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# **Role of Oxidative Stress in Calcific Aortic Valve Disease: From Bench to Bedside - The Role of a Stem Cell Niche**

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Additional information is available at the end of the chapter

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## **1. Introduction**

Calcific Aortic Stenosis is the most common cause of aortic valve disease in developed countries. This condition increases in prevalence with the advancing age of the U.S. population, afflicting 2-3 % by age 65 [1]. Aortic valve replacement is the number one indication for surgical valve replacement in the United States and in Europe. The natural history of severe symptomatic aortic stenosis is associated with 50% mortality within 5 years [2]. Bicuspid aortic valve disease is the most common congenital heart abnormality and it is the most common phenotype of calcific aortic stenosis. The bicuspid aortic valve (BAV) is the most common congenital cardiac anomaly, having a prevalence of 0.9 to 1.37% in the general population [3]. Understanding the cellular mechanisms of tricuspid versus bicuspid aortic valve lesions will provide further understanding the mechanisms of this disease. Currently, there are three fundamental mechanisms defined in the development of aortic valve disease: 1) oxidative stress via traditional cardiovascular risk factors [4-8,6, 7, 9-12], 2) cellular proliferation [13] and 3) osteoblastogenesis in the end stage disease process [14, 15]. Previously, the Wnt/Lrp5 signaling pathway has been identified as a signaling mechanism for cardiovascular calcification [5, 16, 17]. The corollaries necessary to define a tissue stem cell niche: 1) physical architecture of the endothelial cells signaling to the adjacent subendothelial cells: the valve interstitial cell along the valve fibrosa. 2) defining the oxidative-mechanical stress gradient necessary to activate Wnt3a/Lrp5 in this tissue stem niche to induce disease. Recently, the mechanisms of oxidative stress have been identified in the development of calcific aortic valve disease. This chapter will outline the factors important in the role of calcific aortic valve disease.

## 2. The role of lipids in vascular and valvular disease

The role of lipids in vascular atherosclerosis has been defined in the literature for years. Atherosclerosis is a complex multifactorial process which produces a lesion composed of lipids [18, 19], macrophages [20], and proliferating smooth muscle cells [21] apoptosis [22] and extracellular bone matrix production [23] in the vascular wall [24, 25]. The activation of these cellular processes is regulated by a number of pathways. Integrins provide an important role in the regulation of cellular adhesion in atherosclerosis [26]. Another critical regulator of vascular endothelial biology is nitric oxide (NO) [27, 28]. Cholesterol-rich LDL also has a critical role in the onset and further progression of the atherosclerotic lesion via an inactivation of endothelial nitric oxide synthase (eNOS) [22, 29-31] contributing to an abnormal oxidation state within the vessel. In this inflammatory environment, growth factors and cytokines are secreted to induce vascular smooth cell proliferation and recruitment of macrophage cells [32-37] which are important in the development of the atherosclerotic plaque lesion.

Recently, similar risk factors for calcific aortic valve disease have recently been described including male gender, hypertension, elevated levels of LDL, and smoking [38, 39] which mimic those that promote the development of vascular atherosclerosis. Surgical pathological studies have demonstrated the presence of LDL and atherosclerosis in calcified valves, demonstrating similarities between the genesis of valvular and vascular disease and suggesting a common cellular mechanism [40, 41]. Patients who have the diagnosis of familial hypercholesterolemia develop aggressive peripheral vascular disease, coronary artery disease, as well as aortic valve lesions which calcify with age [10, 42]. Rajamannan et al, have shown that the development of atherosclerosis occurs in the aortic valve in a patient with Familial Hypercholesterolemia with the Low density lipoprotein receptor mutation [10]. The atherosclerosis develops along the aortic surface of the aortic valve and in the lumen of the left circumflex artery [10]. This provides the first index case of atherosclerotic aortic valve disease in this patient population. Studies have confirmed in experimental hypercholesterolemia that both atherosclerosis and osteoblast markers are present in the aortic valves [4, 6, 13]. This background provides the foundation for studying valve calcification in an experimental atherosclerotic *in vivo* model.

## 3. Aortic valve calcification

The presence of calcification in the aortic valve is responsible for valve stenosis. Severe aortic stenosis can result in symptomatic chest pain, as well as syncope and congestive heart failure in patients with severe aortic valve stenosis. For years, aortic valve stenosis was thought to be a degenerative process. However, the pathologic lesion of calcified aortic valves demonstrate indicate the presence of complex calcification in these tissues. Furthermore, there are a growing number of descriptive studies delineating the presence of bone formation in the aortic valve [15, 43, 44].

Until recently the etiology of valvular heart disease has been thought to be a degenerative process related to the passive accumulation of calcium binding to the surface of the valve leaflet. Recent descriptive studies have demonstrated the critical features of aortic valve calcification, including osteoblast expression, cell proliferation and atherosclerosis [6, 14, 15, 45] and mitral valve degeneration, glycosaminoglycan accumulation, proteoglycan expression, and abnormal collagen expression [46-49]. These studies define the biochemical and histological characterization of these valve lesions. We and others, have also shown that specific bone cell phenotypes are present in calcifying valve specimens in human specimens [16, 50]. These data provide the evidence that the aortic valve calcification follows the spectrum of bone formation in calcifying tissues.

#### **4. The role of Lrp5/beta-catenin activation in cardiovascular calcification and osteoblast bone formation: Connection with the bone axis**

Bone and cartilage are major tissues in the vertebrate skeletal system, which is primarily composed of three cell types: osteoblasts, chondrocytes, and osteoclasts. In the developing embryo, osteoblast and chondrocytes, both differentiate from common mesenchymal progenitors in situ, whereas osteoclasts are of hematopoietic origin and brought in later by invading blood vessels. Osteoblast differentiation and maturation lead to bone formation controlled by two distinct mechanisms: intramembranous and endochondral ossification, both starting from mesenchymal condensations.

