the regulation of Sox-9 in chondrogenesis. Chondrogenesis is believed to be taking place in diseased mitral valves, as shown by the presence of type II collagen, a product of the process, and Sox 9, a transcription factor involved in initiating it[29]. The presence of these chondrogenic factors detected in diseased valves by IHC suggests that this pathway is active in degenerative valve disease. Interestingly, other developmental processes have been documented in valve diseases, specifically osteogenic pathways documented in calcific aortic valve disease[30]. The presence of indicators of chondrogenesis and osteogenesis in diseased heart valves has lead to the hypothesis that reactivation of developmental pathways may be a primary cause of disease[31]. Another piece of evidence supporting this theory would be the differing distribution of αSMA. In a study by Rabkin-Aikawa et al.,[32] the percent of VICs expressing αSMA in fetal hearts was about 60%, whereas in normal adults it was only 2.5%. In degenerative valves, however, the percentage of VICs expressing αSMA rose to approximately 36%, indicating a similar activated myofibroblast cell phenotype to the fetal valves. After considering these similarities it makes sense that examining the signaling systems involved in early valve development may lead to a greater understanding of the degenerative processes of the diseased state. Other cell processes and

patterns causing the changes found in the disease may be discovered by further investigation of embryonic signaling mechanisms.

An embryonic pathway that has been studied in valve development but not in disease is that of heparin binding epidermal growth factor (HB-EGF), its "sheddase" ADAM17 which is responsible for its release from the cell membrane, and its receptor, the epidermal growth factor receptor (EGFR). ADAM17, also known as TACE, has been identified as the primary regulator of HB-EGF concentrations in valve development, as it cleaves pro-HB-EGF and releases it in an active form so that it can bind its receptor, the EGFR[33]. Knockout mice have shown that interference with this pathway leads to malformed valves[34]. This study also determined that HB-EGF was the most important ligand for the EGFR in valve developmental remodeling, which involves the establishment of the valve layers from the endothelial and mesenchymal cells.[34] Another study found that "HB-EGF plays a positive role in the migratory capability of valve endothelial cells"[35] further supporting its role in directing cells in valve development. Also, HB-EGF signaling is considered to be regulated by BMP signaling, therefore it functions as a secondary process taking place after the EMT initiating mechanisms described earlier, contributing to early valve cell movement as the cardiac cushions remodel into valves[36].

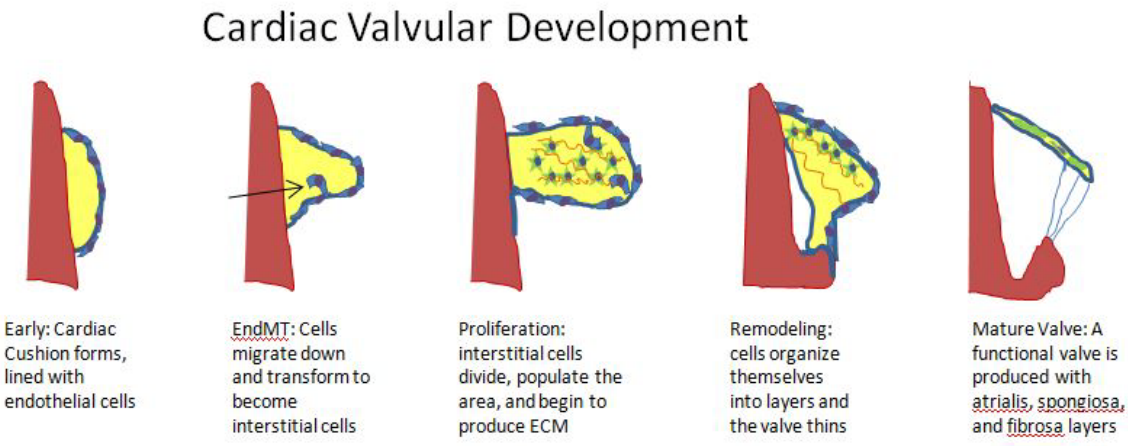


**Figure 2.4 Valvular Morphogenesis from the Early Embryonic State to the Mature Heart Valve**

Figure 2.4 depicts how endothelial cells (blue line) migrate deeper into the cardiac cushion and transform into mesenchymal cells which then form the layers and structure of the adult valve. Much signaling is involved in this process and the fluid dynamics of blood passing through the heart also influences the development of the tissue. Figure inspired by a diagram in Markwald, 2010.[31]

*Pathways related to valve mechanics*

Previous studies have found that HB-EGF to EGFR signaling is influence by both cyclic stretch[37-39], and by increased pressure[40]. This finding relating mechanoreceptors to cell signaling changes has also been replicated in mitral valve studies which exert strain on valves in tissue studies and evaluate differential protein expression[15,41]. These studies show that proteins that are suggestive of degenerative disease can be induced in valves by subjecting them to increased stress and stretch loads, specifically cyclic tensile strain. Therefore even though the specific mechanosensors responsible for responding to these physical changes have not been identified, it is now accepted that increased cyclic strain can cause a DMVD phenotype. This may be the mechanism of initiation of DMVD in individuals with hypertension as discussed earlier, but it is not a complete explanation of the genesis of DMVD in all animals. Also, while the serotonin hypothesis is consistent with these findings,[41] it does not explain specifically how TGFβ appears to cause degenerative changes. A more complete understanding of TGFβ's method of causing cellular reactions may include its interactions with the other developmental pathways and as an initiator of EMT. Also, other signaling mechanisms influenced by mechanical input such as HB-EGF binding to EGFR may work simultaneously with TGFβ signaling, because in some instances, they lead to the same downstream molecule becoming phosphorylated, ERK1/2. (Figure 2.5)

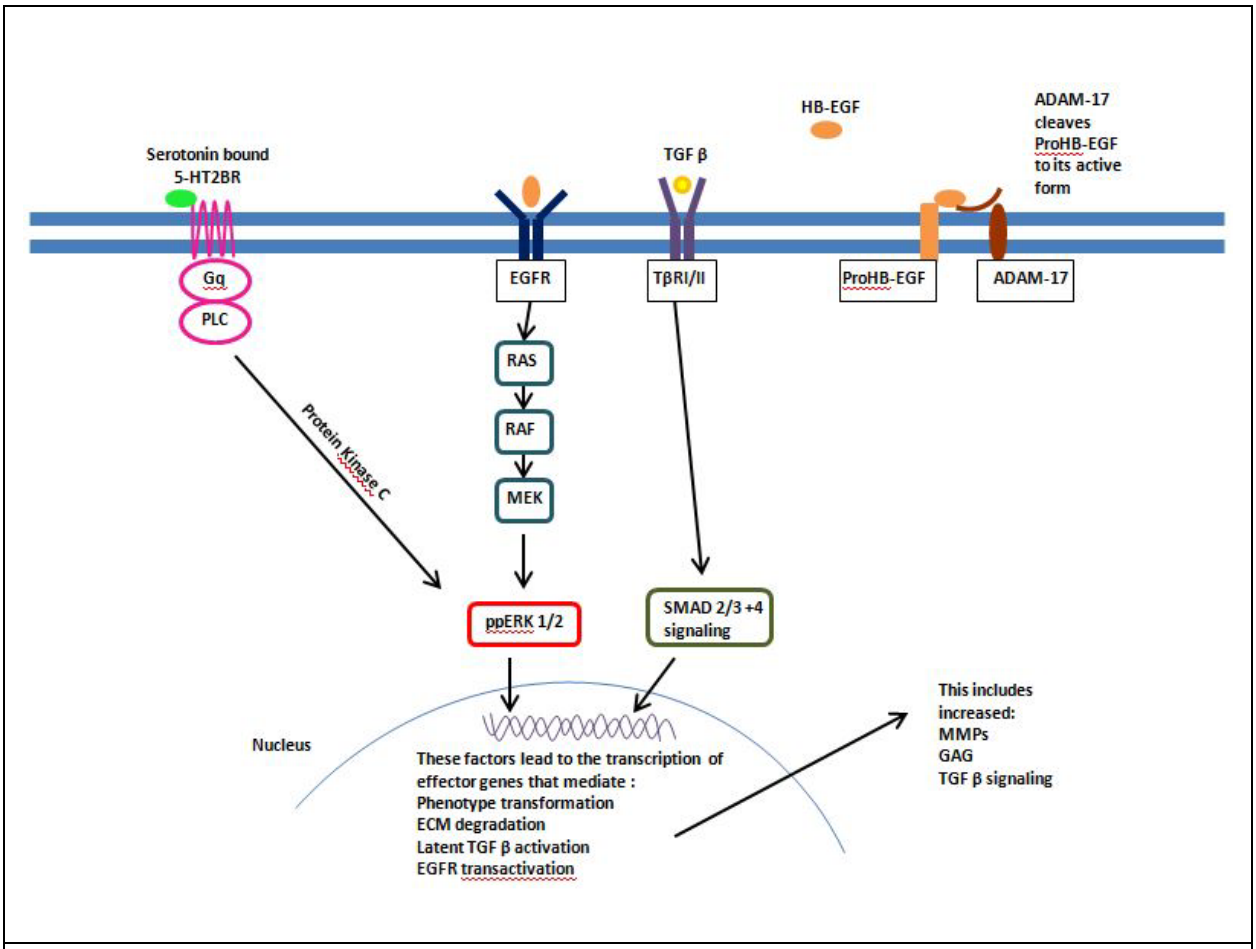


**Figure 2.5 TGFβ and EGFR Signaling**
This figure shows pathways of TGFβ and EGFR signaling. It highlights the common processes between EGFR, TGFβ and serotonin signaling, specifically the activation of ERK ½ (pp=phosphorylated) and the SMADs, and how these messengers lead to the production of effector molecules that change the cell's behavior (such as MMPs, far right). Figure inspired by a diagram by Doyle 2012.[26]

Other proteins that may be relevant to these signaling cascades include the integrins. Integrins are heterodimeric transmembrane proteins with a variety of cellular functions. In relation to TGFβ signaling, integrin αvβ3 has been shown to bind the TβRII portion of the TGFβ receptor and skew TGFβ's affect on the cell from a growth arrest signal to a signal of EMT initiation[42]. This study also found that not only was this integrin expression tied to EMT, but "β3 integrin expression appears essential for TGFβ stimulation of EMT",[42] further underlining its importance to this signaling pathway. In addition to this information, investigators also found that cells expressing integrin β3 significantly increased ERK½ activation after they were stimulated with TGFβ-1, highlighting the mechanism by which this signal is transmitted[42]. This study presents an explanation for the many other cases where integrin αvβ3 has been shown to be upregulated in EMT in cancers[43-45]. EMT induced through integrin αvβ3 has been studied[44] as well as EMT as a process by which malignant tumor cells invade healthy tissue[46]. One way this happens is the cells in a tumor that are able to downregulate normal cell-cell attachment proteins, an early step in EMT, will have less contact inhibition on their growth rates. As these cells proliferate rapidly, they de-differentiate and accumulate mutations. As the pathways of EMT include both the loss of endothelial markers and the gain of motility factors, these cancerous cells have a higher chance of being able to invade local tissues or migrate to distant sites as metastases.

