THE ROLE OF BONE MORPHOGENIC PROTEINS IN HUMAN AORTIC VALVULAR ENDOTHELIAL CELLS

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Knowledge is proud that he has learned so much; wisdom is humble that he knows no more.

William Cowper (1731 AD - 1800 AD), *Discourses*

To Mom and Dad for the love and the support
To Aaron and Marie for the laughter
To Grandpa for the motivation

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LIST OF SYMBOLS AND ABBREVIATIONS

ALK	Activin like receptor
AV	Aortic Valve
BMP	Bone morphogenic protein
BMPR	Bone morphogenic protein receptor
CDK2	Cyclin dependent protein kinase 2
CV-2	Crossveinless 2
EC	Endothelial Cell
EMT	Epithelial-to-mesenchymal-transition
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular Signal regulated kinase
FGF4	Fibroblast growth factor 4
GAG	Glycosoaminoglycans
H&E	Hematoxalin and eosin
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
ICAM1	Inter-cellular adhesion molecule 1
JNK	c-Jun-N-terminal-kinase
KLF2	Kruppel-like factor-2
LDL	Low density lipoprotein
LS	Laminar Shear
MCP1	Monocyte chemoattractant protein 1
MGP	Matrix Gla protein

MMP	Matrix metalloproteinases
NFκB	Nuclear factor-κB
NO	Nitric oxide
Nrf2	NF-E2-related factor 2
OS	Oscillatory Shear
PAEC	Porcine aortic endothelial cells
PAVEC	Porcine aortic valve endothelial cells
PBS	Phosphate buffered saline
RAAVE	Rosuvastatin Affecting Aortic Valve Endothelium
ROS	Reactive oxygen species
SALTIRE	Scottish Aortic Stenosis Lipid-Lowering Therapy, Impact on Regression
SEAS	Simvastatin and ezetimibe in aortic stenosis
siRNA	Small interfering RNA
TGFβ	Transforming growth factor β
TGFβR	Transforming growth factor β receptor
TNFα	Tumor necrosis factor-α
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIC	Valvular interstitial cell

SUMMARY

In the United States alone, there are nearly 49,000 aortic valvular repairs or replacements each year, and this number is expected to rise. Unlike atherosclerosis, the molecular mechanisms contributing to this side-dependent disease development are limited, which contributes to the lack of therapeutic treatments. Once clinically manifested, options for treatment are limited to valvular replacement or repair. Therefore understanding the mechanobiology and cellular responses in aortic valves may provide important information for disease development and possible biomarkers or therapeutic treatments.

Aortic valve disease occurs on one side of the valvular leaflet. The fibrosa side, which faces the aorta, is prone to disease development, while the ventricularis remains relatively unaffected. The hemodynamics is hypothesized to play a role in side dependent disease formation. The fibrosa endothelium is exposed to oscillatory flow while the ventricularis endothelium is exposed to a pulsatile unidirectional flow. Previous work by Dr. Hanjoong Jo's research group has shown that bone morphogenic protein-4 is a mechanosensitve inflammatory cytokine in the vasculature. In the following study, I proposed that mechanosensitive bone morphogenic proteins play a role in side specific aortic valve disease.

Recently, the bone morphogenic proteins (BMPs) have been found in calcified human aortic valves. Furthermore, BMP-4 in vascular endothelial cells is increased by oscillatory shear stress. However, the role of the BMPs in aortic valve endothelial cells and their contribution to aortic valve calcification remains unstudied. Therefore**, the**

overall *objective* of this dissertation was to investigate how disease and hemodynamics affects the BMP pathway and inflammation in human aortic valvular endothelial cells. By understanding how the bone morphogenic proteins are regulated and what roles they play in aortic valve disease, we will have better insight into endothelial cell regulation and contribution in aortic valve pathology. **The *central hypothesis* of this project was that oscillatory flow conditions on the fibrosa side of the aortic valve stimulate endothelial cells to produce BMP-4, which then activates an inflammatory response leading to accumulation of inflammatory cells, calcification, and ultimately valve impairment.** This hypothesis was tested through three specific aims using calcified human aortic valves, non-calcified human aortic valves, and side-specific human aortic valve endothelial cells.

I first worked to establish the importance of the BMPs in the aortic valvular endothelium by looking at two populations of aortic valves: 1) calcified human aortic valves were obtained from patients undergoing valve replacement, and 2) non-calcified valves were obtained from recipient hearts of patients undergoing heart transplantation. Using immunohistochemical techniques, I examined the BMPs, BMP antagonists, and SMADs. Surprisingly, I identified that the ventricularis endothelium had higher BMP expression in both calcified and non-calcified human aortic valves. Furthermore, no disease-dependent BMP expression was detected. Next, I examined the BMP antagonists and found that there was robust BMP antagonist expression in the ventricularis endothelium and very low expression in the fibrosa endothelium. Finally, to determine if the BMP pathway was activated, I stained for the canonical BMP signaling molecule phosphorylated-SMAD 1/5/8 and found increased staining in the endothelium of calcified human aortic valves. Furthermore, a significant increase in SMAD 1/5/8 phosphorylation was seen in the endothelium of calcified fibrosa when compared to the non-calcified

fibrosa. Finally, inhibitory SMAD 6 was significantly increased in the ventricularis endothelium of non-calcified human aortic valves. These findings suggest that preferential activation of BMP pathways, controlled by the balance between the BMPs and their inhibitors, play an important role in side-dependent calcification of human AVs.

I next wanted to examine the role of shear stress in BMP regulation, but before doing so, I needed to examine the endothelial response to fluid shear stress to validate the phenotype of my isolated human aortic valve endothelial cells. KLF2 and eNOS expression in vascular endothelial cells has been shown to be increased by laminar flow and to have anti-inflammatory effects by decreasing VCAM-1 levels. Conversely, oscillatory shear stress has been shown to increase NFkB translocation and increase ICAM-1 and E-selectin. I found laminar shear stress causes human aortic valve endothelial cells align parallel to flow and have robust increases of KLF2 and eNOS and decreases in VCAM-1 levels; however, laminar shear-treated cells had similar levels of NFκB activation as oscillatory treated cells while ICAM-1 and E-selectin was not affected by shear stress. In contrast, oscillatory shear had higher levels of monocytes bound which may be due to eNOS's protective effects under laminar shear and robust VCAM-1 expression in oscillatory shear. These studies suggest differential regulation of human aortic valvular endothelial cells than published reports on human aortic endothelial cells which adds to the growing evidence that valvular endothelial cells are phenotypically different than vascular endothelial cells.

After verifying the shear response of my endothelial cells, I next determined the shear response of the BMPs and BMP antagonists and described BMPs' effect on inflammation. Previously, BMP-4 has been shown *in vitro* and *in vivo* to be increased in endothelial cells exposed to oscillatory flow, while the closely-related BMP-2 has not

been shown to be shear sensitive. In this study I have found that BMPs -2 and -4 are shear sensitive while BMP-6 is not. Furthermore, I have found that follistatin is decreased by laminar flow only in the ventricularis, while MGP1 is decreased in the fibrosa valvular endothelial cells under both oscillatory and laminar flow. Finally, incubation with noggin did not affect monocyte adhesion after shear, suggesting differential regulation of inflammation in human aortic valvular endothelial cells.

By addressing the specific aims of this project, I have investigate disease- and side-dependent valvular endothelial BMP expression *in vivo*, shear regulation of valvular endothelial inflammation *in vitro*, and shear regulation of valvular endothelial BMP expression *in vitro*. My results suggest that the BMP pathway is playing a role in side specific aortic valve disease development; however, regulation of the BMPs does not appear to be shear regulated *in vivo*. Other factors that may be affecting BMP production include including pulsatile pressures, bending stresses, cyclic stretch, and humeral stimuli present in the blood of the patients. However, *in vitro* I have found BMPs -2 and -4 to be shear-regulated in human aortic valvular endothelial cells. Shear-induced inflammation in human aortic valve endothelial cells seems to be VCAM-1-dependent, and BMP-independent. Finally, by identifying factors that are modulated in calcific- and shear-dependent processes, new targets for the early detection of aortic valve disease can be determined and new therapeutics to slow or stop the progression of aortic valve disease may be discovered.

CHAPTER 1

INTRODUCTION

Significance

Aortic valve (AV) disease is a major cause of cardiac related deaths worldwide and is also a strong risk factor for additional cardiac deaths [1-3]. Valvular disease is characterized by the development of stenosis, narrowing of the valve opening area, and/or insufficiency, incomplete closure of the valve, and by the time it is clinically manifested, it is only treatable by valve replacement or repair [4]. With the aging United States population, the most common cardiac disease is calcified aortic valve disease, which is estimated to affect 20% of the population 80 years or older [5]. In 2009, there were nearly 93,000 valve procedures performed in the United States, with 53% of those procedures done on the aortic valve position [6]. Furthermore, worldwide heart valve replacement surgeries are estimated to triple from 290,000 in 2003 to over 850,000 by 2050 [7]. Health care costs due to this disease is estimated to be about 1 billion US dollars annually [8].

Aortic Valve Function and Structure

The aortic valve separates the aorta from the left ventricle. The valve is comprised of three leaflets named for their position in respect to the coronary arteries: the left, right and non-coronary leaflets. The aortic valve cusp is comprised of three distinct layers. The fibrosa, which faces the aorta, is comprised of circumferentially aligned collagen fibers with few elastin fibers [9] and is believed to be the main load-bearing component of the valve (Figure 1.1) [10]. The ventricularis, which faces the left ventricle, is comprised of

radially-aligned elastin fibers with some collagen fibers in random orientation (Figure 1.1) [11]. The spongiosa, which separates the ventricularis from the fibrosa, is comprised of glycosaminoglycans (GAGs) and water (Figure 1.1). The elastin has been described as an organizer of the collagen fibers in the valve, while collagen fibers provide mechanical strength for the valve [10].

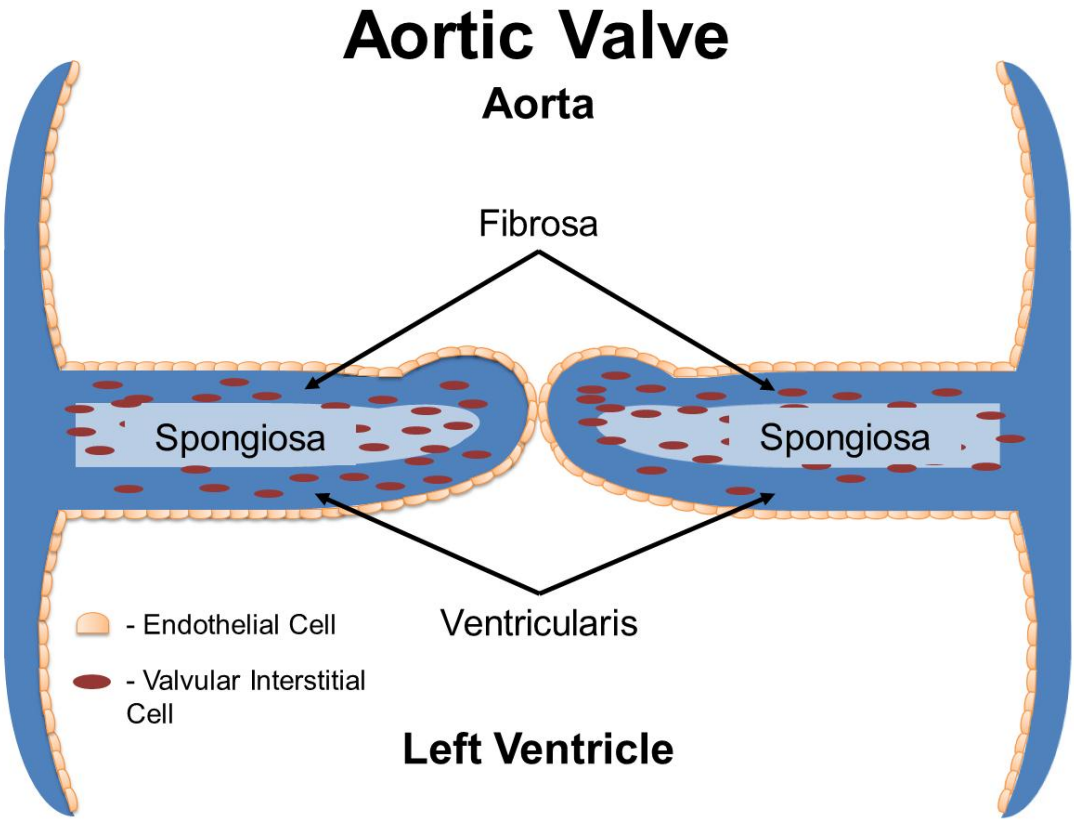

Figure 1.1 Structure of the Aortic Valve. Aortic valve is made of three layers: The fibrosa, the spongiosa and the ventricularis.

Aortic valve interstitial cells (VICs) reside in all three layers. VICs are comprised of several different cell types, including myofibroblasts, fibroblasts and smooth muscle cells (Figure 1.1) [12]. The myofibroblasts, which are characterized by the stress fibers and smooth muscle α-actin, are the most common interstitial cell in diseased valves and are thought to be involved remodeling and regulation of the extracellular matrix [13-14]. Finally,

endothelial cells, which will be described in more detail below, surround and provide a non-thrombogenic barrier for the valve.

Valvulogenisis

Many of the components involved in aortic valve degeneration are also involved in valvulogenesis. There are four stages to valvular formation: endocaridal cushion formation, epithelial-to-mesenchymal transition (EMT), valve primordia, and diversification of cell types / maturation [15]. These stages are described below.

Endocardial Cushion Formation

The first signs of valvular formation in the human embryo occur between embryonic day E31 to E35, with the formation of the endocardial cushion [16]. Bone morphogenic proteins (BMPs) -2 and -4 are major myocardial-derived signals in the formation of the endocardial cushion [17-18]. In the myocardium, BMP expression drives the secretion of hyaluronan and versican, the two components of the cardiac jelly [18-19]. Mice lacking BMP-2 fail to form a proper endocardial cushion because of the lack of extracellular matrix deposition [19-20]. During this time, the endocardium is producing vascular endothelial growth factor (VEGF) which causes endothelial proliferation [15].

EMT

EMT occurs when a portion of the endothelial cells from the endocardium dissociate, move into the cardiac jelly, and turn into a mesenchymal cell type. Numerous proteins have been associated with EMT including BMP-2, transforming growth factor beta (TGFβ), Notch-1, and WNT/β-Catenin [15, 21-22]. All have been shown to play essential roles in EMT in valvulogenisis.

Valve primordia

After EMT and during valve primordia, proliferation of the mesenchymal and endothelial cells occurs. The endothelium proliferates through a VEGF- and extracellular signal regulated kinase (ERK) -dependent processes while the mesenchymal cells proliferate through Wnt/β-catenin, TGFβ, BMP and fibroblast growth factor-4 (FGF-4) signaling [23-27]. Interestingly, mice lacking BMP-6 and -7 develop hypoplastic valves, while mice lacking inhibitory SMAD-6 (inhibitor of BMP signaling) develop hyperplasia demonstrating the balance in BMP signaling necessary for proper valve development [28-30].

Diversification of cell types / maturation

Little is known about the diversification of cell types in the developing aortic valve, however hemodynamics is thought to play a pivotal role [15]. Notch-1 signaling has been localized to the ventricularis side of the valvular leaflet, and is hypothesized to be regulated by shear stress; however, this has not been shown [31-32]. In the spongiosa, BMP-2 signaling is prominent and it drives production of the transcription factor SOX9 and aggrecan in the spongiosa [33]. Less is known about the fibrosa; however, it is hypothesized that Wnt signaling predominates fibrosa maturation [27].

Aortic Valve Diseases

There are two clinical categories of aortic valve diseases that can occur in concert or independently. Aortic stenosis occurs when the maximum valve opening area (experienced during systole) decreases. This causes a larger pressure drop across the valve and when severe, may cause hypertrophy of the heart in order to pump sufficient blood to the body [34]. The causes of aortic stenosis include congenital defects, calcification [35], or bacterial infection of the valve [34]. Diagnosis of aortic stenosis is made when a heart murmur is observed. Patients may experience shortness of breath, chest pain and dizziness [34]. Once diagnosed and if stenosis is severe, the patient can undergo aortic valve replacement.

The second category of aortic valve disease is aortic regurgitation or incompetence. During diastole, in a healthy aortic valve, the three leaflets provide a barrier that stops blood flowing from the aorta into the left ventricle. In patients who have aortic valve regurgitation, the leaflets do not provide an adequate barrier. When the left ventricle is filling, the higher pressure in the aorta causes reverse blood flow across the aortic valve. This change in hemodynamics causes stretching of the heart cavity and hypertrophy of the left ventricle [34]. Causes of this disease include bacterial infection, ischemic heart disease, calcification and congenital defects [34]. As with aortic stenosis, a patient with severe aortic regurgitation may undergo aortic valve replacement [34].

Aortic Valve Calcification

Until recently, aortic valve calcification was thought to be an age-associated disease [36-37], but it is now believed to be an active inflammatory process that is characterized by lipid accumulation, neovascularization, inflammation, calcified nodules and, in some cases, the formation of lamellar bone [36, 38].

Risk Factors

There are many similarities between the pathogenesis of atherosclerosis and aortic valve calcification including potential risk factors for disease development. Risk factors associated with aortic valve disease and atherosclerosis include the increase in age, the male gender, cigarette smoking, diabetes mellitus, hypertension, high total cholesterol, raised low density lipoprotein (LDL) cholesterol, raised triglycerides, low high density lipoprotein (HDL) and raised lipoprotein (a) [39-43]. Other risk factors associated with aortic valve disease include bone diseases such as Paget's and hyperparathyroidism, uraemia and raised serum creatinine and calcium levels [41-42, 44-45].

Genetics

There have been several studies looking at how genetics may be playing a role in calcific aortic valve disease. Increases in promoter polymorphisms in interleukin-10, an anti-inflammatory cytokine, has been associated with calcium content in calcified human aortic valves [46]. Studies have indicated that polymorphisms in the vitamin D receptor are associated with increase calcific aortic valve stenosis while decreasing bone density. It is hypothesized that these polymorphisms are causing calcium mobilization from the bone which then can promote ectopic calcification in the aortic valve [47]. A nonsense mutation in the Notch1 gene causes developmental defects and also plays a role in aortic valve calcification progression [31]. Notch1 is a repressor of Runx2 which is an important osteogenic transcription factor in osteoclasts. It is hypothesized that the defect in Notch1 may an increase in Runx2 and calcium deposition in the aortic valve [31]. Research has also shown that cell cycle genes may also be regulated in aortic

valve calcification. Expression of the p21 gene, a repressor of CDK2 and cell movement from G(1) to G(2), is decreased in calcified human aortic valves, suggesting cell cycle control may be involved in aortic valve sclerosis [48].

Lipids and Statin Treatment

Lipid deposits are often found on diseased valves and are believed to be an initiation point of calcification [49-50]. *In vitro* studies have found several important roles of lipids in the development of aortic valves. Cholesterol has been shown to aid in the precipitation of calcium crystals [51], while 25-hydroxy cholesterol accelerates valvular calcification [52]. Oxidized lipids are believed to stimulate inflammation in a similar manner to the mechanisms seen in atherosclerosis through membrane scavenger receptors [53].

Statins are a class of inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA). By inhibiting HMG-CoA reductase, statins decrease cholesterol production, have anti-inflammatory effects, and preserve endothelial function [54]. In rabbits fed an atherogenic diet, atorvastatin reduces progression of aortic valve calcification through Lrp5 and endothelial nitric oxide (eNOS) pathways [55-56]. Atorvastatin also decreases alkaline phosphotase activity in interstitial aortic cells that have been incubated in an osteogenic media [57]. In the aortic valve, studies have shown that statins limit the formation of calcified nodules and alkaline phosphotase of aortic valve myofibroblasts; however, in osteoblasts statins increase alkaline phosphotase [58]. These data suggest that timing of statin treatment may play an important role in aortic valve disease [36].

Statin treatment in calcific aortic valve disease has had varied outcomes. Three clinical studies have investigated statin's effect on aortic valve disease. The first study performed by the Scottish Aortic Stenosis Lipid-Lowering Therapy, Impact on Regression (SALTIRE), was a prospective study investigating aortic valve stenosis progression and use of atrovastatin. The primary endpoints were aortic-jet velocity and aortic valve calcium score. They found a trend showing slowing in stenosis of the aortic valve, but it was not significant. They concluded that lipid-lowering therapy does not halt progression of aortic valve stenosis [59].

In second clinical study, Rosuvastatin Affecting Aortic Valve Endothelium (RAAVE) trial, aortic valve area decreased in statin-treated and untreated patients; however, the statin-treated patients had half the disease progression of untreated patients, suggesting rosuvastatin has a protective effect. It is important to note that patients in this group had higher aortic valve opening areas than the SALTIRE study, suggesting an earlier stage in aortic valve disease development [60].

The final clinical study, simvastatin and ezetimibe in aortic stenosis (SEAS), was a large scale study (1,873 subjects) looking at the long term (52 months) cardiovascular outcomes of patients receiving simvastatin and ezetimibe. They found no difference between placebo or the statin-treated patient when looking at the end-point of aortic valve-related and cardiovascular events; however this study did find that treatment decreased risk for ischemic events [61].

Based on these studies, it has been concluded that statins may have a beneficial effect on the slowing of aortic valve disease; however, it has not been directly

shown. It is hypothesized that the timing of application of statins may play an important role in its efficacy [36]. Furthermore, its maximal effect may be during the early stages of aortic valve disease progression [36]. Recent studies have supported this hypothesis by showing, in a retrospective study, that statins can reduce aortic valve stenosis only in mild degrees of disease. When disease is more severe, statins had no effect [62].

Neovascularization

Neovascularization in heart valves is a necessary condition for calcification to occur, although it is not sufficient [63]. One reports has found neovascularization in all heart valves that have undergone ossification [64]; however, two reports have shown that vascular density was higher in aortic valves with low to medium calcification, while low vascular density was found in severely stenotic valves [65-66]. The neovessels provide oxygen and nutrients to cells that are beyond the diffusion distance of oxygen. They provide angiogenic factors that have effects on calcifying cells [67], and they also can secrete cytokines, including BMP-2 and BMP-4, that cause osteoprogenitor cells to differentiate in certain contexts [68-71]. Matrix modifying enzymes appear to be important in the neovascularization of these valves. Reports have shown cathepsins V, S, and K to be present in neovessels and to be increased in calcified human aortic valves[72], and further studies have implicated chondromodulin 1 as a necessary factor for the formation of neovessels [73].

Inflammation

As in atherosclerosis, inflammation is an important hallmark of disease development. Sclerotic and calcified aortic valves show an increase in the

inflammatory molecule TGF-β1, matrix tumor necrosis factor-α (TNFα) and also an increase in T-cells and monocyte infiltration [74-78], suggesting an active inflammatory process rather than a passive one [79]. Studies have also shown an increase in the level of vascular cell adhesion molecule 1 (VCAM-1), inter-cell adhesion molecule 1 (ICAM-1) and E-selectin in diseased valves when compared to healthy valves, suggesting endothelial dysfunction that may be caused by aortic valve calcification risk factors [80-81]. Further studies have found that patients with aortic valve calcification have higher levels of circulating E-selectin, and when the calcified valve is replaced, circulating E-selectin levels returns to normal [82]. Further, research investigating the inflammatory Toll-like receptors 2 and 4 found active receptors in normal aortic valve. Activation of these receptors can cause nuclear factor-κB (NFκB) pathway activation while also increasing inflammatory and osteogenic markers [83].

Calcification

Calcium deposition in the aortic valve is a significant indicator of valve disease progression. Most cases of aortic valve calcification are idiopathic in nature [84]. Calcified deposits in the aortic valve primarily occur beneath the fibrosa endothelium [75]. Through immunohistochemical and protein studies, many factors have been shown to be upregulated in calcified human aortic valves. These factors include bone related factors (osteopontin, osteonectin, matrix gla protein, bone morphogenic proteins 2 and 4 and TGF-β1) [64, 79, 85-86] and matrix regulatory factors (matrix metalloproteinases (MMP) 1, 2, 3, 9 and cathepsin S [72, 85, 87]. As previously noted, calcified deposits occur beneath the fibrosa endothelium. Because of its subendothelial presence, it is believed that the VICs present in the

interstitum are responsible for the calcification and are discussed in detail below [88].

Mechanical Forces and the Aortic Valve

The aortic valve is in a complex mechanical environment that includes fluid shear stresses, varying pressures and bending stresses [89]. Changes in one mechanical condition of the valve can cause structural and biological changes in the valve [90-93]. As an aortic valve becomes diseased, the diameter of the valve opening area decreases, and the shear stresses experienced by the valve can change. Clinically, this is manifested by the existence of a heart murmur [34]. In a healthy human heart, the heart beats at a frequency of about 1 Hz, and during diastole the aortic valve experiences a pressure of about 100 mmHg. Although, if the patient is hypertensive, the pressure felt by the aortic valve may be elevated to 200 mmHg [92]. Increases in pressures and frequencies have been shown to affect the biological properties of the valve [92-93]. By virtue of the ventricularis and fibrosa touching each other, the fibrosa during systole is under compression and experiences oscillatory shear stresses while the ventricularis is under tension and experiences pulsatile shear stress [94-96].

***In vitro* Models of Shear Stress**

To determine how fluid shear stress affects the endothelium, two devices have been developed for use in endothelial cell biology: the cone and plate viscometer and the parallel plate flow chamber. The cone and plate viscometer is a circular, Teflon cone with a very small angle (0.5°). When placed in a circular dish it can be rotated to produce fluid flow, and by using Navier-Stokes equations the shear stress profiles can be calculated [97]. The second device that can be used to produce fluid flow is the parallel plate. A roller pump is used to apply steady, uniform laminar flow to the endothelial cells

attached to a glass slide. The height, width, length and pressure drop determine the fluid flow rate and thus the applied shear stress to the endothelial cells [98].

There are draw backs and benefits for both systems. For example the cone and plate shear apparatus can expose many more cells to shear stress when compared to the parallel plate with its small glass slide. Although, this shear system results in a gradient of shear stress, cells at the center of the dish are exposed to lower shear stresses than the cells at the perimeter. Conversely, the parallel plate has a uniform shear stress gradient across the endothelial cell monolayer. Finally, the cone and plate shear apparatus can create a reversal of flow by rotating the cone back and forth. Therefore, this system can expose endothelial cells to both a unidirectional and oscillatory shear stress (Figure 1.2).

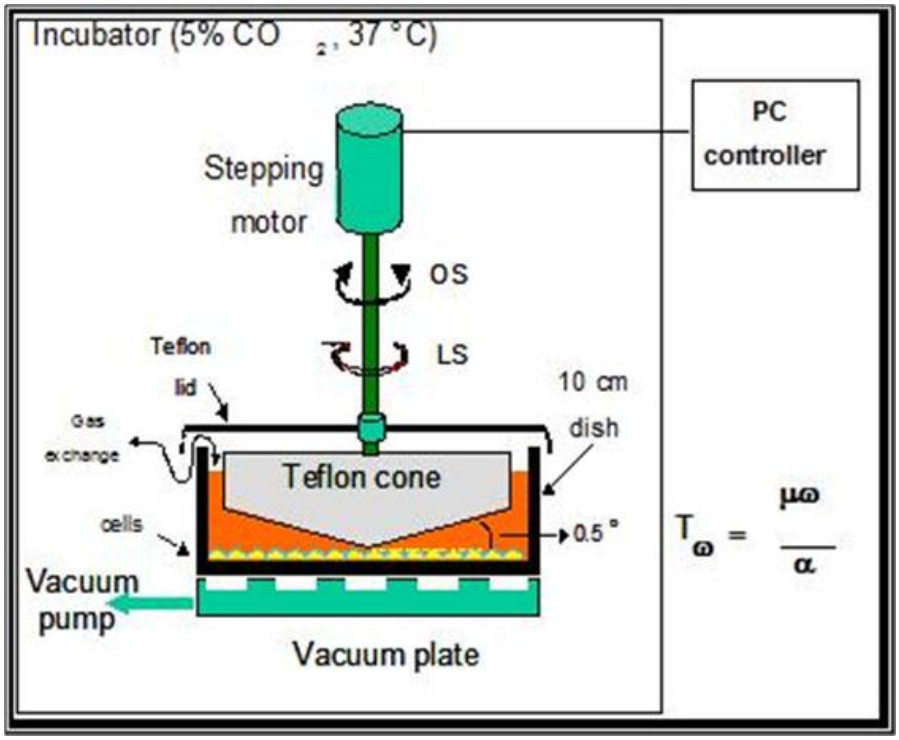

Figure 1.2 Schematic of the cone and plate shear apparatus.