To date only two osteoblast-specific transcripts have been identified: 1) *Cbfa1* and 2) osteocalcin (OC). The transcription factor *Cbfa1* [51] has all the attributes of a 'master gene' differentiation factor for the osteoblast lineage and bone matrix gene expression. During embryonic development, *Cbfa1* expression precedes osteoblast differentiation and is restricted to mesenchymal cells destined to become osteoblast. In addition to its critical role in osteoblast commitment and differentiation, *Cbfa1* appears to control osteoblast activity, i.e., the rate of bone formation by differentiated osteoblasts [51]. We have shown previously that cholesterol upregulates *Cbfa1* gene expression in the aortic valve and atorvastatin decreases the gene expression [6] in an animal model. We have also demonstrated that *Sox9* and *Cbfa1* are expressed in human degenerative valves removed at the time of surgical valve replacement [16]. The regulatory mechanism of osteoblast differentiation from osteoblast progenitor cells into terminally differentiated cells is via a well orchestrated and well studied pathway which involves initial cellular proliferation events and then synthesis of bone matrix proteins, which requires the actions of specific paracrine/hormonal factors and the activation of the canonical Wnt pathway [52].

Genes which code for the bone extracellular matrix proteins in osteoblast cells include alkaline phosphatase (AP), osteopontin (OP), osteocalcin (OC), and bone sialoprotein (BSP). This data supports a potential regulatory mechanism that these matrix proteins play a critical role in the development of biomineralization. To date, many of these markers have been shown to be critical in the extracellular mineralization and bone formation that develops in

normal osteoblast differentiation (Fig.5). Dr. Spelsberg and Dr. Rajamannan have extensive experience in osteoblast cell biology and will contribute to the translational studies in the aortic valve involving the differentiation and mineralization [53, 54].

A link between lipids and osteoporosis have been studied extensively [55-60]. These groups have shown in *in vitro* and *in vivo* studies that lipids decrease bone formation and increase vascular calcification. Hurska's group from the University of Washington have studied this important hypothesis in the LDLR<sup>-/-</sup> mice with renal disease [55]. This studied correlated the important understanding of chronic kidney disease with decreased bone formation rates and increase in vascular calcification. This study demonstrates that accelerated vascular calcification found in patients with end stage renal disease may be related to multifactorial mechanisms including traditional atherosclerotic risk factors and elevated serum phosphate levels. Giachelli has also studied extensively the hypothesis of a sodium phosphate abnormality in the vascular smooth muscle cell [61]. Her group has also shown that osteopontin expression by vascular smooth muscle cells may have an inhibitory effect in the development of calcification [62] which further defines the complexity of the matrix synthesis phase of bone formation. Demer's laboratory has also studied extensively the correlation of lipids with vascular calcification and osteoporosis via inhibition of Cbfa1 in osteoblast cells [60, 63]. This paradoxical finding between the calcifying vascular aorta and osteoporosis is an important link in the hypercholesterolemia hypothesis. The development of cardiovascular calcification is a multifactorial process which includes a number of mechanisms. Studies in the different laboratories provide important evidence towards the development of therapies depending on the patient population i.e. end stage renal disease versus treatment of the traditional risk factors for vascular disease.

Our lab (43) and Towler's laboratory (44) have shown that the Lrp5/Wnt/beta-catenin pathway plays an important role in the development of vascular and valvular calcification. Studies have shown that different mutations in Lrp5, an LDL receptor related protein; develop a high bone mass phenotype and an osteoporotic phenotype (45, 46). In the presence of the palmitoylation of Wnt an active beta-catenin accumulates in the cytoplasm, presumably in a signaling capacity, and eventually translocates to the nucleus via binding to nucleoporins [64], where it can interact with LEF-1/TCFs in an inactive transcription complex [65, 66]. The Wnt/Lrp5/frizzled complex turns on downstream components such as Dishevelled (Dvl/Dsh) which leads to repression of the glycogen synthase kinase-3 (GSK3) [67]. Inhibition of GSK3 allows beta-catenin to accumulate in the nucleus, interacting with members of the LEF/TCF class of architectural HMG box of transcription factors including Cbfa1 involved in cell differentiation and osteoblast activation [68, 69, 70-72] and Sox 9, a HMG box transcription factor, is required for chondrocyte cell fate determination and marks early chondrocytic differentiation of mesenchymal progenitors [73].

To determine a potential signaling pathway for the development of aortic valve disease there are numerous pathways which may be implicated in this disease process [50, 74, 75]. Recent evidence suggests that the Wnt pathway regulates the expression of bone mineral markers in cells responsive to the Wnt pathway. Furthermore the Wnt pathway has been shown to be activated by lipids. Therefore we chose to assess this pathway in our model of

experimental hypercholesterolemia to determine how lipids may be regulating Lrp5 in the aortic valve. This background outlines the potential for lipids in the regulation of aortic valve mineralization via the canonical Wnt pathway.

## **5. Echocardiography and Computerized Tomography (CT) evaluation of the development of calcification and stenosis**

Currently the non-invasive “gold standard” for the diagnosis of aortic valve stenosis is 2-Dimensional doppler echocardiography. It is the test of choice to quantify the severity of valve stenosis and pressure differential across the aortic valve. There are a increasing number of studies which have demonstrated the utility of calculating the volume of calcium and the rate of progression of the disease process in the aortic valve [76-80]. Confirmation of hemodynamic valve stenosis by echo will provide the degree of valve stenosis using ultrasound techniques<sup>4</sup>. MicroCT will assess the degree of calcification within the mineralizing tissues.

