Conclusions

Calcific aortic valve disease is a leading cardiovascular disease in the elderly, and chronic inflammation and calcification play an important role in the progression of this disease. With the increase in the aging population, there is a surge in the incidence of calcific aortic valve disease. However, the cellular and molecular mechanisms responsible for the development and progression of this disease remain incompletely understood. Pharmacological interventions for prevention of aortic valve calcification and its progression to calcific aortic valve disease rely on a thorough understanding of these mechanisms.

Explanted human aortic valve leaflets exhibit evidence of bone-like calcification. Many factors, including shear stress, lipid infiltration, and matrix protein remodeling, likely contribute to aortic valve calcification and stenosis. However, chronic inflammatory changes in aortic valve tissue are believed to play an important role in the development and progression of calcific aortic valve disease, and aortic valve interstitial cells (AVICs), the major cellular components of aortic valve tissue, are involved in the inflammatory process. In this regard, proinflammatory mediators have been shown to induce the expression of pro-osteogenic proteins in AVICs.

Chronic periodontal infection may play a role in the progression of calcific aortic valve disease. In this regard, oral bacteria have been found in diseased aortic valves, and inoculation of rabbits with oral bacteria induces aortic valve lesions. We found that human AVICs express functional Toll-like receptor 4 (TLR4), an important signaling receptor in the innate immune responses and inflammation. Interestingly, stimulation of TLR4 in human AVICs with lipopolysaccharide (LPS) induces the pro-osteogenic response, including the expression of bone morphogenetic protein-2 (BMP-2) and alkaline phosphatase (ALP), and AVICs of stenotic
valves express higher levels of BMP-2 in response to TLR4 stimulation. Examining the molecular mechanism of TLR4-induced pro-osteogenic responses in human AVICs of stenotic valves may provide insights into the pathogenesis of calcific aortic valve disease.

Notch proteins (Notch1–4) are transmembrane receptors expressed on the cell surface. On ligand binding, Notch receptors undergo proteolytic cleavage, leading to the release of their intracellular domains (NICDs) that control cell fate and modulate cell functions. Several studies found that activation of Notch1 promotes osteoblast differentiation. In MC3T3-E1 cells (mesenchymal stem cells), activation of Notch1 by its ligand, Jagged1 or Delta-like 1, promotes osteoblastic differentiation induced by BMP-2. Further, overexpression of NICD1 in human vascular smooth muscle cells increases ALP activity and causes the formation of calcification nodules.

Figure 1. Exaggerated pro-osteogenic response to Toll-like receptor 4 (TLR4) stimulation in aortic valve interstitial cells (AVICs) of stenotic valves is associated with elevated Notch1 levels and enhanced Notch1 cleavage. A–D, AVICs of normal and stenotic valves are treated with lipopolysaccharide (LPS; 200 ng/mL) for 1 to 28 days. A, Representative immunoblots, densitometric data, and ELISA data (from 5 separate experiments using different cell isolates) show that cells from stenotic valves produce higher levels of bone morphogenetic protein-2 (BMP-2) and release greater amounts of this osteogenic factor after LPS stimulation for 24 hours. B, Representative immunoblots, densitometric data, and enzyme activity images (from 5 separate experiments using different cell isolates) show that cells from stenotic valves express higher levels of alkaline phosphatase (ALP) at 24 hours and exhibit greater ALP activity at 14 days of LPS stimulation. C, Representative images of 3 separate experiments and quantitative data show greater formation of calcium deposits (arrow) in AVICs from stenotic valves after LPS stimulation for 4 weeks. D, Representative images of aortic valve tissue show increased number of cells with Notch1 immunoreactivity in stenotic valves and higher density of Notch1 immunohistochemical staining in the interstitial cells of stenotic valves (>20 objective). Immunoblots and densitometric data show that AVICs from 5 stenotic valves have higher levels of Notch1 protein.
study found that heterozygous Notch1-null (Notch1+/−) mice have greater aortic valve calcification than wild-type littermates, suggesting that certain levels of Notch1 protein are required for suppression of aortic valve calcification in mouse. However, the role of Notch1 in regulation of the osteogenic response of human AVICs remains to be investigated.