Shear Stress and Mechanotransduction

As mentioned previously, aortic valve calcification preferentially occurs on the fibrosa of the aortic valve [75, 86]. The fibrosa and ventricularis are exposed to significantly different mechanical forces during the cardiac cycle. The ventricularis experiences a unidirectional pulsatile flow and a stretching force during systole, while the fibrosa is exposed to unstable hemodynamic flow and compression during systole [96]. The preferential disease development in aortic valve calcification is similar to the development of atherosclerosis. In the vasculature, atherosclerosis preferentially occurs in areas of unstable hemodynamic flow, while areas experiencing stable, unidirectional flow are atheroprotected [99]. Furthermore, researchers have found that areas that experience a low mean shear stress preferentially develop atherosclerosis [100].

At the cellular level, the endothelial cells exposed to blood flow feel a drag force, commonly called shear stress. Endothelial cells sense this force through a variety of mechanisms including the actin cytoskeleton, stretch-activated ion channels, G-protein coupled receptors, junctional proteins and integrins [101]. Through biochemical signaling pathways, the cell can change gene transcription, leading to various signaling pathways that may affect cell function and its surroundings [101].

Shear Stress and Inflammation in Vascular Endothelial Cells

When exposed to laminar flow, aortic endothelial cells align parallel to the flow and exhibit increases in atheroprotective genes such as Kruppel-like factor-2 (KLF2), KLF4, and eNOS [98, 102-107]; however, endothelial cells exposed to oscillatory flow express pro-inflammatory molecules such as monocyte chemoattractant protein 1 (MCP1), BMP-4 and inflammatory adhesion molecules [108-112]. Regulation of these inflammatory genes

has been linked to signaling molecules such as p38, ERK, NFκB, and c-Jun-N-terminal-kinase (JNK) [113-118].

In vascular endothelial cells, application of laminar shear causes a robust increase of KLF2 [107]. Interestingly, KLF2 was found not to be induced by other mechanical or biochemical stimuli in endothelial cells [107] and may regulate several anti-inflammatory genes including JNK [119]. Furthermore, KLF2 has been found in protective regions of the vasculature *in vivo* [120], has been found to be upstream of eNOS [121], and helps improve the function of nuclear localization of Nrf2, a powerful anti-oxidant gene transcription factor [122]. In vascular endothelial cells, eNOS is increased by unidirectional flow [123]. Its product, nitric oxide (NO), is a potent vasodilator, promotes vascular health, and is able to reduce intracellular oxidative stress [104-106]. Interestingly, in mice lacking eNOS have the propensity to develop bicuspid aortic valves and eNOS dysfunction was also found in patients with bicuspid aortic valve disease [124-125]. Studies have found that in calcified human aortic valves, increases in reactive oxygen species (ROS) surrounding areas of calcification, are in part due to uncoupled eNOS [126]. Furthermore, in an *in vitro* study, nitric oxide donors supplemented to porcine aortic valve interstitial cells blocked TGFβ-mediated calcified nodule formation [127]. Finally, mRNA analysis of porcine aortic valve endothelium found that eNOS was increased on the fibrosa endothelium when compared to the ventricularis endothelium [128].

High unidirectional shear stress has been shown to inhibit activation of JNK, p38 and NFκB [113-118], while oscillatory flow induces NFκB activation in vivo [129]. The NFκB family members are involved in many physiological and pathophysiological mechanisms including cell differentiation, inflammation, proliferation, apoptosis and atherogenesis [130].

NFκB is a key regulator in shear regulation of inflammatory genes, and the p50/p65 heterodimer binds to shear stress responsive elements [131]. p38 has been shown in to both enhance and repress NFκB activity in endothelial cells [132-133].

Shear Stress and Valvular Endothelial Cells

Valvular endothelial cells are phenotypically distinct from aortic endothelial cells. This finding was first noticed by pathologists observing the aortic valve. They noticed the endothelial cells were aligned perpendicular, not parallel, to the flow as seen in aortic endothelial cells [134]. Studies by Butcher et al. then showed that when exposed to unidirectional shear stress, porcine aortic endothelial cells align parallel to the fluid flow while porcine valvular endothelial cells align perpendicular to the flow. Through the use of immunofluorescent studies, it was also noted the actin cytoskeleton was perpendicular to the flow in porcine aortic valvular endothelial cells. Finally, the group determined alignment of valvular endothelial cells was dependent on Rho-kinase while aortic endothelial cells were dependent on phosphatidylinositol 3-kinase signal and Rho-kinase pathways [135].

Several studies have looked at transcription profiles of endothelial cells *in vitro* and *in vivo*. Peter Davies' research group investigated the mRNA content of pig aortic valve endothelial cells removed from the valve immediately after sacrifice using a special technique [128]. The results show different transcription profiles between the fibrosa and the ventricularis. Briefly, the fibrosa, which experiences unstable flow conditions, has a lower expression of inhibitors of calcification when compared to the ventricularis. Genes downregulated include osteoprotegrin, C-type natriuretic peptide, parathyroid hormone and chordin, which is an inhibitor of the bone morphogenic proteins. Furthermore, they

found that BMP-4, a pro-bone growth cytokine, has a higher expression on the fibrosa compared to the ventricularis endothelium; however, the study found that transcriptional profiles for many proinflammatory cytokines and adhesion molecules were not differentially expressed [128]. Further studies investigating how hypercholesterolemia affects the valvular endothelium in pig; found that BMP-4 mRNA was decreased in hypercholesterolemic pigs. The authors hypothesized that this difference was due to a protective response [136].

A final study that investigated flow dependent transcriptional profiles of aortic valve endothelial cells was performed [137]. The objective of the study was to compare porcine aortic (PAEC) to valvular endothelial cells (PAVEC) and their flow dependent gene expression. The group exposed aortic and valvular endothelial cells to a unidirectional shear stress of 20 dynes/cm^2 or static conditions for 48 hours, collected the mRNA and studied the changes in transcriptional profiles. They found that PAVEC were intrinsically less inflammatory and expressed more genes associated with chondrogenesis while PAEC expressed more osteogenic genes. Finally, they showed that shear stress had a protective effect against calcification [137].

Effects of Stretch on the Aortic Valve

As previously mentioned, hypertension is a strong risk factor for aortic valve disease (odds ratio 1.23-1.74) [138]. A recent study measuring the stretch of the porcine aortic valve in vitro has shown that in super-hypertensive conditions the aortic valve can reach 20% stretch [84], while under normal physiological conditions the aortic valve experiences 10% stretch during diastole [139]. Studies looking at pathological stretch have shown *ex vivo* stretch can alter valvular properties and cellular function. Balachandran et al. have shown that elevated stretch can alter matrix remodeling enzymes including, MMP-1, -2, -

9 and cathepsin S and K. Stretch also affected cellular proliferation and apoptosis [109]. Furthermore, studies have implicated the TGFβ and BMP pathway in stretch induced calcification. Porcine aortic valve leaflets were stretched in an osteogenic media with addition of TGFβ and formed calcified nodules if placed under 20% stretch. However, calcification was inhibited by the BMP antagonist noggin, suggesting BMP involvement in pathological stretch calcification (Balachandran et al. in press).

VICS, TGFβ, and *in vitro* Calcification

Progression of aortic valve disease, specifically sclerosis and calcification, is primarily mediated by VICs [88]. In a normal adult human aortic valve, the VICs are quiescent with little cell proliferation [140-141]; however, as the disease state increases the VICs become more synthetic [140, 142]. One of the predominate hypotheses is that the matrix stiffness and TGFβ play an important role in VIC pathophysiology [143-144]. Recent studies have shown that VICs placed on stiff substrates, mimicking the stiffness of sclerotic tissues, adopt myofibroblastic phenotype and form calcified nodules [139]. The primary mechanism of myofibroblast differentiation is believed to be the mechanical tension experienced by the cell and TGFβ [145]; however, if VICs are placed on a compliant substrate, calcified nodules formed but were the cells were less responsive to TGFβ and had more osteogenic marker expression. These results suggest differentiation of VICs is, in part, due to matrix stiffness[139].

Bone Morphogenic Proteins

BMPs were first found as a bone growth and repair molecule [146]. They are members of the transforming growth factor beta superfamily (TGF-β), which includes TGF-βs, inhibitins, bone morphogenic proteins, growth differentiation, activins and myostatin [147].

Currently, there have been over 30 different BMPs identified, while the BMP2/4 and BMP5/6/7 classes are the best characterized. BMPs are important in embryonic development, cartilage and bone formation, cell differentiation, valvulogenisis, and apoptosis [147-148].

BMP-4 is synthesized as a 408-amino acid precursor that is proteolytically cleaved by a pre-proconvertase [149-150] which then leaves a C-terminal mature protein (116 amino acids) that has seven conserved cysteine residues. BMP-4 is then secreted in its active form, must homodimerize before binding to its receptor [151].

BMPs were first implicated in vascular biology when they were found in atherosclerotic plaques [152], although the sources of the BMPs were never investigated. BMPs in endothelial cells have been shown to have important roles. BMP-6 has been shown to have angiogenic properties [153-154]. BMP-2 mRNA and protein is upregulated during hypoxia and incubation with VEGF in microvascular endothelial cells, while TNF-α mRNA levels are also increased in capillary endothelial cells [155]. In endothelial cells, BMP-4 has been shown to be involved in apoptosis [156], and BMP-4 produced by endothelial cells can modulate mineral deposition in calcifying vascular cells [71]. BMP-2, -4, and -6 have all been identified as important players in valvulogenesis. BMP-2 and -4 are important in endocardial cushion formation, EMT, and cellular proliferation during heart valve development [15].

Dr. Hanjoong Jo's lab has investigated BMP-4 extensively in endothelial cells through DNA microarrays and functional studies and has reported several important findings. BMP-4 expression is upregulated in endothelial cells exposed to disturbed flow, inducing and inflammatory response through a NFκB and NADPH oxidase-dependent

mechanisms, which then lead to monocyte adhesion that can be inhibited through the use of BMP-4-specific small interfering RNA (siRNA) [110-111]. Further, infusion of exogenous BMP-4 increases systolic blood pressure in an endothelium-dependent mechanism, while infusion of noggin or apocynin, an inhibitor of the NADPH oxidase, completely blocked the BMP-4 effects [157]. Recent studies have shown porcine aortic valve leaflets increase ICAM-1 and VCAM-1 under altered shear stress *ex vivo*. Addition of the BMP inhibitor noggin decreased expression of these inflammatory molecules [158].

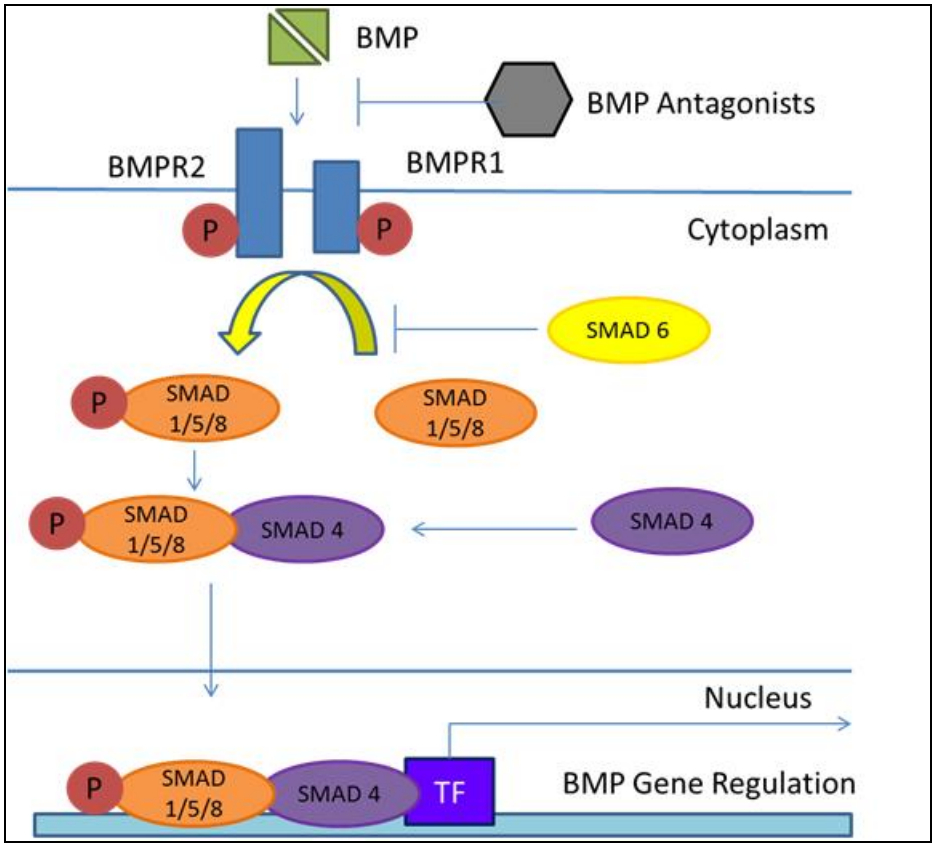

Figure 1.3 Bone Morphogenic Protein Signaling Pathway

Bone Morphogenic Protein Antagonists

BMP antagonists bind to the BMPs with varying degrees of affinity. Once bound, they inhibit the interaction of the BMPs with their cognate receptors [159-164]. BMP antagonists include, noggin, crossveinless 2 (CV-2, also known as BMPER), chordin, follistatin, DAN and matrix Gla protein-1 (MGP-1) [165]. In cultured vascular endothelial cells, BMP antagonists noggin, follistatin and MGP-1, which are co-expressed with BMP-4, provide a negative feedback mechanism inhibiting BMP-4's inflammatory effect [165]. Furthermore, follistatin and noggin are found in advanced atherosclerotic lesions [165]. In porcine aortic valve leaflets, the mRNA of the BMP antagonist chordin was increased on the ventricularis endothelium [128]. Mice lacking noggin at the embryonic stage, have increased thickening of endocardial cushion due to cell proliferation [166]. Finally, uncarboxylated MGP is decreased in the plasma of patients that have aortic valve calcification than the healthy cohort [167]. It is hypothesized that this decrease in uncarboxylated MGP is due to decreased release of MPG from the vascular wall. In the aortic valve, little research has looked at BMP antagonist expression at the VIC or endothelial cell level.

BMP Receptors

The BMPs and TGFβ have two types of specific signaling receptors: bone morphogenic protein receptor-1 (BMPR) and BMPR-II, or transforming growth factor β receptor-1 (TGFβR) and TGFβR-II, respectively, and both are required for signaling. Once the ligand is bound to its receptors, the active domain of the type II receptor phosphorylates the type I receptor, which in turn phosphorylates the R-SMADs (SMAD 1, 2, 3, 5, 8) [168-170]. The functional role of BMPRs in vasculature is not well-studied and in the valve is non-existent. Loss of BMPR-II in vascular smooth muscle cells results in familial primary pulmonary hypertension in humans [171]. In endothelial cells, transfection with

constitutively active mutants of activin like receptor 2 (ALK2), ALK3 and ALK6 have been shown to stimulate expression of id gene (inhibitor of differentiation gene, a well-known BMP target) and angiogenic responses [172]. Finally, BMPRIa knockout mice have impaired valve development [15].

Intracellular BMP Signaling

SMAD 2/3 and SMAD 1/5/8 are canonical mediators of TGFβ and BMP signaling, respectively. When BMPR-1 or TGFβR-1 is phosphorylated, its kinase activity is activated and it then can phosphorylate SMAD 1/5/8 or 2/3, respectively [173]. These phospho-SMADs then bind with co-SMAD 4 and are then translocated into the nucleus, regulating a wide range of gene expression. SMAD 6, an inhibitory SMAD, can block the R-SMADs from being phosphorylated by competing for activation through the type I receptors [168-170]. SMAD6 was shown to be induced by laminar shear stress in vascular endothelial cells [174]. Moreover, SMAD6 deficiency was shown to cause cardiac valve hyperplasia in mice [28], demonstrating its importance in AV biology. Furthermore, SMAD6 deficient mice have extensive ectopic calcification throughout the vasculature [28].

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CHAPTER 2

SPECIFIC AIMS

Project Significance

Aortic valve (AV) disease is a major cause of cardiac-related deaths worldwide and is a strong risk factor for additional cardiovascular events[1-3]. In 2009, there were nearly 93,000 valve procedures performed in the United States, with 53% of those procedures done on the aortic valve position[4]. With the aging population in the United States, the incidence of aortic valve disease is rising. While gross pathological changes and surgical treatments of diseased valves have received much attention, the molecular mechanisms underlying aortic valve inflammation, calcification, and subsequent valve dysfunction are not well understood and remain vastly understudied [5]. Aortic valve disease is diagnosed by severe symptoms at which only invasive surgical repairs or replacements are treatment options [5]. By understanding the mechanism by which aortic valve disease occurs, we may determine early biomarkers of disease and possible therapeutic targets and therefore may slow or stop further disease progression.

AV leaflets function under complex hemodynamic conditions, including pulsatile pressures, unidirectional and disturbed fluid flows, bending stresses, and cyclic stretch [6]. The vascular endothelium is a critical sensor and mediator of hemodynamic and humoral stimuli. Similar to the vascular endothelium, where atherosclerosis preferentially occurs in areas of disturbed flow, AV calcification and sclerosis primarily occur in a side-dependent manner [7-11]. The fibrosa endothelium experiences disturbed flow conditions throughout the cardiac cycle and is prone to accelerated AV calcification [6, 12-13]. Conversely, the ventricularis endothelium, which is located toward the left ventricle,

experiences pulsatile flow during systole and remains relatively unaffected [12-13]. Recent work studying porcine AV endothelium has shown distinct side-dependent gene and protein expression profiles [14]. The aortic side of porcine AV endothelium showed propensity for calcification while the ventricularis endothelium was protected [14].

Bone morphogenic proteins (BMPs) are likely to play an important role in the development and progression of aortic valve disease. BMPs are members of the transforming growth factor β (TGFβ) superfamily [15], and are now known to play important roles in embryonic development, patterning, cartilage formation, and cell differentiation [16-17]. More specifically, BMP-4 is a mechanosensitive and proinflammatory cytokine in vascular endothelial cells [18-19]. In valvular endothelial cells, BMP-4 expression is decreased on the fibrosa endothelium as compared to the ventricularis endothelium of healthy porcine aortic valves *in vivo*[14]. Further, BMP-4 was also found to be decreased by laminar flow in porcine aortic valve endothelial cells *in vitro*; however, BMPs and BMP antagonists have been never been studied in the endothelium of human aortic valves in the context of disease [7]. In the following study, I investigate endothelial expression of BMPs, BMP antagonists, and SMAD activation in calcified and non-calcified human aortic valves. To provide more mechanistic insight into the shear response of valvular endothelial cells, I also investigated inflammatory responses as well as presence of BMP members and activation of the BMP pathway post-shear *in vitro*. In the following study, I investigate disease- and side-dependent valvular endothelial BMP expression *in vivo*, shear regulation of valvular endothelial inflammation *in vitro*, and shear regulation of valvular endothelial BMP expression *in vitro*.

Project Objective

The goal of this project was to investigate how disease and hemodynamics affects the BMP pathway and inflammation in human aortic valvular endothelial cells. Understanding the mechanisms by which BMPs and the BMP pathway are regulated in valvular endothelial cells may provide important insight into the initiation, progression, and offer possible therapeutic targets of aortic valve disease.

Overall Hypothesis

Oscillatory flow conditions on the fibrosa side of the aortic valve stimulate endothelial cells to produce BMP-4, which then activates an inflammatory response leading to accumulation of inflammatory cells, calcification, and ultimately valve impairment. This hypothesis was tested through three specific aims using calcified human aortic valves, non-calcified human aortic valves, and side-specific human aortic valve endothelial cells.

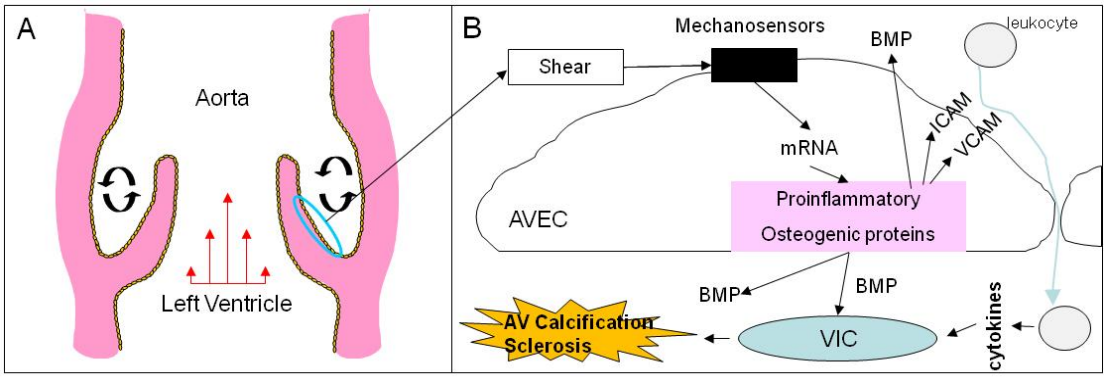

Figure 2.1 Overall Hypothesis

Specific Aim 1

Characterization of BMPs, BMP antagonists, and SMADs in Calcified and Non-Calcified Human Aortic Valve Endothelial Cells.

Hypothesis: Side-dependent expression of bone morphogenic proteins (BMPs) and BMP antagonists in the endothelium of ventricularis and fibrosa is associated with human AV calcification.

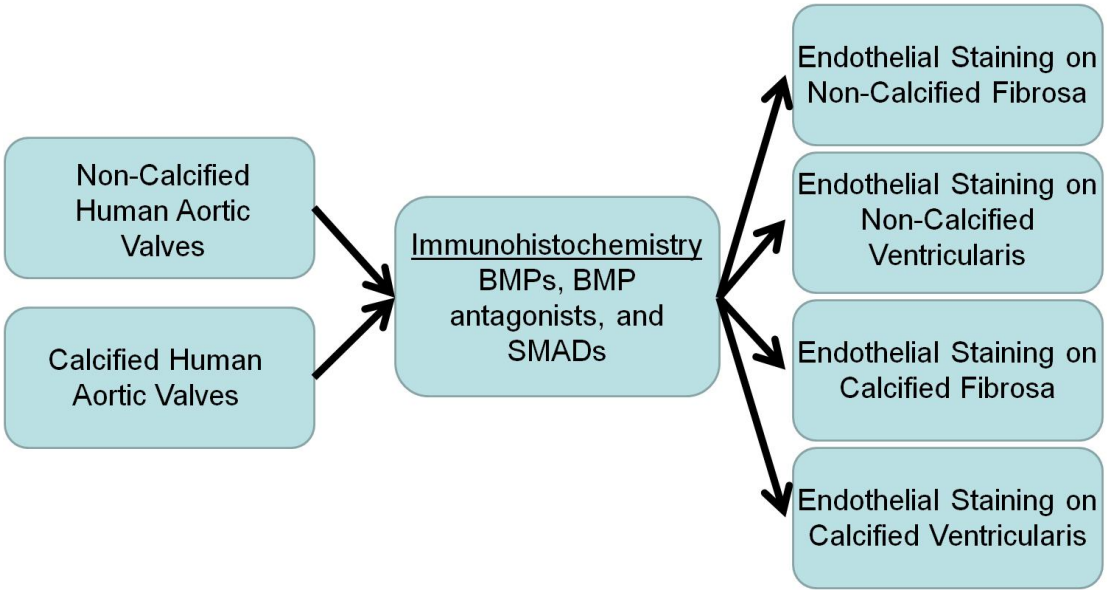

Figure 2.2 Experimental layout of Specific Aim 1

To determine whether AV calcification is connected with the BMPs or BMP antagonists, immunohistochemical studies were performed on human AVs looking at the following markers: BMP, BMP antagonists, and SMAD levels in calcified and non-calcified human aortic valve endothelium.

Specific Aim 2

Characterization of anti- and pro-inflammatory shear responses in human aortic valve endothelial cells.

Hypothesis: Oscillatory shear stress increases the pro-inflammatory phenotype of human aortic valve endothelial cells.

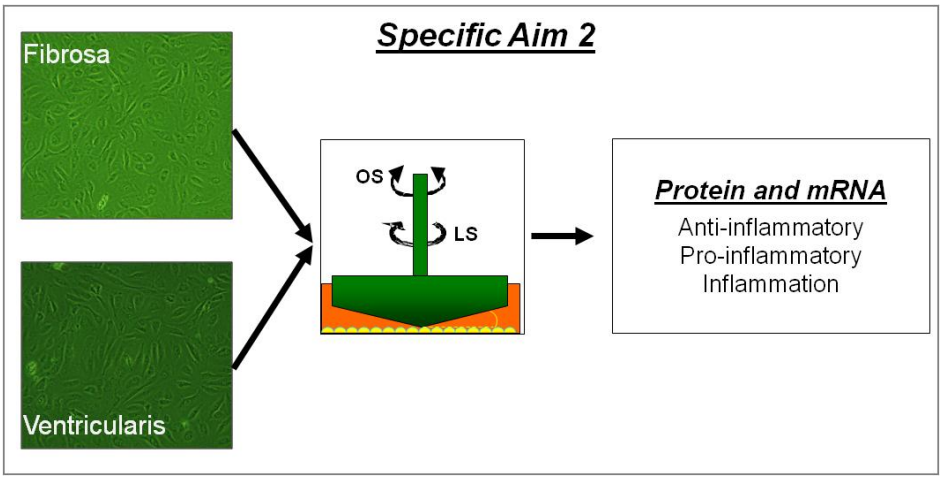

Figure 2.3 Experimental layout of Specific Aim 2

Characterization data from Specific Aim 1 showed an increase in SMAD 1/5/8, a canonical BMP activation marker, in calcified human aortic valves. Before examining shear regulation of the BMPs *in vitro*, I wished to establish the shear response of human aortic valve endothelial cells to laminar and oscillatory shear stress by looking at anti- and pro-inflammatory molecules. In these studies, laminar and oscillatory shear stresses were applied to side-specific endothelial cells and then assayed for anti- and pro-inflammatory proteins at the mRNA and protein levels using quantitative PCR and western blot techniques respectively. Subsequently, a monocyte adhesion assay was used to determine the functional inflammation state of the human aortic valvular endothelial cells.

Specific Aim 3

Characterization of BMPs and BMP antagonists under shear stress and their role in inflammation in human aortic valve endothelial cells.

Hypothesis: Oscillatory shear stress induces BMP-4 protein expression and this increase contributes to the inflammatory response of human aortic valve endothelial cells.