## **6. Development of future medical therapies for calcific aortic stenosis**

The natural history studies of valvular aortic stenosis as defined by clinical and histopathologic parameters have provided landmark developments towards the understanding of this disease. HMG CoA reductase inhibitors may provide an innovative therapeutic approach by employing both lipid lowering and possibly non-lipid lowering effects to forestall critical stenosis in the aortic valve. Our laboratory has shown that atorvastatin has a number of effects in the aortic valve including: 1) inhibition of foam cell accumulation [6], 2) inhibition of Cbfa1 activation [6], 3) eNOS enzymatic activation [11] and 4) attenuation of Lrp5 receptor activation [81]. Statins have potent LDL lowering effects via inhibition of the rate-limiting step in cholesterol synthesis. There are a number of experimental models which demonstrate the potential for treating the vasculature with statins to inhibit matrix formation [24, 25], cellular proliferation [6] and vascular aneurysm formation [82]. Although valve replacement is the current treatment of choice for severe critical aortic stenosis, future insights into the mechanisms of calcification and its progression may indicate a role for lipid lowering therapy in modifying the rate of progression of stenosis.

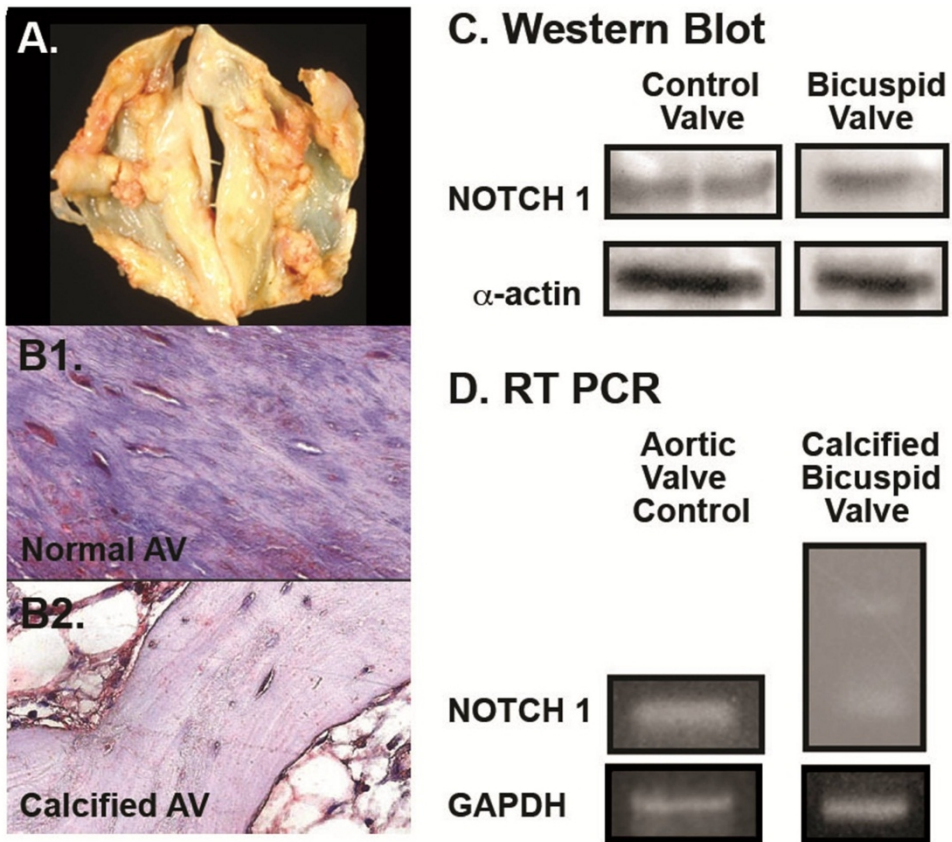
There are a growing number of retrospective studies demonstrating that statins may have benefits in slowing the progression of aortic stenosis [83-85]. A recent clinical trial by Cowell et al, demonstrated that high dose atorvastatin did not slow the progression of aortic stenosis in patients [86]. However, the timing of the initiation of the statin therapy was at a later stage of aortic valve disease. A clinical trial in Portugal called RAAVE- Rosuvastatin Affecting Aortic Valve Endothelium demonstrated prospectively that statins slow progression in CAVD in an open label study. In the RAAVE study we found a change in aortic valve area (AVA) in the control group was  $-0.10 \pm 0.09$  cm<sup>2</sup> per year versus  $-0.05 \pm 0.12$  cm<sup>2</sup> per year in the

Rosuvastatin group ( $p=0.041$ ). In addition there was an increase in peak aortic valve velocity was  $+0.24\pm 0.30$  m/sec/yr in the control group as compared to the increase in  $+0.04\pm 0.38$  m/sec/yr in the Rosuvastatin group ( $p=0.007$ ), indicating that in this prospective hypothesis driven study we found by echocardiography a slowing of progression in the aortic valve disease. SALTIRE initiated atorvastatin in patients who had more advanced aortic stenosis as defined by the mean aortic valve area  $1.03$  cm<sup>2</sup> as compared to the average aortic valve area in RAAVE of  $1.23$  cm<sup>2</sup> as the baseline aortic valve area prior to treatment with Rosuvastatin [87]. The investigators of RAAVE hypothesize that the beneficial effect of the statin was secondary to the early initiation of treatment. Furthermore, the SALTIRE investigators recently acknowledged the potential of medical therapies may be found if the treatment of this disease is initiated earlier in the disease process [86]. The studies planned in this application should lead to an important understanding of the molecular and cellular mechanisms of aortic valve disease. Furthermore, the experimental approach will also correlate the development of valve calcium by MicroCT and hemodynamic progression by echocardiography in this important disease process.