Integrin αvβ3's role in cell migration capability[47] as well as its part in the EMT process seems to account for its presence in invasive cancers, and it may play a role in EMT in heart valve tissue because of its relation to TGFβ signaling.

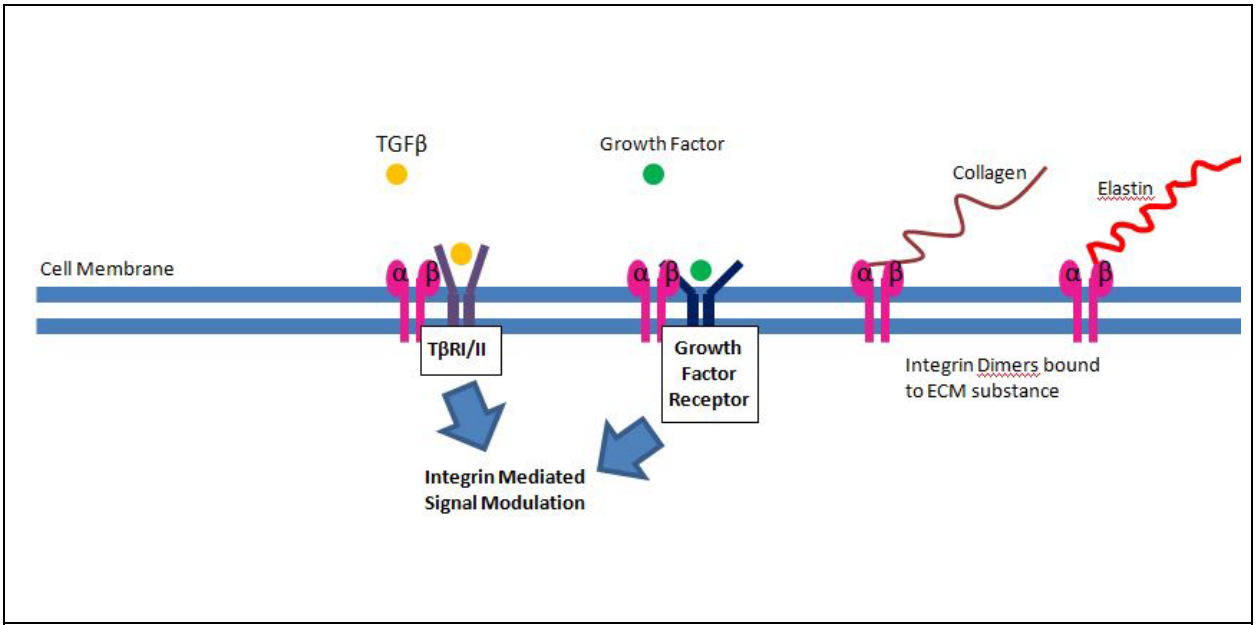


**Figure 2.6 Integrin Influence on Signaling**

Figure 2.6 illustrates how integrins (pink) influence the signaling of TGFβ and EGFR because they are coupled to the respective receptors. Integrin roles in ECM-cytoskeletal interactions (far right) are a pathway by which mechanical forces influence cell signaling. Integrin β3's interactions with TβRI and II change the course of TGFβ signaling. Figure inspired by a diagram by Doyle 2012.[26]

*Cell density and transformation in the diseased valve*

A recent finding described by Disatian et al. is the increased cellularity present in degenerative valves. Her work showed that diseased valves have increased cell density, especially in the atrialis layer[48] but increased cell division does not seem to be the cause of this result[21] because Ki-67, a marker of active cell division was not found to be increased in diseased areas. These areas of increased cell density seem to produce, or at least colocalize with, the distinctive degenerative nodules and thickening. Two theories to explain the increased cell density were proposed, that there is decreased cell death in the area or that cells may be migrating from other areas to the points of disease. To determine the cause of this increased cellularity, another study investigating apoptosis in these areas was performed. This research used the TUNEL assay to examine the apoptosis pathway and found that "an anti-apoptosis mechanism does not explain the increase in cell density seen in canine myxomatous mitral valves"[49]. Also, data from other protein arrays shows an abundance of migratory proteins in degenerative valve tissue, indicating that cell migration is the more likely cause of the increased cell density[22].

The molecule CD31 also known as platelet endothelial cell adhesion molecule-1, is a specific identifier of endothelial cells, and has been used to study their location[50]. Endothelial cells of heart valves are subjected to strong mechanical forces such as shear stress and are often damaged in degenerative valves. A study by Corcoran et al. using electron microscopy observed denudation of endothelial cells on areas of degenerative valves presumably because of mechanical damage[51]. The remaining endothelial cells adjacent to these denuded areas showed an increase in microappendages which they suggest indicates protective and repair

responses. While this damage would seem to be caused by the increased stresses and pressures diseased valves face, these repair mechanisms may contribute to the worsening of the disease by causing the thickening which impairs the proper function of the valve.

Whichever way the valve becomes damaged initially, discovering the cell mechanisms by which DMVD propagates is integral to developing a treatment or prevention for it. Studying markers for signaling cascades such as serotonin, TGFβ, and Notch are one way of trying to understand how and why the valve cells transform and begin to express MMPs, αSMA, and other proteins which directly cause the degenerative changes. Perhaps altering these signals could alter the course of the disease as the valve becomes progressively more dysfunctional. After examining elements that the signaling molecules discussed above have in common as well as the hypothesis that developmental pathways are involved in degenerative disease, we decided to determine if EndMT occurs in degenerative valves. CD31 is the primary molecule of interest as it should indicate endothelial cell locations. If endothelial cells are detected migrating into deeper cell layers, then this would indicate that they are transforming and undergoing EndMT. Also, if this EndMT occurs at areas of the valve where activated myofibroblasts are expressing increased αSMA, then this would support the possibility that EndMT is responsible for increased cell density in diseased areas. Because TGFβ, the primary initiator of EMT, has already been shown to be increased in diseased valves, other signaling processes present in developmental EMT such as HB-EGF will also be studied, as well as molecules such as integrins which direct TGFβ signal transduction.

# Chapter 3

## Hypothesis and Objectives

The hypotheses of this thesis were:

Hypothesis 1: Differential protein expression in degenerative (myxomatous) mitral valves compared to healthy valves reflects cell signaling mechanisms and cellular processes that mediate the valve degeneration.

Objective 1.1: To evaluate targeted protein abundance patterns of cell membrane receptors and ligands present in healthy and degenerative mitral valves.

Hypothesis 2: Pathologic changes in degenerative mitral valves are mediated in part by endothelial to mesenchymal cell transformation (EndMT) and epidermal growth factor (EGF) signaling.

Objective2.1: To identify spatial expression patterns by immunohistochemistry of differentially expressed proteins associated with EndMT and valve endothelial cell markers within the tissue matrix of healthy and degenerative mitral valves.

Objective 2.2: To determine if EGF signaling molecules correlate spatially with the process of EndMT.

# Chapter 4

## Differential Protein Abundance in Healthy and Diseased Mitral Valve Tissue

Degenerative mitral valve disease (DMVD), also known as myxomatous mitral valve disease, is widespread among dogs with prevalence as high as 58% of the population over eight years old[52]. It is also the most commonly seen cardiac disease in canines[53]. Indications such as a left-sided systolic heart murmur or mitral valve regurgitation as diagnosed by echocardiogram, along with predisposing factors such as advanced age, hypertension, or breed predisposition, provide strong evidence for the diagnosis of degenerative mitral valve disease. Breeds such as Cavalier King Charles Spaniels have been shown to have a genetic predisposition for developing DMVD both earlier and more frequently than other dogs,[5] and studies have attempted to identify these breeds and understand what they have in common in order to narrow down genetic causes[7]. The disease affects both humans and dogs and the degenerative valves from these species exhibit many histological similarities. Because canine mitral valve disease has been shown to be a good model for studying human mitral valve degeneration[3], and degenerative canine valves are more accessible than human valves, examination of these samples can yield important insights into the cellular processes involved in the initiation and progression of DMVD.

Multiple cell signaling pathways have been implicated in degenerative mitral valve disease, and cell membrane proteins play a key role in these cellular interactions. These signaling pathways likely mediate important cellular processes in degenerative mitral valves such as interstitial cell phenotype transformation, increases in cell density, and changes in extracellular matrix[22]. Cell phenotype transformation is the change from a normal interstitial

cell into an “activated myofibroblast” which has been shown to express catabolic enzymes implicated in the pathological remodeling process present in degenerative valves[21]. Cellular signaling mechanisms may be responsible for this transformation, and knowing the presence and magnitude of cell membrane proteins may help connect causes and effects in the disease process. Examples of signaling pathways that have been implicated in DMVD include TGFβ, serotonin, and developmental signaling processes[17,31]. However this disease process is complex and likely involves many interactive signaling pathways mediating intricate cellular processes. One approach to identifying other signaling cascades is to use a “systems biology” or “omics” approach to determine differential expression or abundance patterns between normal and diseased tissue. Previous differential expression studies in canine degenerative MVD have included a differential transcriptomic study[54] and a shotgun proteomic study[22]. These studies have provided important clues into cellular processes and associated signaling mechanisms that mediate disease progression.

The objective of this study was to further characterize cellular processes and signaling pathways in canine DMVD by using a targeted signaling protein array to compare protein abundance patterns of specific membrane receptors present in normal and degenerative mitral valves. I hypothesized that the results of this study would provide further insight into complex cellular processes that mediate canine DMVD.

*Materials and Methods*

Septal mitral valve leaflets (n=3) were excised from deceased dogs with advanced DMVD presented to the CSU Veterinary Teaching Hospital necropsy facility. These samples, termed “degenerative” group, showed the characteristic signs of late-stage (Whitney grade 3)

degenerative mitral valve disease including large coalescing nodules, ruptured and elongated chordae tendinae, thickened and opaque valve leaflets, and a regurgitant jet "scar" or mark on the endocardium of the left atrium[52]. Normal septal mitral valve leaflets, termed "normal" group, were obtained from young healthy beagles sacrificed for other studies. Normal mitral valves (Whitney grade 0) were smooth, translucent, and thin. All samples were processed by first removing blood clots and other contaminants with repeated rinsing in phosphate buffered saline. Samples were then frozen at -80°C for storage. After thawing, samples were washed again with PBS and leaflets were cropped such that all samples were of similar weight by using a sensitive scale.