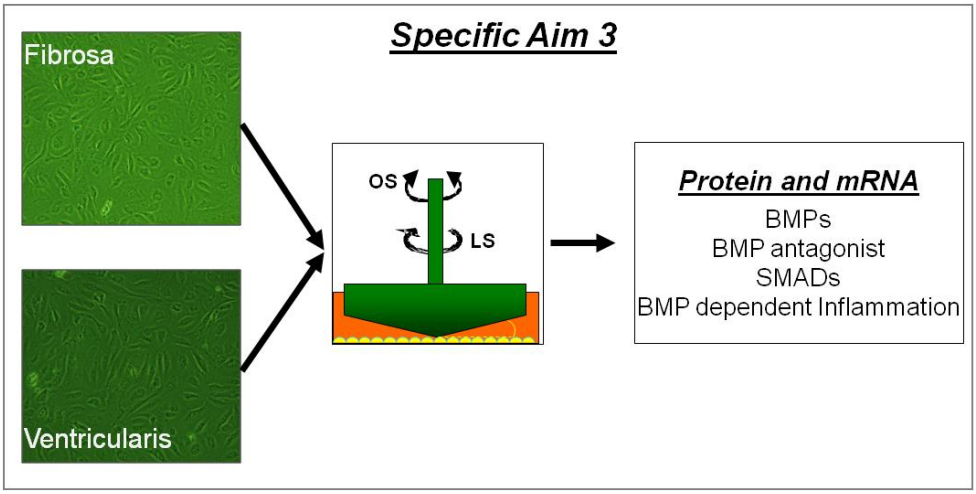

Figure 2.4 Experimental layout of Specific Aim 3

In Specific Aim 1, BMP-dependent SMADs were activated on the fibrosa of calcified human aortic valves. Specific Aim 2 characterized the inflammatory response of human aortic valvular endothelial cells. To determine if the BMPs and their antagonists were regulated by shear, I used quantitative PCR and western blot analysis to determine mRNA and protein levels, respectively. Finally, to determine if BMPs play a role in functional inflammation of the human aortic valve endothelial cells, a monocyte adhesion assay with addition of noggin was employed. Unlike vascular endothelial cells, BMPs do not affect functional inflammation in human aortic valve endothelial cells *in vitro*.

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CHAPTER 4

SHEAR RESPONSE AND INFLAMMATION IN HUMAN AORTIC VALVE ENDOTHELIAL CELLS

In Chapter 3, I identified that bone morphogenic proteins (BMPs) and their antagonists were expressed in the valvular endothelium and that BMP pathway activity was significantly increased in calcified valves and was highest on the fibrosa side of calcified cusps. In this chapter, my goal was to determine the shear response of valvular endothelial cells by looking at shear responsive elements such as endothelial nitric oxide synthase (eNOS) and Kruppel-like factor 2 (KLF2). I also investigated valve endothelial cell inflammation. More specifically, I aimed to determine which inflammatory transcription factors are affecting the endothelial cells. For the first time, I have characterized the shear and inflammation responses of human aortic valve endothelial cells.

Summary

Background. ICAM1, VCAM1 and E-Selectin have shown to be increased in sclerotic aortic vales. These molecules attract inflammatory cells (monocytes, T-cells) from the peripheral blood and as they accumulate, the disease progresses. The role of fluid shear stress on the regulation of anti- and pro-inflammatory responses in human aortic valves, however, remains understudied. I hypothesized that oscillatory shear would cause an increase in inflammation markers (ICAM-1, VCAM-1) while laminar shear would increase anti-inflammatory transcription factors/molecules such as KLF2 and eNOS.

Methods and Results. To test this hypothesis *in vitro*, OS or unidirectional LS was applied to side-specific human aortic valvular ECs. Known shear responsive gene levels were examined through immunoblotting and quantitative PCR (qPCR) while the shear effect on inflammation was determined through a monocyte adhesion assay. I confirmed that both fibrosa and ventricularis ECs exhibit an increase of KLF2 and eNOS under laminar shear when compared to oscillatory shear while seeing the endothelial cells aligned parallel to flow. However, phospho-p38 and NFkB, known inflammatory transcription factors, were not increased by oscillatory shear. This trend continued for ICAM-1 and E-selectin. However, VCAM-1 mRNA and protein was decreased by laminar flow, I hypothesize it is due to increased NO production. Finally, an increase (2-fold) in monocyte binding under oscillatory shear stress when compared to laminar shear stress was shown.

Conclusions. For the first time, I describe human aortic valve endothelial cell anti- and pro-inflammatory function under fluid shear stress. I found that laminar shear stress increased KLF2 and eNOS while decreasing VCAM-1 and monocyte adhesions. Furthermore, understanding the interaction between reactive oxygen species, nitric oxide production (eNOS), and inflammation (VCAM-1) may give us insight and offer possible avenues for pharmaceutical intervention to stop or slow the progression of aortic valve disease.

Introduction

Aortic valve calcification is the number one pathology responsible for an aortic valve replacement procedure in the western world. In fact, 20% of people over the age of 80 are believed to have calcification of the aortic valve [1]. This disease's hallmark is ectopic

calcification and is characterized by the accumulation of inorganic phosphates, calcium, bone-related proteins (bone morphogenic proteins (BMPs), osteopontin, osteonectin, transforming growth factor β (TGFβ)), inflammatory cells (monocytes, leucocytes and T-cells), and osteoblast like cells [2-9]. While the gross pathology, surgical replacement, and clinical manifestations of aortic valve disease have received much attention, the cellular mechanisms leading to aortic valve degeneration has received little [10].

Inflammation plays a central role in numerous pathologies. During the initial stages of atherosclerosis, the endothelial cells that line the blood vessel exhibit an upregulation of inflammatory molecules including inter-cellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and E-Selectin [11-12]. These molecules attract inflammatory cells (monocytes, T-cells) from the peripheral blood and as they accumulate, the disease progresses[2-5]. Aortic valve lesions have similar cellular presences, with the majority of peripheral blood cells being T-cell and macrophages [2-5]. Further, ICAM1, VCAM1 and E-Selectin have shown to be increased in sclerotic aortic vales[13]. Integration of this data suggests an active inflammatory response, similar to atherosclerosis, occurs in degenerative aortic valve disease [14-15].

A second similarity between atherosclerosis and aortic valve calcification is disease location. Atherosclerosis preferentially occurs in areas that are exposed to disturbed, oscillatory, or low mean shear stress [16-17]. Aortic valve calcification also occurs in a side-dependent fashion, where the fibrosa, which faces the aorta and experiences oscillatory flow conditions, develops calcified lesions more readily than the ventricularis [3].

When exposed to laminar flow, aortic endothelial cells align parallel to the flow and exhibit increases in athero-protective genes such as Kruppel-like factor 2 (KLF2), KLF4, and endothelial nitric oxide synthase eNOS [18-24]; however endothelial cells exposed to oscillatory flow express pro-inflammatory molecules such as monocyte chemo-attractant protein 1 (MCP1), BMP-4 and inflammatory adhesion molecules [25-29]. Regulation of these inflammatory genes has been linked to signaling molecules such as p38, extracellular signal regulated kinase (ERK), nuclear factor-κB (NFκB), and c-Jun-N-terminal-kinase (JNK) [30-35].

In vascular endothelial cells, application of laminar shear causes a robust increase of KLF2 [24]. Interestingly KLF2 was found not to be induced by other mechanical or biochemical stimuli in endothelial cells [24] and may regulate several anti-inflammatory genes including JNK [36]. Furthermore, KLF2 has been found in protective regions of the vasculature *in vivo* [37], has been found to be upstream of eNOS [38], and helps improve the nuclear localization of NF-E2-related factor 2 (Nrf2), a powerful anti-oxidant gene transcription factor [39]. In vascular endothelial cells, eNOS is increased by unidirectional flow [40]. Its product, nitric oxide (NO), is a potent vasodilator, promotes vascular health, and is able to reduce intracellular oxidative stress [21-23]. Interestingly, in mice lacking eNOS have the propensity to develop bicuspid aortic valves and eNOS dysfunction was also found in patients with bicuspid aortic valve disease [41-42]. Studies have found that in calcified human aortic valves, increases in reactive oxygen species (ROS) surrounding areas of calcification, are in part due to uncoupled eNOS [43]. Furthermore, in an *in vitro* study, supplemented NO donors to porcine aortic valve interstitial cells (VICs) blocked TGFβ-mediated calcified nodule formation [44]. Finally, mRNA analysis of porcine aortic valve endothelium found that eNOS was increased on the fibrosa endothelium when compared to the ventricularis endothelium [45].

In this study, I investigate the shear response and regulation of anti- and pro-inflammatory genes in side specific aortic valve endothelial cells. I hypothesize that oscillatory shear will induce pro-inflammatory genes including ICAM1, VCAM1, and E-Selectin while laminar shear induces eNOS and KLF2 production.

Materials and Methods

Cell Isolation

Side-specific endothelial cells were harvested from patients undergoing heart transplants at Emory University according to an IRB-approved protocol. Valves were excised from the heart and washed three times in Hank's Buffered Saline Solution (HBSS). They were then incubated in a 5X antibiotic solution (Gibco) for 30 minutes. The leaflets were then oriented so the fibrosa or ventricularis endothelium were facing the same direction. A 600 units/mL solution of collagenase type II (Worthington 4176) was incubated on the valve for 5 minutes. Leaflets were then washed with growth media and the collagenase/growth media mixture was collected in a centrifuge tube. Leaflets were then scraped two times in succession (lightly and then harder) with a sterile scalpel. Between scrapes the leaflets were rinsed with growth media and the solution was placed in separate collection tubes. The leaflets were then placed in a new dish and washed. The same procedure of collagenase and scraping was then performed on the opposite side. Cells were spun down at 1000 RPM for 5 minutes and plated in a 12-well dish and sequentially expanded into a 6-well and T-75 dish. The cells were then sorted using 5 μg/mL of DiL-Acetylated-LDL (Biomedical Technologies Inc.) and the BD FACS Aria Cell Sorter.

Human Aortic Valve Endothelial Cell Culture and Shear Stress Studies

Endothelial cells obtained from human aortic valves were cultured in growth medium [MCDB131 (Cellgro®) containing FBS (Cellgro®, 10%), bovine ECGS (1%), L-Glutamine (Gibco, 1%), Penicillin-Streptomycin (Gibco, 1%), hydrocortisone (Sigma, 0.001 mg/mL), hFGF (R&D, 0.002 μg/mL), hEGF (Invitrogen, 0.01 μg/mL), IGF (Invitrogen, 0.002 μg/mL), VEGF (R&D, 0.001 μg/mL), ascorbic acid (Sigma, 50 μg/mL)] and used between passages 4 and 5. Confluent endothelial cell monolayers were grown in 10 cm dishes and were exposed to an average ventricularis unidirectional laminar shear level (20 dynes/cm^2) or an OS bidirectional flow (± 5 dynes/cm^2) for 24 hours using a Teflon cone and plate viscometer (α =0.5°) as described previously. As a control, cells were cultured in a no flow (static) conditions. One hour before shear studies, media was replaced [MCDB131 (Cellgro®) containing FBS (Cellgro®, 2.5%), L-Glutamine (Gibco, 1%), Penicillin-Streptomycin (Gibco, 1%)].

Western Blots

Following shear exposure, cells were rinsed three times with phosphate buffered solution (PBS) and then lysed using a cell lysis buffer (RIPA) supplemented with phosphotase (Sigma) and protease (Roche) inhibitor cocktails. Following a modified Lowery protein assay, equal amounts of total protein were resolved by SDS-PAGE as previously described [46]. Protein was transferred from the SDS-gel to an immobilio-P-membrane (Millipore, PVDF) and probed with anti-BMP4 (Santa Cruz, 1:1000), anti-SMAD 1/5/8 (Cell Signaling, 1:1000), anti-phosphorylated SMAD 1/5/8 (Cell Signaling, 1:1000), or anti-actin (Santa Cruz, 1:1000). A secondary antibody conjugated to alkaline phosphate was used to detect protein levels by chemiluminescence method [46].

Immunohistochemistry Studies

After 24 hours of shear, endothelial cells were washed 3 times in phosphate buffer solution (PBS) and were fixed in 4% formaldehyde for 10 minutes. After fixing the cells were washed in PBS, permeabilized for 15 minutes with 0.05% Triton X, washed again, and then blocked with 10% donkey serum for 30 minutes. Cells were then incubated with an anti-phospho-NFκB (SAB) primary antibody for 2 hours, followed by incubation with a specific secondary antibody and Hoechst counterstain. Cells were then imaged at a 63x original magnification and imaged using a Zeiss confocal microscope.

Quantitative PCR

Following shear exposure, cells were washed three times with ice cold PBS. Total RNA was then isolated using RNeasy Mini Kit (Qiagen). 1 µg of total RNA was reverse transcribed using random hexamer primers and a SuperScript®III First Strand Kit (Invitrogen). The resulting cDNA was then amplified using real time PCR (ABI Step One Plus) and a master mix containing, gene-specific forward and reverse primers (IDT, Table 1), ROX reference Dye (Stratagene, 1:50), and 2x Brilliant II SYBR® Green QPCR master mix (Stratagene). The PCR conditions were 2 minutes at 56°C, 10 minutes at 95°C, 40 cycles of 30s at 95°C and 1 minute at 60°C, with a melting curve of 15 seconds at 95°C and 1 minute at 60°C. All values were normalized to 18S (Ambion).

TABLE 4.1 qPCR Primer Sequences		
Gene	**Primer Sequence**	
ICAM-1	Forward	5' - ACCGGAAGGTGTATGAACTGAGCA - 3'
	Reverse	5' - TGGCAGCGTAGGGTAAGGTTCTT - 3'
E-Selectin	Forward	5' - CCACTGGGAAACTTCAGCTACAA - 3'
	Reverse	5' - ACGAACCCATTGGCTGGAT - 3'
VCAM-1	Forward	5' - GGAATTAACCAGGCTGGAAGAAGCAG - 3'
	Reverse	5' - AAGCCCTGGCTCAAGCATGTCATA - 3'
eNOS	Forward	5' - AATCCTGTATGGCTCCGAGA - 3'
	Reverse	5' - GGGACACCACGTCATACTCA - 3'
Klf2	Forward	5' - AGACCTACACCAAGAGTTCGCATC - 3'
	Reverse	5' - CATGTGCCGTTTCATGTGCAGC - 3'

Monocyte Binding

Monocyte binding was done under static conditions using purchased THP1 Monocytes (ATCC) as described previously. THP1 monocytes, at a concentration of $1x10^6$ cells/mL were incubated with a fluorescent dye 2';7'-bis(carboxyethyl-5) (6)-carboxyfluroscein-AM (BCEFC, Molecular Probes, 1 mg/mL) in serum-free RPMI (Cellgro®) for 30 minutes at 37°C. During which, endothelial cells, which were exposed to shear stress for 24 hours, with or without noggin (R&D, 50 ng/mL), were washed with RPMI media before addition of monocytes (6 million). Monocytes were incubated with endothelial cells for 30 minutes at 37°C to allow binding. Unbound monocytes are removed by washing with HBSS with calcium and magnesium (Cellgro®). Bound monocytes were then quantified by counting the number of monocytes bound per viewing area (5x Original Magnification). Images were captured using an epifluorescent microscope (Zeiss).

Statistics

Data is presented was mean ± standard error with n's representing number of replicates. Student's t-test was used to establish significance between groups. $P<0.05$ was considered statistically significant with at least 3 independent experiments.

Results

Shear response of HAVEC

Fibrosa and ventricularis endothelial cells were placed under unidirectional or oscillatory shear for 24 hours. After shear both fibrosa and ventricularis endothelial cells aligned parallel to the laminar flow; however endothelial cells under oscillatory flow maintained the cobblestone morphology (Figure 4.1).

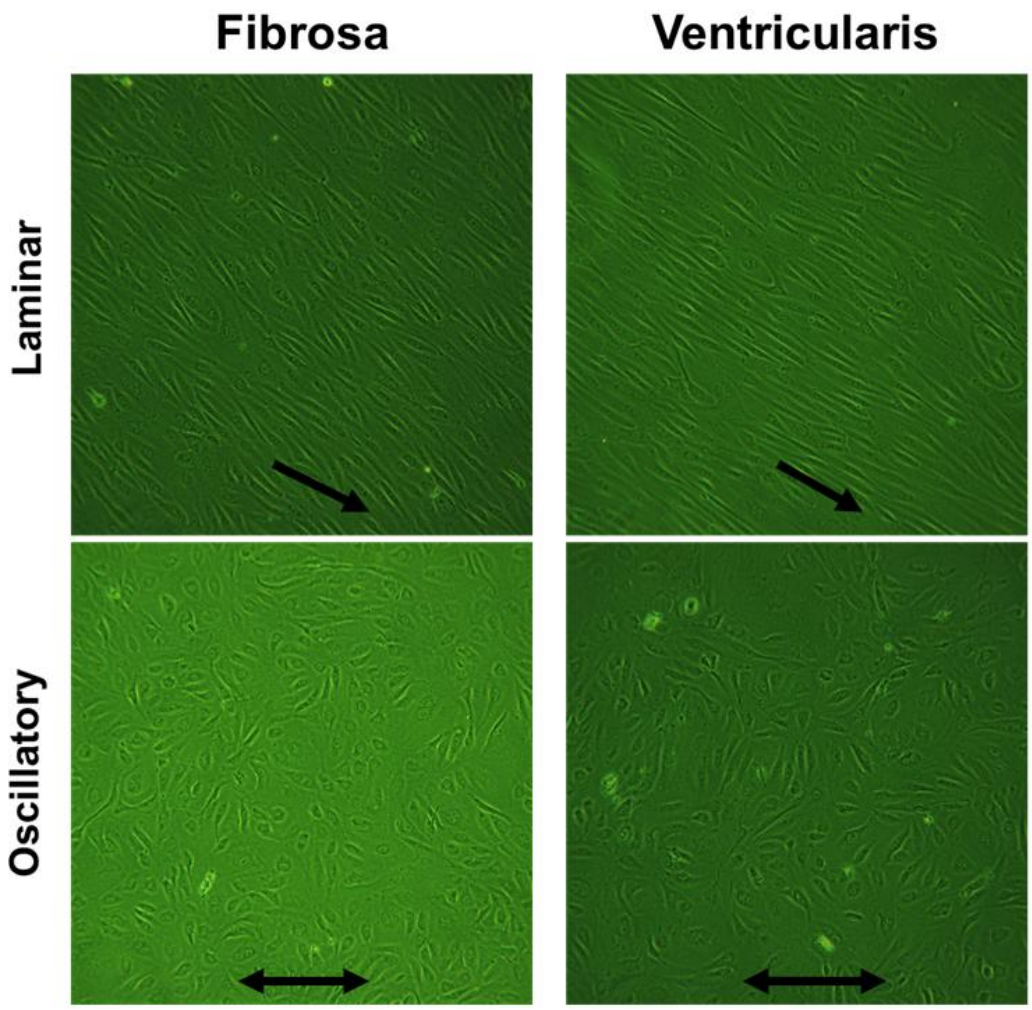

Figure 4.1 Cell alignment of human aortic valve endothelial cells after 24 hours of shear.

Endothelial Nitric Oxide Synthase (eNOS) and Kruppel-like Factor 2 (KLF2) are increases by laminar flow in human aortic valvular endothelial cells

KLF2 and eNOS mRNA was increased by laminar flow in both fibrosa and ventricularis endothelial cells (Figure 4.2a, b). To further examine the responsiveness to shear, eNOS protein was investigated. The total eNOS and the activated phosphorylated eNOS were significantly upregulated by laminar shear 2-fold when compared to static and oscillatory shear (Figure 4.2c, d). Interestingly, mRNA levels of eNOS were

upregulated in ventricularis cells under laminar flow when compared to the ventricularis. However, protein levels between the two cell types were similar (Figure 4.2b, c, d).

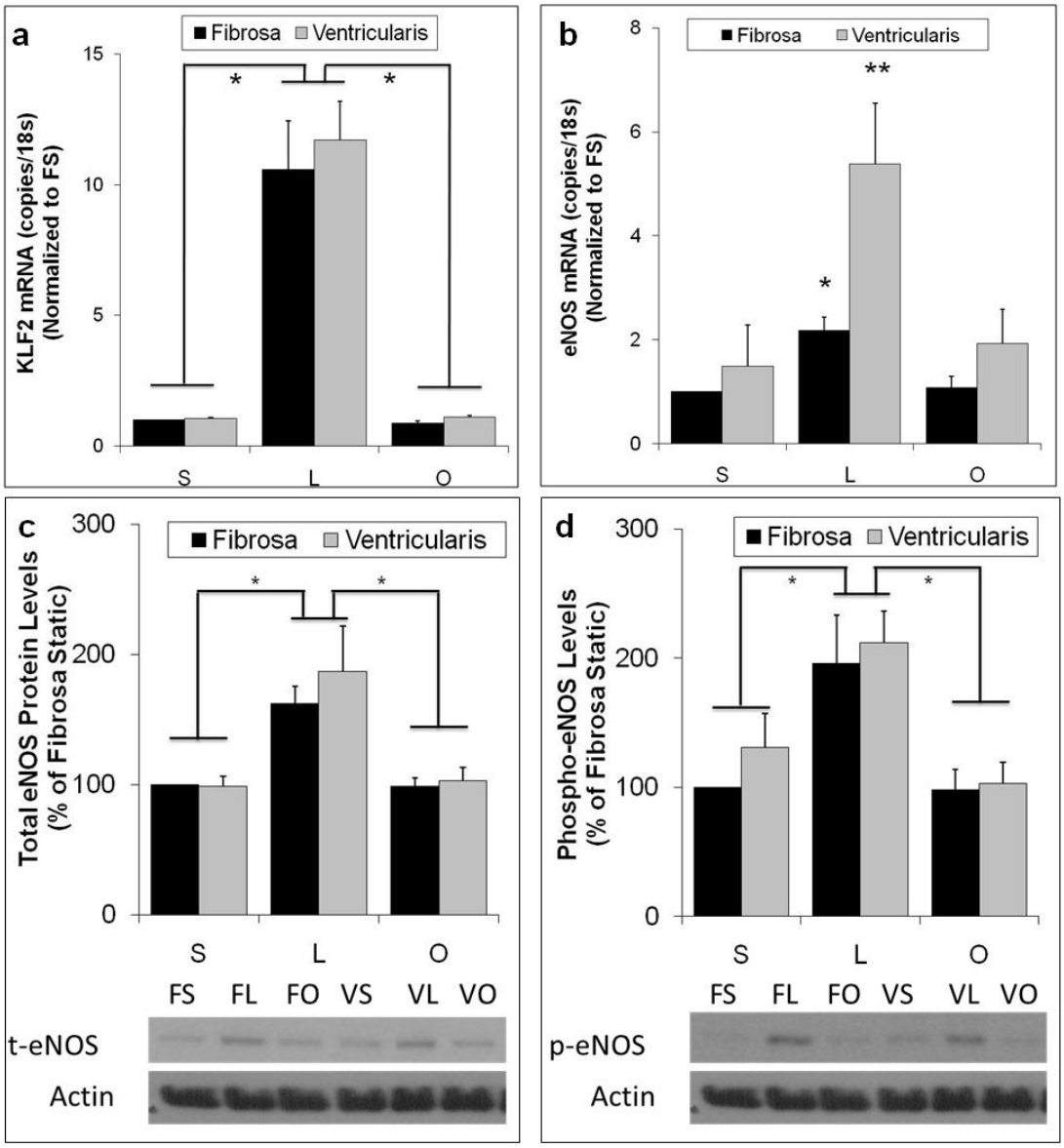

Figure 4.2 Laminar shear induces KLF2 mRNA and eNOS mRNA and protein expression. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA or protein was collected. (a) and (b) KLF2 and eNOS mRNA was analysis respectively. (a)* p<0.05 (b) *p<0.05 against FS, VL, and FO. ** p<0.05 against VS and VO. n- 4 from 3 different patients. (c) and (d) Total and phosphorylated eNOS protein analysis respectivly.* n- 4 from 4 different patients. * p<0.05. (Means ± SE).*

NFkB and p38 are not reduced by laminar flow

To give insight into inflammatory pathway, known inflammatory signaling molecules were examined. After 24 hours of shear, no significant differences were detected between ERK and JNK activation (data not shown). A 4-fold increase of phosphorylated p38 was detected under laminar shear while no difference was detected with total p38 levels (Figure 4.3a). The final inflammatory pathway examined was the NFkB pathway. Total IκB was examined and showed a 2-fold increase under laminar flow (Figure 4.3b); however, when investigating the active subunit of NFkB (phosphorylated-p65), little change seen when comparing laminar flow to oscillatory flow (Figure 4.3c). To determine if IκB was blocking translocation of phospho-p65 into the nucleus, cells were stained using a specific phosphoralated-p65 antibody to investigate translocation into the nucleus. Equal levels of p-p65 were seen in the nucleus cells after laminar and oscillatory flow in both cell types (Figure 4.3d, e).

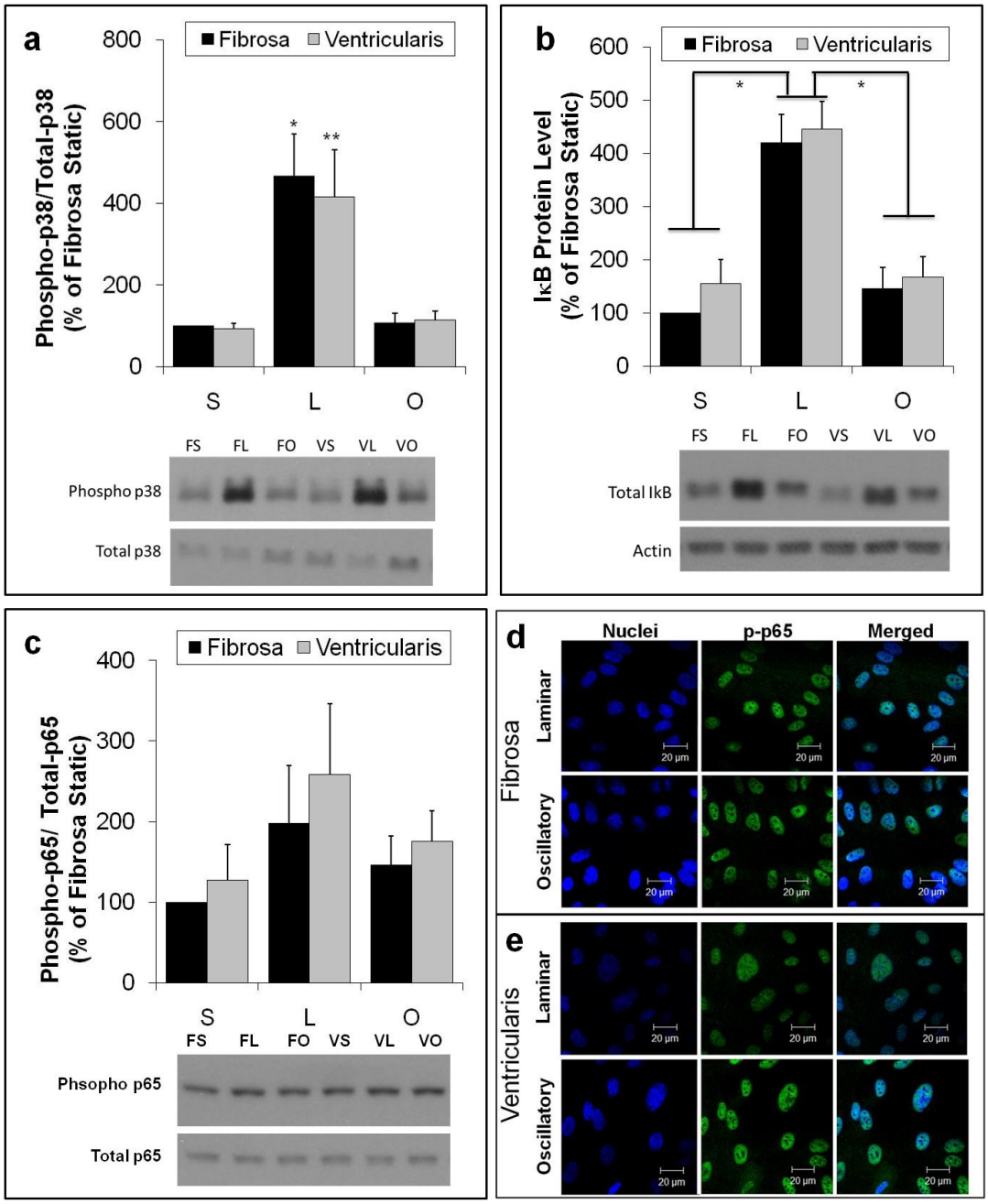

Figure 4.3 Phosphorylated-p65 and phosphorylated-p38 levels are not reduced by laminar flow. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular protein was collected. (a) and (b) phospho-p38 and IkB protein levels respectively. n- 4 from 4 different patients. In (a) * p<0.05 against FO and ** p <0.08 against VO. In (b) * p<0.05. (c) Phosphorylated p65 protein analysis. n=3 from 3 different patients. (d) and (e) immuocytochemistry of phosphorylated-p65 in laminar or oscillatory treated side-specific endothelial cells. (Means ± SE).*

VCAM-1 Levels are decreased by laminar flow

VCAM-1 mRNA and proteins were downregulated by laminar shear by 2-fold (Figure 4.4b, d). No differences were seen between fibrosa and ventricularis endothelial cells. No changes in ICAM-1 mRNA were detected in either endothelial cell type; however, at the protein level, ICAM-1 was only significantly higher in the fibrosa endothelial cells under laminar flow when compared to fibrosa oscillatory (Figure 4.4a, c). Furthermore, ICAM-1 and E-selectin were increased by laminar flow (Figure 4.4a, c Figure 4.5a). Because of the conflicting results seen between ICAM-1, E-Selectin and VCAM-1, I sought to determine the inflammation state of the aortic valve endothelial cell by performing a monocyte adhesion assay. In both fibrosa and ventricularis endothelial cells, oscillatory shear significantly increased (2-fold) the amount of monocyte adhesion compared to oscillatory shear demonstrating functional inflammation in human aortic valve endothelial cells (Figure 4.5b).