The bicuspid aortic valve (BAV) is the most common congenital cardiac anomaly, having a prevalence of 0.9 to 1.37 % in the general population [3]. The natural history of the BAV is progressive stenosis that typically occurs at a faster rate than tricuspid aortic valves requiring earlier surgical intervention in the BAV patients [2, 3]. With the decline of acute rheumatic fever, calcific aortic stenosis has become the most common indication for surgical valve replacement. Despite the high prevalence of aortic stenosis, few studies have investigated the mechanisms responsible for aortic valve disease. The cellular mechanism for the development of this disease is not well known. Previously, we and others have demonstrated that aortic valve calcification is associated with an osteoblast bone-like phenotype [14, 15]. This bone phenotype is regulated by the canonical Wnt pathway in experimental cardiovascular calcification [5, 17]. We have also shown that the canonical Wnt/Lrp5 pathway is upregulated in diseased human valves from patients with valvular heart disease [16]. These studies implicate that inhibition of the canonical Wnt pathway provides a therapeutic approach for the treatment of degenerative valvular heart diseases. A recent study [88], discovered that a loss of function mutation in Notch1 was associated with accelerated aortic valve calcification and a number of congenital heart abnormalities. Normal Notch1 receptor functions to inhibit osteoblastogenesis [89, 90]. Evaluation of Notch1 gene and protein expression in human bicuspid calcified valves compared to normal aortic valves removed at the time of surgical valve replacement is shown in Figure 1, Panel A. Notch1 protein expression was decreased in the BAV compared to controls by immunohistochemistry and Western Blot expression Figure 1, Panel B1 and B2, and C. RNA expression by RTPCR indicates a spliced Notch1 receptor in the diseased valves as compared to controls as shown in Figure 1, Panel D. This Notch1 splicing may be the regulatory switch important for the activation of the Wnt pathway and downstream calcification in these diseased valves [5, 17, 90].

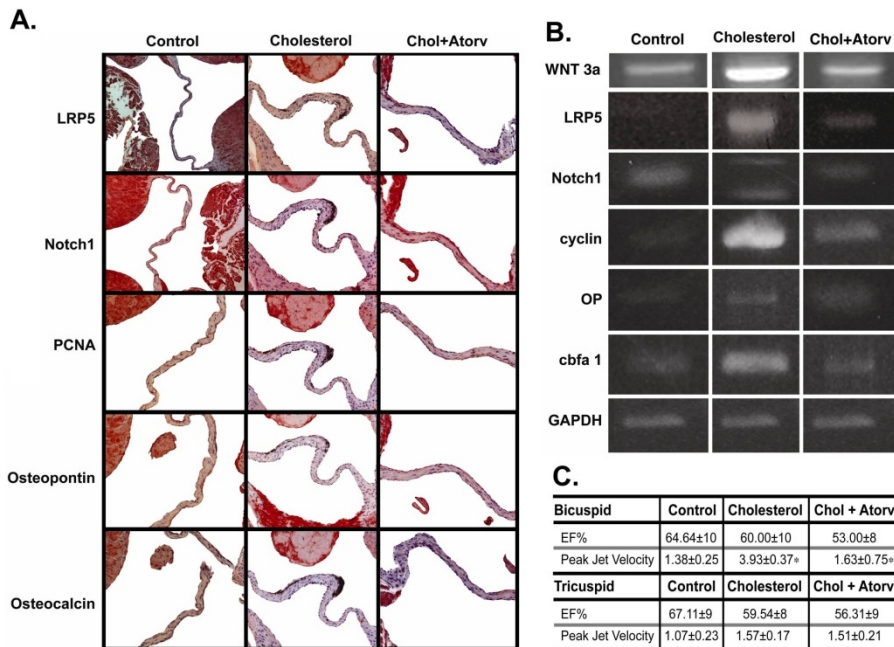
Risk factors for the development of calcific aortic valve disease (CAVD) have been elucidated in a number of epidemiologic databases [38]. The risk factors for CAVD are similar to those of vascular atherosclerosis which include: elevated LDL, hypertension, male gender, smok-

ing and increased body mass index [38]. The elucidation of these risk factors have provided the experimental basis for hypercholesterolemia as a method to induce aortic valve disease [4-8]. Furthermore, studies have shown that the eNOS<sup>-/-</sup> mouse is a novel mouse model which develops anatomic bicuspid aortic valves (BAV) [91]. To understand if eNOS<sup>-/-</sup> mice with the BAV phenotype, develops accelerated stenosis earlier than tricuspid aortic valves via the Lrp5 pathway activation, eNOS<sup>-/-</sup> mice were given a cholesterol diet versus cholesterol and atorvastatin. The Visual Sonics mouse echocardiography machine was used to screen for the BAV phenotype. Echocardiography hemodynamics was also performed to determine the timing of stenosis in bicuspid vs. tricuspid aortic valves eNOS<sup>-/-</sup> mice on different diets.