To isolate the proteins from the raw valve tissue, the valves were minced to increase surface area and then digested with lysis buffer to break down the cells and release proteins from the rest of the cellular debris. Next they were centrifuged at 15000xg for 10 minutes, which isolated the protein containing supernate. A Modified Lowry protein assay was performed on the samples which involved the reaction of protein with cubric sulfate and tartrate resulting in the formation of tetradentate copper-protein complexes. The next step was the addition of Folin-Ciocalteu Reagent which was reduced in proportion to the chelated copper complexes. This produced a blue colored product measurable at 750nm. A known quantity of bovine serum albumin was diluted in lysis buffer at varying concentrations to produce the standard against which the unknowns were compared. 40µL of each standard and unknown sample were added to microplate wells, and 200 µL of Modified Lowry Reagent was then pipetted into each well. This mixture was incubated at room temperature for exactly ten minutes, and then 20 µL of Folin-Ciocalteu Reagent was added to each well. This was then

incubated at room temperature for thirty minutes and absorbance was measured using a spectrophotometer plate reader. The standard curve was obtained by plotting average values for the BSA standard against its concentration in mg/mL, and the curve was then used to determine protein concentration of the unknown samples. The protein concentrations from this assay were used to calculate the appropriate volumes needed to load equal masses of protein in the next procedure, the protein array.

The procedure for R&D Systems' "Proteome Profiler" antibody array was followed, including loading of sample, adding and washing with particular buffers, antibodies, and fluorescence, and then obtaining images from the chemiluminescence imager. Reagents and samples were prepared according to the manufacturer's specifications, and then the procedure was started using one diseased valve and one healthy valve. 2.0 mL of 1X array buffer was pipetted into each well of the 4-well multi-dish, this served as a blocking buffer. Tweezers were used to place the membranes in individual wells, and the container was incubated for one hour on a rocking platform shaker. 250 µg of sample was added to 300µL of array buffer and adjusted to obtain a final volume of 3 mL with array buffer. The array buffer was then removed from the wells and 1.5 mL of the prepared 3 mL sample mixture was added to both Part N and Part C of the multi dish. This was incubated overnight on ice with a rocking platform shaker. After incubation, each array was washed three times with 1X wash buffer for 10 minutes. For the part N array, 30 µL of detection antibody cocktail N was diluted to 1.5 mL with 1X array buffer and added to the well containing the N membrane. For the part C array, 30 µL of detection antibody cocktail C was diluted to 1.5 mL with 1X array buffer 8/1 and added to the well containing the C membrane. The membranes were then incubated for two hours at room

temperature on a rocking platform shaker. After incubation, each array was again washed three times with 1X wash buffer for 10 minutes. The Streptavidin-HRP was diluted in 1X array buffer, and 2 mL was added to each of the wells, covering the membrane. This was incubated on a rocking platform shaker for thirty minutes. After incubation, each array was washed three times with 1X wash buffer for 10 minutes. Following these last washes, 1mL of chemi reagent mix was pipetted onto each set of membranes. Images of the membranes were then obtained using a chemiluminescence imager. After assessing the results, the procedure was repeated twice, for a total of three pairs of membranes for both the degenerative and normal group. Overall 6 valves were used, each valve generating two membranes, the Nonhematopoetic and Common Analytes arrays, and each of these had two spots per antibody tested leading to the collection of a large number of data points.

After this, measurements of the spots that contained the 119 antibodies being tested were obtained on the computer image processing program ImageJ. Darker spots correlated to an increased level of antibody binding, and therefore a more positive result of protein expression. After quantifying the degree of visibility of the spots, they were normalized against IgM. This was a way of calibrating the sample loads by relating them to a housekeeping protein and positive control. Therefore, the different sample results could be compared. A Mann-Whitney test was then used to determine the statistical significance of the differences between healthy and diseased samples for each protein. Healthy and diseased data points were also averaged to provide a ratio to show biological significance.

*Results*

Valves were collected from three dogs with normal mitral valves and three dogs with degenerative mitral valves. The mitral valves used for the “normal” group of this study came from one year old beagles collected from a research facility. These animals underwent euthanasia for an orthopedic research project and no gross morphologic cardiac abnormalities were present. The valves used for the “degenerative” group were obtained from dogs euthanized for other health reasons. The first animal was a 15 year old male castrated Chow, the second a 13 year old male castrated Border Collie, and the third a 10 year old male castrated Greyhound. No animals were euthanized for the purposes of this study. The average age of the diseased group dogs was 12.7 and the average age of the healthy group was 1.

The protein array revealed several proteins that had differential abundance between the groups. Most differences involved a protein having increased abundance in the diseased valves, while a few proteins were less abundant. (Figure 4.1)

Based on the Mann Whitney statistical test and using a p value of .05, 25 proteins had significantly different abundance levels when comparing the two groups. The ratios of diseased to normal expression indicated that 18 proteins were increased, and a cutoff of twice the normal group’s average abundance level was considered biologically significant. (Table 4.1) Smaller differences were not considered biologically relevant.

Integrin β3/CD61 was the most biologically significant protein among the proteins with a p value of less than 0.05. It had a ratio of 4.778 when comparing abundance of the degenerative group to the normal group. Integrin αv (the other half of the common integrin αvβ3 heterodimer) was measurable in 2 of the 3 diseased valves. However, because integrin αv

was undetectable in all normal valves, statistical significance could not be tested. Nevertheless, the presence of both parts of the heterodimer in the degenerative valves suggests that αvβ3 is the form of the integrin that is upregulated.

Matrix metalloproteinase 2 (MMP-2) was found to have a p value of 0.003948 and biological significance of 2.4922 and was of interest because of the links found between it and integrin β3[47]. Also, Matrix metalloproteinases or MMPs (a group of zinc requiring matrix degradation enzymes) have been previously shown to play a role in degenerative valve disease[55].

Other data of note from Table 1 include the related group of proteins Epiregulin, ADAM 17, and HB-EGF, all with significant p-values and biological significance. Epiregulin and HB-EGF are both ligands for the epidermal growth factor receptor (EGFR). ADAM 17 is a regulator of these ligands; it is a membrane bound enzyme that when exposed to its substrate, such as Pro-HB-EGF, cleaves it to HB-EGF so this active form is released for further signaling[33].

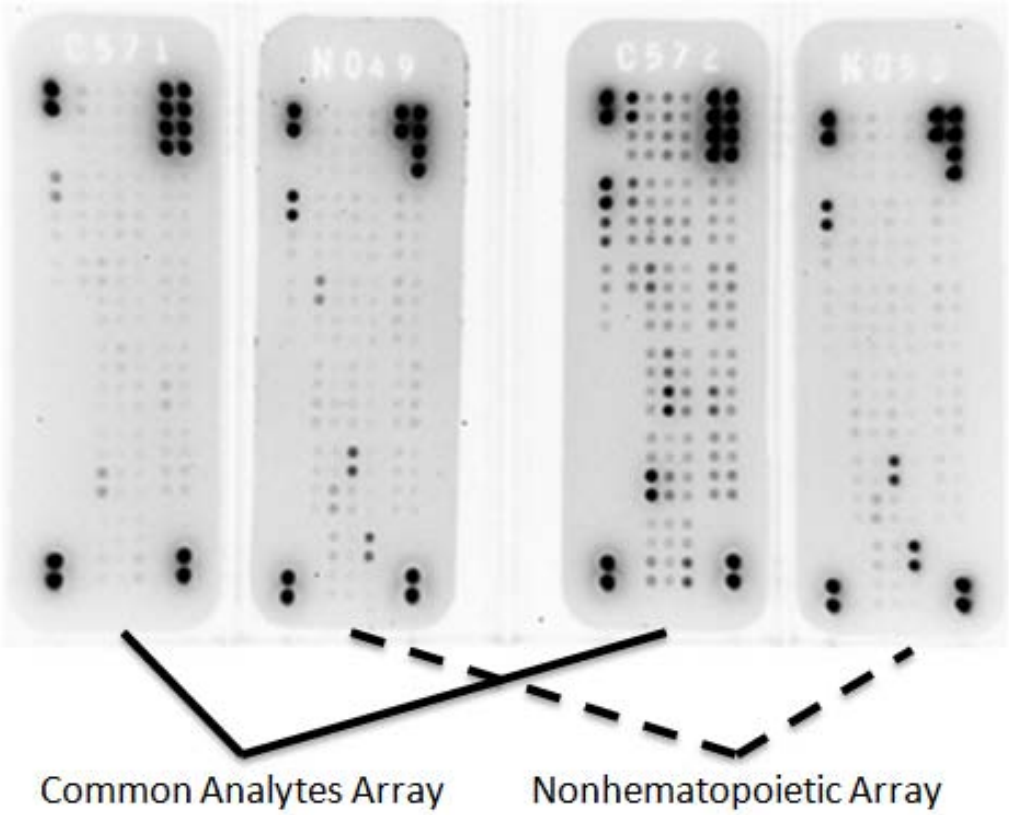


**Figure 4.1 Representative Chemiluminescent Images Obtained from Protein Array Membranes**

The two membranes on the left were from a normal mitral valve, the two on the right from a degenerative one. The many dark spots on the degenerative Common Analytes Array membrane are indicative of upregulated proteins compared to the normal membrane.