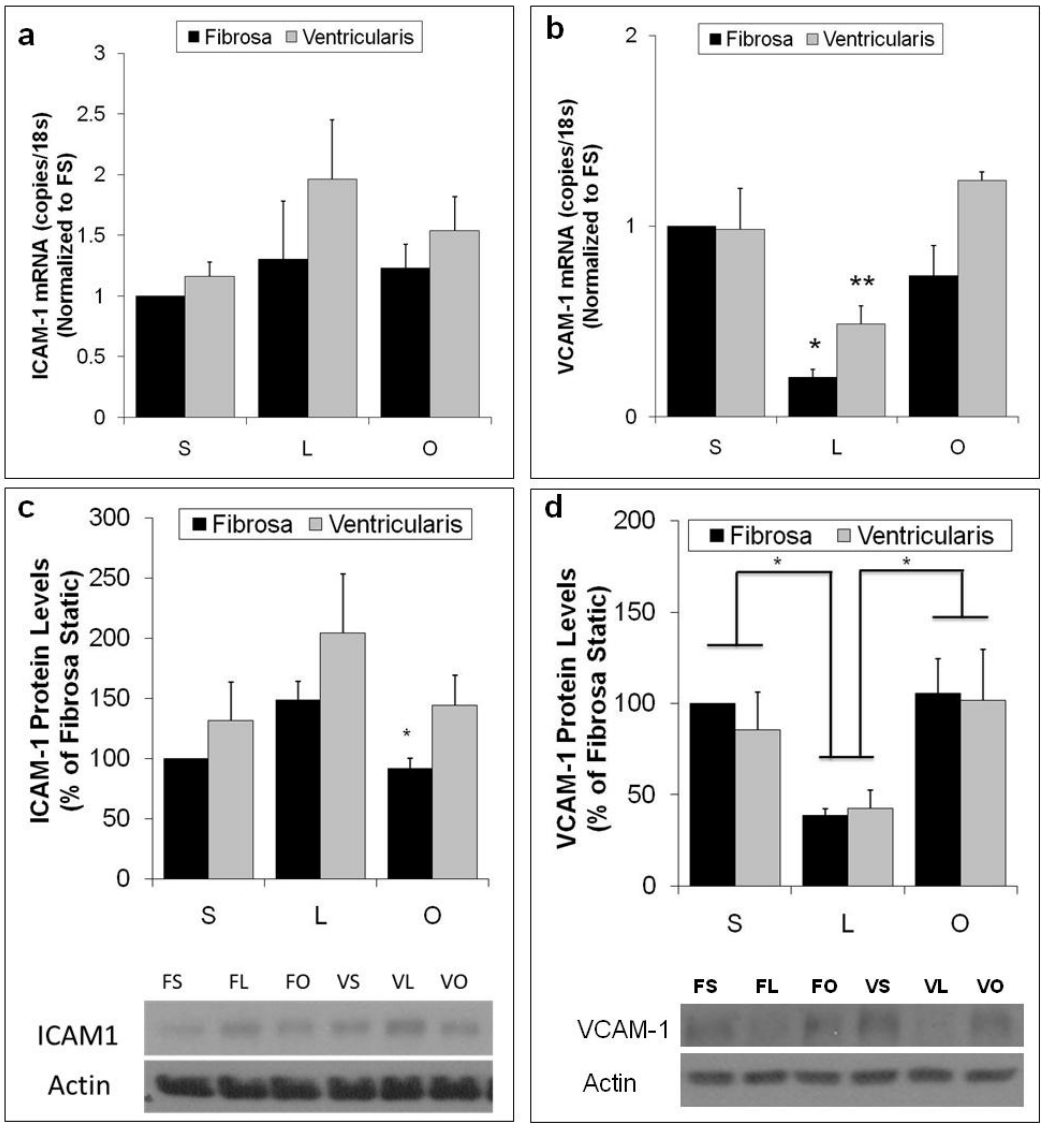

Figure 4.4 Laminar shear decreases VCAM-1 in human aortic valve endothelial cells. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA or protein was collected. (a) and (b) ICAM-1, and VCAM-1 mRNA analysis respectively. * p<0.05 against FS, and FO. ** p<0.05 against VS and VO. n= 4 from 3 different patients. (c) and (d) ICAM-1 and VCAM-1 protein analysis respectively. n= 4 from 4 different patients. (c) * p<0.05 against FL. (d) * p<0.05. (Means ± SE).*

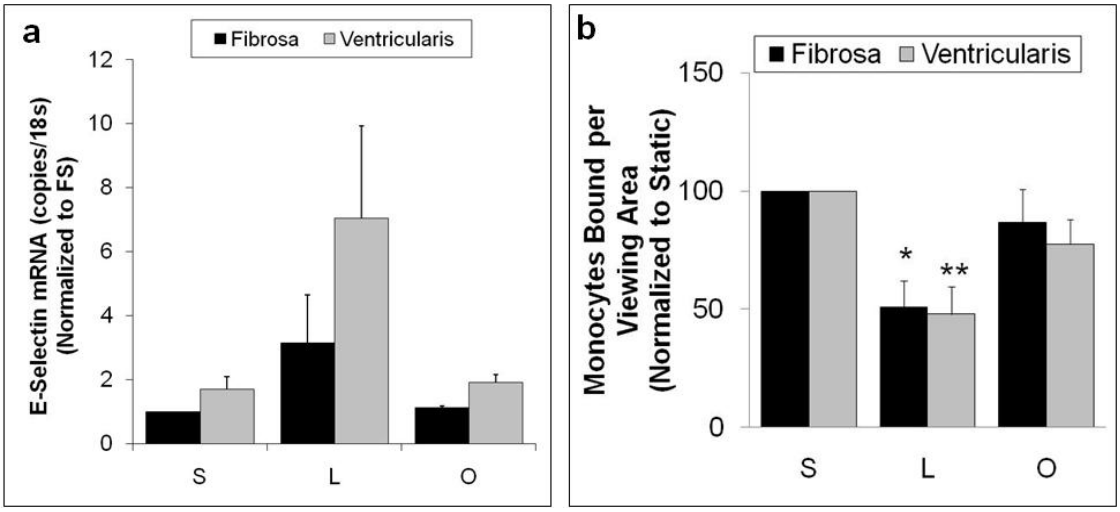

Figure 4.5 Laminar shear decreases monocyte adhesion in human aortic valve endothelial cells. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA. (a) E-selectin mRNA analysis, n=4 with 3 different patients. (b) After 24 hours of shear, fluorescently labeled monocytes were incubated with the conditioned endothelial cells. Monocytes were counted per viewing area. * p<0.05 against FO. ** p<0.05 against VO. n=5. Error bars are standard error. (Means ± SE).*

Discussion

Vascular endothelial cells are critical mediators in the health of the vascular system[47]. They secrete vasoactive compounds that can regulate vascular tone and mechanical properties[47]. Recently, studies have found that valvular endothelial cells can have similar effects on the valvular interstitium and play a critical role of the mechanical properties of the valvular leaflet [47]. Furthermore, studies investigating calcified valvular stenosis have found increased levels of endothelial inflammatory adhesion molecules [13], leukocyte infiltrate [3, 48], and increased levels of reactive oxygen species surrounding areas of valvular calcification [43, 49]. Because of the correlation of the preferential side development of aortic valve disease and the hemodynamics experienced on the fibrosa, I tested the hypothesis that the oscillatory flow experienced on the fibrosa side of the

valvular leaflet causes an increase in inflammatory molecules while unidirectional flow blocks inflammation.

I first tested the shear responsiveness of the fibrosa and ventricularis endothelial cells by looking at cell alignment, and known shear-sensitive genes. Porcine aortic valve endothelial cells were shown to align perpendicular to flow [50]. In fibrosa and ventricularis human aortic valve endothelial cells, I found that cells aligned parallel to the fluid flow (Figure 4.1). To further determine the shear responsiveness of these endothelial cells, transcription factor KLF2 was examined at the mRNA level. In vascular endothelial cells, application of laminar shear causes a robust increase of KLF2 [24]. Interestingly KLF2 was found not to be induced by other mechanical or biochemical stimuli in endothelial cells [24] and may regulate several anti-inflammatory genes including JNK [36]. Furthermore, KLF2 has been found in protective regions of the vasculature *in vivo* [37], has been found to be upstream of eNOS [38], and helps improve the nuclear localization of Nrf2, a powerful anti-oxidant gene transcription factor [39]. I have found that laminar shear increases KLF2 in both fibrosa and ventricularis endothelial cells (Figure 4.2a). In order to mimic physiological conditions, I investigated fibrosa cells under oscillatory flow compared to the ventricularis cells under laminar flow. In this case, I see a dramatic increase in KLF2 mRNA in the ventricularis cells suggesting a more anti-inflammatory state is present on the ventricularis side of the aortic valve.

I next looked downstream of KLF2 at eNOS. In vascular endothelial cells, eNOS is increased by unidirectional flow. Its product, NO, is a potent vasodilator, promotes vascular health, and is able to reduce intracellular oxidative stress [21-23]. Interestingly, in mice lacking eNOS have the propensity to develop bicuspid aortic valves and eNOS dysfunction was also found in patients with bicuspid aortic valve disease [41-42]. Studies

have found that in calcified human aortic valves, increases in reactive oxygen species (ROS) surrounding areas of calcification, are in part due to uncoupled eNOS [43]. Furthermore, in an *in vitro* study, supplemented nitric oxide donors to porcine aortic valve interstitial cells blocked TGFβ-mediated calcified nodule formation [44]. Finally, mRNA analysis of porcine aortic valve endothelium found that eNOS was increased on the fibrosa endothelium when compared to the ventricularis endothelium [45]. I found that laminar shear increases total and phosphorylated eNOS levels (Figure 4.2b-c). It remains to be seen if this endothelial specific NOS can affect ROS surrounding calcified nodules *in vivo*, however recent *ex vivo* studies have found endothelial produced NO can relax valvular interstitial cells [47]. The differences between the porcine *in vivo* results to my *in vitro* findings suggest factors other than shear stress may be playing a role.

After determining the shear response at the transcriptional and translational level of two molecules known to be upregulated by unidirectional laminar shear, I then investigated four inflammatory signaling molecules, JNK, p38, ERK1/2 and NFκB. High unidirectional shear stress has been shown to inhibit activation of JNK, p38 and NFκB [30-35], while oscillatory flow induces NFκB activation *in vivo* [51]. I was surprised to find that p38 activation, measure by phosphorylated-p38, was increased by laminar flow (Figure 4.3a). This increase was not different between the fibrosa and ventricularis and further investigation into the mechanism by which p38 is upregulated may provide fundamental information in regulation of this map kinase. Interestingly, reports have found p38 is essential in cytoskeletal remodeling, and endothelial cells under laminar flow fail to align with the flow when p38 is inhibited [52-53]. I found that the inhibitor of NFκB, IκB, was increased two-fold by laminar flow (Figure 4.3b); however the level of activated NFκB, phosphorylated p65, was not decreased by laminar flow when compared to oscillatory

flow (Figure 4.3c). To determine if this transcription factor translocates into the nucleus in this environment, I performed phosphorylated p65 immunostaining. I found that all phosphorylated p65 was located in the nucleus (4.3d, e). Because of these results I decided to look downstream of these transcription factors by looking endothelial adhesion molecules.

ICAM-1, VCAM-1 and E-selectin are important regulators of inflammation in the vasculature. In vascular endothelial cells ICAM-1 and E-selectin are increased by oscillatory flow [28, 54] while unidirectional flow has little effect on either adhesion molecule [55-56]. In contrast, VCAM-1 is decreased by unidirectional laminar flow while not being affected by oscillatory flow [57-58]. As previously mentioned, ICAM-1, VCAM-1, and E-selectin are all found in calcified human aortic valves. Furthermore, in porcine valvular endothelial cells, laminar shear was found to decrease VCAM-1 mRNA levels. My results found a small increase between ICAM-1 protein levels in laminar or oscillatory shear stress conditions (Figure 4.4a, c). This result is correlated with my data concerning NFκB activation, as ICAM-1 has a NFκB responsive element in its promoter [59]. VCAM-1 has also been shown to be responsive to NFκB [59];however, unlike ICAM-1, VCAM-1 had a promoter that is responsive to NO and Nrf2 [60]. I found VCAM-1 protein and mRNA was decreased by laminar flow in fibrosa and ventricularis endothelial cells (Figure 4.4b, d). In conjunction with endothelial expression of eNOS, I hypothesize that the NO produced by eNOS under laminar shear effects VCAM-1 expression and overrides NFkB. To determine the overall inflammatory state of the valvular endothelial cells, a monocyte adhesion assay was performed to determine the inflammatory state of the valvular endothelial cells. In both cell types, oscillatory shear significantly increased the number of monocyte bound to the endothelium when compared to laminar

conditioned cells (Figure 4.5b). With consideration to the above ICAM-1 and VCAM-1 data, it is likely that VCAM-1 is responsible for the monocyte adhesion in these cells, although this needs to be tested.

It is important to emphasize that the cells used in this study come from a diseased population, some of which had vascular inflammation at the time of cell isolation. Inflammatory signaling molecules, i.e. p38 and NFkB, and endothelial cell adhesion molecules, i.e. ICAM-1 and E-selectin, may be affected by the patients' medical condition at the time of harvest. Furthermore, the *in vitro* conditions that I use in these experiments are approximations of the hemodynamic and humeral stimuli experienced *in vivo*.

This study, for the first time, chronicles the effect of shear stress on side specific human aortic valve endothelial cells. Both cell types have similar patterns of shear upregulation for KLF2 and eNOS. These valvular endothelial cells have an unexpected response to laminar shear stress with ICAM-1 and E-Selectin, which may be due to p38 and NFκB activation. VCAM-1 mRNA and protein was decreased in laminar flow, in a similar manner to published reports, possibly due to the anti-inflammatory effect of NO. Finally, it appears that VCAM-1 is responsible for the oscillatory shear-induced monocyte binding, although direct testing needs to be conducted. Finally, this information is critical in understanding the mechanisms by which aortic valve disease occurs. Understanding the inflammatory phenotype and pathways can help in developing a successful tissue engineered aortic valve. Furthermore, understanding the interaction between reactive oxygen species, nitric oxide production, and inflammation may give us insight and offer possible avenues for pharmaceutical intervention to stop or slow the progression of aortic valve disease.

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Chapter 3

PREFERENTIAL ACTIVATION OF SMAD 1/5/8 ON THE FIBROSA ENDOTHELIUM IN CALCIFIED HUMAN AORTIC VALVES

The overall objective of this dissertation is to examine the production and regulation of the bone morphogenic proteins (BMPs), the BMP antagonists, and the SMAD proteins in human aortic valve endothelial cells. In Specific Aim 1, I sought to determine endothelial BMP expression in calcified and non-calcified human aortic valves *in vivo*. I then sought to determine the shear response of human aortic valve endothelial cells *in vitro* by investigating well known shear responsive genes and endothelial inflammation. Finally, in Specific Aim 3, I investigated the shear regulation of the BMPs, the BMP antagonists, and BMP dependent inflammation. The use of an *in vitro* shear system was used in Specific Aims 2 and 3 to generate shear stress profiles to simulate the oscillatory and unidirectional laminar flow seen by the valve endothelium. For the first time, I have characterized valvular endothelial BMP and BMP expression *in vivo*. Furthermore, I have also characterized shear dependent regulation of the BMP, BMP antagonists, and inflammation in human aortic valve endothelial cells *in vitro.*

Summary

Background. Aortic valve (AV) calcification preferentially occurs on the fibrosa-side while the ventricularis-side remains relatively unaffected. Here, I tested the hypothesis that side-dependent expression of bone morphogenic proteins (BMPs) and BMP antagonists in the endothelium of ventricularis and fibrosa is associated with human AV calcification.

Methods and Results. Human calcified AVs obtained from AV replacement surgeries and non-calcified AVs from heart transplantations were used for immunohistochemical studies. I found that BMP2/4/6 expression was significantly higher on the ventricularis endothelium compared to the fibrosa in both calcified and non-calcified AV cusps. However, BMP antagonists (crossvienless-2 and noggin) expression was significantly higher on the ventricularis endothelium compared to the fibrosa in both disease states. While phospho-SMAD-1/5/8 (a canonical BMP pathway) level was high in the calcified fibrosa endothelium, phospho-SMAD-2 (a canonical TGFβ pathway) was not different in any groups. Moreover, significant expression of inhibitory SMAD-6 expression was found only in the non-calcified ventricularis endothelium.

Conclusions. SMAD1/5/8 is preferentially activated in the calcified fibrosa endothelium of human AVs. These findings suggest that preferential activation of BMP pathways may be controlled by the balance between the BMPs and their inhibitors, leading to side-dependent calcification of human AVs.

Introduction

Aortic valve (AV) stenosis is a major cause of cardiac related deaths worldwide, and remains a strong risk factor for cardiac-related death [1-3]. While gross pathological changes and surgical treatments of the diseased valves have received much attention, the molecular mechanisms underlying AV inflammation, calcification, and subsequent valve dysfunction are not well understood and remain vastly understudied [4]. AV calcification is characterized by the accumulation of calcium, inorganic phosphates, extracellular matrix proteins, bone-related factors [5-7], and osteoblast-like cells [5, 8] in the

fibrosa, or aortic side, of the valve cusp [7, 9]. Surprisingly, few studies have investigated AV endothelial cell involvement in those patients with aortic stenosis.

AV leaflets function under complex hemodynamic conditions, including pulsatile pressures, unidirectional and disturbed fluid flows, bending stresses, and cyclic stretch [10]. The vascular endothelium is a critical sensor and mediator of hemodynamic and humoral stimuli. Similar to the vascular endothelial system, where atherosclerosis preferentially occurs in areas of disturbed flow, AV calcification and sclerosis primarily occur in a side-dependent manner [11-15]. The fibrosa endothelium experiences disturbed flow conditions throughout the cardiac cycle and is prone to accelerated AV calcification. Conversely, the ventricularis endothelium, which is located toward the left ventricle, experiences unidirectional pulsatile flow during systole and remains relatively unaffected. Recent studies using porcine AV endothelium have shown distinct side-dependent gene and protein expression profiles. The aortic side endothelium showed propensity for calcification (increased bone morphogenic protein 4 (BMP-4) while decreasing BMP antagonist chordin), while the ventricularis endothelium was protected [16]. Dr. Robert M. Nerem's lab has also shown that unidirectional laminar shear stress decreases BMP-4 mRNA and protein expression in cultured porcine AV endothelial cells [11]. In addition, *ex vivo* studies using normal porcine AV leaflets have shown that altered shear conditions induces inflammation by a BMP-4-dependent pathway [17].

BMPs are members of the transforming growth factor β (TGFβ) superfamily. Initially discovered as inducers of bone formation [5], the BMPs are now known to play important roles in embryonic development, patterning, cartilage formation, and cell differentiation[18-19]. Dr. Hanjoong Jo's lab has shown that BMP-4 is a mechanosensitive and proinflammatory cytokine in vascular endothelial cells [20-21]. Furthermore, BMP-4

infusion induced hypertension in mice in a NADPH oxidase-dependent manner [22]. In addition, BMP-2 and -4 expression are increased in calcified human AVs and human atherosclerotic lesions [23-24].

BMP antagonists bind to the BMPs with varying degrees of affinity. Once bound, they inhibit the interaction of the BMPs with their cognate receptors [25-30]. BMP antagonists include, noggin, crossveinless 2 (CV-2, also known as BMPER), chordin, follistatin, DAN and matrix Gla protein-1(MGP-1) [31]. In porcine aortic valve leaflets, the mRNA of the BMP antagonist chordin was increased on the ventricularis endothelium [16]. Finally, uncarboxylated MGP is decreased in the plasma of patients that have aortic valve calcification than the healthy cohort [32].

The BMPs and TGFβ have two types of specific signaling receptors: BMPR-I and BMPR-II, or TGFβR-1 and TGFβR-II, respectively, and both are required for signaling. Once the ligand is bound to its receptors, the active domain of the Type II receptor phosphorylates the type I receptor, which in turn phosphorylates the R-SMADs (SMAD 1, 2, 3, 5, 8) [33-35]. SMAD2/3 and SMAD1/5/8 are canonical mediators of TGF-β and BMP signaling, respectively. These phospho-SMADs then bind with co-SMAD 4 and are then translocated into the nucleus, regulating a wide range of gene expression. SMAD 6, an inhibitory SMAD, can block the R-SMADs from being phosphorylated by competing for activation through the type I receptors [33-35].

At present, it is not known whether BMPs and BMP antagonists play a role in human AV calcification. I hypothesized that the fibrosa endothelium, exposed to disturbed flow conditions, upregulates BMP expression while downregulating BMP antagonists, contributing to side-specific human AV calcification. My study using calcified and non-

calcified human AVs suggests that side-dependent activation of the BMP pathway is regulated by the balance between BMPs and BMP antagonists in endothelium, contributing to the preferential AV calcification in the fibrosa.

Materials and Methods

Human AV Procurement

AVs were received from two patient populations according to the IRB approved protocols at Emory University with informed consent. Calcified human AVs were obtained immediately following valve replacement surgeries in 16 patients at Emory University Hospital Midtown. Fifteen patients had tri-leaflet valves, while 1 patient had a bicuspid AV. Non-calcified AV (n=6, all tri-leaflet AV) were harvested from recipient patients undergoing heart transplantation at Emory University Hospital. Patient demographics are presented in Table 1. Immediately following harvesting, the AVs were photographed, washed in ice-cold phosphate buffered saline (PBS), and cusps were individually snap-frozen in optimal cutting temperature (O.C.T.) compound (Tissue-Tek). Valves were then sectioned (7μm) in the radial direction to include the base and free edge (tip), stored at -80°C and used for immunohistochemical staining studies.

Table 3.1 Patient Characteristics

	Calcified	Non-Calcified
Number of Patients	16	6
Age (mean ± SD)	66.4 ± 16.28	54.7 ± 8.5
Female	7 (43.8%)	1 (16%)
Bicuspid valves	1 (6.25%)	0 (0%)
Ejection Fraction (mean ± SD)	0.498 ± 0.139	NA
NYHA * (mean ± SD)	2.2 ± 1.1	NA
Congestive Heart Failure	9 (56.25%)	6 (100%)
Diabetes mellitus	2 (12.5%)	3 (50%)
Hemoglobin A1C (mean ± SD)	6 ± 1.18	NA
Dyslipidemia	6 (37.5%)	NA
Hypertension	12 (75%)	2 (33.3%)
Last Creatinine Level (mean ± SD)	1.12 ± 0.33	NA
Dialysis	0 (0%)	NA

* NYHA - New York Heart Association, NA – not available

Histochemistry and Immunohistochemistry

Hematoxalin and eosin (H&E for general histology), Verhoeff Van Giessen (elastin), and Alizarin Red (calcification) staining was carried out for histomorphometric analysis. Immunohistochemical studies were carried out as previously described [31] using specific antibodies as following: endothelial marker (von Willebrand Factor, (Dako, 1:400)), BMPs (BMP-2 (Lifespan Biosciences, 1:100), BMP-4 (Biovision, 1:25), and BMP-6 (Santa Cruz. 1:25)), BMP antagonists (noggin (LabFrontier, 1:100, CV-2 (R&D, 1:100, MGP-1 (ABCAM, 1:100) and DAN (R&D, 1:25), phospho-SMAD-1/5/8 (Cell Signaling, 1:200) and phospho-SMAD-2 (Cell Signaling, 1:100), and inhibitory SMAD (SMAD-6, (Lifespan Biosciences 1:25) Rhodamine Red X antibody (Jackson Labs) was used as a secondary antibody with a Hoechst dye nuclear counter staining. Fluorescent images were taken with a Zeiss Axioskopepifluorescence microscope using a 10X objective. Briefly, valve sections were allowed to thaw to room temperature for 10 minutes, and

then fixed in ice cold acetone for 5 minutes. After fixing, slides were allowed to dry for 10 minutes, washed three times for 5 minutes in PBS, incubated with 10% antibody specific serum for 1 hour, and then overnight with the primary antibody in a 10% antibody specific serum. Valve section was then washed three times for 5 minutes in PBS, incubated with the Rhodamine Rex X antibody for 1 hour, washed three times for 5 minutes in PBS, and then incubated with Hoechst dye for 8 minutes. After incubation slides were washed three times for 5 minutes in PBS and then mounted using Dako mounting media.

Image Analysis

Three cross-sectional images were obtained from each AV section, where endothelial layer was present based on Hoechst staining. Digital images were then blindly graded for endothelial staining intensity from 0 (no positive staining) to 5 (most intense positive staining) by three individuals. The grades of the three cross-sections were averaged to determine the staining intensity of each antibody examined. The fibrosa and ventricularis endothelia were separately graded.

Statistical Analysis

All data are reported by mean ± SE with n signifying the number of different AV leaflets stained. Significant differences were determined by ANOVA using a Tuckey posthoc testing. All p-values <0.05 were considered significant.

Results

Immunohistochemical examination of AVs

H&E staining for general histology (Figure 3.1a, e), Alizarin Red (Figure 3.1b, f) for calcification and Verhoeff Van Giessen (Figure 3.1c, g) for elastin was carried out with calcified and non-calcified human AVs. All human AVs (n=6 patients) obtained from the heart transplantation patients were negative for Alizarin Red staining (Figure 3.1a, c), suggesting that they were not calcified. In contrast, all calcified AVs (n=16 patients) obtained from AV replacement surgeries were confirmed by Alizarin Red staining (Figure 3.1b, f). To examine the presence of an intact endothelium, von Willebrand factor staining was performed on the AV leaflet (Figure 3.1d, h). Intact endothelium was confirmed on valves used in my study.

Side-dependent expression of BMPs and BMP antagonists in human AV

Robust expression of BMPs 2, 4, and 6 was observed in all the tested AV endothelium. However, unlike my original hypothesis, BMP-2, -4, and -6 expression was higher in the ventricularis endothelium than fibrosa endothelium (Figures 3.2, 3.3, and 3.4). BMP-2 and BMP-4 expression was significantly higher in the non-calcified ventricularis endothelium compared to the fibrosa endothelium of both calcified and non-calcified AVs (Figures 3.2 and 3.3 a-g). BMP-6 expression was significantly higher in the calcified ventricularis endothelium than the fibrosa endothelium (Figure 3.4 a-h). There was no difference in expression levels of all three BMPs in the ventricularis endothelium of calcified and non-calcified AVs. The same was true for the fibrosa endothelium of calcified and non-calcified AVs (Figures 3.2, 3.3, and 3.4).