**Figure 1.** Histology of the aortic valves from human bicuspid calcified valves compared to normal aortic valves removed at the time of surgical valve replacement; **Panel A.** Bicuspid Aortic Valve Removed from patient at the time of surgical valve replacement. **Panel B1.** Notch1 Immunohistochemistry of a Normal Aortic Valve. **Panel B2.** Notch1 Immunohistochemistry of a Bicuspid Aortic Valve. **Panel C.** Notch1 protein expression was decreased in the BAV compared to controls by immunohistochemistry and Western Blot expression. **Panel D.** Notch1 RNA expression was decrease in the BAV as compared to Control aortic Valve.

Figure 2 demonstrates the characterization of the eNOS phenotype as defined by histology, RTPCR and echocardiography. In Figure 2, Panel A is the histology for BAV, Figure 2, Panel B is the semi-quantitative RTPCR from the BAV eNOS<sup>-/-</sup> mice, and echocardiographic data for the bicuspid vs. tricuspid aortic valves Figure 2, Panel C. We measured Notch1, Wnt3a and downstream markers of the canonical Wnt pathway by protein and RNA expression. Notch1 protein was diminished and the RNA expression demonstrates a similar spliced variant with lipid treatments which was not present with the control and atorvastatin treatment. Cholesterol diets increased the members of the canonical Wnt pathway and Atorvastatin diminished these markers significantly (p<0.05).



**Figure 2.** Characterization of the Bicuspid Aortic valve from the eNOS<sup>-/-</sup> bicuspid aortic valves. **Panel A.** Immunohistochemistry stain for Lrp5, Notch1, Proliferating Cell Nuclear Antigen, Osteopontin and Osteocalcin from the eNOS<sup>-/-</sup> aortic valves on the control, Left column, control diet; middle column, cholesterol diet; right column, cholesterol diet plus atorvastatin. In each panel, the aortic valve leaflet is in the center. (All frames 20X magnification) **Panel B.** RTPCR for Wnt3a, Lrp5, Notch1, cyclin1, Osteopontin and Cbfa1 from the eNOS<sup>-/-</sup> aortic valves on the control, A1, Cholesterol A2, and the Cholesterol + Atorvastatin diets A3. **Panel C.** Echocardiographic results of the tricuspid versus bicuspid eNOS<sup>-/-</sup> null mice.

BAV is a complex model to study the mechanisms of calcification. The importance of cell-cell communication within a stem cell niche is necessary for the development of valvular heart disease. The two corollaries necessary for an adult stem cell niche is to first define the physical architecture of the stem-cell niche and second is to define the gradient of proliferation to differentiation within the stem-cell niche. The endothelial lining cell located along the

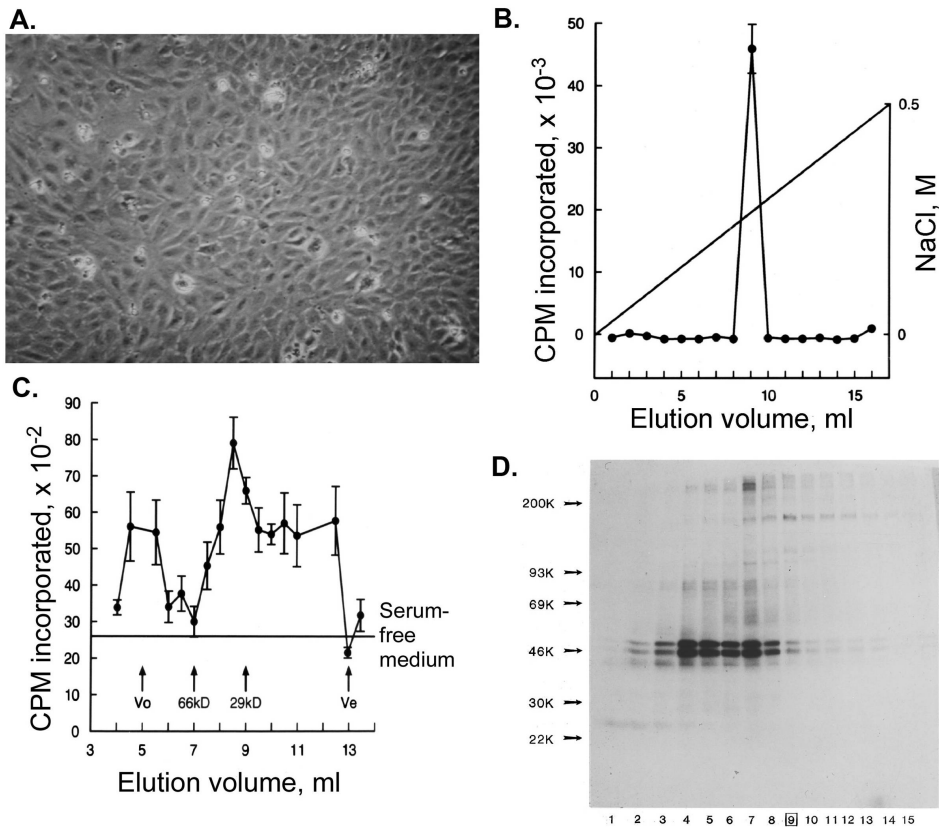


aortic surface is responsible for the secretion of a growth factors [92]. These cells interact with the subendothelial cells that are resident below the endothelial layer of cells. These cells have been characterized as myofibroblast cells [75, 93, 94].