**Table 4.1 Differentially Abundant Signaling Proteins in Canine Normal and Degenerative Mitral Valves**

18 of 119 proteins tested were found to be statistically significant with a p value of <.05 when comparing the quantified protein expression of the two groups (degenerative and normal) with the Mann-Whitney Test and biologically significant with an abundance ratio of >2.0.

| Protein | Biological Significance (Diseased/Control) | P-value |
|---|---|---|
| Integrin β3/CD61 | 4.778 | 0.0040 |
| ICAM-2/CD102 | 3.282 | 0.0163 |
| Pref-1/DLK-1/FA1 | 3.254 | 0.0104 |
| Epiregulin | 2.710 | 0.0040 |
| Stabilin-1 | 2.697 | 0.0040 |
| Endoglin/CD105 | 2.657 | 0.0040 |
| LOX-1/SR-E1 | 2.650 | 0.0250 |
| TACE/ADAM17 | 2.633 | 0.0065 |
| EMMPRIN/CD147 | 2.544 | 0.0374 |
| MMP-2 (total) | 2.492 | 0.0040 |
| CD155/PVR | 2.240 | 0.0040 |
| IL-1 RII | 2.216 | 0.0040 |
| HB-EGF | 2.191 | 0.0104 |
| SREC-1 | 2.113 | 0.0104 |
| TIMP-3 | 2.097 | 0.0374 |
| Lipocalin-2/NGAL | 2.085 | 0.0250 |
| JAM-A | 2.063 | 0.0250 |
| VAP-1 | 2.016 | 0.0374 |

*Discussion*

This protein array study indicated distinct protein differences between healthy mitral valves and those exhibiting signs of degenerative processes. The importance of these specific protein differences is still unknown, but understanding their relation to each other and known mechanisms active in degenerative disease yields interesting hypotheses.

Integrin β3 was the most upregulated protein among all those studied. Integrins are heterodimeric integral membrane proteins with a variety of cellular functions. The most likely heterodimer for this protein to be a part of would be αvβ3 which has roles in cellular adhesion and migration and also influences TGFβ signaling by binding to its receptor on the cell surface[42]. TGF-β, a cytokine involved in ECM regulation, has been shown to have increased expression in degenerative valves[18]. Integrin β3 has also shown to be upregulated in tissues where cells are undergoing transformative processes such as endothelial to mesenchymal cell transformation (EndMT)[43-44]. The presence of a high abundance of this protein in degenerative mitral valve tissue could have a few interpretations. Integrinβ3 may be mediating changes of the extracellular matrix that occur in diseased valves because it facilitates cellular movement, it may be contributing to cellular transformation by influencing TGFβ signaling, or it may simply be upregulated as a result of other transformative processes such as those affecting interstitial cells. Interstitial cells in degenerative mitral valve tissue undergo phenotype transformation, as shown by the expression of markers for smooth muscle cells[21]. This cellular type change could be responsible for some of the protein differences observed in this array.

Integrin αvβ3 is not widely expressed in normal tissues, but it is known to be increased in activated smooth muscle cells [56]. The increase in myofibroblasts in degenerative mitral valve

tissue could account for this finding. Also there is evidence that cyclic strain induces increased levels of integrin αvβ3 in vascular smooth muscle cells[57], which may have relevance to other findings where cyclic strain induced degenerative type change in canine mitral valves[15].

The upregulation of MMP-2 is consistent with prior data showing that catabolic matrix enzymes occur at a higher concentration in the diseased state of the mitral valve[55]. MMPs also generally correlate with disease progression, indicating that they play a role in facilitating the disease process, especially the dissolution of the stratification of the normal valve layers[12].

The data showing an increased presence of HB-EGF, ADAM 17, and Epiregulin may have significance in our understanding of cell signaling in degenerative disease. HB-EGF and ADAM17 are both important molecules involved in the development of mammalian heart valves, as shown by the results of mouse knockout models for both these proteins[34,36,38]. When either of these molecules are absent in mutants, deposition of both GAG and collagen are increased. While this seems to be a paradoxical effect compared to our results of its increase in DMVD, it still underlies the importance of this signaling in the valve tissue and emphasizes it's involvement in valve homeostasis. The pathway including HB-EGF and ADAM 17 is a good example of a developmental pathway that initiates the EndMT process in early heart valve development[35]. Other developmental pathways have been implicated in degenerative mitral valve disease, such as BMP and Wnt signaling [29]. Therefore, the increased abundance of these ligands (HB-EGF and Epiregulin) and their regulating enzyme(ADAM17) which activate the EGF receptor may be yet another dormant developmental pathway that is re-activated in the diseased state.

The large age difference between dog groups is a result of the samples that were available, and some protein expression changes could have been due to this age difference. However, degenerative valve disease is age-linked[52], therefore it is difficult to age match when conducting these studies and so we acknowledge this possible confounding factor.

*In conclusion,* the differently expressed proteins identified by this protein array are likely relevant to degenerative valve disease. Some identified proteins, like the significant expression of MMP-2, are consistent with previously described findings and therefore provide a degree of validation to the rest of the data. Other results, such as those of the EGF-related proteins, have not been previously identified in degenerative valves, but importantly are known to play roles in early developing valves such as the initiation of EndMT. This pattern of developmental pathway proteins increasing in abundance in degenerative valves has been previously reported, but not with these proteins specifically. The significantly increased abundance of integrin αvβ3 is also a new finding with more than one possible interpretation, including phenotype transformation of interstitial cells to activated myofibroblasts, TGFβ signaling and EndMT. Therefore next steps of this study could include a more targeted examination of these proteins with the specific goal of examining their spatial relationship with known or previously unknown pathologic processes in degenerative mitral valves such as myofibroblast transformation and EndMT.

# Chapter 5

## Cells in Degenerative Mitral Valve Tissue Undergo Endothelial to Mesenchymal Cell Transformation

To understand the causes of degenerative mitral valve disease, it is important to investigate the changes and patterns occurring at the cellular level. The disease is characterized by increased expression of α smooth muscle actin (αSMA) in valvular interstitial cells (known as myofibroblast activation or transformation), an exuberant deposition of glycosaminoglycan (GAG), and a loss of proper valve architecture where the normally distinct layers of atrialis, spongiosa, and fibrosa become intermixed. This breakdown of cell layers contributes to valves becoming thickened, nodular, and less elastic, therefore leading to loss of mechanical integrity.

Many mechanisms of this degradation of the extracellular matrix (ECM) have been identified, such as increased matrix metalloproteinases (MMPs) and changes in cellular activity resulting in overproduction of GAG. For instance TGFβ, a cytokine involved in ECM regulation, has been shown to have increased expression in diseased valves[18]. Also, specific areas of pathologic change visible on histological examination show phenotype transformation (myofibroblast activation) with increased cell density but not increased cell division.[21] One possible explanation for increased cellular density is that cells may be migrating from other areas rather than simply dividing. Understanding why these areas have increased cell density in diseased valves could help explain the physical changes in degenerative valves, such as nodules and irregularities of valve stratification. Connecting these known observations with protein expression patterns reported in the previous chapter leads to a hypothesis that could explain cellular changes previously observed in degenerative mitral valve disease (DMVD). The

hypothesis of this study is that endothelial to mesenchymal cell transformation (EndMT), which is known to play a central role in heart valve development, is recapitulated in degenerative valve disease and could explain previous observations in degenerative valves such as increased density of myofibroblasts.

EndMT is a multi-faceted process that takes place in wound healing, development (notably heart valve formation), and cancer progression. The process involves a cell receiving a signal to change phenotype after which it begins to lose characteristics that define it as an endothelial cell, such as adhesion molecules, and gain characteristics that define it as a mesenchymal cell. During this change, it detaches from the endothelial cells around it and migrates through the basement membrane into the tissue interstitum where it begins to express mesenchymal cell markers, which include αSMA[25]. To investigate if EndMT is occurring, two molecules were selected that should provide evidence that this cell migration pattern is present. The endothelial cell marker CD31, also known as platelet endothelial cell adhesion molecule-1, is a widely accepted marker of endothelial cells[50]. Endothelial cells should normally only be present in a thin monolayer on the surface of heart valve tissues. If endothelial cells deviate from this pattern as shown by the indicator CD31, this would be evidence of endothelial cell migration into the interstitum, a critical step in EndMT.

Myofibroblast transformation of interstitial cells, identified by αSMA expression, has been previously reported to occur in "cellular clusters" most often close to the atrialis surface of degenerative mitral valves. Co-localization of endothelial cell markers such as CD31 and/or

signaling molecules associated with EndMT with these cellular clusters could provide evidence for EndMT in the degenerative process.

Much research has been done showing that the process of EndMT is involved in the development of the heart valve. By undergoing EndMT in the diseased state, the valvular cells may have reactivated dormant developmental pathways. Supporting this theory, other pathologic processes that mimic developmental pathways have been identified in diseased heart valves, including BMP and Wnt signaling[29]. The pathway including heparin binding epidermal growth factor (HB-EGF) and its sheddase, ADAM 17, is a good example of an essential developmental pathway that initiates EMT in early heart valve development as endothelial cells migrate and begin to form the cell layers and extracellular matrix of an adult valve[35].

The proteins HB-EGF and ADAM 17 were selected for examination because they were identified by a previous protein array as significantly increased in diseased valves, and because of their known role in EndMT. These proteins were examined for co-localization with myofibroblast transformation, identified by expression of αSMA, and migrating endothelial cells, identified by CD31. Co-localization of these proteins and cellular phenotype markers could provide evidence of EndMT in degenerative mitral valves.

*Materials and Methods*

1. *Tissue Collection*

Mitral valves were obtained post mortem from healthy dogs (N=8) and dogs considered to have DMVD (N=13). Valves were also obtained from 6 or 7 year old research beagles euthanized for another study and were confirmed to have normal mitral valve function by echocardiogram. The diseased valves were collected from dogs of various breeds that died or were euthanized for reasons unrelated to this study and underwent necropsy. These dogs were determined to have degenerative mitral valve disease by gross postmortem inspection of their valves, and were classified as either intermediate or severe. Valves and portions of papillary muscle and chordae tendinae were removed from the heart with surgical scissors and rinsed in phosphate buffered saline for approximately two hours. Valves were then sliced to fit in cassettes, fixed in 10% buffered formalin for 1-2 hours at room temperature, stored in 70% EtOH overnight, and embedded in paraffin blocks. 5µm slices were mounted on microscope slides for immunohistochemical staining. All valves were stained with Movat pentachrome stain (American Mastertech Scientific) and assessed for morphology to determine if microscopic degenerative changes were consistent with the gross examination and classification of the valves.