Bacterial lipopeptide and LPS have been found to induce Notch1 activation in macrophages. Inhibition of γ-secretase, which processes Notch1 to release NICD1, reduces LPS-induced expression of proinflammatory cytokines in macrophages. We recently found that a cross-talk between TLR4 and Notch1 plays a role in mediating the expression of proinflammatory mediators by human AVICs. Currently, the role of Notch1 in TLR4-mediated pro-osteogenic response in human AVICs is unknown. The purposes of this study are to determine the role of Notch1 in augmentation of the pro-osteogenic response to TLR4 stimulation in AVICs of stenotic human aortic valves and to elucidate the underlying mechanisms.

Figure 1 (Continued). E. Representative immunoblots and densitometric data (from 5 separate experiments using different cell isolates) show that TLR4 stimulation induces Notch1 cleavage in AVICs of normal and stenotic valves. Cells of stenotic valves generate higher levels of Notch intracellular domain 1 (NICD1). F. Representative images of immunofluorescence staining show that TLR4 stimulation induces Notch1 (Cy3) translocation from the cell surface (fluorescein isothiocyanate) to perinuclear regions and into the nuclei (DAPI) in AVICs of stenotic valves. P<0.05 vs normal cells receiving the same treatment. *P<0.05 vs corresponding control; and †P<0.05 vs normal cells receiving the same treatment.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Augmented Expression of BMP-2 and ALP by AVICs of Stenotic Valves Is Associated With Elevated Notch1 Levels and Enhanced Notch1 Cleavage in Response to LPS Stimulation

To determine the effect of TLR4 stimulation on the pro-osteogenic response in AVICs of normal and stenotic valves, we analyzed cellular Runx2, BMP-2, and ALP levels and calcium deposits after treatment with LPS. Runx2 levels were elevated in AVICs from both normal and diseased valves after LPS stimulation. However, no difference in Runx2 levels between normal and diseased cells was observed (Figure II in the online-only Data Supplement). In contrast, the levels of BMP-2 and ALP, with or without stimulation with TLR4 agonist LPS for 24 hours, were higher in AVICs of stenotic valves than those in AVICs of normal valves (Figure 1A and 1B). Similarly, BMP-2 levels were higher in the supernatants of AVICs from diseased valves, particularly after LPS stimulation (Figure 1A). Further, AVICs of diseased valves exhibit higher ALP activity (Figure 1B) after 14 days of LPS stimulation and greater calcium deposits (Figure 1C) after 4 weeks of LPS stimulation.

As shown in Figure 1D, the number of Notch1-positive cells and Notch1 immunoreactivity are greater in stenotic aortic valve. Similarly, Notch1 protein levels were higher in AVICs isolated from diseased valves than those in cells isolated from normal valves (5 samples in each group are shown in Figure 1D; results of additional samples are shown in Figure I in the online-only Data Supplement). TLR4 stimulation induced Notch1 cleavage in AVICs of either normal valves or stenotic valves. NICD1 was detected by immunoblotting at 4 hours after LPS stimulation, and NICD1 accumulation was evident with prolonged stimulation. Interestingly, markedly higher levels of NICD1 were found in AVICs of stenotic valves (Figure 1E). Notch1 translocation to perinuclear regions and into the nuclei was observed in both normal and diseased AVICs after TLR4 stimulation with LPS (only images of diseased cells are shown in Figure 1F). Therefore, the augmented pro-osteogenic response to TLR4 stimulation in AVICs of stenotic valves is associated with elevated cellular Notch1 protein levels and enhanced Notch1 cleavage in response to TLR4 stimulation.

Notch1 Plays an Important Role in Mediating BMP-2 and ALP Expression in AVICs of Stenotic Valves

To determine the role of enhanced Notch1 activation in the augmented pro-osteogenic response in AVICs of stenotic valves, we applied DAPT, a γ-secretase inhibitor, to inhibit the generation of NICD1. We confirmed that DAPT inhibits Notch1 cleavage (Figure III in the online-only Data Supplement). Interestingly, treatment with DAPT markedly reduced BMP-2 and ALP levels in cells of either normal or diseased valves. However, a greater reduction was observed in AVICs of stenotic valves, resulting in diminished differences between AVICs of normal and stenotic valves (Figure 2A). More importantly, treatment with DAPT reduced calcium
deposits in cells of diseased valves after a chronic exposure to LPS (Figure 2A).