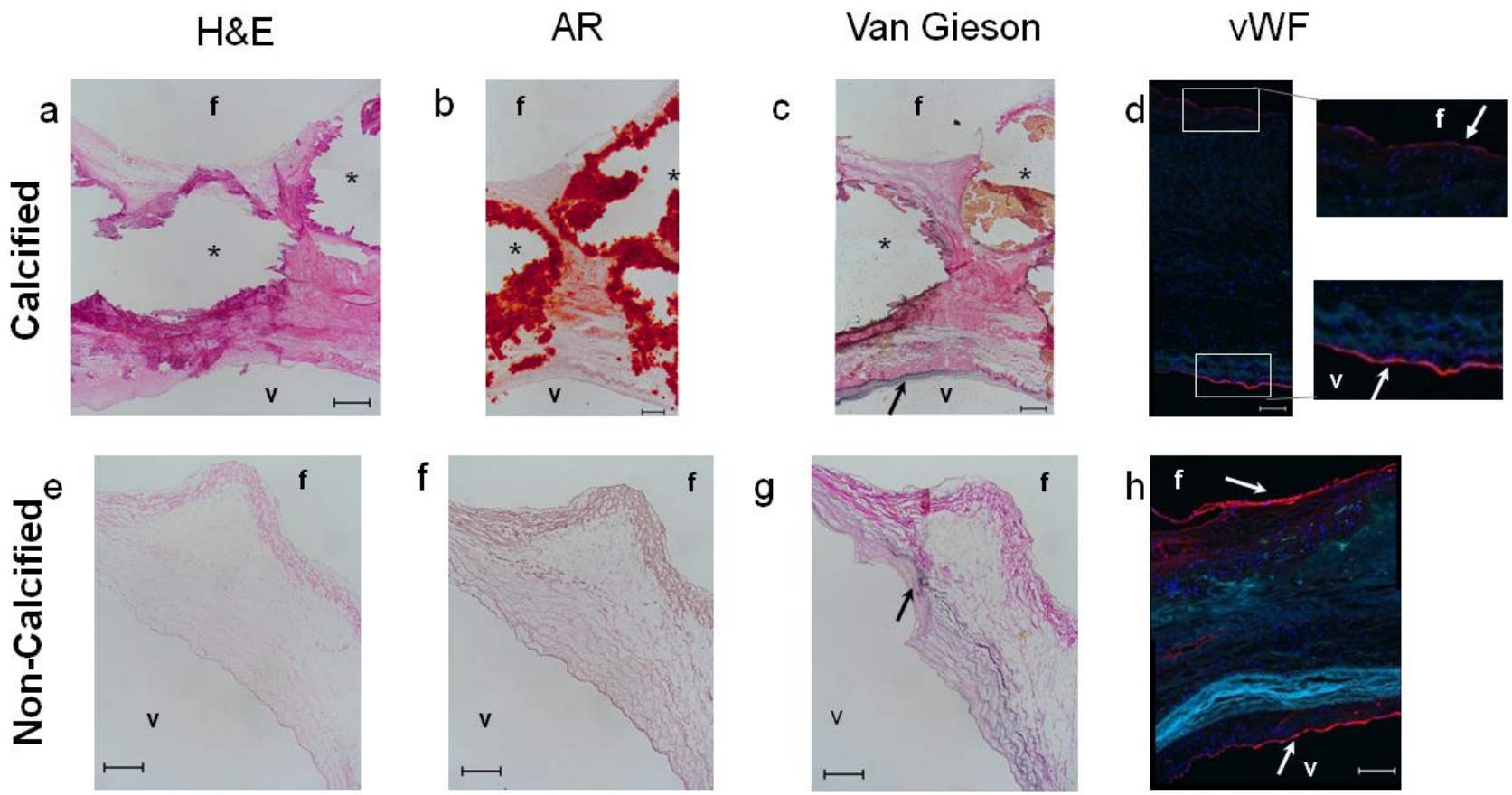

Figure 3.1 Calcification and endothelial staining of AV cusps. *Valves were obtained from either heart transplant (non-calcified) or valve replacement (calcified) surgeries, snap frozen and sectioned. Sections were then stained for H&E (**a**, **e**), alizarin red (**b**, **f**), Verhoeff Van Giessen (**c**, **g**) or von Willebrand factor (**d**, **h**). Representative staining (n=12 patients) shows side-specific calcification (*) in calcified leaflets (**b**, **f**), while maintaining an intact endothelial layer (**d, h**: arrows). Verhoeff Van Giessen stain was used to stain for elastin (shown in black, arrows) to help in leaflet orientation (**c**, **g**). f: fibrosa, v: ventricularis*

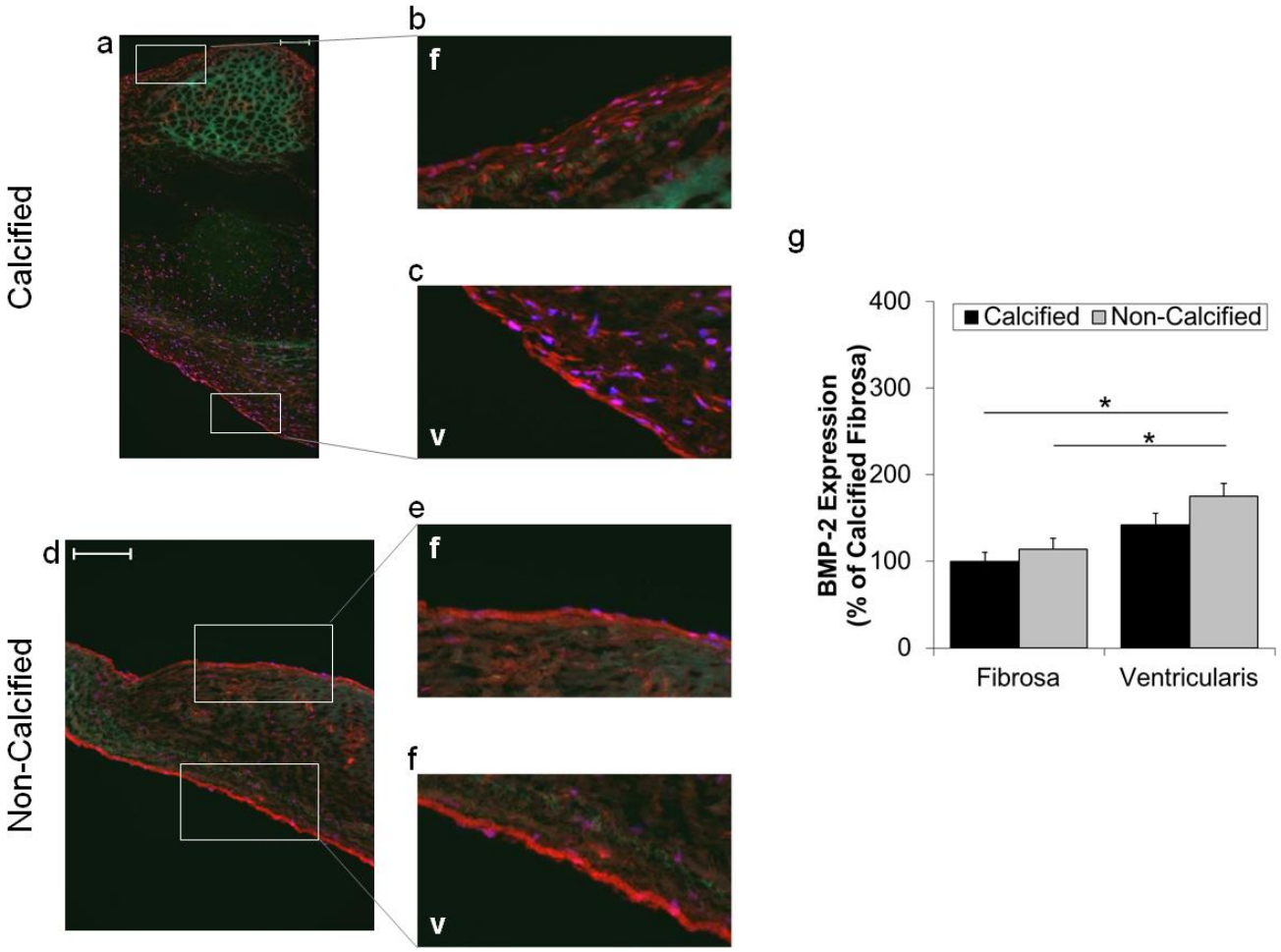

Figure 3.2 BMP-2 expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for BMP-2 (**a-f**) and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for each BMP-2 (**g**) (mean ± SEM). For BMP-2, n=13 calcified and n=13 non-calcified.*p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

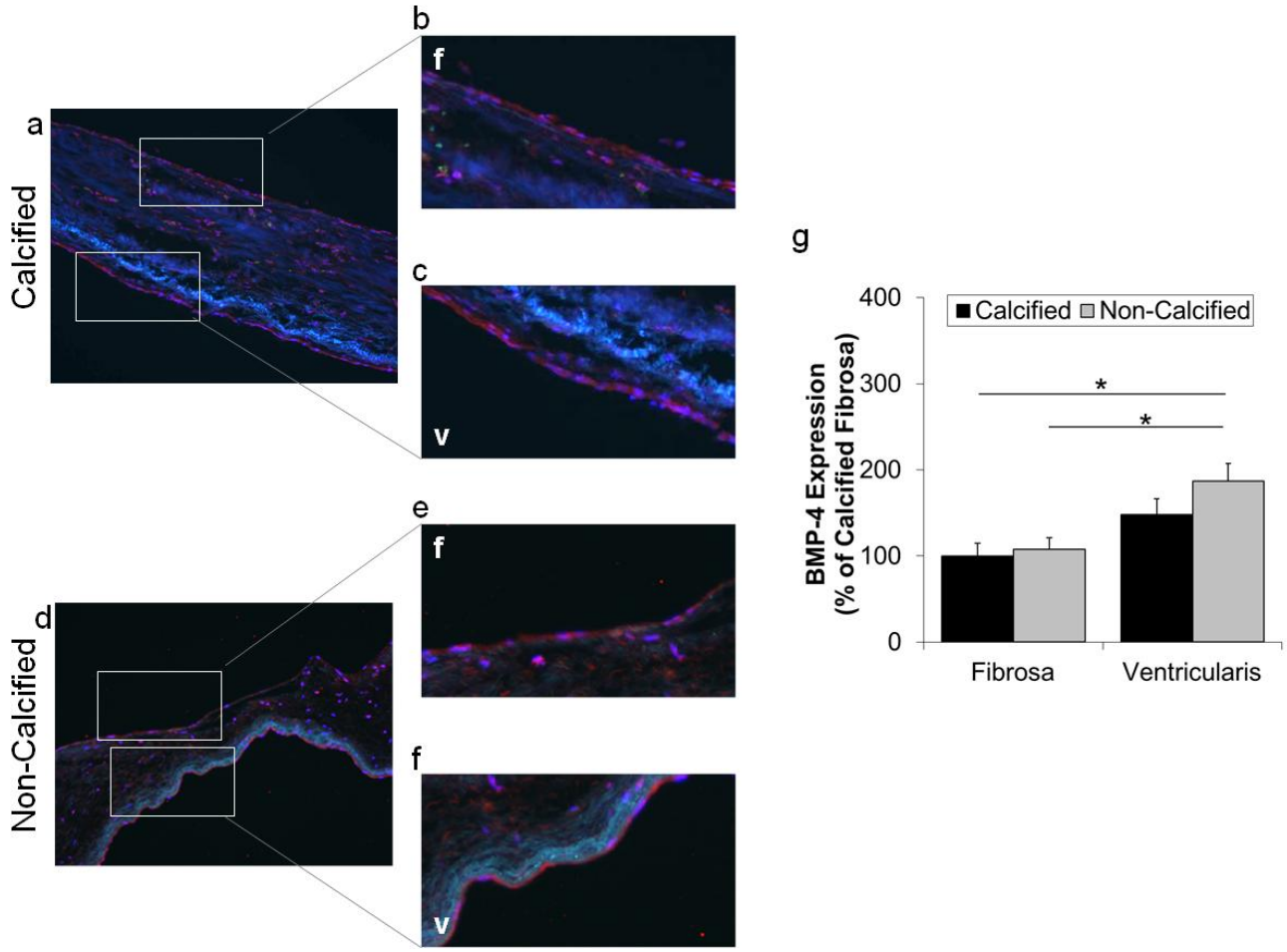

Figure 3.3 BMP-4 expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for BMP-4 (**a-f**) and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for each BMP-4 (**g**) (mean ± SEM). For BMP-4, n=9 calcified and n=8 non-calcified.*p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

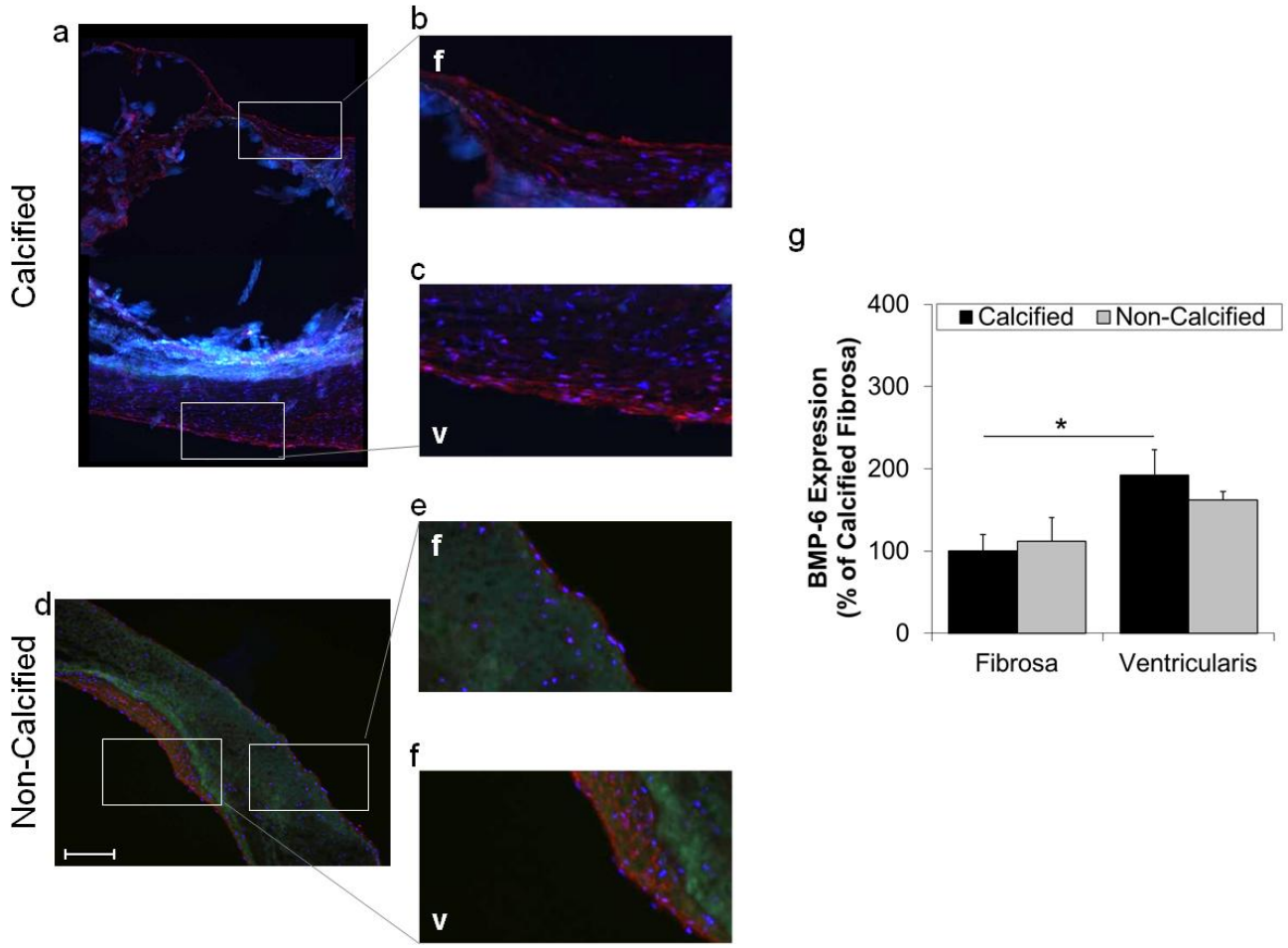

Figure 3.4 BMP-6 expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for BMP-6 (**a-f**) and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for each BMP-6 (**g**) (mean ± SEM). For BMP-6,* n=12 calcified and n=11 non-calcified.**p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

Next I examined whether BMP antagonists (CV-2, noggin, DAN, follistatin, chordin, and MGP-1) were expressed in a side-dependent manner in human AV endothelium. CV-2 and noggin expression was significantly lower in the fibrosa endothelium both in calcified and non-calcified AVs (Figure 3.5a-g). Furthermore, I found that CV-2 expression was significantly reduced in the calcified ventricularis endothelium than the non-calcified ventricularis endothelium (Figure 3.5g), while this disease-dependency was not observed for noggin (Figure 3.6g). DAN expression was not significantly different in the endothelium; although a trend of decreased staining was seen between the calcified and non-calcified fibrosa endothelium (Figure 3.7a-g). At this time, none of the available antibodies that I examined resulted in specific staining patterns for follistatin, chordin, and MGP-1.

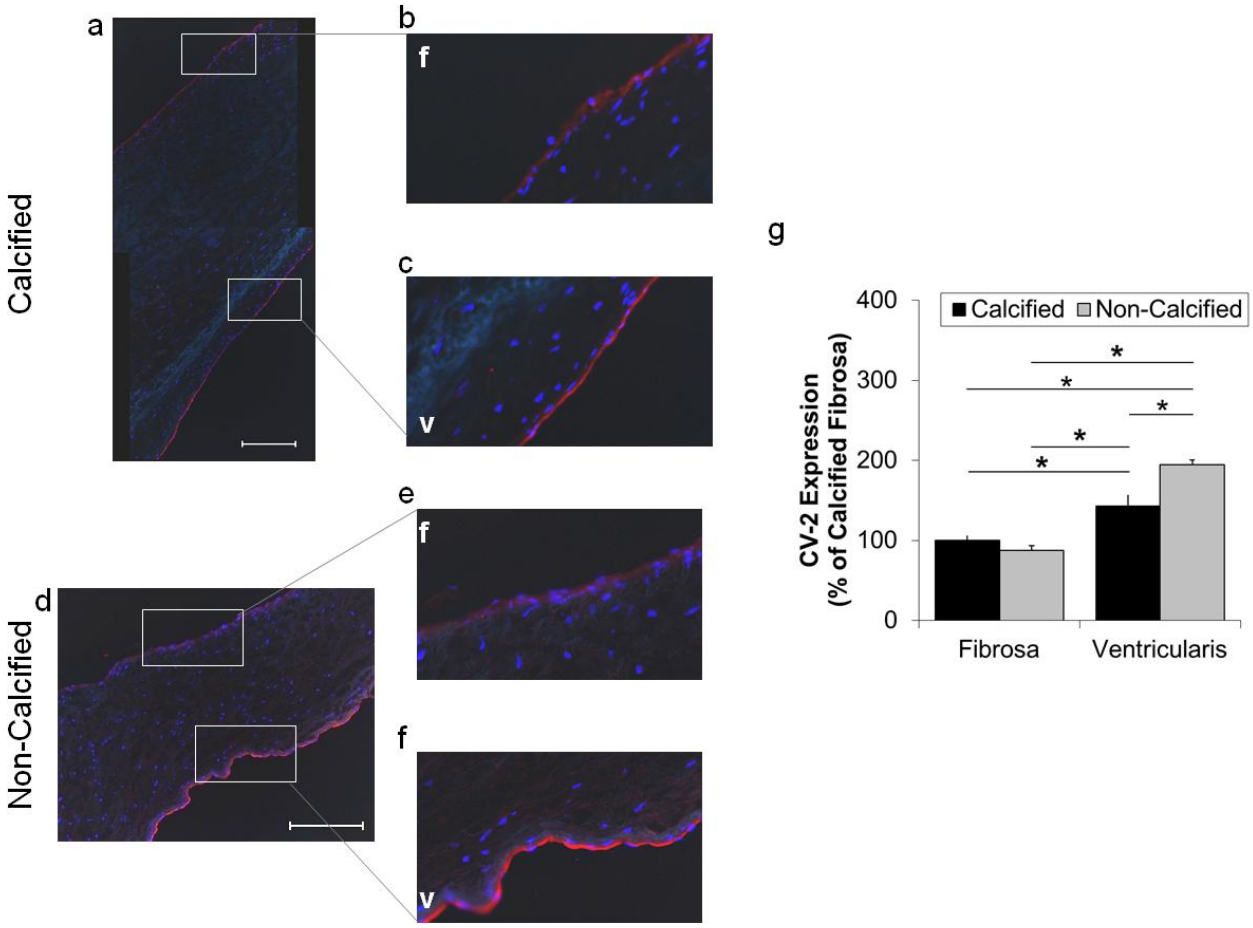

Figure 3.5 CV-2 expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for CV-2 (**a-f**), and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for each antagonist (**g**) (mean ± SEM). For CV-2, n=20 calcified and n=14 non-calcified. *p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

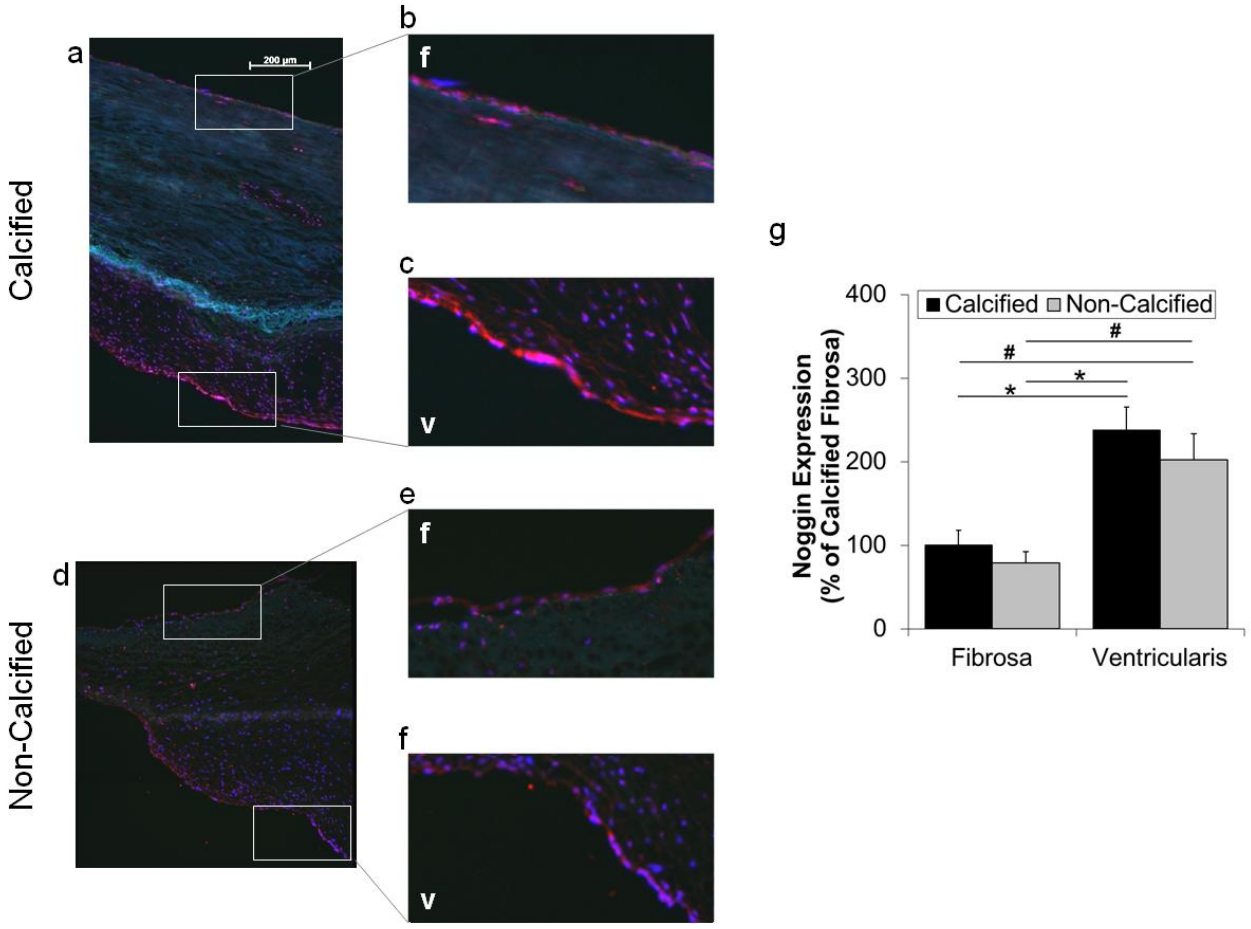

Figure 3.6 Noggin expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for noggin (**a-f**) and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for noggin (**g**) (mean ± SEM). For noggin, n=14 calcified and n=6 non-calcified. *p<0.05, #<0.06. **v** and **f** denote ventricularis and fibrosa respectively.*

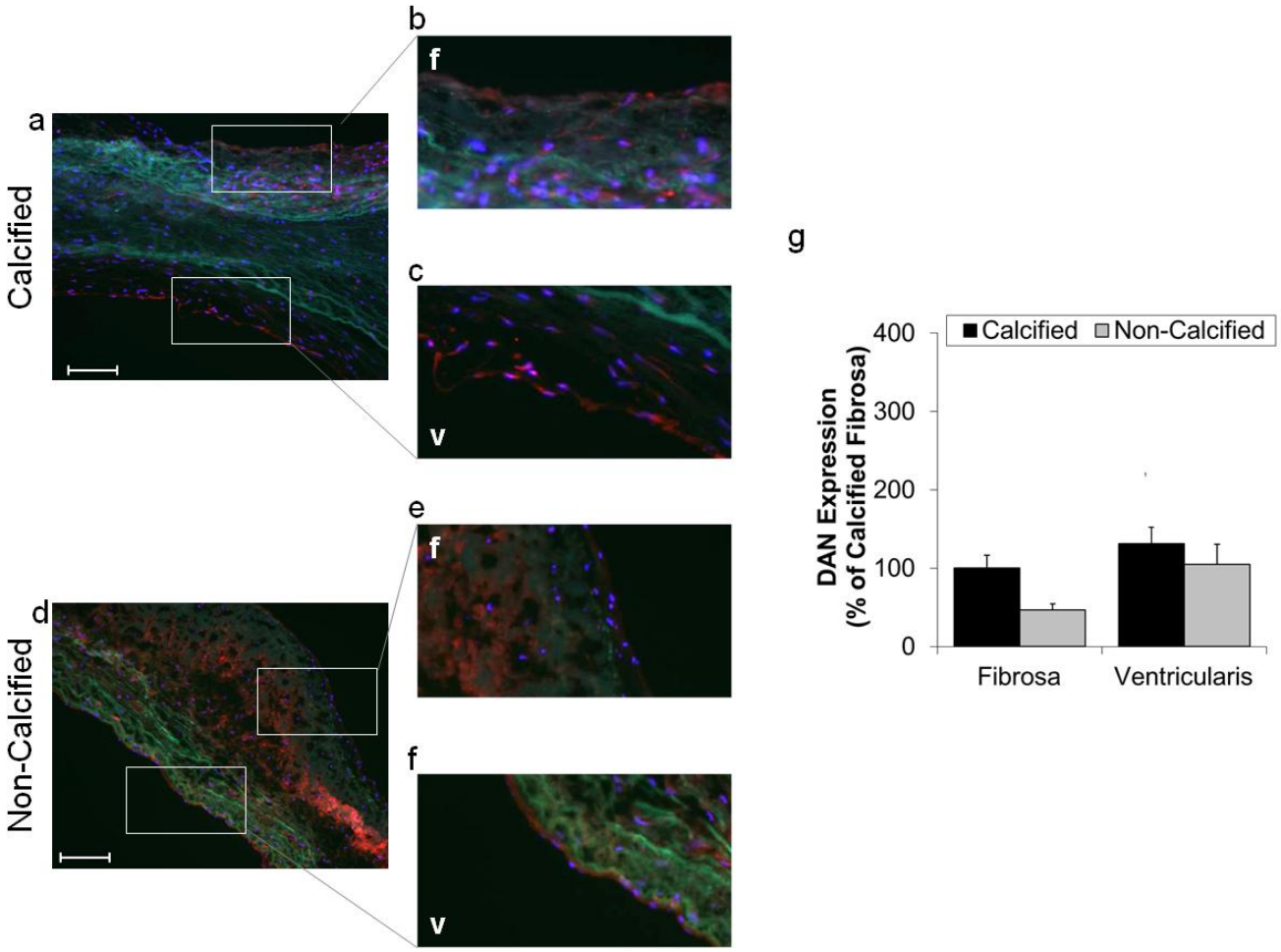

Figure 3.7 DAN expression in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for DAN (**a-f**) and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for DAN (**g**) (mean ± SEM). For DAN, n=10 calcified and n=8 non-calcified. *p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

Side-dependent activation of a BMP-sensitive SMAD pathway in human AV

Above staining results indicated that while BMPs were modestly decreased in the calcification-prone fibrosa-endothelium, the BMP antagonists (CV-2 and noggin) seemed to be much more abundant in the ventricularis endothelium. To determine whether the relative abundance of the BMP antagonist over the BMPs were affecting the side-dependent BMP pathway activation, I examined the level of phosphorylated SMAD 1/5/8 (phospho-SMAD 1/5/8) - a BMP activation pathway marker- in AV endothelium. Intense phospho-SMAD 1/5/8 staining was observed only in the calcified fibrosa endothelium (Figure 3.8). In contrast, non-calcified AV endothelia in either fibrosa or ventricularis showed only faint levels of phospho-SMAD 1/5/8 (Figure 3.8).