2. *Immunohistochemistry*

Slides were stained using immunohistochemistry to determine the location of endothelial cells and myofibroblasts and the activity of EGFR and integrin signaling. Endothelial cells were identified by positive staining for the marker CD31 (PECAM-1) (Dako, Carpenteria, CA, USA). Myofibroblasts were identified by α smooth muscle actin (αSMA) (Santa Cruz

Biotechnologies, CA, USA). The antigens HB-EGF (Santa Cruz Biotechnologies, CA, USA) and ADAM 17 (also known as TACE) (Sigma-Aldrich, St. Louis, MO, USA) were used to identify EGFR signaling, and integrin β3 (Abcam, Cambridge, MA, USA) was stained for as an indicator of cell migration and activation. To begin staining, slides were soaked in xylenes for 15 minutes for deparrafinization and rehydrated through graduated alcohol baths. Antigen retrieval was performed using a decloaking chamber at 95ºC for 40 minutes (Biocare Medical, Concord, CA, USA) and either citrate or pH 9 decloaking fluid (Target retrieval solution S1699, Dako). The slides were incubated with a background reducing agent (Biocare Sniper, Biocare Medical, Concord, CA, USA) for 5 minutes and then incubated with primary antibody overnight in a humidified chamber. Primary antibody was diluted at varying concentrations to obtain optimal staining balance (1:10 for CD31, 1:50 for HB-EGF, 1:200 for ADAM 17, 1:300 for Integrin β-3 and 1:100 for αSMA). The following day, sections were blocked with peroxidase for 15 minutes followed by incubation with secondary antibody (Dako mouse HRP link) for 30 minutes. Slides were then stained with DAB+ chromagen and counterstained with hematoxylin (Dako) then dehydrated through graduated alcohol baths. Sections were rinsed with Tris-buffered saline containing .01% Tween 20 (TBS-T) between each step. The distribution patterns of the various proteins were reviewed and analyzed with light microscopy.

3. *Quantitative Analysis*

αSMA staining was quantified by counting the number of positive staining "cellular clusters" per histologic section of each whole valve. This excludes the normal thin layer of positive αSMA staining previously identified as smooth muscle cells close to the surface of the atrialis layer or actin staining of vessels or papillary muscle that may have been included in the

sample. It is defined by distinct staining of cells in a clumped or dispersed distribution extending into the spongiosa or fibrosa layers as described by Han 2008[23] and Disatian 2008[21]. This abnormal staining is not seen often in healthy valves, but is clearly visible in degenerative valves such as shown in Figure 5.3. Differences between healthy, intermediate, and severe groups were compared with ANOVA and pairwise comparison tests.

Semi quantitative analysis of cell staining for ADAM 17 and HB-EGF was assessed by assigning each sample a number 0-4 correlating to the approximate percentage of cells that stained positive in an average microscope field area of the valve tissue. The number correlates to percentages as follows: 0=no visible staining, 1= 1-25%, 2=26-50%, 3=51-75%, 4=76-100%. In this way the quantity of cells expressing each molecule can be assessed without taking into account stain intensity, which varies significantly among samples and batches of slides undergoing IHC. Results of staining between groups were compared using box plots and the Kruskall-Wallace test.

CD31 and integrin β3 staining were observed by comparing photomicrographs obtained using the Axiocam camera and Axiovision software. Positive staining in areas of co-localization were identified and examined for evidence of cellular patterns.

*Results*

1. *Animals*

Mitral valves were obtained post mortem from dogs (N=8) confirmed to have normal mitral valves by echocardiography and gross examination, and from dogs with degenerative mitral valve disease based on gross examination of their valves (N=11) or gross examination and

echocardiogram (N=2). The degenerative valve samples were sorted into two groups, intermediate (N=6) and severe (N=7), based on gross post mortem criteria. The degenerative valves were collected from dogs of various ages and breeds (see Table 5.1 and Figure 5.1) that underwent necropsy. The median age of dogs in the intermediate group was 11.5 years (range 6 to 14). The median age of dogs in the severe group was 10 years (range 8 to 17). All dogs were either considered small dogs (<15 Kg) (N=13), or medium sized dogs (between 15 and 35 Kg) (N=8). The cause of death or euthanasia was mitral valve disease in one dog with severe disease, and unrelated to heart disease in all other cases.

**Table 5.1 Breed and Gender of Sampled Dogs**

This table provides summary data of dogs whose mitral valves were sampled and studied.

| Breed and Gender of Dogs According to Group | | | | | |
|---|---|---|---|---|---|
| Control | Gender | Intermediate | Gender | Severe | Gender |
| Beagle | F | Beagle | F | Cavalier King Charles Spaniel | M |
| Beagle | F | Beagle | M | Miniature Poodle | M |
| Beagle | F | Border Collie | M | Mixed Breed | F |
| Beagle | M | English Spaniel | F | Australian Shepherd | M |
| Beagle | M | Mixed Breed | F | Beagle | F |
| Beagle | M | Pointer | M | Beagle | M |
| Beagle | M | | | Labrador Retriever | M |
| Beagle | M | | | | |

F indicates female; M indicates male

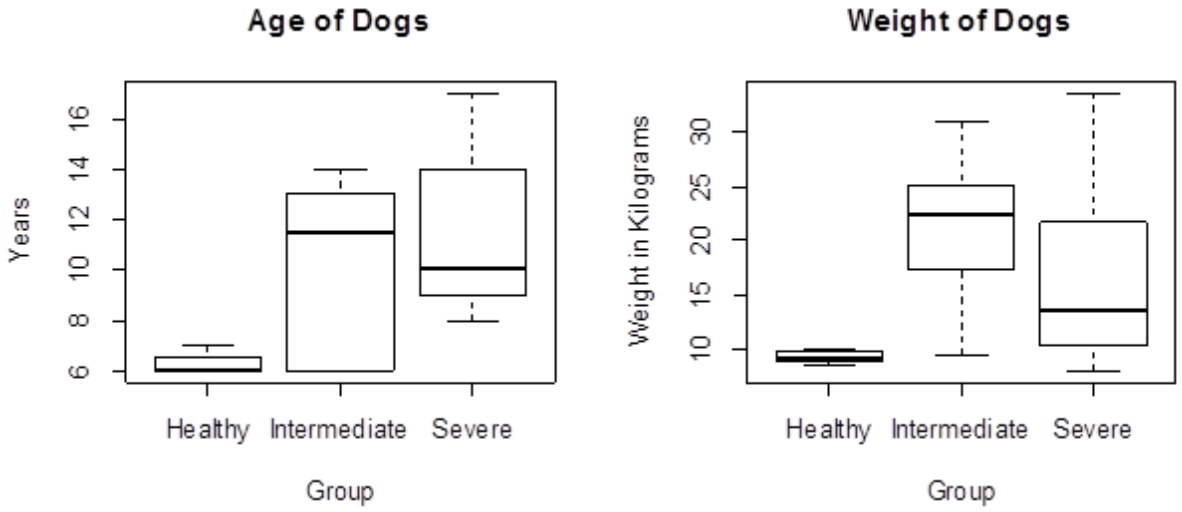


**Figure 5.1 Ages and Weights of Dogs**

The ages of the dogs included as diseased in this study were slightly higher than the ages of the control group. The weights of the dogs included in the diseased groups were also generally higher, but all dogs were small or medium size and no dog weighed more than 34 Kg.

2. *Histology*

Movat staining of normal mitral valves showed a clear stratified structure consistent with previous studies. Atrialis, spongiosa, and fibrosa layers were identifiable and nodules and valve thickening were absent. Intermediate stage valves showed some disorganized black elastin fibers in the spongiosa layer as well as less distinct staining of the yellow collagen areas in the fibrosa. Severely diseased valves showed substantially increased amounts of GAG staining as well as loss of valve stratification, especially towards the distal end of the leaflet. (Figure 5.7 A-C)

3. *Immunohistochemistry*

αSMA staining was found in all mitral valves stained, but the distribution and amount of positive cells varied between the groups. Normal mitral valves had minimal evidence of positive αSMA myofibroblast clusters. The mean number of myofibroblast clusters per histologic section of the normal, intermediate degenerative, and severe degenerative groups were 1.13, 3.17, and 6.86 respectively. Statistical analysis showed that all groups were significantly different (Table 5.2). αSMA stained myofibroblasts were generally present near the atrial surface and towards the endothelium of the normal valves in a smooth, coherent pattern (Figure 5.3 A). In intermediate degenerative valves, myofibroblast type cells identified by positive αSMA staining were more often present in deeper valve layers, but were generally dispersed and evenly distributed compared to the severe valves. The severe valves often exhibited aggregates of αSMA near the valve surface (Figure 5.6 C), and streaks originating at the surface and descending into the interstitum (Figure 5.5 C).These streaks co-localized with at least one other of our molecules of interest in nearly all cases, and often co-localized with most

of them (Figures 5.5 and 5.6). Also, αSMA staining was very prevalent and widespread throughout all valve layers in some of the diseased valves (Figure 5.3 C).

Positive CD 31 staining was found on sections of the endothelium of most normal and intermediate-stage valves and all severely degenerative valves. This staining was usually present on involutions or folds of the valve tissue, and was also present in blood vessel walls in papillary muscle sections, showing normal endothelial cell staining. Many edges of the valve tissue did not stain well for CD31 which may have been due to loss of the surface endothelial cells during tissue processing. In many intermediate and all but one severe group valve, CD31 staining was also found in deeper layers of the valve, and positive cells appeared to be descending into the atrialis and spongiosa layers (Figure 5.2 B and Figure 5.3 E and F). These positive CD31 areas did not exhibit visual characteristics of capillaries or immune cells, which would be possible explanations for their location. This staining was usually present on areas specifically identified as degenerative based on Movat staining most often co-localized with αSMA positive myofibroblast clusters.

Cells positive for HB-EGF were found in all mitral valves and the staining was widely distributed in most samples. Although not significantly increased in the diseased valve groups when analyzed with a Kruskall-Wallis test, the amount of HB-EGF staining showed a clear upward trend compared to the healthy valves as presented in Figure 5.4. HB-EGF often stained more heavily in areas of αSMA myofibroblast staining and areas of increased cellular density (Figure 5.6 B), but did not clump like CD 31 or αSMA. It did however form streaks similar to CD31 as shown in Figure 5.5 (B) and co-localized with ADAM 17 both at the surface of valves

and deeper within the matrix (Figure 5.7). HB-EGF positive streaks were found in the middle layer of many healthy valves (Figure 5.7 G). ADAM 17 staining was usually found in a pattern similar to that of HB-EGF. That is, staining was often widespread and no “clumping” was found, however staining was increased in severely degenerative valves compared to normal valves (Figure 5.4) and in areas that appeared to have active cellular change occurring such as in Figure 5.7. Also, the molecule integrin β3 was detected in many abnormal locations on the diseased valves and tended to co-localize with αSMA (Figure 5.6 D) especially where cell density was increased.

**Table 5.2 P Values for α-Smooth Muscle Actin Staining of Normal, Intermediate Degenerative and Severely Degenerative Mitral Valves** : After the one way ANOVA indicated that the data were from different distributions (P value= .00000825), pairwise comparisons using Student's t tests with pooled standard deviation were conducted. This table shows p values for comparisons of the three groups.

| | Healthy | Intermediate |
|---|---|---|
| Intermediate | p=0.03035 | |
| Severe | p=0.0000019 | p=0.00064 |

All groups were significantly different because $p < \alpha = .05$.