To further determine the role of Notch1 in mediating the pro-osteogenic response to TLR4 stimulation, we performed Notch1 knockdown in AVICs of diseased valves. Treatment with Notch1 small interfering RNA reduced cellular Notch1 protein levels by 70% (P<0.05; Figure 2B). Cells with Notch1 knockdown had lower levels of BMP-2 and ALP after TLR4 stimulation (Figure 2B). Further, we stimulated normal cells cultured on Jagged1-coated plates and found that the expression of BMP-2 and ALP in response to LPS stimulation was augmented by Jagged1 (Figure 2C). Together, these results demonstrate that Notch1 plays an important role in mediating the pro-osteogenic response to TLR4 stimulation in AVICs and...
that enhanced Notch1 activation is responsible for augmentation of the pro-osteogenic response in AVICs of stenotic valves.

**Notch1 Modulates ERK1/2 and Nuclear Factor-κB Phosphorylation in Human AVICs**

The ERK1/2 and nuclear factor-κB (NF-κB) pathways are involved in osteoblast differentiation and function. Our previous study found that these 2 pathways play a role in mediating BMP-2 expression by human coronary artery endothelial cells exposed to oxidized low-density lipoprotein. To further determine the mechanism underlying the augmented pro-osteogenic response to TLR4 stimulation in AVICs of stenotic valves, we examined ERK1/2 and NF-κB phosphorylation after treatment with LPS for 1 to 24 hours. As shown in Figure 3, AVICs of stenotic valves exhibited enhanced ERK1/2 and NF-κB p65 phosphorylation at all time points examined. It is particularly interesting that ERK1/2 and NF-κB p65 phosphorylation were maintained at high levels at 8 and 24 hours in cells of diseased valves, whereas it declined at these time points in cells of normal valves. The enhanced ERK1/2 and NF-κB phosphorylation in AVICs of stenotic valves correlates with enhanced Notch1 activation. It is possible that enhanced Notch1 activation plays a role in maintaining or sustaining ERK1/2 and NF-κB activation.

To determine the role of Notch1 in ERK1/2 and NF-κB phosphorylation, we treated AVICs of stenotic valves with DAPT or Notch1 small interfering RNA and then stimulated the cells with LPS for 1 to 8 hours. We found that Notch1 ligand, Jagged1, enhanced phosphorylation of mitogen-activated protein kinase kinases 1/2 (MEK1/2), the upstream kinase of ERK1/2, after LPS treatment (Figure 4D). The results suggest that Notch1 enhances ERK1/2 phosphorylation in human AVICs through modulation of MEK1/2 activation. Indeed, we observed that the MEK1/2-ERK1/2 pathway is involved in modulation of NF-κB activation.

Both ERK1/2 and NF-κB Pathways Are Involved in Mediating BMP-2 and ALP Expression in AVICs of Diseased Valves

We applied MEK1/2 inhibitor PD98059 to determine the role of the ERK1/2 pathway in the expression of BMP-2 and
ALP in AVICs of stenotic valves. Figure 5A shows that inhibition of the ERK1/2 pathway attenuated BMP-2 and ALP expression after LPS treatment for 24 hours. In addition, inhibition of the ERK1/2 pathway suppressed the formation of calcium deposits in cells treated with LPS for 4 weeks (Figure 5A).

We treated the cells of stenotic valves with SN50 (a cell-permeable NF-κB inhibitory peptide) and SN50M (control peptide) 1 hour before LPS stimulation and examined cellular BMP-2 and ALP levels. Figure 5B shows that NF-κB inhibition with SN50 markedly reduced BMP-2 and ALP levels, whereas the control peptide SN50M had no effect. Similarly, treatment with SN50 reduced calcium deposits in cells of diseased valves after a chronic exposure to LPS (Figure 5B). Together, these data demonstrate that both ERK1/2 and NF-κB pathways are involved in mediating BMP-2 and ALP expression, as well as osteogenic changes in AVICs of stenotic valves, and indicate that they play a role as downstream mediators in upregulation of the pro-osteogenic response by Notch1.