To test the hypothesis that BAV disease develops secondary to a stem cell niche process, the physical cell-cell communication needed to be established [95]. In the aortic valve the communication for the stem cell niche would be between the aortic valve endothelial cell and the adjacent myofibroblast cell located below the aortic lining endothelial cell. Conditioned media was produced from untreated aortic valve endothelial cells for the microenvironment that activates signaling in the myofibroblast cell. A mitogenic protein (Wnt3a) was isolated from the conditioned media and then tested directly on the responding mesenchymal cell, the cardiac valve myofibroblast [93, 96,95]. This transfer of isolated protein to the adjacent cell was necessary to determine if the cell would proliferate directly in the presence of this protein. This system is appealing because the responding mesenchymal cell is isolated from the anatomic region adjacent and immediately below that of the endothelial cells producing the growth factor activity along the fibrosa surface. Very little is known regarding the characterization of the endothelial cell conditioned media. These experiments test the corollary that the physical architecture described above is necessary for disease development in the aortic valve.

Figure 3 demonstrates the isolation and characterization of the Wnt3a from the conditioned media microenvironment. Figure 3, Panel A, is light microscopy of aortic valve endothelial cells isolated from the aortic surface of the aortic valve. The results of the mitogen assays for fractions eluting from a DEAE- Sephadex column are shown in Figure 3, Panel B. It can be seen that the mitogenic activity appeared as a single peak eluting at approximately 0.25 M NaCl. The material eluting from DEAE- Sephadex was then applied to Sephadex G-100; the results of mitogen assays on fractions eluting from such a gel filtration column are shown in Figure 3, Panel C. It can be seen that under these native, non-denaturing conditions the bulk of the mitogenic activity eluted as a peak corresponding to standard proteins of 30- 40,000 molecular weight. A SDS denaturing protein gel was run on each sample from the eluted proteins and the bulk of activated protein correlated with the protein peak at 46kd as shown in Figure 3, Panel D. The protein size and charge determination is similar to that previously characterized as Wnt3a [97]. This material lost all activity when heated to 100°C for 5 minutes; disulfide bond reduction with dithiothreitol also abolished all mitogenic activity; and treatment with trypsin destroyed all activity, implicating a protein structure.

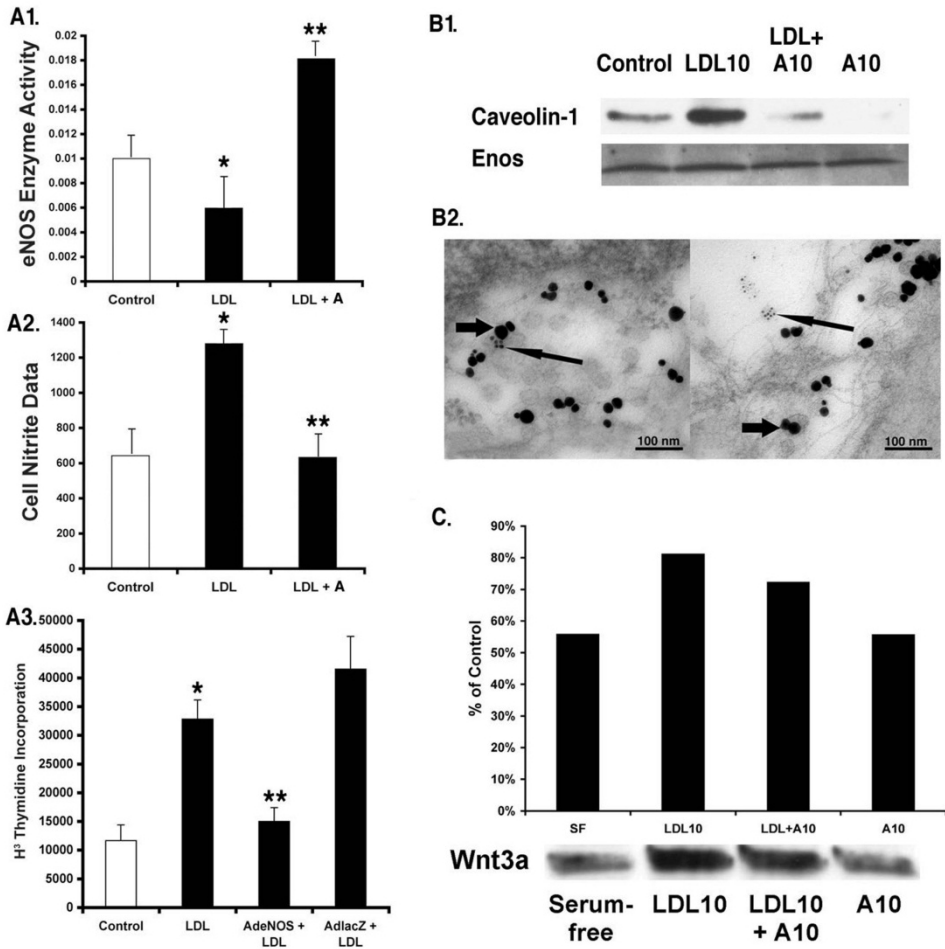
The second corollary for identifying a stem cell niche is to define the gradient responsible for the proliferation to differentiation process. The main postulate for this corollary stems from the risk factor hypothesis for the development of aortic valve disease. If traditional atherosclerotic risk factors are necessary for the initiation of disease, then these risk factors are responsible for the gradient necessary for the differentiation of myofibroblast cells to become an osteoblast calcifying phenotype [5, 17, 62, 75, 94, 95, 98, 99]. If traditional risk factors are responsible for the development of valvular heart disease, then an oxidative stress mechanism is important for the development of a gradient in this niche.



**Figure 3.** Protein Isolation and Characterization of Aortic Valve Endothelial Cell Conditioned Media; **Panel A.** Light Microscopy for Aortic Valve Endothelial Cells. **Panel B.** Cell Proliferation for fractions eluting from a DEAE- Sephadex column. ( $p < 0.001$ ) **Panel C.** Fractions from DEAE- Sephadex to characterize weight with Sephadex G-100. ( $p < 0.001$ ) **Panel D.** Southern Blot for Protein Expression of Fractions isolated from the DEAE-Sephadex column.