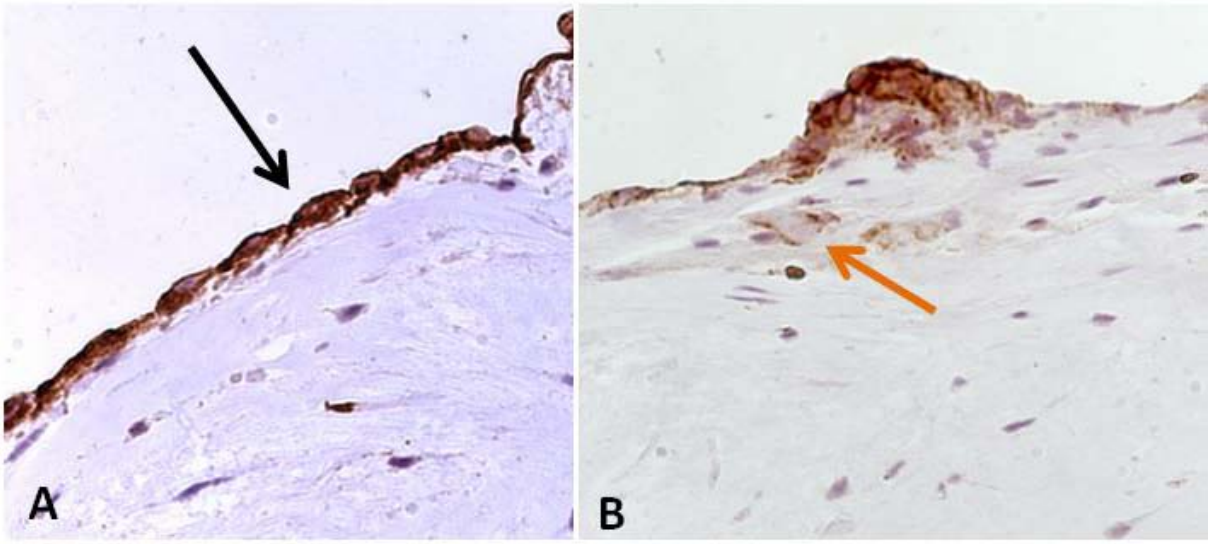


**Figure 5.2 Immunohistochemistry for CD31 of Canine Mitral Valves**
Photomicrograph A is a high power view of endothelial cells (black arrow) on a normal area of mitral valve. These cells are squamous shaped, regular, and have clear boundaries and nuclei. Photo B shows a degenerative area of mitral valve where an irregularity on the valve surface (brown lump) has stained positive for CD31. These cells are rounder, bunched, and have abnormal shapes compared to the cells in A. The area adjacent to the abnormality appears to lack normal endothelial cells. CD31 staining in cells below the surface was also evident (orange arrow). This cell is unlikely to be a macrophage which also expresses CD31 because these cells are rarely found in degenerative mitral valve tissue. [21] Magnification 40x.

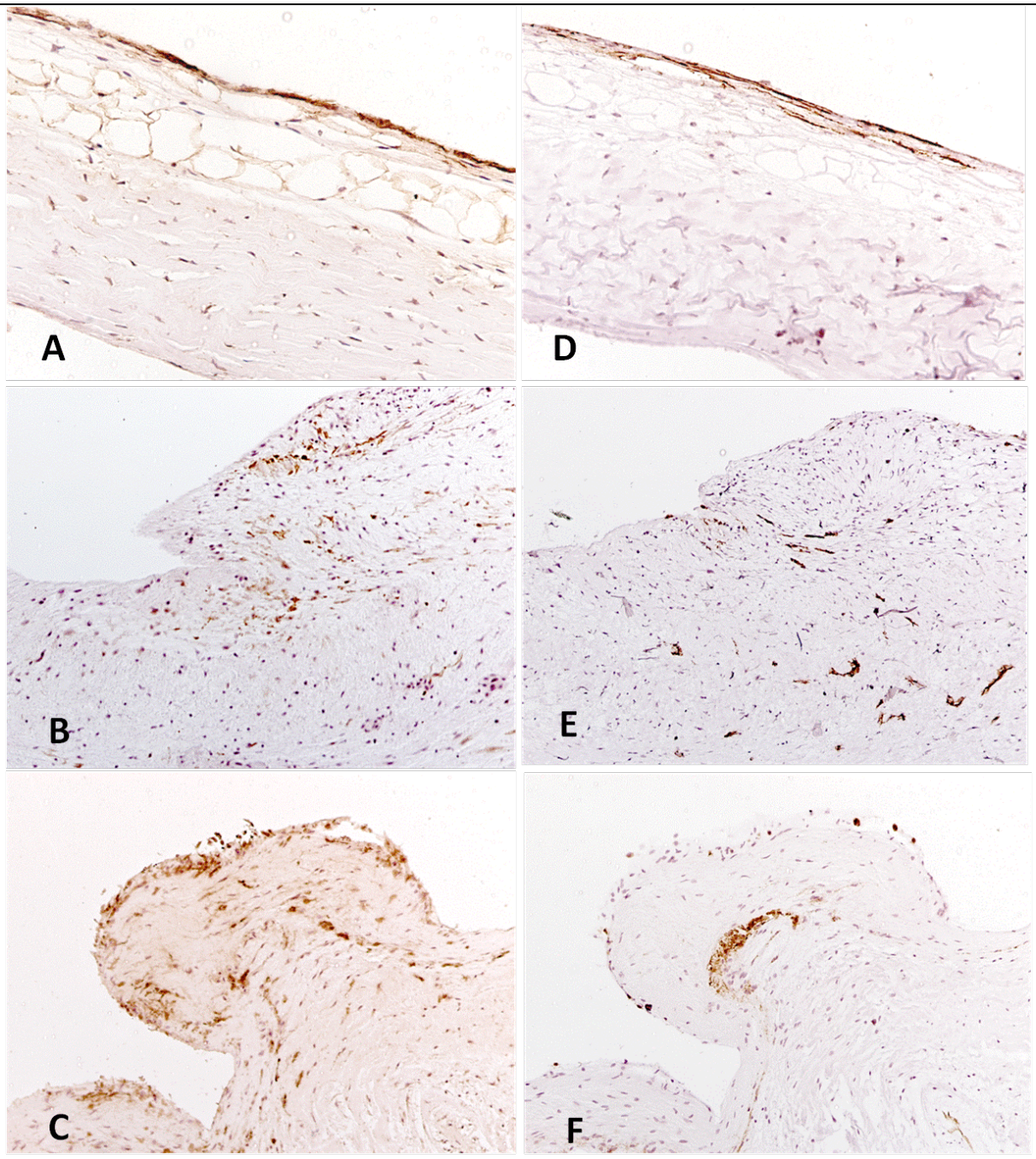


**Figure 5.3 αSMA and CD31 Immunohistochemistry of Normal, Intermediate Degenerative, and Severely Degenerative Canine Mitral Valves**

Photomicrographs of sequential tissue sections of normal (A, D), intermediate degenerative (B,E), and severely degenerative (C, F) mitral valves stained for αSMA (A-C) or CD31(D-F). Brown staining indicates the presence of the target molecule, while purple shows the hematoxylin counter stain. Magnification 20x for A and D, 10x for B, C, E, and F.

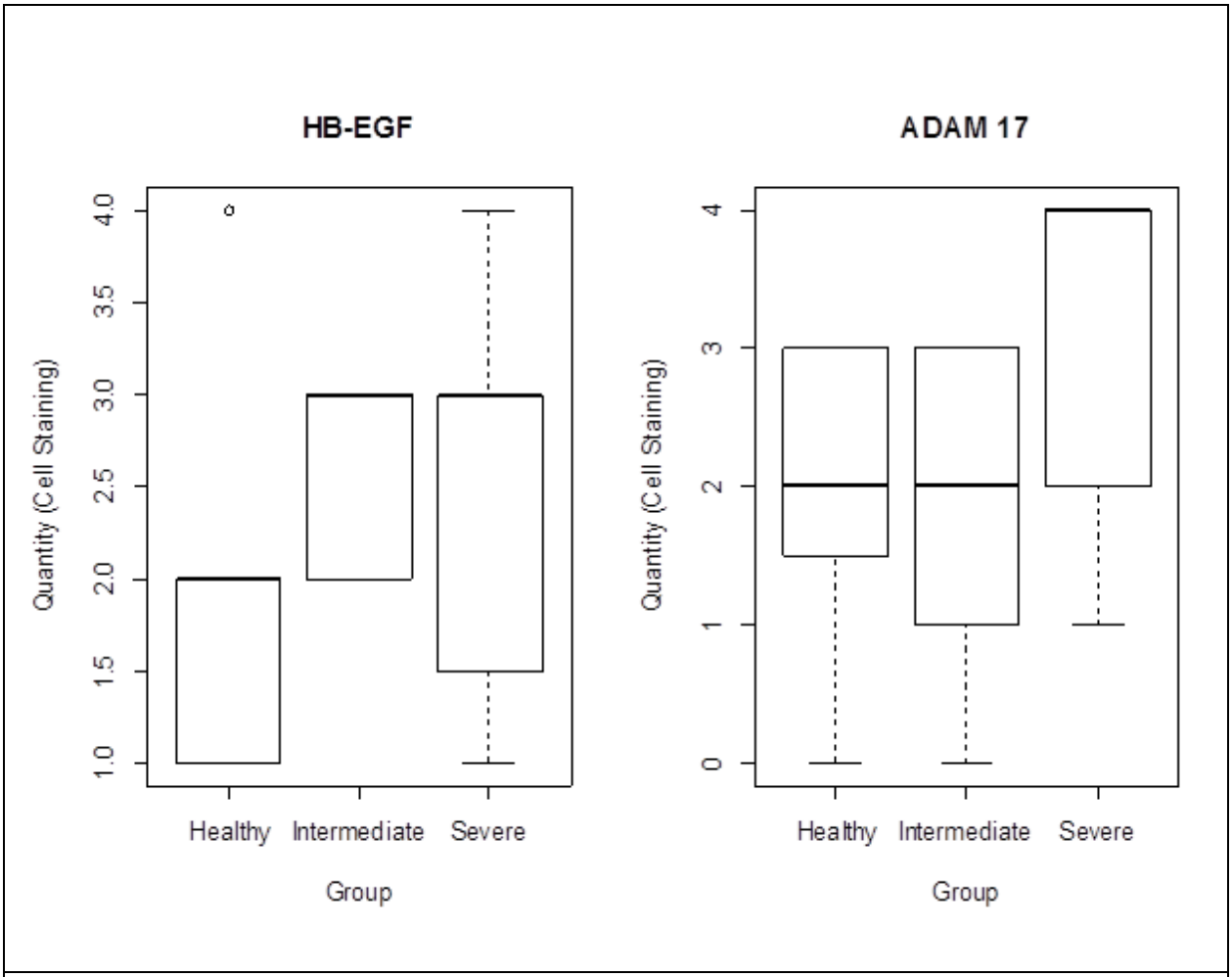


**Figure 5.4 Semi Quantitative Analysis of HB-EGF and ADAM17 Immuno-Staining of Normal, Intermediate Degenerative, and Severely Degenerative Mitral Valves**

HB-EGF and ADAM 17 were both detected on most valves stained. HB-EGF shows an increase in staining in the intermediate and severe groups, while ADAM 17 only had increased staining in the severe group as the median (bold line) is 4 compared to the other groups' median of 2.