The observation that the ERK1/2 pathway plays a role in the mechanism of NF-κB phosphorylation in AVICs of diseased valves (Figure 4E) raised a question whether the ERK1/2 pathway contributes to the mechanism of pro-osteogenic response through NF-κB or it also has a NF-κB-independent effect. We examined the effect of PD98059 on BMP-2 and ALP levels in diseased AVICs treated with NF-κB inhibitor SN50. After stimulation with LPS for 24 hours, the levels of BMP-2 and ALP were comparable in cells treated with SN50 alone and cells treated with SN50 plus PD98059 (Figure VI in the online-only Data Supplement). Thus, the ERK1/2 pathway seems to modulate BMP-2 and ALP expression in diseased AVICs primarily through modulation of NF-κB activation.

Discussion
Calcific aortic valve disease affects a large number of people aged ≥65 years, and progressive valvular calcification causes heart failure and results in the second most common cardiovascular surgery performed. With the emerging longevity, calcific aortic valve disease is becoming an increasingly important healthcare issue. Currently, pharmacological intervention of the progression of this disease is unavailable because of limited knowledge of the underlying mechanism. Chronic inflammatory and calcification processes play an important role in the progression of this disease. However, the cellular and molecular mechanisms responsible for the development and progression of this disease remain incompletely understood.

The pro-osteogenic response of AVICs plays a critical role in aortic valve calcification and the progression of calcific aortic valve disease. We previously reported that human AVICs exhibit a pro-osteogenic response, characterized as the expression of pro-osteogenic proteins, to TLR4 stimulation. The results of the present study show that elevated cellular Notch1 levels and enhanced Notch1 activation play a major role in augmentation of the pro-osteogenic response to TLR4 stimulation in AVICs of stenotic valves. Notch1 has a novel role in modulation of the phosphorylation of ERK1/2 and NF-κB, 2 important mediators of the AVIC pro-osteogenic response, and the ERK1/2 pathway seems to mediate the pro-osteogenic response primarily through modulation of NF-κB activation.
Figure 4. Notch1 modulates ERK1/2 and nuclear factor-κB (NF-κB) phosphorylation. A, Aortic valve interstitial cells (AVICs) of stenotic valves are treated with DAPT and then stimulated with lipopolysaccharide (LPS) for 1 to 8 hours. Representative immunoblots and densitometric data (n=5) show that inhibition of Notch1 with DAPT markedly reduces ERK1/2 and NF-κB p65 phosphorylation. B, AVICs of stenotic aortic valves are treated with Notch1 small interfering RNA (siRNA) and then stimulated with LPS for 1 to 4 hours. Representative immunoblots and densitometric data (from 5 separate experiments using different cell isolates) show that Notch1 knockdown reduces ERK1/2 and NF-κB p65 phosphorylation. C, AVICs of normal valves are cultured on Jagged1-coated plates and stimulated with LPS for 1 to 8 hours. Representative immunoblots and densitometric data (from 5 separate experiments using different cell isolates) show that Notch1 ligand, Jagged1, enhances ERK1/2 and NF-κB p65 phosphorylation. D, AVICs of normal valves were cultured on Jagged1-coated plates and then treated with LPS for 1 to 8 hours. A representative immunoblot of 3 separate experiments shows that activation of Notch1 with Jagged1 enhances MEK1/2 phosphorylation in AVICs exposed to LPS. E, AVICs of stenotic valves were treated with PD98059 and then stimulated with LPS for 1 to 8 hours. Representative immunoblots of 5 separate experiments show that PD98059 suppresses ERK1/2 phosphorylation, and inhibition of the ERK1/2 pathway with PD98059 attenuates NF-κB p65 phosphorylation. *P<0.05 vs corresponding control; and ‡‡P<0.05 vs LPS alone or vehicle+LPS. DMSO indicates dimethyl sulfoxide.
stimulation.\textsuperscript{21} We observed in the present study that the augmented pro-osteogenic response to TLR4 stimulation in diseased AVICs is at least partly because of elevated Notch1 levels and augmented Notch1 activation. The novel findings of the present study highlight a potential role for Notch1 in augmentation of the osteogenic response to proinflammatory stimulation in human AVICs. Interestingly, Notch1 mutation has been reported in families with aortic valve developmental deficiency and high incidence of calcific aortic valve disease.\textsuperscript{27} It is likely that the developmental deficiency in aortic valves makes them susceptible to the valvular disease in people with Notch1 mutation.