As a comparison, phospho-SMAD 2 levels, a canonical TGFβ signaling activation marker, was used. Overall, I did not observe any statistically significant differences in phospho-SMAD 2 levels in any of the AV endothelial groups. However, I found a trend for lower phospho-SMAD 2 levels in the non-calcified fibrosa endothelium ($p<0.1$) compared to the non-calcified ventricularis endothelium (Figure 3.9).

Lastly, I examined the level of inhibitory SMAD 6. SMAD 6 expression was significantly higher in the non-calcified ventricularis endothelium compared to non-calcified fibrosa endothelium and endothelium of calcified valves (Figure 3.10).

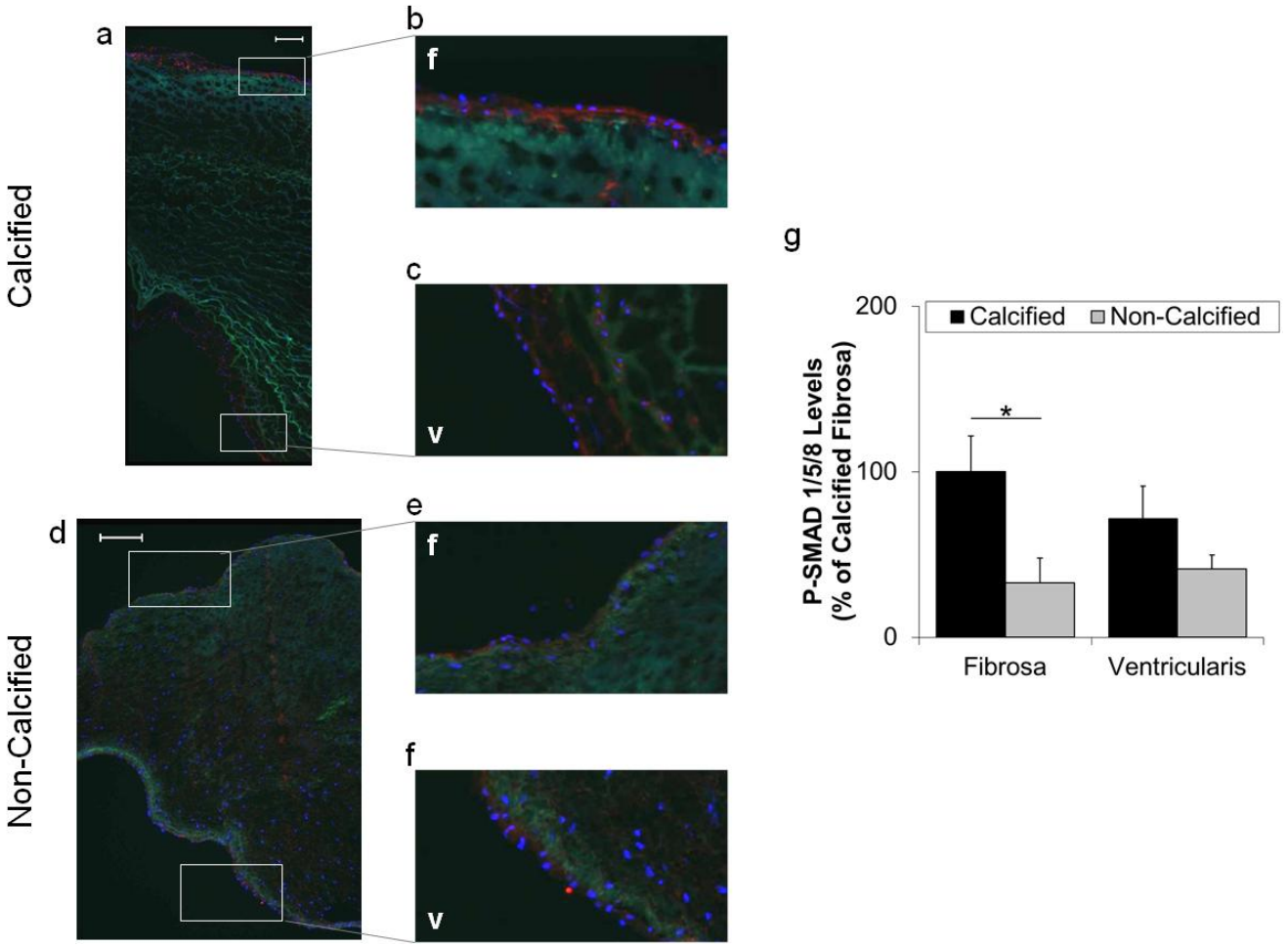

Figure 3.8 Phospho-SMAD 1/5/8 level is high in calcified fibrosa endothelium. *Calcified and non-calcified AV sections were stained for phospho-SMAD 1/5/8 (**a-f**), and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for phospho-SMAD 1/5/8 (**g**) (mean ± SEM). For phospho-SMAD 1/5/8, n=13 calcified and n=12 non-calcified. *p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

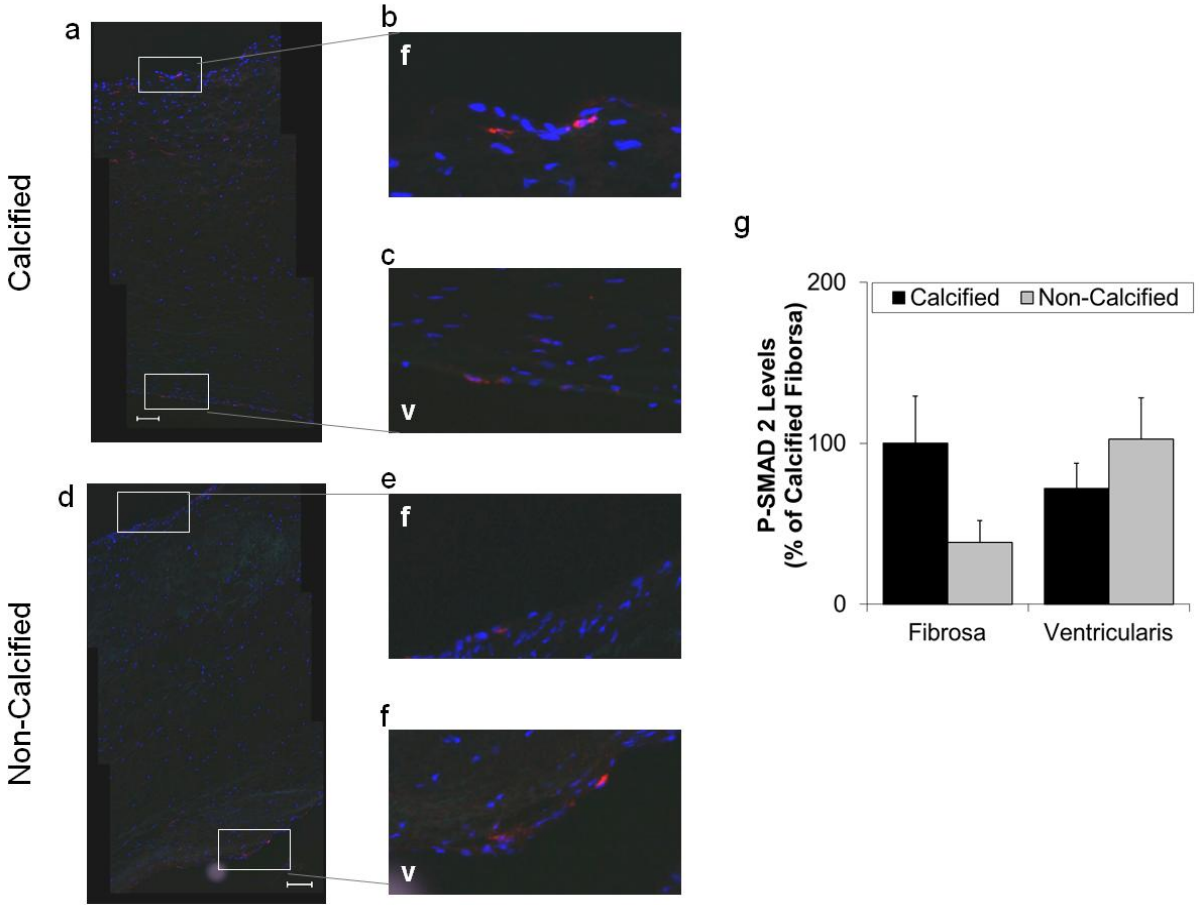

Figure 3.9 Phospho-SMAD 2 levels in the fibrosa and ventricularis endothelium. *Calcified and non-calcified AV sections were stained for phospho-SMAD 2 (**a-f**), and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for phospho-SMAD 2 (**g**) (mean ± SEM). For phospho-SMAD 2, n=13 calcified and n=12 non-calcified. For phospho-SMAD 2, n=14 calcified and n=13 non-calcified. For SMAD 6, n=22 calcified and n=15 non-calcified. *p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

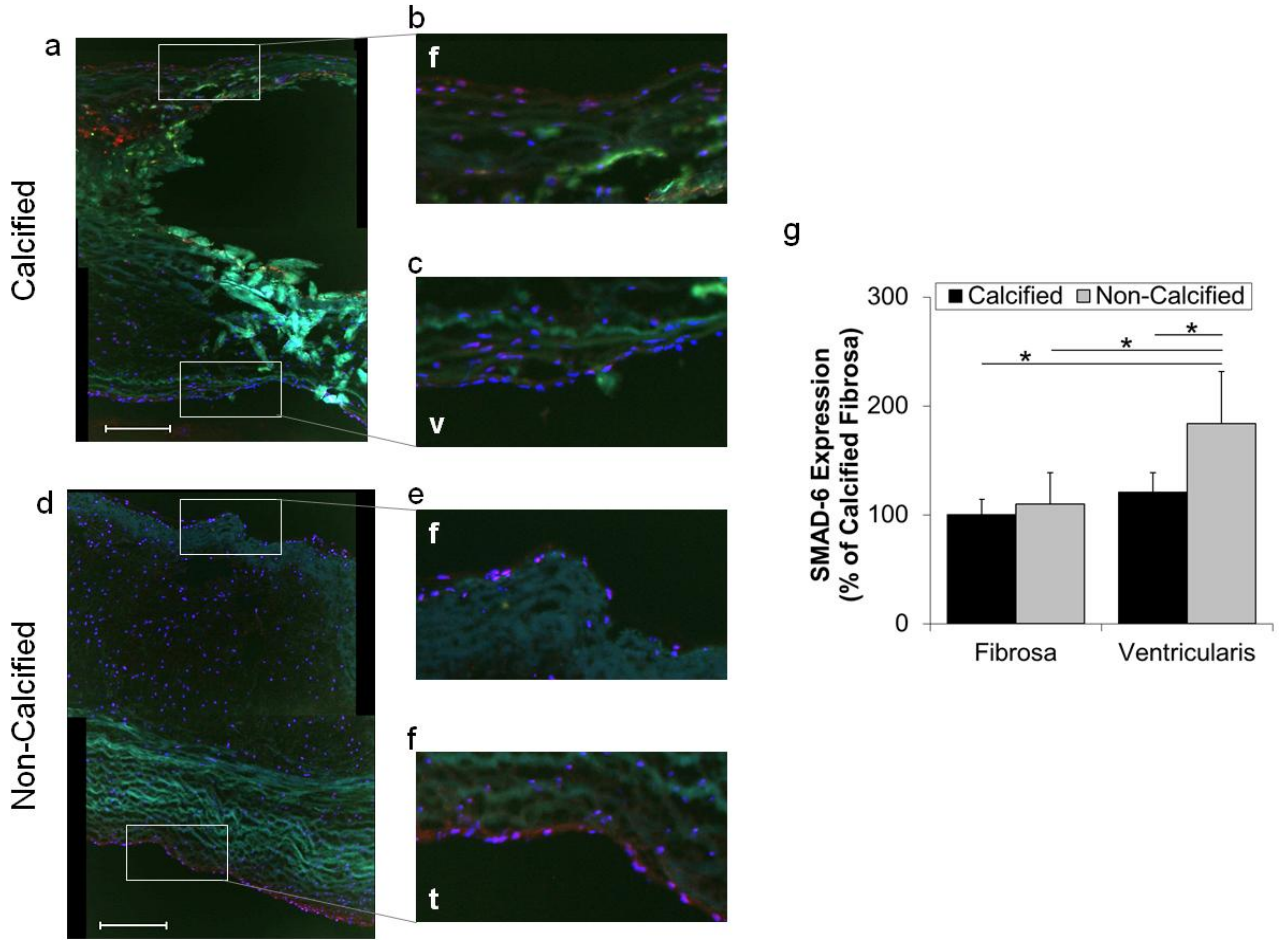

Figure 3.10 Inhibitory SMAD 6 level is highest in the ventricularis endothelium of non-calcified valves. *Calcified and non-calcified AV sections were stained for phospho-SMAD 6 (**a-f**), and a rhodamine-labeled secondary antibody. Shown are representative images. Bar graphs show staining intensities of fibrosa- and ventricularis-endothelium for phospho-SMAD 6 (**g**) (mean ± SEM). For SMAD 6, n=22 calcified and n=15 non-calcified. *p<0.05. **v** and **f** denote ventricularis and fibrosa respectively.*

Discussion

AV calcification and sclerosis primarily occur in the fibrosa, while the ventricularis is relatively unaffected [7, 9]. However, the specific mechanisms underlying this side-dependent AV disease is unclear. A potential mechanism is the different hemodynamic environment that is experienced by each side of the valvular leaflet. The fibrosa endothelium is exposed to disturbed flow conditions including oscillatory flow whereas the ventricularis endothelium is exposed to pulsatile laminar flow conditions [10]. This suggests that oscillatory shear experienced by the fibrosa endothelium may be responsible for the pro-osteogenic environment leading to the side-dependent calcification. This hypothesis has been supported by previous studies using mainly normal porcine AVs and cultured porcine AV endothelial cells.

Previously, Dr. Hanjoong Jo's lab have shown that oscillatory shear stress induces BMP-4 expression in vascular endothelial cells, and this BMP-4 expression leads to inflammatory response in a NFκB and NADPH oxidase-dependent manner [21]. In humans, BMP-2 and -4 are found in atherosclerotic plaques, endothelium overlying advanced atherosclerotic lesions, and in calcified regions of AVs [23-24]. Furthermore, in normal pig AVs, BMP-4 mRNA and protein levels are higher on the fibrosa endothelium [11, 16]. Also, laminar shear inhibits expression of BMP-4 in cultured pig AV endothelial cells [11]. Based on these findings, I hypothesized that the fibrosa endothelium exposed to oscillatory flow would express a high level of BMPs, which in turn leads to side-dependent calcification of the AV. However, my results did not support my initial hypothesis. I found that BMP-2 and -4 expression was higher on the non-calcified ventricularis endothelium compared to fibrosa endothelium of both calcified and non-calcified AV (Figures 3.2, 3.3, and 3.11). BMP-6 expression also seemed to be higher on the ventricularis endothelium compared to the fibrosa endothelium of both calcified

and non-calcified AVs (Figure 3.4). These results indicate that BMP expression levels in the diseased (both calcified and non-calcified) human AV endothelium do not correlate with the side-dependent AV calcification. It is important to note that the previous studies used normal pig AV endothelium to demonstrate the higher BMP-4 levels in the fibrosa endothelium than the ventricularis [11, 16]. However, a recent study showed that BMP-4 levels were decreased in the fibrosa endothelium of the hypercholesterolimic pig AVs compared to the normal pig AV [15]. This surprising result in diseased pigs is consistent with my current finding in diseased human AVs. Therefore, I next tested an alternative hypothesis that decreased expression of BMP antagonists is responsible for the preferential calcification in human AVs.

In cultured vascular endothelial cells, BMP antagonists noggin, follistatin and MGP-1, which are co-expressed with BMP-4, provide a negative feedback mechanism inhibiting BMP-4's inflammatory effect [31]. Furthermore, follistatin and noggin are found in advanced atherosclerotic lesions [31]. In healthy pig AVs, chordin mRNA levels are higher on the ventricularis endothelium than the fibrosa endothelium [16]. Here, I found that CV-2 and noggin were differentially expressed in the human AVs (Fig. 3.5, 3.6, and 3.11). Noggin and CV-2 expression levels were lower in the fibrosa endothelium than the ventricularis in both calcified and non-calcified AVs. Furthermore, CV-2 expression was highest in the non-calcified ventricularis. These results suggest that abundant levels of BMP antagonists in the ventricularis endothelium, especially in non-calcified human AVs, provide an anti-calcific environment. This result is also consistent with the previous result showing higher chordin expression on the ventricularis endothelium of normal pig AV compared to the fibrosa [16].

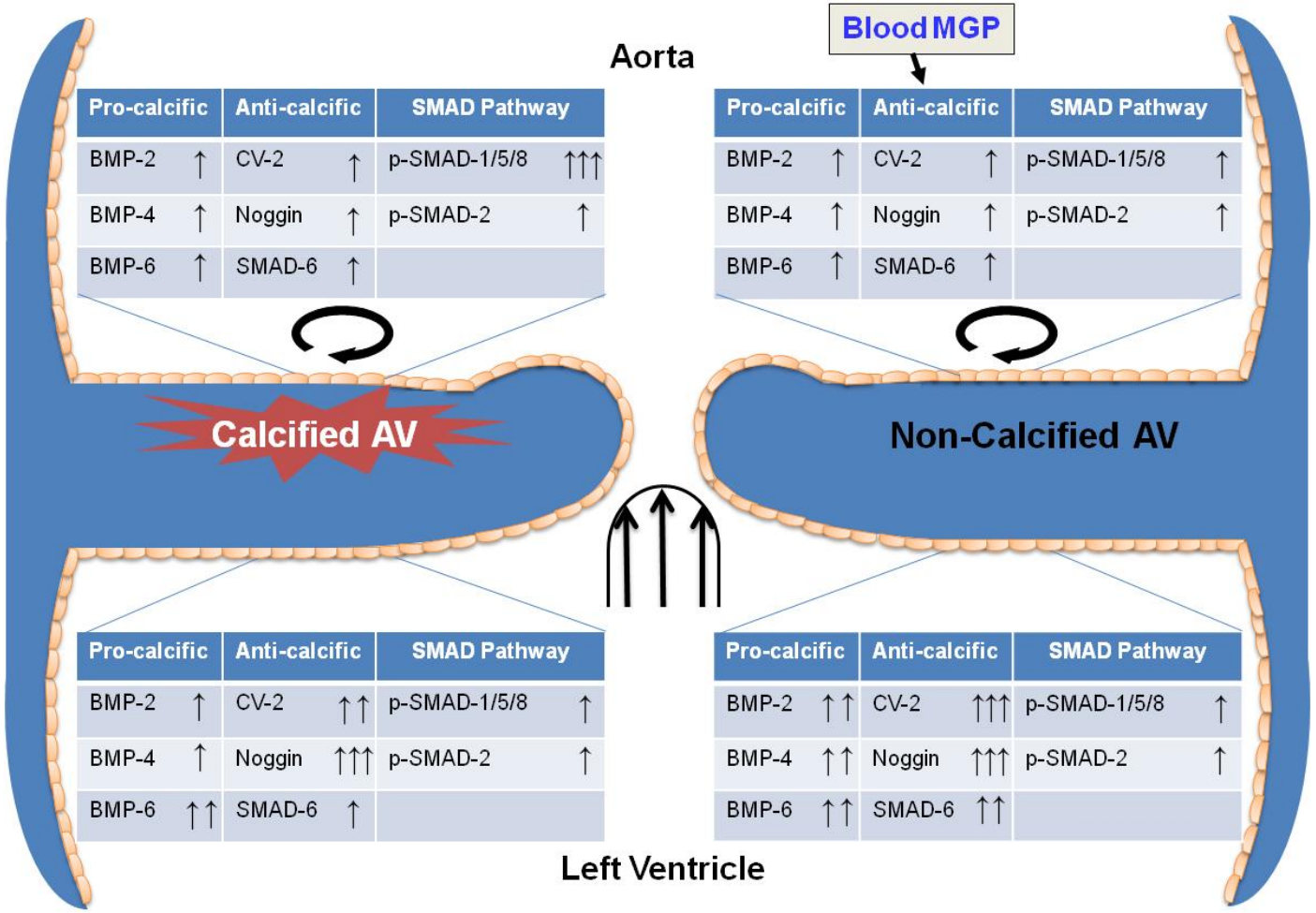

Figure 3.11 A schematic summary of the results. *Shown in the left is a calcified human AV and the right is a non-calcified human AV. Endothelial expression of BMP, BMP antagonists, inhibitory SMAD and phospho-SMADs in a side-dependent manner, summarizing the results are also shown. Pulsatile laminar shear in the ventricularis and oscillatory shear in the fibrosa sides are shown.*

Inhibitory SMAD6 could play an important role in the side-dependent AV calcification. SMAD6 was shown to be induced by laminar shear stress in vascular endothelial cells [36]. Moreover, SMAD6 deficiency was shown to cause cardiac valve hyperplasia in mice [37], demonstrating its importance in AV biology. This led us to hypothesize that side-dependent expression of SMAD6 would play a role in fibrosa calcification. Consistent to this hypothesis, I found that SMAD 6 expression levels were highest in the non-calcified ventricularis endothelium, contributing to the anti-calcific environment on the ventricularis-side of the AV (Figure 3.10 and 3.11).

Since my findings thus far suggested that the preferential calcification on the fibrosa may be correlated with decreased endothelial BMP antagonists and the inhibitory SMAD levels, I examined whether BMP pathways were activated in the AV endothelium. My result showed that phospho-SMAD 1/5/8, the canonical BMP pathway activation marker, was significantly activated in the calcified fibrosa endothelium compared to non-calcified fibrosa endothelium (Fig. 3.8). In contrast, I found no significant differences in phospho-SMAD 2 levels among all groups (Fig. 3.9), indicating that there was no differential activation of the canonical TGFβ signaling pathway. These findings clearly demonstrate the correlation between the phospho-SMAD1/5/8 activation in the endothelium and calcification in the fibrosa-side in human AVs.

It was somewhat surprising to find that BMP, BMP antagonists, and SMAD 6 levels were similar between the fibrosa endothelium of calcified and non-calcified human AVs, while phospho-SMAD 1/5/8 levels on the calcified fibrosa endothelium was significantly higher than the non-calcified fibrosa. There are several potential mechanisms that may explain my unexpected findings. First, a recent study found that patients with AV

calcification have significantly lower levels of circulating uncarboxylated MGP (ucMGP) than the healthy cohort [32]. They hypothesized that the low levels of ucMGP is because of the lack of release of MGP into the circulation from the vascular wall due to consumption of MGP. The deficiency of circulating MGP in calcified AV patients may tip the balance in favor of the BMPs (Figure 3.11), promoting a pro-calcific environment compared to the non-calcified AV patients. Second, activities of some BMP antagonists such as noggin are subject to post-translational regulations [38-39]. The hydrophobic ring of Pro-35 of noggin inserts into a hydrophobic pocket on BMP-7 and that point mutation of Pro35Arg reduces its binding affinity to BMP-7 [39]. ROS is capable of oxidizing proline residues [40]. Importantly, ROS level is known to be increased in the calcified regions, but not in the non-calcified regions of human AVs [41]. In addition, I have shown that OS produces ROS production by the BMP4-dependent mechanisms in vascular endothelial cells [20-21]. Therefore, I propose that OS-dependent production of ROS in the fibrosa endothelium and the adjacent regions may oxidatively modify the BMP4 antagonists which could reduce their binding affinities to BMPs. Third, some BMP antagonists (e.g. follistatin, chordin, MGP-1) that I could not examine due to the lack of specific antibodies for immunostaining studies may also be responsible for my finding. Fourth, expression of the BMP receptors may also contribute to the observed difference.

It is important to emphasize that the non-calcified AVs used for this study were obtained from recipient hearts following heart transplantations. Therefore, these samples were from heart failure patients, not from a “healthy” subject population, and should not be viewed as non-diseased AVs although they were not calcified.

In summary, I showed that BMP pathways are preferentially activated in the calcified fibrosa endothelium human AVs. This side- and disease-dependent activation of BMP pathway correlates with the deficiency of BMP antagonists and an inhibitory SMAD in the fibrosa endothelium. These findings suggest that preferential activation of BMP pathways is controlled by the balance between the BMPs and their inhibitors play an important role in side-dependent calcification of human AVs.

Acknowledgments

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CHAPTER 5

SHEAR-REGULATION OF BONE MORPHOGENIC PROTEINS, BONE MORPHOGENIC PROTEIN ANTAGONISTS AND THEIR CONTRIBUTION TO INFLAMMATION IN HUMAN AORTIC VALVE ENDOTHELIAL CELLS

In Chapter 3, I identified that bone morphogenic proteins (BMPs) and their antagonists are expressed in the valvular endothelium and that BMP pathway activity is significantly increased in calcified valves and is expressed highest on the fibrosa side of calcified cusps. In Chapter 4, I found that human endothelial cells isolated side-specifically from the ventricularis and fibrosa endothelium were shear-responsive *in vitro*, and had distinct inflammatory shear responses from each other. In this chapter, my goal was to: 1) determine the shear responsiveness of BMPs and BMP antagonists and 2) establish the functional importance of BMPs in valvular endothelial cell inflammation. For the first time, I have characterized the shear response of the BMPs in human aortic valve endothelial cells, and have found that increased expression of BMPs, unlike aortic endothelial cells, does not induce inflammation as measured by a monocyte binding assay.

Summary

Background. Previous studies indicate that bone morphogenic proteins (BMPs) may play a role in aortic valve disease. Specifically, porcine valvular endothelial cells (ECs), like porcine aortic, decrease their production of BMP4 when exposed to laminar shear stresses *in vitro*. The role of fluid shear stress on the regulation of BMPs in human

aortic valves, however, remains understudied. I hypothesized that OS would cause an increase in BMP4 production and enhance inflammation in valvular ECs in a BMP-dependent process.