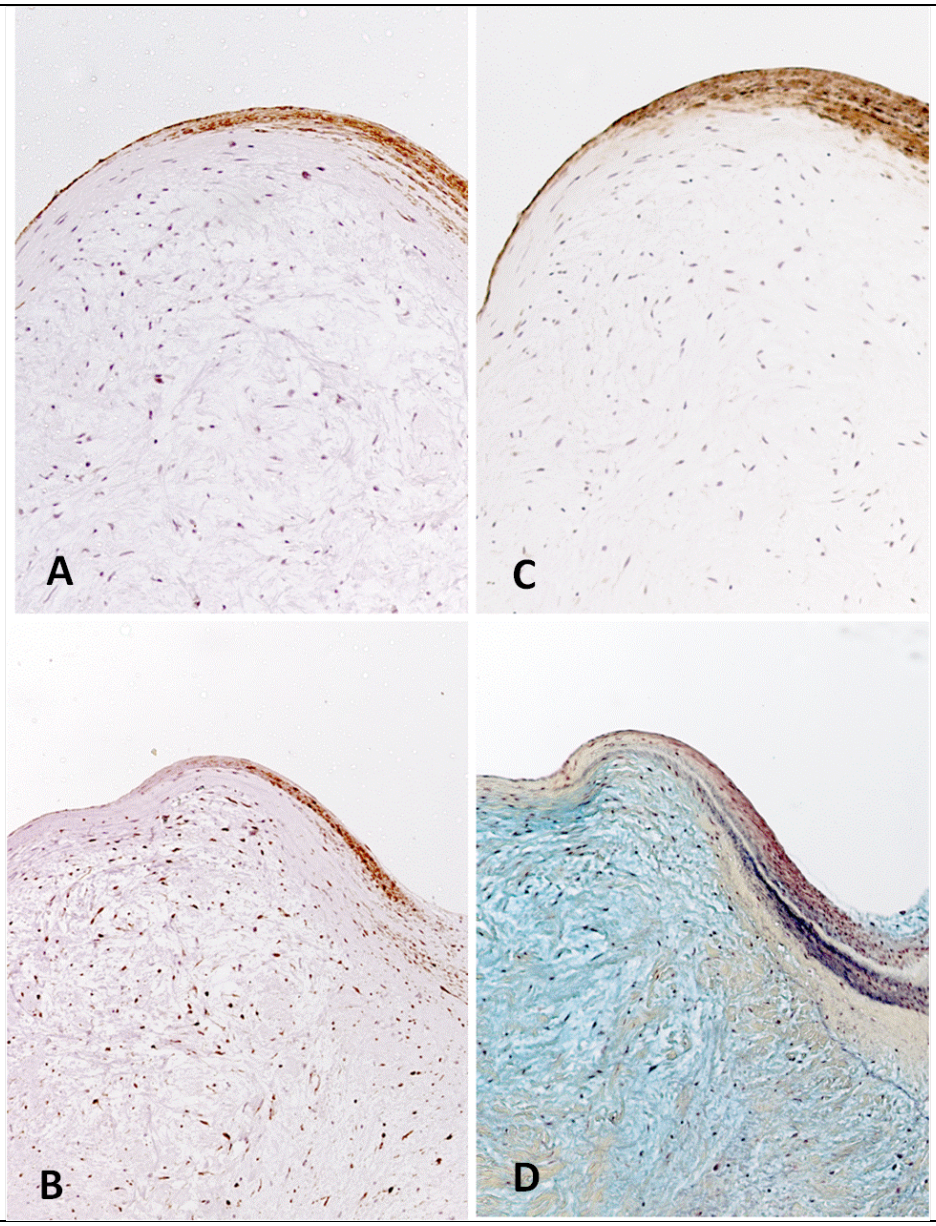


**Figure 5.5 Immunohistochemistry of Sequential Sections of a Severely Degenerative Mitral Valve Stained for CD31** (A), **HB-EGF** (B), **αSMA** (C), **and Movat Pentachrome** (D).
Brown staining on A shows CD 31 present on the endothelium progressing deeper into the valve matrix from left to right. HB-EGF and αSMA are also present in this area, and the blue staining of GAG found in D as well as the whorled pattern of the interstitial cells indicates that below this area is more evidence of degenerative disease. For Movat staining, collagen = yellow, glycosaminoglycan = blue, elastin = black. From right to left, the disruption of the black elastin layer of the atrialis is also an indicator of degenerative change. Magnification 10x.

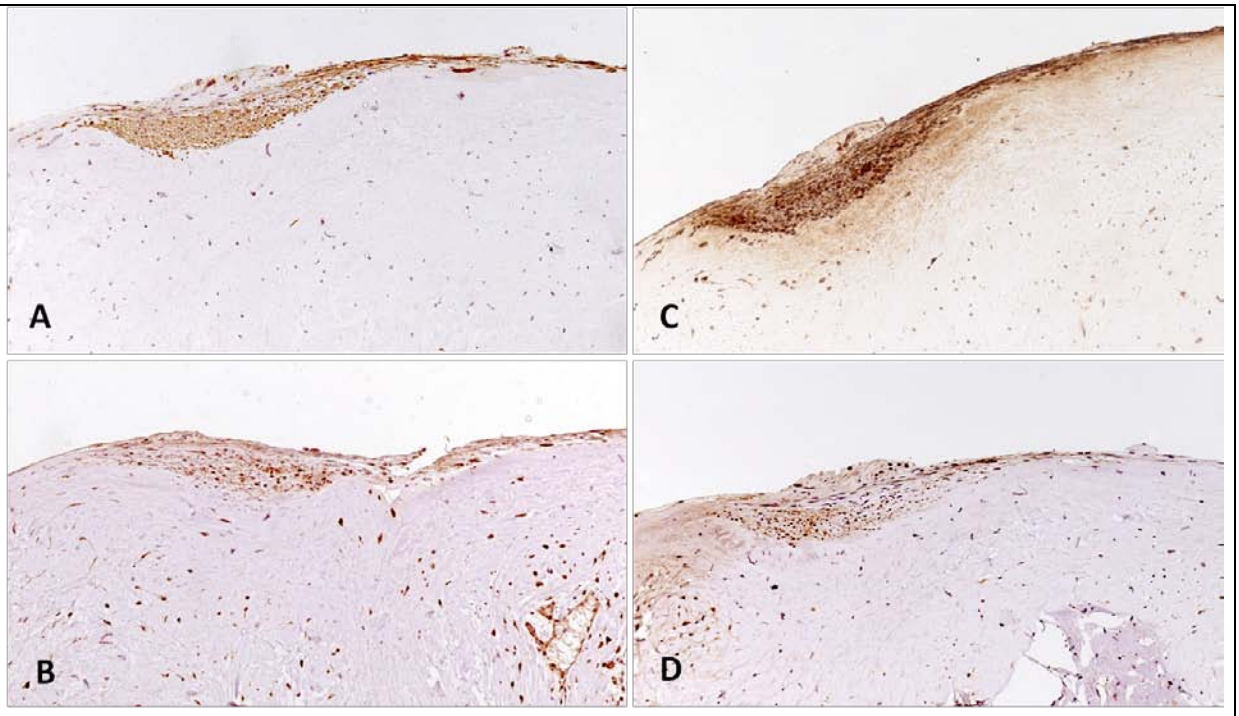


**Figure 5.6 Immunohistochemistry of Sequential Sections of a Severely Degenerative Mitral Valve Stained for CD31** (A), **HB-EGF** (B), **αSMA** (C), **and Integrin β3** (D).
Positive staining of this area (dark brown) indicates co-localization of these molecules of interest. Magnification 10x.

**Table 5.3 Semi Quantitative Analysis of HB-EGF and ADAM 17 Immunohistochemistry Staining in Canine Mitral Valves**

| | HB-EGF | ADAM 17 |
|---|---|---|
| Healthy group mean | 1.88 | 2.00 |
| Intermediate group mean | 2.67 | 1.83 |
| Severe group mean | 2.43 | 3.00 |
| Kruskal-Wallis test p value | 0.2036 | 0.1683 |

The group differences were not significant because the p values were larger than .05