\begin{figure}[h]
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\caption{The ERK1/2 and nuclear factor-\kappa B (NF-\kappa B) pathways mediate bone morphogenetic protein-2 (BMP-2) and alkaline phosphatase (ALP) expression in aortic valve interstitial cells (AVICs) of stenotic valves. A, AVICs of stenotic aortic valves are treated with PD98059 (25 \textmu mol/L) 1 hour before treatment with lipopolysaccharide (LPS). Representative immunoblots and densitometric data (from 5 separate experiments using different cell isolates) show that inhibition of the ERK1/2 pathway attenuates BMP-2 and ALP expression after LPS treatment for 24 hours. Representative images of 3 separate experiments and quantitative data show that inhibition of the ERK1/2 pathway with PD98059 reduces calcium deposits (arrow) in AVICs of stenotic valves after LPS treatment for 4 weeks. B, AVICs of stenotic aortic valves are treated with a cell-permeable NF-\kappa B inhibitory peptide (SN50, 100 \textmu g/mL; the same concentration of SN50M for control) 1 hour before treatment with LPS. Representative immunoblots and densitometric data (from 5 separate experiments using different cell isolates) show that inhibition of NF-\kappa B reduces BMP-2 and ALP expression after LPS treatment for 24 hours. Representative images of 3 separate experiments and quantitative data show that inhibition of NF-\kappa B reduces calcium deposits (arrow) in AVICs of stenotic valves after LPS treatment for 4 weeks. *P<0.05 vs control; and ‡P<0.05 vs LPS alone or SN50M+LPS. DMSO indicates dimethyl sulfoxide.}
\end{figure}
Notch1 Augments the Pro-Osteogenic Response in AVICs of Stenotic Valves Through Modulation of ERK1/2 and NF-κB Activation

The ERK1/2 and NF-κB pathways are involved in osteoblast differentiation and function. Our previous study found that these 2 pathways play a role in mediating BMP-2 expression by human coronary artery endothelial cells exposed to oxidized low-density lipoprotein. In the present study, we found that ERK1/2 inhibition attenuates BMP-2 and ALP expression after LPS treatment, and NF-κB inhibition seems to have a greater impact. These data demonstrate that both ERK1/2 and NF-κB pathways are involved in the augmented pro-osteogenic response in AVICs of stenotic valves and indicate that they may play a role as downstream mediators in the upregulation of the pro-osteogenic response by Notch1.

A previous report found an effect of Notch1 signaling on NF-κB and protein kinase B activation in macrophages treated with LPS. Our recent study shows that Notch1 signaling has a profound effect on NF-κB phosphorylation in human AVICs and that Notch1–NF-κB interaction is enhanced in AVICs of stenotic valves. Interestingly, the results of the present study show that DAPT, which inhibits Notch1 activation, and Notch1 small interfering RNA not only attenuate NF-κB phosphorylation but also suppress ERK1/2 phosphorylation. The effect of Notch1 inhibition and knockdown on ERK1/2 phosphorylation is more profound at 4 and 8 hours after LPS stimulation. AVICs of diseased valves exhibit significant Notch1 activation at these time points. Further, activation of Notch1 with Jagged1 enhances ERK1/2 and NF-κB phosphorylation in AVICs of normal valves after TLR4 stimulation. Because Notch1 upregulates the phosphorylation of ERK1/2 and NF-κB, and these 2 factors are involved in mediating the pro-osteogenic response, it is reasonable to propose that Notch1 augments the pro-osteogenic response in AVICs of stenotic valves through modulation of ERK1/2 and NF-κB activation.