Nitric oxide is important in terms of the mechanism in adult disease processes and also in the developmental abnormalities such as the bicuspid aortic valve phenotype in the eNOS null mouse. To answer this question of the role of oxidative stress and nitric oxide in the aortic valve, I performed *in vitro* experiments to determine eNOS enzymatic and protein regulation in the presence of lipids and attenuation with Atorvastatin. We have previously published that eNOS is regulated in the aortic valve in an experimental hypercholesterolemia model of valvular disease [11]. Figure 4, demonstrates the eNOS regulation in the endothelial cells in the presence of lipids with and without Atorvastatin. A number of standard assays were performed to measure eNOS functional activity. Figure 4, Panel A1, tests for eNOS enzymatic activity in the aortic valve endothelial cells (AEC) in the presence of LDL with and without Atorvastatin. eNOS enzymatic activity was decreased in the presence of lipids and Atorvastatin improved functional enzyme activity. Figure 4, Panel A2, shows re-

sults for tissue nitrites measured in the endothelial cells providing indirect evidence for the enzyme activity. There was an increase in nitrites with lipid treatments and attenuation with Atorvastatin. This increase in nitrite levels correlates with a decrease in the functional activity of the eNOS enzyme in the aortic valve endothelium.



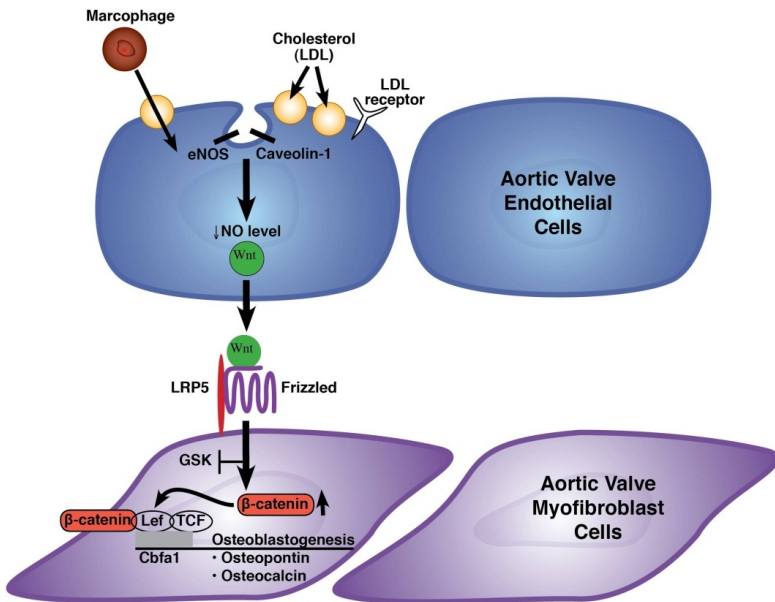
**Figure 4. Evidence for eNOS regulation and Wnt3a Secretion from Aortic Valve Endothelial Cells.** \* $p < 0.001$  for control compared to cholesterol, \*\* $p < 0.001$  for cholesterol compared to cholesterol + Atorvastatin. **Panel A1.** eNOS enzymatic activity in the aortic valve endothelial cells (AEC) in the presence of LDL with and without atorvastatin. **Panel A2.** Cell Nitrite activity in the aortic valve endothelial cells (AEC) in the presence of LDL with and without atorvastatin. **Panel A3.** Thymidine incorporation in LDL treated media, compared to AdeNOS treated myofibroblast cells, versus control LacZ virus. **Panel B1.** Caveolin-1 and eNOS protein expression isolated from the lipid with and without atorvastatin treated cells as shown by Western Blot. **Panel B2.** Electron microscopy immunogold labeling for eNOS and Caveolin-1 localize in the aortic valve endothelial cells in caveolae. **Panel C.** Wnt3a Immunoprecipitate from Conditioned Media treated with LDL with and without Atorvastatin.

The proof of principle experiment to test the importance of eNOS enzymatic activity is an overexpression experiment to determine if eNOS is able to inhibit cell proliferation, an early cellular event in the development of aortic stenosis [13]. Experiments were performed to overexpress eNOS to determine if eNOS overexpression in the aortic valve endothelial cells would regulate cell proliferation. The *in vitro* myofibroblast cells were directly transduced with an eNOS adenoviral gene construct. Thymidine incorporation was measured to test if overexpressing eNOS can inhibit cellular proliferation. Figure 4, Panel A3, eNOS overexpression inhibits the cell proliferation in the oxidized LDL treated cells induced as compared to the LacZ control treated cells.

A key regulator of eNOS function is caveolin-1 which is expressed in aortic valve endothelial cells<sup>19</sup>. Caveolin-1 upregulation in the presence of lipids inactivates eNOS enzymatic function and further promotes oxidative stress [100, 101]. Experiments were performed to localize the expression of Caveolin1 and eNOS in the aortic valve endothelial cell caveolae. A well defined mechanism to inactivate eNOS enzymatic activity is functional binding of eNOS with caveolin1 in the presence of lipids [29, 95, 102]. Figure 4, Panel B1, demonstrates that Caveolin-1 is upregulated in the lipid treated cells and decreases with atorvastatin treatment with no change in the eNOS protein expression as shown by Western Blot. Figure 4, Panel B2, demonstrates the ultrastructural evidence by immunogold labeling for eNOS and Caveolin-1 present in the aortic valve endothelial cells in caveolae, similar to previously reported data [9, 11]. This caveolin1 upregulation is indirect evidence in addition to the direct data of a decrease in the enzyme activity, that caveolin-1 may play a similar role in AEC found in the aortic valve similar to the vascular endothelium.