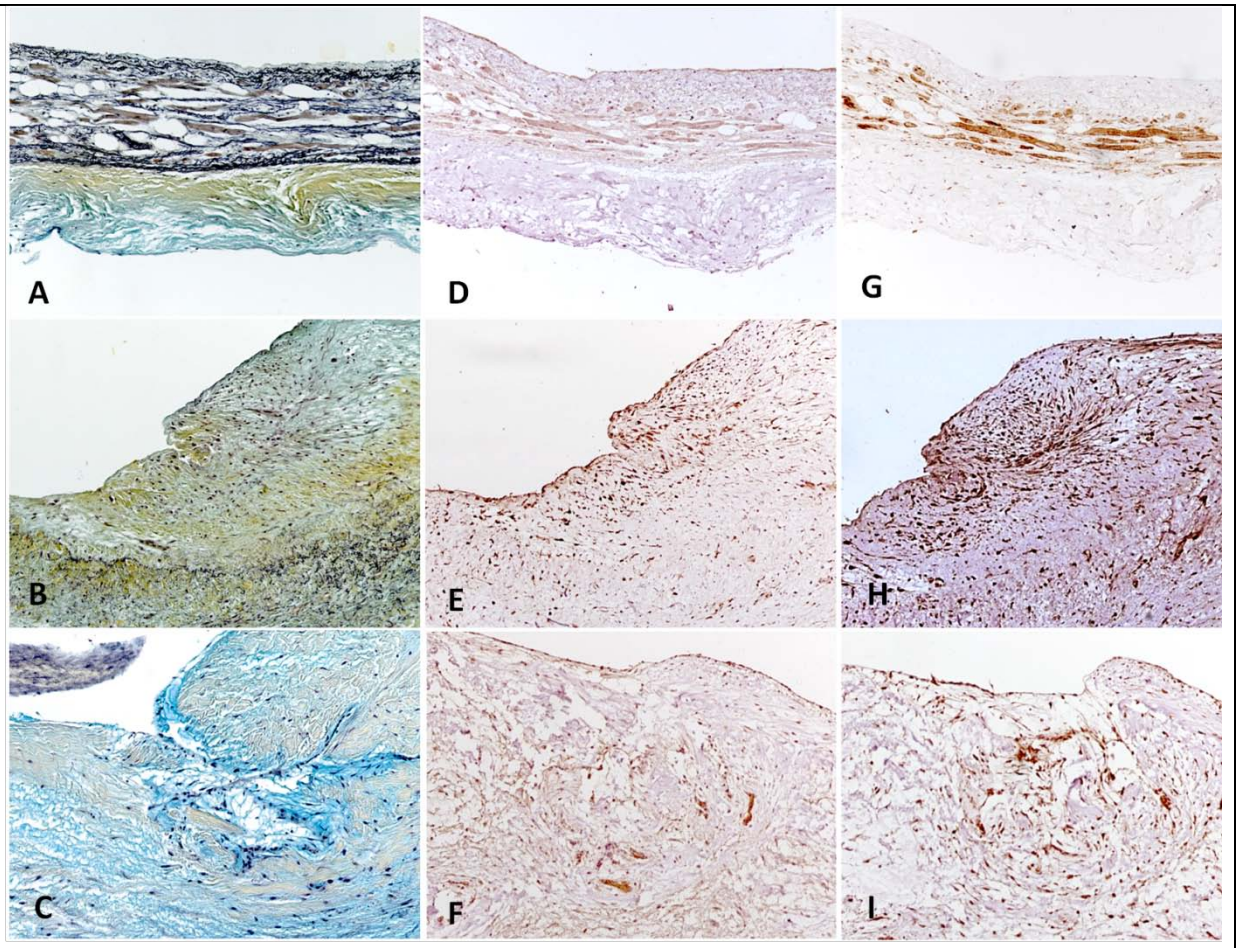


**Figure 5.7 Movat** (A-C)**, ADAM 17**(D-F)**, and HB-EGF**(G-I) **Immuno-Staining of Normal**(A,D,G)**, Intermediate Degenerative** (B,E,H)**, and Severely Degenerative**(C,F,I)**Mitral Valves**.
For Movat staining, Collagen = yellow, Glycosaminoglycan = blue, elastin = black. Clear layers are visible in A, less distinct in B, and nonexistent in C which has distinct blue GAG buildup. D has minimal positive brown staining, while the presence of ADAM17 is markedly increased on the surface of E and deeper in the matrix of F. HB-EGF appears to be present in organized streaks in G. In both H and I there is dark staining consistent with ADAM17 leading from the surface down into the matrix. Magnification 10x.

*Discussion*

As reported previously, degenerative mitral valves exhibited cellular clusters of αSMA positive interstitial cells consistent with myofibroblast transformation. Although not quantified in this study, these abnormal areas subjectively had higher cell density compared to the surrounding tissue and compared to normal mitral valves. Other studies have not been able to identify evidence of active cell division based on sparse staining of the marker of active dividing cells, Ki67[21,49,58]. Therefore, a mechanism for increased cellular density has not yet been identified.

As expected the cells on the valve surface were positive for the endothelial cell marker, CD 31. Many valves showed areas of negative staining for CD31 on the valve surface consistent with denudation of the endothelium. It is not clear from this study whether endothelial denudation was an ante-mortem pathological change or a post-mortem artifact associated with valve processing. The fact that both normal and degenerative valves showed evidence of endothelial denudation is more supportive of the latter interpretation. An important finding of this study was the presence of CD31 positive cells deep within the valve interstitum of degenerative mitral valves. These CD31 positive cells were not associated with vessels. This finding supports the presence of endothelial cells that have migrated into the valve interstitum and provides evidence that EndMT is occurring in degenerative mitral valves. As interstitial CD31 positive cells were strongly associated with areas of high cell density myofibroblasts further supports EndMT and provides a possible mechanism for the increase in cellular density in these areas.

Interstitial CD 31 staining was more often seen in severely degenerative valves than in intermediate valves or normal valves, but was still found in some of these samples. This suggests that EndMT could be an early cellular change in the disease process which progresses with severity.

ADAM17, HB-EGF, and integrin β3 all showed increased staining in degenerative valves compared to normal valve samples. While it is difficult to specify the extent to which each of these proteins affects degenerative valve disease, understanding the roles that they are known to play in cell interactions yields some interesting possible explanations which support the EndMT hypothesis.

The co-localization of CD31 and other proteins targeted such as HB-EGF, ADAM 17, Integrin β3, and αSMA provides further evidence that EndMT is occurring. First, αSMA staining identifies areas of cellular transformation where myofibroblasts become clumped and descend into deeper cell layers beneath the atrialis [21]. This cellular activity is a possible result of EndMT. Next, the presence of integrin β3 in these areas as either a marker of cellular migration or a product of activated smooth muscle cells [56,59] further indicates cellular phenotype transformation. In addition to these markers, the cell signaling proteins HB-EGF and ADAM 17 present a possible initiating signal for EndMT. These proteins have previously been identified in EndMT in mitral valve development [34,36,60] and therefore the possibility of reactivating their signaling in the diseased state seems reasonable.

It is unknown if activated EGFR signaling interacts with other possible initiators of EndMT, like TGFβ signaling. Both of these receptors are influenced by integrins, and TGFβ's

receptor requires modulation of integrin β3 to induce EndMT[42]. In addition, the non-canonical pathway of TGFβ signaling has the same cellular reaction as EGFR signaling, that is the activation of ERK1/2 and subsequent production of MMPs[26]. Also, if TGFβ is initiating EndMT in degenerative mitral valves, increased levels may be cause by serotonin signaling (Figure 2.2).

While some of the demographics of the dogs used in this study varied between groups, such as the dog weights and ages, effort was made to choose samples which were similar to the control group to minimize the chance of differences being the consequence of size, age, and breed variability. Because of this, the valves should be reasonably comparable and histological differences should be attributed to the valves' disease state. Also, 14 of the 21 dogs used in this study belonged to breeds designated as having a higher risk of MMVD by Table 1 from a study by Parker et al. [7]. Therefore most disease changes in the sample valves should be consistent with previously described canine DMVD.

*In conclusion* this study demonstrated that cells expressing the endothelial marker CD31 are present beneath the normal surface layer in both clumps and individual cells in degenerative valves. This finding suggests that endothelial cells have migrated into the valve interstitum in a pattern resembling the EndMT process apparent in developing valves and provides the first evidence of EndMT in mitral valve degeneration. This process could explain the increased cellular density in degenerative areas on mitral valves EndMT in degenerative mitral valves may be influenced by integrin β3 or may be initiated, as in the developing valve, by EGFR ligands such as HB-EGF.

# Chapter 6

## Conclusions and Future Directions

Results of the above studies highlight the relevance of certain signaling molecules and cell markers in degenerative mitral valve tissue. While searching for an explanation for the areas of increased cell density in degenerative valves described in previous literature, the hypothesis of cellular movement and migration from specific signaling patterns was generated. The array of signaling proteins provided a baseline for the investigation of molecules that had significance to DMVD, and analysis of these proteins' functions helped support the theory that EndMT contributes to the areas of increased cell density. The notably increased abundance of the molecule integrin αvβ3 supported the hypothesis of cell movement and migration because it is upregulated in actively moving cells, and it has also been described to be upregulated in EMT. The array also revealed molecules whose increased abundance could be responsible for altered signaling in degenerative valves. These molecules, the EGFR ligand HB-EGF and its partner molecule ADAM17, have known roles in EndMT in early developing valves, and therefore support another theory of DMVD, that of the recurrence of developmental pathways in the degenerative process.

The immunohistochemical study results show a more complete picture of where endothelial cells are located in normal and degenerative valves as well as their relationship to activated myofibroblasts. This information shows a direct correlation between endothelial cells migrating into the interstitum via EndMT and areas of increased cell density populated by αSMA positive myofibroblasts. Therefore the structural change of abnormal cell clusters typical of degenerative valves appears to be caused, at least in part, by EndMT. While EndMT is a

complex process that could be initiated by many different mechanisms, the co-localization of HB-EGF and ADAM17 with these degenerative areas indicates that the EGFR signaling cascade plays a role.

This study provides first evidence of EndMT in degenerative mitral valve disease. However, this information seems to bring up more questions than it answers. For example, what is the temporal relationship between EndMT and the structural changes that occur in diseased valves? Does it originally contribute to nodule formation or is it a healing and remodeling attempt by the tissue as a result of increased strain on the valve after it has already degenerated and developed a regurgitant leak? Also, perhaps most importantly, can it be altered by blocking signaling to slow degenerative valve changes?

All these important questions have the potential to be answered in future experiments. Future studies should start with the staining of cells with both αSMA and CD 31 with different indicator colors. This would serve as more definitive evidence that cells are transforming because they would be expressing both endothelial and mesenchymal markers. If samples could be compared to the timeline of DMVD more definitively with additional history of the progression of valve disease in the patients such as echocardiograms of all the animals, it could provide more indications of how soon EndMT is happening after degeneration starts.

Next, using a method such as lineage tracing would be a way to track the movement of individual cells while they are still alive in tissue culture and determine how quickly EndMT happens in valves. Variables that influence EndMT could be modified, and their effects quantified. In this way HB-EGF could be tested to determine if increasing its concentration or

that of ADAM17 has a positive effect on EndMT, further supporting the hypothesis presented above that it is a primary initiator of the mechanism in DMVD. Monoclonal antibodies that block EGF signaling by not allowing HB-EGF to bind could also be used to sort through the causes and effects of these signaling processes.

In addition to these proposed studies, an experiment using an inducible model of DMVD such as a cyclic valve stretcher could be performed with the goal of determining if strain induces EndMT and if so, which molecules initiate it. This would provide valuable information that could lead to explanations of the underlying cause of degenerative mitral valve disease in dogs.

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