The observation that Notch1 mediates ERK1/2 phosphorylation is novel. However, the underlying mechanism is not fully understood. Because ERK1/2 is phosphorylated by MEK1/2, and MEK1/2 activation requires its phosphorylation, we examined the effect of Notch1 ligand, Jagged1, on MEK1/2 phosphorylation. The results show that Jagged1 enhances MEK1/2 phosphorylation in normal cells exposed to LPS. Further, inhibition of MEK1/2 abrogates the augmented ERK1/2 phosphorylation in AVICs of diseased valves.
valves. The results indicate that Notch1 activation leads to ERK1/2 phosphorylation through activation of MEK1/2.

The mechanism underlying the effect of Notch1 on NF-κB phosphorylation seems to involve molecular interaction of NICD1 with IκB kinase. In addition, ERK1/2 is reported to modulate NF-κB phosphorylation in several cell types. We found in the present study that Notch1 activation plays a role in the enhanced ERK1/2 phosphorylation in AVICs of diseased valves and that inhibition of the ERK1/2 pathway attenuates NF-κB phosphorylation. Thus, Notch1 activation also enhances NF-κB phosphorylation through the ERK1/2 pathway. The observation that the ERK1/2 pathway plays a role in the mechanism of NF-κB phosphorylation in AVICs of diseased valves raised a question whether the ERK1/2 pathway contributes to the mechanism of pro-osteogenic response through NF-κB or it also has a NF-κB-independent effect. To address this question, we examined the effect of PD98059 on BMP-2 and ALP levels in diseased AVICs treated with NF-κB inhibitor SN50. Our results show that the levels of BMP-2 and ALP after LPS stimulation were comparable in cells treated with SN50 alone and cells treated with SN50 plus PD98059. It seems that the ERK1/2 pathway modulates BMP-2 and ALP expression in diseased AVICs primarily through modulation of NF-κB activation.

Calcific aortic valve disease is becoming an increasingly important issue in clinical cardiology because of the aging of our population. However, pharmacological intervention for prevention of the progression of calcific aortic valve disease is currently unavailable because of the limited knowledge of the underlying mechanism of this disease. The pro-osteogenic response of AVICs is known to play a critical role in aortic valve calcification and the progression of calcific aortic valve disease. The results of the present study obtained in human AVICs of diseased valves indicate that Notch1 and its downstream signaling pathways may be potential targets for suppression of AVIC pro-osteogenic reprogramming and for prevention of the progression of calcific aortic valve disease.

Limitations
One of the limitations of this study is its relatively small sample size. In addition, cultured AVICs may have certain different characteristics than cells in vivo. However, we found in a previous study that TLR4 levels are comparable in freshly isolated human AVICs and cells of passage 6.

Conclusions
The present study demonstrates that Notch1 protein levels are elevated in AVICs of stenotic valves. Enhanced Notch1 activation plays an important role in augmentation of the pro-osteogenic response to TLR4 stimulation through modulation of ERK1/2 and NF-κB activation. These novel findings provide mechanistic insights into AVIC pro-osteogenic response and highlight the potential role of the Notch1-regulated signaling network in the progression of calcific aortic valve disease.

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Disclosures
None.

References
The results of the present study show that elevated cellular Notch1 levels and enhanced Notch1 activation play a major role in augmenting the osteogenic response to proinflammatory stimulation in the interstitial cells of stenotic human aortic valves. Notch1 modulates the phosphorylation of ERK1/2 and nuclear factor-κB, 2 important mediators of the osteogenic response, and the ERK1/2 pathway mediates the osteogenic response primarily through modulation of nuclear factor-κB activation. These novel findings provide mechanistic insights into the osteogenic response in human aortic valve cells and highlight the potential role of the Notch1-regulated signaling network in the progression of calcific aortic valve disease. These findings may improve the understanding of the inflammatory mechanism underlying calcific aortic valve disease and suggest therapeutic targets for prevention of the progression of this cardiovascular disease via suppression of pro-osteogenic reprogramming in aortic valve interstitial cells.
Notch1 Promotes the Pro-Osteogenic Response of Human Aortic Valve Interstitial Cells via Modulation of ERK1/2 and Nuclear Factor-κB Activation
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Materials and Methods