Methods and Results. To test this hypothesis *in vitro*, OS or unidirectional LS was applied to side-specific human aortic valvular ECs. BMP, BMP antagonists, and SMAD levels were examined through immunoblotting and quantitative PCR (qPCR) while the BMPs' effect on inflammation was determined through a monocyte adhesion assay in the presence or absence of a BMP antagonist, noggin. I confirmed that both fibrosa and ventricularis ECs exhibit a 2-fold increase of BMP-4 protein and BMP-2 mRNA under OS when compared to LS while seeing no changes in BMP-6 mRNA. The BMP antagonist, crossveinless-2, was not regulated by shear stress, while follistatin was decreased by LS in ventricularis ECs but not fibrosa ECs. Finally, no difference in monocyte adhesion was seen between noggin-treated and untreated ECs.

Conclusions. These results demonstrate differences between fibrosa and ventricularis ECs under different shear conditions while also demonstrating differences between human aortic valvular ECs and human aortic ECs. The differences described here may have important implications in disease development and in the design of a tissue engineered heart valve.

Introduction

Until recently, aortic valve calcification was thought to be an age-associated disease [1-2], but it is now believed to be an active inflammatory process that is characterized by lipid accumulation, neovascularization, inflammation, calcified nodules and, in some cases, the formation of lamellar bone [1, 3]. Aortic valve disease and atherosclerosis share many similarities and risk factors. Atherosclerosis preferentially develops in areas that experience disturbed or low oscillating flow conditions such as the common carotid bifurcation, coronary arteries, and the abdominal aorta, while areas that experience laminar flow conditions remain athero-protected [4-5]. As with the vessel wall in atherosclerosis, the aortic valve disease preferentially occurs on the fibrosa-side of the valvular cusp were it experiences oscillatory fluid shear stresses [6-7]. In contrast, the ventricularis, which experiences a pulsatile unidirectional flow, remains relatively unaffected [7].

The vascular endothelium is a critical mediator of mechanical and humoral stimuli and is an active participant in vascular biology. Fluid shear stress plays a critical role in the physiological state of the endothelial cell [8]. When endothelial cells are subjected to a unidirectional fluid shear stress, they align parallel to the fluid flow and secrete several factors that promote endothelial cell survival and vascular wall health; however, when endothelial cells experience oscillatory fluid shear stress, they do not align to the flow, secrete inflammatory cytokines, and promote leukocyte migration through the expression of adhesion molecules[9-20]. One of the inflammatory cytokines secreted by endothelial cells that is of interest is BMP-4 [18-19].

The BMPs are members of the transforming growth factor β (TGFβ) superfamily of proteins and were originally discovered as a bone growth and repair protein[21-22];

however this family of proteins and their antagonists have been shown to have diverse and important roles in embryonic development, dorsal ventral formation, chondrogenesis, and cell differentiation [22-23]. Functionally, BMPs are secreted in an active from and bind to specific receptors thereby inducing phosphorylation of SMAD 1, 5, or 8. SMAD 1, 5, or 8 then becomes a transcription factor, regulating BMP-related genes[24-28]. In the vascular endothelium, BMP4 has been shown *in vitro* and *in vivo* to be increased in endothelial cells exposed to oscillatory flow, while the closely related BMP2 has not been shown to be shear sensitive[18-19]. I have shown that BMP4 is a mechanosensitive and proinflammatory cytokine in vascular endothelial cells [18-19]. Furthermore, it is known that BMP4 infusion induced hypertension in mice in a NADPH oxidase-dependent manner [29]. Moreover, BMP-2 and -4 expression is increased in calcified human AVs and human atherosclerotic lesions [30-31].

The BMP antagonists serve as a regulator of BMP activity, and have important functions in endothelial cell biology. BMP antagonists are secreted glycol proteins which include noggin, DAN, crossveinless 2 (CV-2), follistatin, matrix Gla protein (MGP), chordin and gremlin[32]. Each antagonist has a different affinity for BMPs [32]. Most BMP antagonists bind to BMPs to block signal transduction; however CV-2 can act either as an antagonist or an agonist in endothelial cells [33].

Here, I hypothesize that disturbed flow conditions, which are see on the aortic side of the valve, will 1) cause an increase in BMPs (specifically BMP4), and 2)decrease antagonist expression. These phenomena will cause an increase in the inflammatory state of the aortic valve ECs. My results indicate that OS increases BMP-2 and -4 and also causes a modest increase in phosphorylated SMAD 1/5/8, a marker of BMP pathway activation. Finally, it appears that in aortic valve ECs, BMPs are not playing a role in inflammatory

activation as indicated by a monocyte binding assay. These results give us important information regarding differential shear regulation of the BMPs in aortic valve endothelial cells and may have implications in the pathogenesis of aortic valve disease.

Materials and Methods

Cell Isolation

Side-specific endothelial cells were harvested from patients undergoing heart transplants at Emory University according to an IRB-approved protocol. Valves were excised from the heart and washed three times in Hank's Buffered Saline Solution (HBSS). They were then incubated in a 5X antibiotic solution (Gibco) for 30 minutes. The leaflets were then oriented so the fibrosa or ventricularis endothelium were facing the same direction. A 600 units/mL solution of collagenase type II (Worthington 4176) was incubated on the valve for 5 minutes. Leaflets were then washed with growth media and the collagenase/growth media mixture was collected in a centrifuge tube. Leaflets were then scraped two times in succession (lightly and then harder) with a sterile scalpel. Between scrapes the leaflets were rinsed with growth media and the solution was placed in separate collection tubes. The leaflets were then placed in a new dish and washed. The same procedure of collagenase and scraping was then performed on the opposite side. Cells were spun down at 1000 RPM for 5 minutes and plated in a 12-well dish and sequentially expanded into a 6-well and T-75 dish. The cells were then sorted using 5 μg/mL of DiL-Acetylated-LDL (Biomedical Technologies Inc.) and the BD FACS Aria Cell Sorter.

Human Aortic Valve Endothelial Cell Culture and Shear Stress Studies

Endothelial cells obtained from human aortic valves were cultured in growth medium [MCDB131 (Cellgro®) containing FBS (Cellgro®, 10%), bovine ECGS (1%), L-Glutamine

(Gibco, 1%), Penicillin-Streptomycin (Gibco, 1%), hydrocortisone (Sigma, 0.001 mg/mL), hFGF (R&D, 0.002 μg/mL), hEGF (Invitrogen, 0.01 μg/mL), IGF (Invitrogen, 0.002 μg/mL), VEGF (R&D, 0.001 μg/mL), ascorbic acid (Sigma, 50 μg/mL)] and used between passages 4 and 5. Confluent endothelial cell monolayers were grown in 10 cm dishes and were exposed to an average ventricularis unidirectional laminar shear level (20 dynes/cm^2) or an OS bidirectional flow (± 5 dynes/cm^2) for 24 hours using a Teflon cone and plate viscometer (α =0.5°) as described previously. As a control, cells were cultured in a no flow (static) conditions. One hour before shear studies, media was replaced [MCDB131 (Cellgro®) containing FBS (Cellgro®, 2.5%), L-Glutamine (Gibco, 1%), Penicillin-Streptomycin (Gibco, 1%)].

Western Blots

Following shear exposure, cells were rinsed three times with phosphate buffered solution (PBS) and then lysed using a cell lysis buffer (RIPA) supplemented with phosphotase (Sigma) and protease (Roche) inhibitor cocktails. Following a modified Lowery protein assay, equal amounts of total protein were resolved by SDS-PAGE as previously described [34]. Protein was transferred from the SDS-gel to an immobilio-P-membrane (Millipore, PVDF) and probed with anti-BMP4 (Santa Cruz, 1:1000), anti-SMAD 1/5/8 (Cell Signaling, 1:1000), anti-phosphorylated SMAD 1/5/8 (Cell Signaling, 1:1000), or anti-actin (Santa Cruz, 1:1000). A secondary antibody conjugated to alkaline phosphate was used to detect protein levels by chemiluminescence method [34].

Quantitative PCR

Following shear exposure, cells were washed three times with ice cold PBS. Total RNA was then isolated using RNeasy Mini Kit (Qiagen). 1 μg of total RNA was reverse transcribed using random hexamer primers and a SuperScript®III First Strand Kit

(Invitrogen). The resulting cDNA was then amplified using real time PCR (ABI Step One Plus) and a master mix containing, gene-specific forward and reverse primers (IDT, Table 1), ROX reference Dye (Stratagene, 1:50), and 2x Brilliant II SYBR® Green QPCR master mix (Stratagene). The PCR conditions were 2 minutes at 56°C, 10 minutes at 95°C, 40 cycles of 30s at 95°C and 1 minute at 60°C, with a melting curve of 15 seconds at 95°C and 1 minute at 60°C. All values were normalized to 18S (Ambion).

TABLE 5.1 qPCR Primer Sequences

Gene	Primer Sequence	
BMP-2	Forward	5' - AGGGCATCCTCTCCACAAAAG - 3'
	Reverse	5' - CCACCCCACGTCACTGAAGT - 3'
BMP-4	Forward	5' - ATGAAGCCCCCAGCAGAAGT - 3'
	Reverse	5' - AGGGCTCACATCAAAAGTTTCC - 3'
BMP-6	Forward	5' - GGAAGCATGAGCTGTATGTGAGTT - 3'
	Reverse	5' - GTTGAGTGGGAAGGAGCATTCT - 3'
Chrodin	Forward	5' - AAGGACCTGGAGCCGGAACT - 3'
	Reverse	5' - AGTCCGCCAACCTCACATTG - 3'
Crossveinless 2	Forward	5' - TTCTTGCCATCCTTGCGTAGT - 3'
	Reverse	5' - GGCATCCAGACACCCTTTAATTT - 3'
Follistatin	Forward	5' - GAAAACCTACCGCAATGAATGTG - 3'
	Reverse	5' - ACATCCCGACAAGTCTTTTTACATC - 3'
MGP	Forward	5' - CCTCACAGCCTTCCACTAACATC - 3'
	Reverse	5' - TCAGGCTCTTCATGGTTTCGT - 3'
Noggin	Forward	5' - GGCCAGCACTATCTCCACATC - 3'
	Reverse	5' - GCAGCGTCTCGTTCAGATCCT - 3'

Monocyte Binding

Monocyte binding was done under static conditions using purchased THP1 Monocytes (ATCC) as described previously [18-19]. THP1 monocytes, at a concentration of $1x10^6$ cells/mL were incubated with a fluorescent dye 2';7'-bis(carboxyethyl-5) (6)-

carboxyfluroscein-AM (BCEFC, Molecular Probes, 1 mg/mL) in serum-free RPMI (Cellgro®) for 30 minutes at 37°C. During which, endothelial cells, which were exposed to shear stress for 24 hours, with or without noggin (R&D, 50 ng/mL), were washed with RPMI media before addition of monocytes (6 million). Monocytes were incubated with endothelial cells for 30 minutes at 37°C to allow binding. Unbound monocytes are removed by washing with HBSS with calcium and magnesium (Cellgro®). Bound monocytes were then quantified by counting the number of monocytes bound per viewing area (5x Original Magnification). Images were captured using an epifluorescent microscope (Zeiss).

Statistics

Data is presented was mean ± standard error with n's representing number of replicates. Student's t-test was used to establish significance between groups. $P<0.05$ was considered statistically significant with at least 3 independent experiments.

Results

Laminar shear decreases intracellular BMP4 levels

Laminar shear stress (LS) in both the ventricularis and fibrosa endothelial cells decreased pro- and mature-BMP4 by two-fold when compared to oscillatory shear stress (OS). No significant difference was seen between cells isolated from either side, or between OS and static conditions. Interestingly, a side-specific difference in BMP-4 levels exists at the transcription level. LS caused a 2.5-fold decrease in BMP-4 mRNA of ventricularis endothelial cells when compared to OS, which corroborates with the protein levels; however, no BMP-4 mRNA differences were observed when comparing laminar shear to OS in fibrosa endothelial cells. As a comparison, BMPs -2 and -6 shear-dependent mRNA expression levels were investigated in HAVEC. Interestingly,

BMP-2 mRNA expression was upregulated (2-fold) by OS in both fibrosa and ventricularis endothelial cells (Figure 5.2a). Although, not significant, a modest increase in BMP-2 mRNA levels was seen in ventricularis endothelial cells when compared to fibrosa endothelial cells. BMP-6 mRNA was not altered by the different shear conditions (Figure 5.2b). Protein levels were not detected in the cell lysate due to the secretory nature of these proteins.

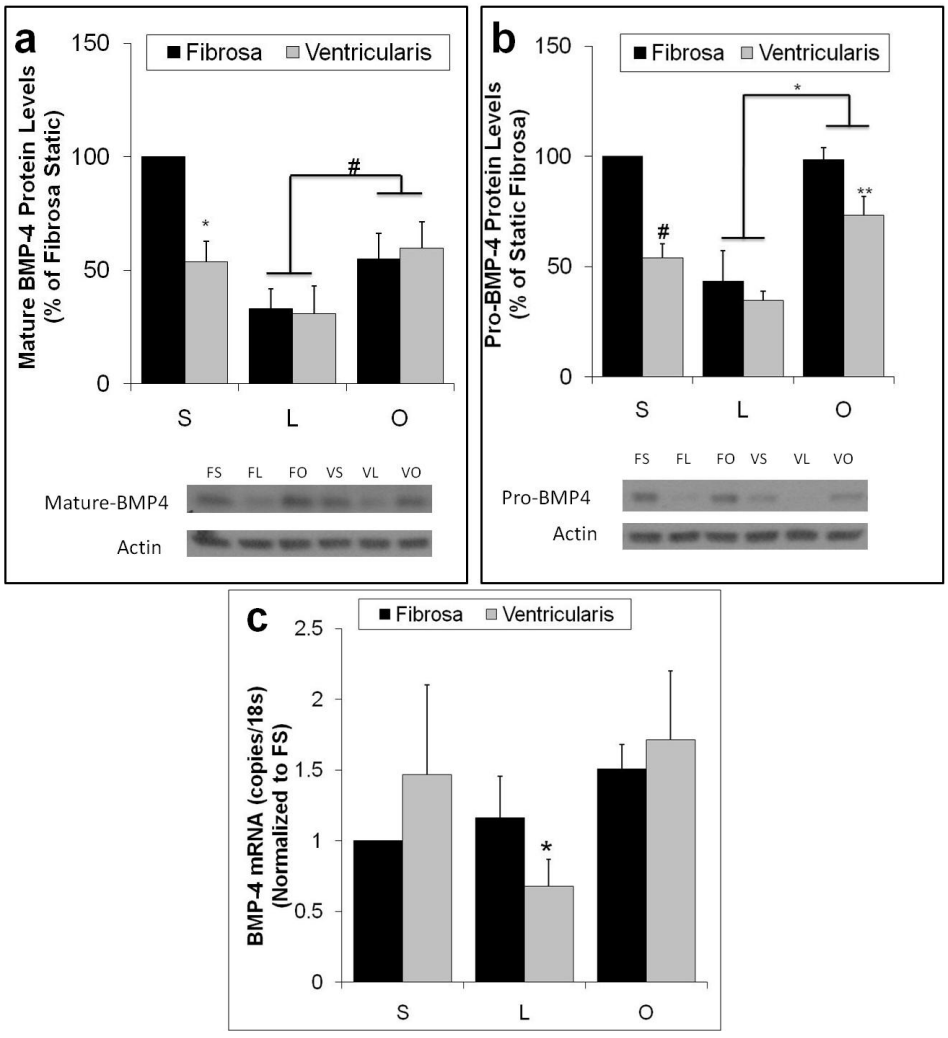

Figure 5.1 Shear regulation of BMP-4 in human aortic valvular endothelial cells. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA or protein was collected. (a) and (b) mature and pro-BMP-4 were examined respectively. n=4 (a) * p<0.05 against FS. # p<0.10. (b) * p<0.05. # p<0.05 against FS. ** p<0.05 against FO. (c) BMP-4 mRNA level.*p<0.05 against VO and FO. n=4 from 3 different patients. (Means ± SE).*

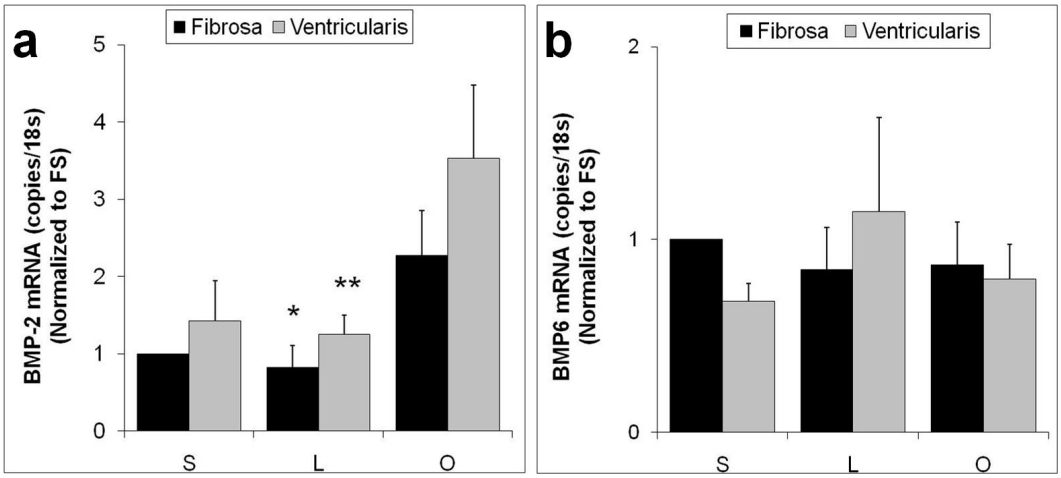

Figure 5.2 Shear regulation of BMPs -2 and -6 in human aortic valvular endothelial cells. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA was collected. (a) and (b) BMP-2 and BMP-6 mRNA levels were examined respectively. (a) * p<0.05 against V O and FO. ** p<0.05 against VO. n=4 from 3 different patients. (Means ± SE).*

Differential regulation of BMP antagonists

I found differential regulation of BMP antagonists on the fibrosa and ventricularis sides of the aortic valve. I found a trend of an increase by LS of the BMP antagonist CV-2 in both endothelial cell types. Follistatin mRNA showed a different regulation pattern when compared to that of CV-2. In fibrosa endothelial cells, I saw no shear regulation of follistatin. In the ventricularis endothelial cells LS caused significant reduction (4 fold) in follistatin mRNA when compared to OS. The BMP antagonist, MGP, in fibrosa endothelial cells was significantly reduced (2-fold) by shear, both laminar and oscillatory shear, when compared to static conditions; no difference was seen between LS and OS in the fibrosa endothelial cells. There was no shear regulation of MGP in the ventricularis endothelial cells. Noggin and chordin were also investigated, but mRNA of these antagonists was near the detection limit of qPCR.

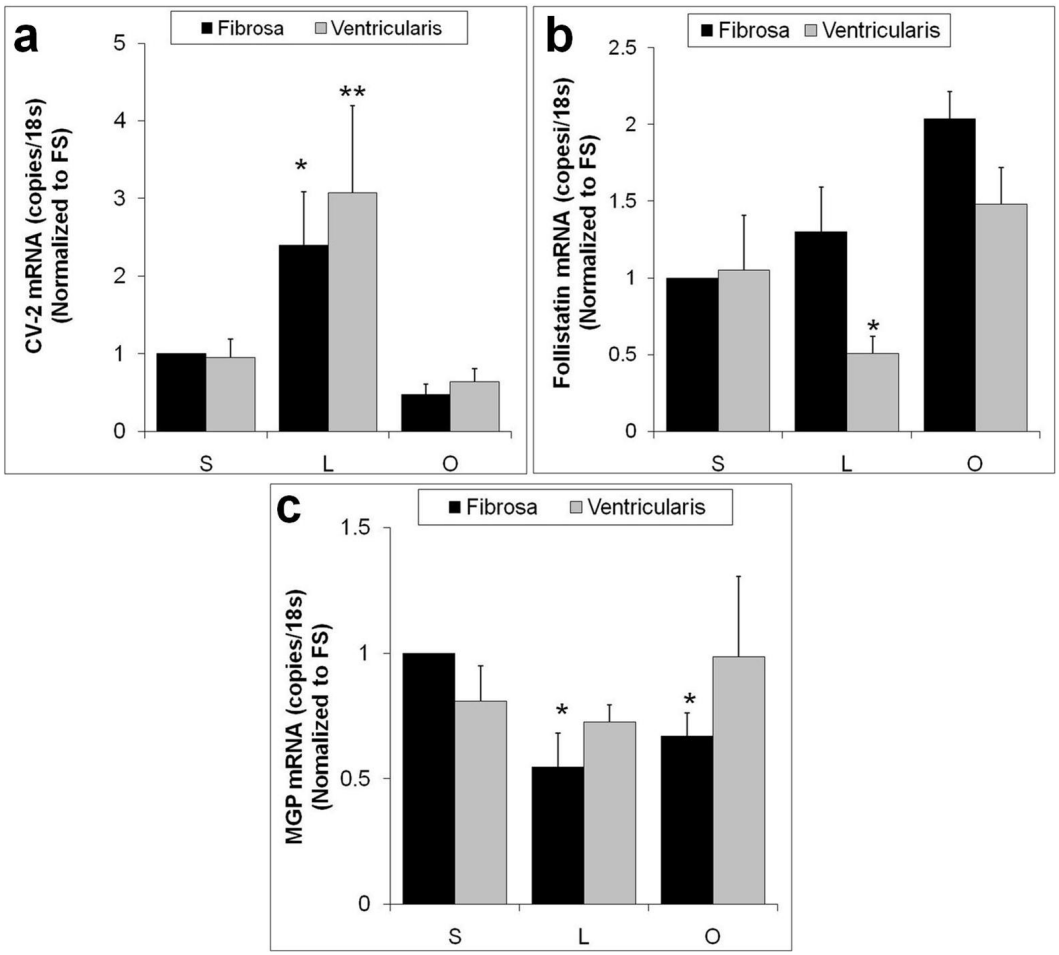

Figure 5.3 Shear regulation of CV2, follistatin and MGP in human ventricularis endothelial cells. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular mRNA was collected. (a) CV-2 mRNA levels were examined and an increase was seen under laminar flow. * p<0.05 against FO. ** p<0.09 against VO. n=4 from 4 different patients (b) Follistatin mRNA levels were examined and laminar shear decreased follistatin in ventricularis endothelial cells but not fibrosa. * p<0.05 against VO. n=4 from 3 different patients. (b) MGP mRNA levels were examined and shear decreased MGP in fibrosa endothelial cells but not ventricularis. * p<0.05 against FS. n=4 from 3 different patients. (Means ± SE).*

Laminar shear reduces SMAD 1/5/8 signaling in aortic valve endothelial cells in vitro

To determine the effect of BMPs on the endothelium phosphorylated SMAD 1/5/8, a marker of BMP pathway activation, was examined (Figure 5.4a). LS significantly downregulated phosphorylation of SMAD 1/5/8 in both cell types when compared to static. However, no difference was detected when comparing laminar versus oscillatory flow. Additionally, no shear regulation was found of total SMAD 1/5/8 protein levels.

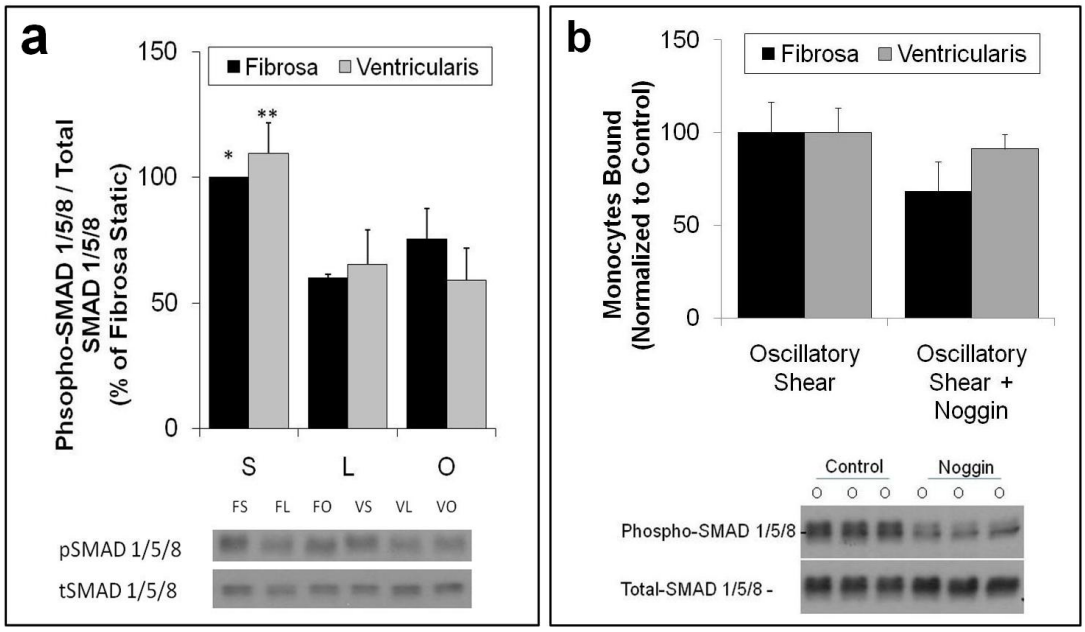

Figure 5.4 BMP pathway activation and BMP dependent monocyte adhesion. *Fibrosa (F) and ventricularis (V) endothelial cells were shear for 24 hours in static (S) laminar (L) or oscillatory shear conditions (O). After shear cellular protein was collected. (a) and phospho- and total-SMAD 1/5/8. Laminar shear decreased phospho-SMAD 1/5/8 levels when compared to static after densiometric analysis. *p<0.05 n=4 against FO. ** p<0.05 n=4 against VL and VO. (b) During oscillatory shear, endothelial cells were incubated with noggin for 24 hours. A monocyte adhesion assay was then performed. No difference was detected between the oscillatory shear control and the oscillatory + noggin. n= 4 from 4 different patients. (Means ± SE).*

BMPs do not play a role in endothelial inflammation

To test the BMPs' effect on endothelial cell inflammation, noggin was added for the duration of the shear and a monocyte adhesion assay was used to access the

inflammatory state of the endothelial cell. To first assess noggin's ability to block BMP signaling in valvular endothelial cells, the cells were sheared for 24 hours with and without noggin. Western blot analysis showed significantly less phosphorylated SMAD 1/5/8 in OS-conditioned endothelial cells treated with noggin than OS-conditioned endothelial cells treated without noggin. In fibrosa and ventricularis endothelial cells exposed to OS, noggin had no effect on the number of monocytes bound.

Discussion

AV calcification and sclerosis primarily occur in the fibrosa, while the ventricularis side is relatively unaffected [7, 35]; however, the specific mechanisms underlying this side-dependent AV disease is unclear. A potential mechanism is the different hemodynamic environment that is experienced by each side of the valvular leaflet. The fibrosa side of endothelium is exposed to disturbed flow conditions including oscillatory flow whereas the ventricularis endothelium is exposed to pulsatile laminar flow conditions [6]. This suggests that oscillatory shear experienced by the fibrosa endothelium may be responsible for the pro-osteogenic environment leading to the side-dependent calcification. This hypothesis has been supported by previous studies using mainly normal porcine AVs and cultured porcine AV endothelial cells [36].

Previously, I have shown that oscillatory shear stress induces BMP-4 expression in vascular endothelial cells, and this BMP-4 expression leads to inflammatory response in a NFκB and NADPH oxidase-dependent manner [18-19]. In humans, BMP-2 and -4 are found in atherosclerotic plaques, endothelium overlying advanced atherosclerotic lesions, and in calcified regions of AVs [30-31]. Furthermore, in normal pig AVs, BMP-4 mRNA and protein levels are higher on the fibrosa endothelium [36-37]. Also, laminar shear inhibits expression of BMP-4 in cultured pig AV endothelial cells [36]. Based on these

findings, I hypothesized that the fibrosa endothelium exposed to oscillatory flow would express a high level of BMPs, which in turn leads to side-dependent calcification of the AV; however, my results in Chapter 3 did not confirm my initial *in vivo* hypothesis. To determine if BMP decrease on the fibrosa side of the valvular leaflet was due to the oscillatory shear stress, I examined BMPs, BMP antagonists, and SMAD levels in human AV endothelial cells *in vitro*.