Experiments were performed to determine if Wnt3a secretion changes in the microenvironment of the aortic valve endothelial cells with and without lipids. Figure 4, Panel C, demonstrates that Wnt3a protein concentration in the conditioned media in the presence of LDL with and without Atorvastatin. There is a significant increase in the protein with the lipids and attenuation of this protein secretion with the Atorvastatin treatments. This experiment tests the effects of lipids regulating the development of a "Wnt3a" gradient in the microenvironment. If LDL increases Wnt3a secretion into the conditioned media or the microenvironment of the diseased aortic valve, this further contributes to the activation of the canonical Wnt pathway in the subendothelial space of the aortic valve.

The final experiment to test the importance of a stem cell niche to activate the cellular osteoblast gene program in the subendothelial layer cells was to test for the gene expression of the Wnt/Lrp5 pathway in the myofibroblast cells. The stem cell niche is a unique model for the development of an oxidative stress communication within the aortic valve endothelium. As shown in Figure 5, oxidative stress contributes to the release of Wnt3a into the subendothelial space to activate Lrp5/Frizzled receptor complex on the extracellular membrane of the myofibroblast. This trimeric complex then induces glycogen synthase kinase to be phosphorylated. This phosphorylation event causes  $\beta$ -catenin translocation to the nucleus.  $\beta$ -catenin acts as a coactivator of osteoblast specific transcription factor Cbfa1 to induce mesenchymal osteoblastogenesis in the aortic valve myofibroblast cell.



**Figure 5.** Schematic Modeling for Calcification in the Aortic Valve Stem Cell Niche.

Adult tissues stem cells are a population of functionally undifferentiated cells, capable of (i) homing (ii) proliferation, (iii) producing differentiated progeny, (iv) self-renewing, (v) re-generation, and (vi) reversibility in the use of these options. Within this definition, stem cells are defined by virtue of their functional potential and not by a specific observable characteristic. This data is the first to implicate a cell-cell communication between the aortic valve endothelial cell and the myofibroblast cell to activate the canonical Wnt pathway. Lrp5 is important in normal valve development [103], in this stem cell niche, reactivation of latent Lrp5 expression [5, 16], regulates osteoblastogenesis in these mesenchymal cells. The two corollary requirements necessary for an adult stem cell niche is to first define the physical architecture of the stem-cell niche and second is to define the gradient of proliferation to differentiation within the stem-cell niche. The aortic valve endothelial cell communicates with the myofibroblast cell to activate the myofibroblast to differentiate to form an osteoblast-like phenotype [14]. This concept is similar to the endothelial/mesenchymal transition critical in normal valve development [104]. This data fulfills these main corollaries of the plausibility of a stem cell niche responsible for the development of valvular heart disease. Within a stem cell niche there is a delicate balance between proliferation and differentiation. Cells near the stem-cell zone are more proliferative, and Wnt likely plays a role in directing cell differentiation. Stem cell behavior is determined by the number of its stem cell neighbors, which in the valve is the endothelial cell. This assumption is aimed at simply describing the fact that cytokines, secreted by cells into the micro-environment are capable of activating quiescent stem cells into differentiation [105].

The important inhibitor in this model is Notch1. Notch1 plays a roll in cellular differentiation decisions. In the osteoblast cell, it serves as an inhibitor of osteoblast differentiation [89, 90]. In the aortic valve, it serves to turn off bone formation via the cell-cell crosstalk between the endothelial and the myofibroblast cells. Normal Notch1 receptor functions to maintain normal valve cellular composition and homeostasis. In the presence of lipids, Notch1 is spliced and therefore activates osteoblastogenesis. In turn, the Wnt3a is secreted and binds to Lrp5 and Frizzled on the extracellular membrane to regulate the osteoblast gene program. This developmental disease process follows a parallel signaling pathway that has been observed in the normal embryonic valve development that has been well delineated by previous investigators [104]. A similar cell-cell communication is necessary for the development of valve disease.

This study provides the correlates described in the mathematical modeling by Agur [106]. This mathematical model has demonstrated the principal that the universal properties of the stem cells can be described in a simple discrete model as derived from hemopoietic stem cell behavior [106]. The transition of hemopoietic stem cells from quiescence into differentiation, is governed by their cell-cycling status, by stimulatory hormones secreted by neighboring cells into the micro-environment and by the level of amplification of stem-cell population [105, 107]. The model of Agur, defines the corollaries necessary to identify a stem cell niche, first the physical architecture of the stem cell niche and second the gradient necessary to regulate the niche. In the BAV the gradient is defined by the niche's microenvironment. The initiation of event of oxidative stress inhibits normal endothelial nitric oxide synthase function, activates notch1 splicing which in turn induces Wnt3a secretion to activate bone formation within the valve [5, 17], [99].

The model proposed in the study as described in Figure 5, provides the cellular architecture for the development of this disease process. This model does not take into account other cytokine/growth factor mediated mechanisms that have been shown to also be important in this disease process [108]. However, understanding CAVD from a development disease perspective will provide a foundation for understanding this and other development disease processes. Clinical trials in the field of CAVD are demonstrating variable results [86, 87]. The possible differences in the published trials are secondary to the timing of therapy and the biological targeting of the lipid levels in these patients. Future medical therapies targeting stem cell niche mediated diseases provides a novel model system to test and to translate clinically for patients in the future.

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