Materials

Notch1 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against human Runx2 was purchased from Novus Biologicals (Littleton, CO). Antibodies against Notch1, NICD1, phosphorylated NF-κB p65, total NF-κB p65, phosphorylated ERK1/2, total ERK1/2, phosphorylated MEK1/2, total MEK1/2 and β-actin were purchased from Cell Signaling, Inc. (Beverly, MA). Antibody against BMP-2 was purchased from ProSci, Inc. (Poway, CA). Antibody against ALP was purchased from ABCAM (Cambridge, MA). Recombinant Jagged1 and BMP-2 ELISA kit were purchased from R&D System (Minneapolis, MN). Kit for NF-κB DNA-binding assay was purchased from Active Motif (Carlsbad, CA). SN50 and SN50M were purchased from ENZO Life Science Inc. (Farmingdale, NY). PD98059 were purchased from EMD Millipore (Billerica, MA). Medium 199 was purchased from Lonza (Walkersville, MD). DAPT, LPS (E. coli 0111:B4) and all other chemicals/reagents were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

Cell isolation and culture

Normal tricuspid aortic valves were collected from the explanted hearts of 9 male patients (age 58±9.6 years) undergoing heart transplantation, and stenotic tricuspid aortic valves were obtained from 9 male patients (age 61±7.8 years) undergoing aortic valve replacement. All patients gave informed consent for the use of their valves for this study. This study was approved by the COMIRB of the University of Colorado Denver.

AVICs were isolated and cultured as previously described \(^1\). Briefly, valve leaflets were subjected to sequential digestions with collagenase. Cells were collected by centrifugation and cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B and 10% fetal bovine serum. When the cells reached 80 to 90% confluence, they were subcultured on plates and chamber slides for the experiments. Cells from passage 4 to 6 were used for this study.
We previously reported that AVIC isolations and cultures are absent of endothelial contamination \(^1\). We confirmed that all cultures used in the present study are negative for von Willebrand factor staining. In addition, all cells express vimentin, and approximately 75% of cells are myofibroblasts that express alpha-smooth muscle actin.

Cellular Notch1 protein levels were analyzed in untreated cells from all 9 isolates of each group. Cells from the first 5 isolates of each group were utilized for the experiments outlined below.

Cells stimulated with LPS (0.2 \(\mu\)g/ml) for 1 to 24 h were analyzed for Notch1 activation (NICD1 generation), and NF-\(\kappa\)B and ERK1/2 phosphorylation. BMP-2 and ALP levels were examined in cells stimulated with LPS (0.2 \(\mu\)g/ml) for 24 h. To determine the role of Notch1 in ERK1/2 and NF-\(\kappa\)B phosphorylation, and in BMP-2 and ALP expression, cells were treated with DAPT (50 \(\mu\)mol/L) or Notch1 siRNA (60 nM) prior to stimulation with LPS. To determine the effect of Notch1 activation, cells were cultured on plates coated with Jagged1 (5.0 \(\mu\)g/ml) and stimulated with LPS. To determine the role of ERK1/2 and NF-\(\kappa\)B in BMP-2 and ALP expression, cells were treated with ERK1/2 inhibitor PD98059 (25 \(\mu\)M) or NF-\(\kappa\)B inhibitor SN50 (100 \(\mu\)g/ml; the same concentration of SN50M as control) 1 h prior to stimulation with LPS. Additional cells were treated with ERK1/2 inhibitor PD98059 (25 \(\mu\)M) to determine the role of ERK1/2 in NF-\(\kappa\)B phosphorylation.

**Immunoblotting**

Immunoblotting was applied to analyze Notch1, NICD1, BMP-2, ALP, phosphorylated ERK1/2, total ERK1/2, phosphorylated NF-\(\kappa\)B p65, total NF-\(\kappa\)B p65 and \(\beta\)-actin. Cells were lysed in a sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue and 10% glycerol). Protein samples were separated on gradient (4-20%) minigels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California). The membranes were blocked with 5% non-fat dry milk solution, and then incubated with primary antibody against Notch1, NICD1, BMP-2, ALP, phosphorylated ERK1/2, total ERK1/2, phosphorylated NF-\(\kappa\)B p65, total NF-\(\kappa\)B p65, phosphorylated
MEK1/2, total MEK1/2 or β-actin. After washing with TPBS [phosphate-buffered saline (PBS) containing 0.05% Tween 20], the membranes were incubated with a peroxidase-linked secondary antibody specific to the primary antibody. Following further washes, membranes were treated with enhanced chemiluminescence reagents. Then, the membrane was exposed on X-ray film. NIH Image J software was used to analyze the area and density of protein band.