Based upon previous results, I hypothesized that the oscillatory flow conditions would cause an increase BMP-4 expression similar to that of human vascular endothelial cells and porcine valvular endothelial cells. Not surprisingly I found that BMP-4 protein, both pro- and mature-forms, was decreased by LS *in vitro.* Furthermore, no differences were seen between fibrosa and ventricularis endothelial cells. A physiological comparison of OS-conditioned fibrosa endothelial cells to LS-conditioned ventricularis cells showed results contrary to my *in vivo* findings (Chapter 3); BMP-4 was increased in the fibrosa endothelial cells. Further, I examined BMPs -2 and -6 mRNA expression. My previous *in vitro* results suggested, like BMP-4, ventricularis endothelial cells should have more BMP-2 and -6. No reports have suggested shear regulation of BMPs -2 or -6; however, I found that BMP-2 mRNA in both fibrosa and ventricularis endothelial cells was increased by OS, while no regulation of BMP6 was seen. Next I compared *in vivo* and *in vitro* BMP antagonist levels.

It is important to study BMP antagonists' role in aortic valves for the following reasons. In cultured vascular endothelial cells, BMP antagonists, noggin, follistatin and MGP-1, which are co-expressed with BMP-4, provide a negative feedback mechanism inhibiting BMP-4's inflammatory effect [32]. Furthermore, follistatin and noggin are found in advanced atherosclerotic lesions [32]. In healthy pig AVs, chordin mRNA levels are higher

on the ventricularis endothelium than the fibrosa endothelium [37]. Furthermore, in patients with aortic valve calcification circulating uncarboxylated MGP is decreased when compared to the healthy cohort[38]. The authors hypothesized that the lower levels of circulating MGP may be because it is being used up in the vascular wall and therefore has less secretion into the circulation. In Chapter 3, I found that noggin and CV-2 were differentially expressed in the human AVs. Noggin and CV-2 expression levels were lower in the fibrosa endothelium than the ventricularis in both calcified and non-calcified AVs. MGP expression was not affected by shear in ventricularis endothelial cells; however, in fibrosa endothelial cells, shear exposure, LS or OS, decreased this BMP antagonist's expression. mRNA expression of follistatin, which I could not find an appropriate antibody for immunohistochemical studies, was differentially regulated by shear in the ventricularis endothelial cells but not the fibrosa endothelial cells. The final antagonist for which I detected mRNA was CV-2. CV-2 mRNA expression was not regulated by shear, but was a trend of an increase was observed of laminar shear increasing CV-2 mRNA. Finally, noggin was examined but only low mRNA levels were detected and thus not shown here. Synthesis of these results suggest that BMP antagonists can provide a negative feedback mechanism similar to what is found in vascular endothelial cells in which the antagonists help regulate BMP action. In order to assess the effect the antagonists are having on the valvular endothelium, I then looked at the canonical BMP pathway activation.

The BMPs have two types of specific signaling receptors: BMPR-I and BMPR-II, and both are required for signaling. Once the ligand binds to its receptors, the active domain of the type II receptor phosphorylates the type I receptor, which in turn phosphorylates the R-SMADs (SMAD 1, 5, 8) [25-27]. SMAD1/5/8 is the canonical mediator of BMP signaling. This phospho-SMAD then binds with co-SMAD 4 and translocates to the

nucleus thereby regulating a wide range of gene expression [25-27]. I found that in fibrosa and ventricularis endothelial cells, phospho-SMAD 1/5/8 levels are decreased by laminar flow when compared to static, but were not significantly different from oscillatory shear. Further, no differences were detected between the fibrosa and ventricularis endothelial cells.

Finally, in Chapter 4 I found that laminar shear decreased the inflammation state of the aortic valve leaflet. Considering that laminar shear decreases BMP-2 and -4 both fibrosa and ventricularis endothelial cells, I hypothesized that BMPs are playing an active role in inflammation in the aortic valve endothelial cells. Recent studies have shown that BMP-4 in vascular endothelial cells is partly responsible for the oscillatory shear induced inflammatory response [18-19]. BMP-4 works though a NFκB and NADPH oxidase to induce monocyte binding though ICAM-1 [18-19]. Furthermore, a recent report using porcine aortic valves has shown increased ICAM-1, VCAM-1 and BMP-4 on the fibrosa endothelium exposed to pulsatile fluid flow. ICAM-1 and VCAM-1 expression was reduced when valve leaflets were incubated with the BMP antagonist noggin indicating BMP dependent inflammation [39]; however, in valvular endothelial cells, no modulation in monocyte adhesion when blocking the BMP pathway with noggin was observed.

During valvulogenisis the BMPs, BMP antagonists, and SMADs play important roles in epithelial-to-mesenchymal transition (EMT), cardiac cushion formation, and valve primordia[40-54]. Further, if the BMPs, BMP antagonists, BMP receptors, or SMADs are removed during valvulogenisis, valve deformation occurs [40-54]. Endocardial endothelial cells will transform into a mesenchymal cell when exposed to BMP [45]. Furthermore, EMT in adult porcine aortic valvular endothelial cells has been shown to occur by

addition of TGFβ [55]. My results suggest that the canonical BMP and BMP-dependent inflammatory pathways are not activated by shear in valvular endothelial cells which is unlike in vascular endothelial cells. I hypothesize that valvular endothelial cells maintain tight control of the BMP pathway in order to stop EMT from occurring. The mechanism behind the relationship of BMPs and EMT needs to be determined. Possible regulation markers to investigate include the inhibitory SMADs, other BMP antagonists, or the BMP receptors in the context of EMT.

Several differences are seen when comparing the *in vitro* results to the *in vivo* results presented in Chapter 3. There are potential mechanisms that may explain these differences. First, in the aortic valve, endothelial cells are in close proximity to the interstitial cells that lay beneath the endothelium. This proximity allows for communication between the two cell types that is not present in my *in vitro* system. Second, the hemodynamics used in my studies are simplified. The aortic valve functions under complex hemodynamic conditions, including pulsatile pressures, unidirectional and disturbed fluid flows, bending stresses, and cyclic stretch. My studies are using a time-average shear stress of 20 dynes/cm^2, while, in the heart, the shear stress experienced by the ventricularis of the aortic valve experience a maximum shear stress of80 dynes/cm^2 [39]. Finally, endothelial cells are harvested from diseased hearts. This condition may have adverse effects on the valvular endothelial cells. Furthermore, the plasma components of the blood may have contributed to the *in vivo* endothelial phenotype I saw, and thus affecting BMP signaling *in vivo*.

In summary, this chapter shows for the first time that BMPs -2 and -4 expression is regulated by shear stress in human aortic valvular endothelial cells. I also found shear regulation of follistatin and MGP; however this regulation of follistatin was only detected

in ventricularis endothelial cells. Unlike aortic endothelial cells, CV-2 was not regulated by shear. No noggin or chordin mRNA was detected in my endothelial cells, which was detected in vascular endothelial cells. Finally, I found that the BMPs had no effect on inflammation in human aortic valve endothelial cells through the addition of noggin. I hypothesize that this is due to tight control of the BMPs in order to stop EMT from occurring. These results offer us important functional information about how shear stress affects BMP signaling in valvular endothelial cells.

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CHAPTER 6

DISCUSSION

Limitations

There are several limitations in the work presented here and are described in detail below.

Lack of proper control for immunohistochemical staining of human aortic valves

The valves used in the immunohistochemical presented here are categorized by their calcification state; however, the non-calcified valves are taken from older patient populations who have severe cardiomyopathy. The hemodynamics, in this patient population, are significantly altered and the shear and strain felt by the valvular leaflet will, in turn, be different than a healthy patient population. Finally, this patient population has significant pharmaceutical use, which I could not control for, that may affect the endothelial gene and protein responses seen in this study.

Finally, in my study, I did not separate the differences in the base, belly or free edge on the valvular leaflet. I also did not perform BMP, BMP antagonist, or SMAD analysis based on the leaflet position. The fluid profiles, and disease profiles can and do differ between leaflet position and location on the leaflet and may provide for an interesting study in the future.

Cell source and the vitro studies

The cells isolated and used in these studies come from a diseased population. The environment from which these endothelial cells come from may have an effect on the

endothelial cell phenotype that I see in my study. Furthermore, i*n vivo* the endothelial cells are in close proximity to the valvular interstitial cells, and cells present in the blood. The cross talk between these two cells types may play an important role in the response of the endothelial cells[1]. Furthermore, the components, i.e. cytokines and platelets, of the blood may have important signaling functions that are not present in the in my *in vitro* studies[2], and may explain differences between Chapters 3 and 5. Finally, the protein coating used in these studies was exclusively gelatin, a hydrolyzed form of collagen. *In vivo* the basement membrane is a mix of type I collagen, type IV collagen, and fibronectin. It is likely that difference in these matrix molecules can modulate endothelial function in my culture system in a similar matter as vascular endothelial cells [3].

Shear Stress Application

The shear patterns used in these studies are different from the cyclic pattern of shear stress seen on the ventricularis and fibrosa endothelium *in vivo*. For the atheroprotective waveform used in this study, I used the reported average physiological shear magnitude of 20 dynes/cm^2 [4-5]; however, in *ex vivo* studies have now detailed the shear stress waveform over the cardiac cycle and report that shear stress can reach 80 dynes/cm^2 during peak systole, and during diastole is at a resting state of 0 dynes/cm^2 [6]. The shear stress conditions used in this thesis was a simplified model to determine the effect of shear stress on the valvular endothelium.

Inflammatory Pathway Analysis was limited

In Chapter 4, I investigated several transcription factors that I believed may be responsible for the inflammation induced in human aortic valvular endothelial cells; however, other transcription factors, such as AP1, Egr-1, and Nrf2, may play an important role in the shear response of human aortic valvular endothelial cells.

Furthermore, inflammatory pathway analysis may also be hindered due to heterogeneous patient characteristics. To determine shear stress' effect on inflammation, ideally cells from a healthy patient should be used. Due to the lack of this subject population, analysis may have to be performed on porcine aortic valvular endothelial cells.

Lack of a Model System of Aortic Valve Calcification

The *in vitro* and *in vivo* data from this dissertation provides molecular insight by which BMPs and their antagonists function in human aortic valvular endothelial cells exposed to shear stress. However, the disconnect between the *in vivo* data present in chapter 3 and the *in vitro* data in chapter 5 may be explained using a mouse model system which are not used in my studies. Mouse model systems enable manipulation of disease state in a tightly controlled *in vivo* environment.

Summary

The overall objective of this dissertation was to investigate the disease- and shear-dependent endothelial expression of the BMPs and their contribution to inflammation. By understanding the expression and effect of the BMPs on the valvular endothelium, I will have better insight into the role of the endothelium and the BMP family in the pathogenesis of aortic valve disease. *The central hypothesis of this dissertation was that oscillatory flow conditions on the fibrosa side of the valve stimulate endothelial cells to produce BMP-4, which then activates an inflammatory response leading to accumulation of inflammatory cells, calcification, and ultimately valve impairment.* This hypothesis was tested in the following three specific aims using diseased human aortic valves, and cultured side-specific human aortic valve endothelial cells from transplanted hearts.

- **Specific Aim 1: Characterization of the BMPs, BMP antagonists, and SMADs in Calcified and Non-Calcified Human Aortic Valve Endothelial Cells.**

- **Specific Aim 2: Characterization of anti- and pro-inflammatory shear responses in Human Aortic Valve Endothelial Cells**

- **Specific Aim 3: Characterization of BMPs and BMP antagonists under shear stress and their role in inflammation in human aortic valve endothelial cells**

To establish the importance of BMPs in the endothelium of aortic valves, two populations of valves were used: calcified and non-calcified human aortic valves. Previously, I have shown that oscillatory shear stress induces BMP-4 expression in vascular endothelial cells, and this BMP-4 expression leads to inflammatory response in a NFκB and NADPH oxidase-dependent manner [7]. In humans, BMP-2 and -4 are found in atherosclerotic plaques, endothelium overlying advanced atherosclerotic lesions, and in calcified regions of AVs [8-9]. Furthermore, in normal pig AVs, BMP-4 mRNA and protein levels are higher on the fibrosa endothelium [10-11]. Also, laminar shear inhibits expression of BMP-4 in cultured pig AV endothelial cells [11] however, my results showed that the BMP-2 and -4 are significantly higher in the ventricularis endothelium of non-calcified human aortic valves when compared to the fibrosa of either disease state. Furthermore, BMP-6 endothelial expression was significantly higher in the ventricularis of calcified valves when compared to the fibrosa of either disease state. Because of these results I investigated BMP antagonist expression, and found that the BMP antagonists, noggin and CV-2, were highest in the ventricularis endothelium when compared to the fibrosa

regardless of disease state. To determine if the BMP antagonist levels were sufficient to inhibit BMP pathway activation, phosphorylated SMAD 1/5/8 was examined. Phosphorylated SMAD 1/5/8 levels were significantly increased on the fibrosa endothelium of calcified valves compared to the fibrosa endothelium of non-calcified valves. The BMP results differ from previously mentioned reports of BMP-4 mRNA expression in porcine aortic valve endothelium. It is important to note that both valve populations are from a diseased population. Furthermore, a recent study has shown that pigs fed an atherogenic diet have decreased BMP-4 mRNA in the valve endothelium [12]. The authors hypothesize that this decrease is a protective mechanism[12]. A similar mechanism may be at work in my study. My results suggest that the increased levels of BMP antagonists may play an important protective role in the ventricularis, by inhibiting BMP signaling, as seen through SMAD 1/5/8. Finally, circulating levels of the BMP antagonist, MGP, are decreased in patients with aortic valve calcification, and may help explain why phosphorylation of SMAD 1/5/8 is seen in the fibrosa endothelium of calcified valves but not non-calcified valves [13]. These findings suggest that preferential activation of BMP pathways, controlled by the balance between the BMPs and their inhibitors, play an important role in side-dependent calcification of human AVs.

I next wanted to examine the role of shear stress in BMP regulation, but before doing so, I needed to examine the endothelial response to fluid shear stress to validate the phenotype of my isolated human aortic valve endothelial cells. Previous studies in porcine and canine have indicated that valvular endothelial cells should align perpendicular to flow [11, 14-15]. KLF2 and eNOS expression in vascular endothelial cells has been shown to be increased by laminar flow and to have anti-inflammatory effects by decreasing VCAM-1 levels [16-19]. Conversely, oscillatory shear stress has been shown to increase NFkB translocation and increase ICAM-1 and E-selectin [7, 20-24]. I

found laminar shear stress causes human aortic valve endothelial cells align parallel to flow and have robust increases of KLF2 and eNOS and decreases in VCAM-1 levels; however, laminar shear-treated cells had similar levels of NFκB activation as oscillatory treated cells while ICAM-1 and E-selectin were not affected by shear stress. In contrast, oscillatory shear had higher levels of monocytes bound which may be due to eNOS's protective effects under laminar shear and robust VCAM-1 expression in oscillatory shear [16]. Future studies looking at valvular endothelial cells from a healthy subject population will need to be done to confirm the results shown here.

After verifying the shear response of my endothelial cells, I next determined the shear response of the BMPs and BMP antagonists and described BMPs' effect on inflammation. Previously, BMP-4 has been shown *in vitro* and *in vivo* to be increased in endothelial cells exposed to oscillatory flow, while the closely-related BMP-2 has not been shown to be shear sensitive. Previously, I have shown that BMP4 is a mechanosensitive and pro-inflammatory cytokine in vascular endothelial cells [7, 23]. In this study I have found that BMPs -2 and -4 are shear sensitive while BMP-6 is not. Furthermore, I have found that follistatin is decreased by laminar flow only in the ventricularis, while MGP1 is decreased in the fibrosa valvular endothelial cells under both oscillatory and laminar flow. Finally, incubation with noggin did not affect monocyte adhesion after shear, suggesting differential regulation of inflammation in human aortic valvular endothelial cells.

Conclusions

By addressing the specific aims of this project, I have investigated disease- and side-dependent valvular endothelial BMP expression *in vivo*, shear regulation of valvular endothelial inflammation *in vitro*, and shear regulation of valvular endothelial BMP expression *in vitro*. My results suggest that the BMP pathway is playing a role in side specific aortic valve disease development; however, regulation of the BMPs does not appear to be shear regulated *in vivo*. Activation of the canonical BMP pathway in endothelial cells of calcified human aortic valves may enhance the pro-osteogenic environment on the fibrosa side of the valvular leaflet leading to increased disease. Other factors that may be affecting BMP production include pulsatile pressures, bending stresses, cyclic stretch, and humeral stimuli present in the blood of the patients. However, *in vitro* I have found BMPs -2 and -4 to be shear-regulated in human aortic valvular endothelial cells. Shear-induced inflammation in human aortic valve endothelial cells seems to be VCAM-1-dependent, and BMP-independent. Finally, by identifying factors that are modulated in calcific- and shear-dependent processes, new targets for the early detection of aortic valve disease can be determined and new therapeutics to slow or stop the progression of aortic valve disease may be discovered.

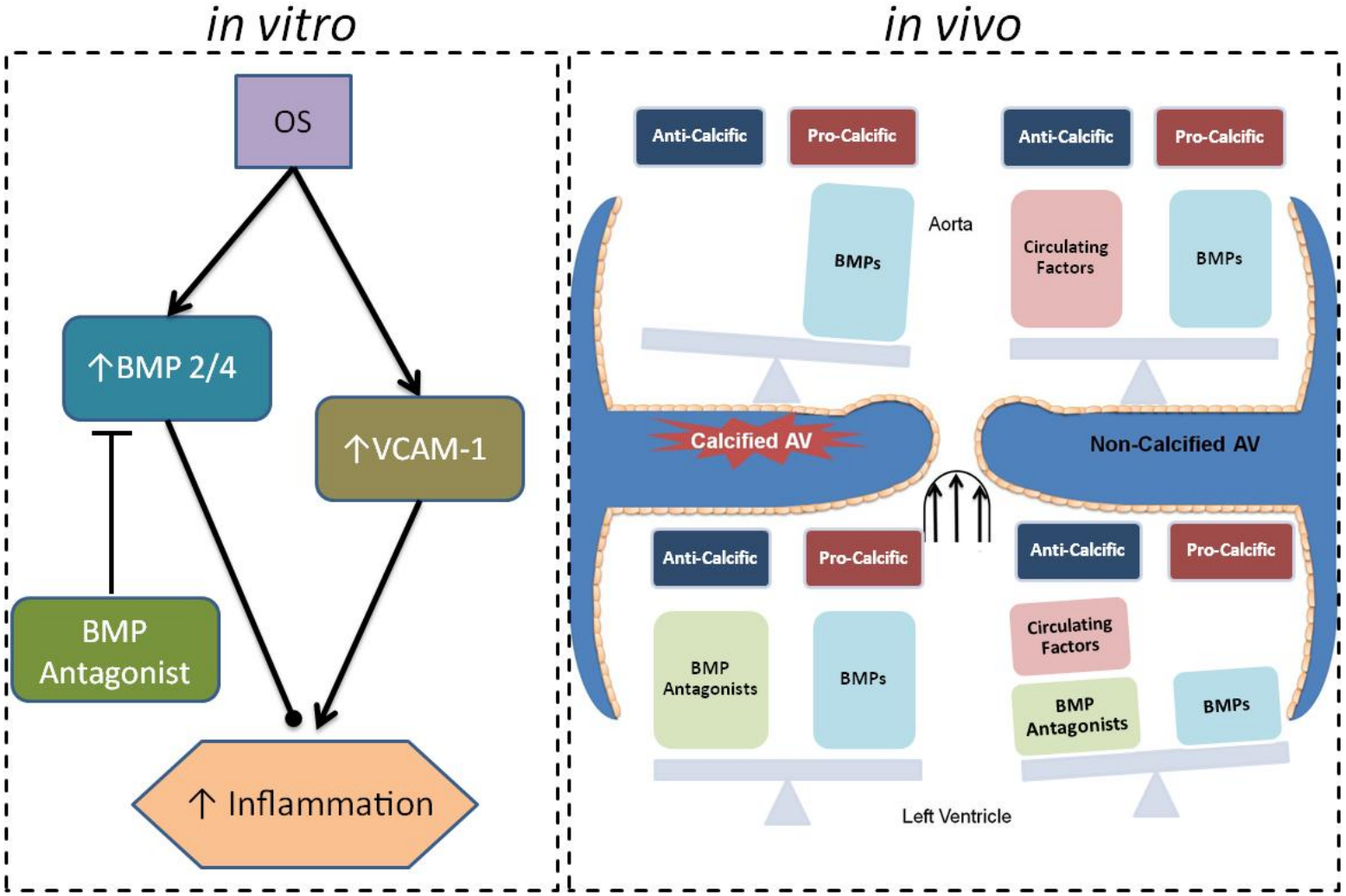

Figure 6.1 Overall summaries of project findings.

Future Directions

The lack of mechanistic insight into the pathogenesis of aortic valve disease, and the endothelium's role in this disease provides many new avenues for future research. The findings described in this dissertation have elucidated possible roles for the endothelium, the BMPs, and their antagonists and provide a basis for future research in aortic valve disease.

Microarray Analysis of mRNA and microRNA in calcified human aortic valves

The current paradigm in treatment of aortic valve disease is either the replacement or repair of the aortic valve. Biomarkers for the early detection and treatment of aortic valve are lacking. Discovering one pharmaceutical drug that may stop or even slow the degeneration of aortic valve disease would improve the outlook for patients with this disease. The results presented in Chapter 3 investigate endothelial expression of the BMPs and BMP antagonists in aortic valve disease. This is just one family of proteins that may be involved endothelial inflammation and osteogenisis. To gain further understanding of other possible contributors to aortic valve disease, microarray studies of side specific human calcified human aortic valve endothelial cells can be used. By using a trizol-based assay endothelial-enriched mRNA and microRNA can be collected from both sides of the valvular leaflet (Figure 6.1). microRNAs are 18-22 nucleotide segments which bind to the 3' UTR of mRNA thereby inhibiting translation or signaling mRNA for degradation [25]. It is hypothesized that microRNAs regulate 30-50% of all mRNAs [25]. Further, it is known that microRNAs play an important role in the cardiovascular system and that some microRNAs are shear-regulated [26-28]. To date, microRNA's role in human aortic valvular endothelial cells remains unknown and could uncover a possible mechanism by which aortic valve disease occurs [25]. Moreover, by performing mRNA and miRNA arrays, new biomarkers for aortic valve disease may be

discovered. These biomarkers may be clinically relevant in two ways: First, if it is a secreted protein it may be used as an early indicator of aortic valve disease, allowing physicians to detect disease development before it is clinically manifested. Second, possible targets for pharmaceutical intervention may present themselves in the study.

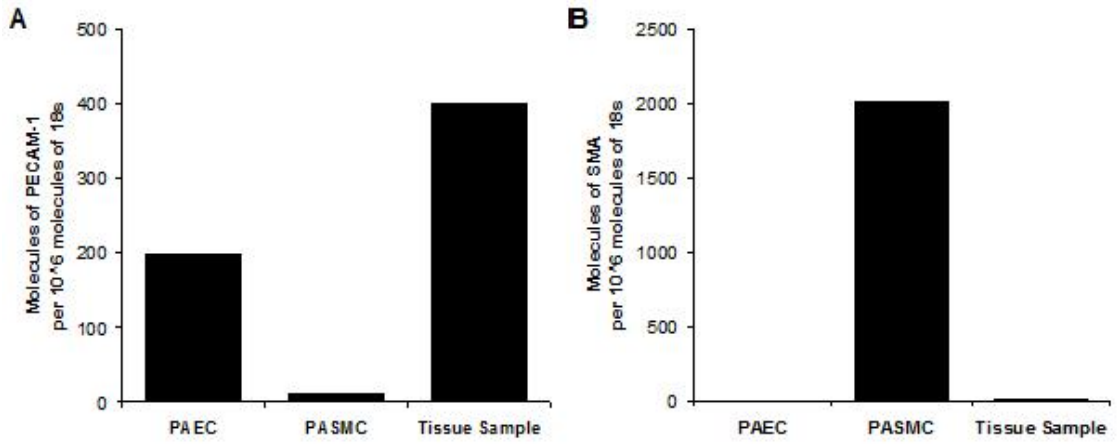

Figure 6.2 Isolation of endothelial specific mRNA from porcine valve cusps. *RNA was isolated from three sources, porcine aortic endothelial cells, porcine aortic smooth muscle cells, and fresh pig aortic valves using a QIAzol Lysis Reagent. Quantitative PCR was then performed using and endothelial marker (**A**), PECAM-1 and an interstitial cell marker (**B**), αSMA. PECAM-1 and αSMA were normalized by 18s. mRNA isolated from porcine valve tissue shows endothelial specific markers while lacking interstitial cell marker.*

Involvement of Reactive Oxygen Species and Nitric Oxide in aortic valve disease

Recent studies looking at calcified human aortic valve disease have suggested involvement of both reactive oxygen species and nitric oxide synthesis in aortic valve calcification [29-30]. In Chapter 4 I presented that laminar shear stress increases phosphorylated e-NOS in human aortic valve endothelial cells. Mouse knockouts may provide important information of possible mechanisms by which aortic valve disease occurs, specifically the role of eNOS and reactive oxygen species. eNOS knockout mice have the propensity to develop bicuspid aortic valves [31]. Furthermore, research has

shown patients with bicuspid aortic valves there was a significant endothelial eNOS protein levels than patients with a tricuspid aortic valve [32].

By using mice in the ApoE-/- background deficient in eNOS and feeding them a high cholesterol diet, I can induce both an oxidative and hyperlipidemic conditions. Using a sensitive osteogenic probe (OsteoSense® 680) I can look at calcification development in these mice at 20 weeks [33]. There is also conflicting literature about the role of NADPH oxidase in aortic valve disease [34]. One study has found that reactive oxygen species is due to the uncoupling of eNOS while NOX2 and NOX4 are decreased [29]. A second study was not able to confirm this result [30]. Therefore to address this discrepancy, it may be beneficial to look at NOX1 deficient and over expressing mice in an ApoE-/- background. Without NOX1, the mice will have reduced superoxide levels while still having a hypercholesterolimic phenotype. With an overexpressed NOX1, the mice will have higher superoxide levels while having a hypercholesterolimic phenotype. By using two double knockout mice and an overexpressing NOX1 mouse, I discover new information the involvement of nitric oxide and reactive oxygen species.

Total BMP and BMP antagonists in human aortic valves

In Chapter 3, I presented data looking at endothelial expression of the BMPs and the BMP antagonists in calcified and non-calcified human aortic valves. Previous research has found increased levels of BMP-2 and -4 at the whole valve level [9]; however, it may be useful to look at total cellular (endothelial and interstitial cell) expression and location of the BMPs and BMP antagonists in calcified human aortic valves as little is known about interstitial cell secretion of the antagonists, and how they vary throughout the valve. By understanding the side dependent secretion of the BMPs and their

antagonists, it will give us insight into the valvular interstitial cell, BMP, and BMP antagonist involvement in the calcification of the human aortic valve.

Inflammatory Transcription Factor Signaling in Human Aortic Valve Endothelial Cells in vivo

In Chapter 4 I investigated many signaling pathways that potentially activated in human aortic valve endothelial cells under shear stress. Recent studies investigating high cholesterol diet in pigs has seen a significant shift in inflammatory signaling at the mRNA level [12], however very little is known of *in vivo* inflammatory signaling in calcified and non-calcified human aortic valves, and what transcription factors are important in the degeneration of the aortic valve. By using specific antibodies for NFkB, p38, and JNK I may be able to elucidate possible inflammatory processes that are increased in calcified human aortic valves.

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