**NF-κB DNA-binding assay**

NF-κB (p65) DNA-binding activity in cell lysate was measured by an assay kit according to the manufacturer’s instructions. The kit contains 96-well plates to which oligonucleotides containing the NF-κB binding site have been immobilized. Activated NF-κB binds to the oligonucleotides and is detected with a specific antibody. The amount of active NF-κB was determined using a microplate reader.

**ELISA for BMP-2**

Cell culture supernatants were collected. BMP-2 levels were analyzed using an ELISA kit following the manufacturer’s protocol.

**ALP activity staining**

Cells were fixed, and histochemical staining for ALP activity was performed as previously described. Briefly, cell monolayers were washed with PBS and fixed for 10 minutes in 4% paraformaldehyde, followed by incubation at room temperature for 30 minutes with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate, 0.5% N,N-dimethylformamide, 2 mM MgCl2, and 0.6 mg/ml of fast blue BB salt in 0.1 M Tris-HCl, pH8.5. Excessive dye was removed by washing with PBS. ALP
activity staining was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan).

Alizarin red S staining

Alizarin red S staining for calcium deposits was performed as described previously \(^2\)\(^-\)\(^4\). Briefly, cell monolayers were washed three times with PBS and fixed for 10 min with 4% paraformaldehyde, followed by incubation with 0.2% Alizarin red S solution (pH 4.2) for 30 min. Excessive dye was removed by washing with distilled water. Alizarin red S staining was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan). To quantitatively analyze Alizarin red stain, wells were rinsed with distilled water, and Alizarin red S stains were bleached with 10% acetic acid at 85°C. Supernatant was spectrophotometrically analyzed at 450 nm \(^5\).

Immunofluorescent staining

Notch1 was localized using immunofluorescent staining as described previously \(^6\). After permeabilization with a methanol/acetic acid mixture, cells on chamber slides were fixed with 4% paraformaldehyde, incubated with rabbit polyclonal antibody against human Notch1 overnight at 4°C. After washing with PBS, cells were incubated with Cy3-tagged secondary antibody against rabbit IgG (imaged on the red channel). Nuclei were stained with bis-benzimide (DAPI, imaged on the blue channel), and glycoproteins on cell surfaces with Alexa 488-tagged wheat germ agglutinin (imaged on the green channel). Microscopy was performed with a Leica DMRXA digital microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) equipped with Slidebook software (I. I. I. Inc., Denver, CO).

Gene silencing

Notch1 silencing was performed using the method described previously \(^7\). Cells were cultured in antibiotic-free growth medium until 60% confluent. The cells were incubated with a mixture of siRNA
specific to human Notch1 (60 nM) and transfection reagent (6 μl per ml medium) in antibiotic- and serum-free medium for 6 h. After transfection, cells were incubated in growth medium for 48 h, and then stimulated with LPS. Control cells were treated with scrambled siRNA and transfection reagent.

**Statistical analysis**

Data are presented as mean ± standard error (SE). Student’s t-test was used for comparison between two groups. For more than two groups, significance was determined using one-way analysis of variance (ANOVA) with comparison between groups by post hoc Bonferroni/Dunn test. Statistical significance was defined as \( P \leq 0.05 \). Non-parametric Mann-Whitney U test was performed to confirm the difference of 2 group comparison. For multiple group comparisons, non-parametric Kruskal-Wallis test was performed to confirm the differences.
References


Figure I. AVICs from stenotic valves have elevated Notch1 levels. Levels of Notch1 expression in AVICs of normal and stenotic valves from 4 additional pairs of donors were examined with immunoblotting. The immunoblot shows that the levels of Notch1 protein in stenotic AVICs are higher than those of normal AVICs. The results are consistent with the results presented in Figure 1D.

Figure II. Runx2 levels are comparable in AVICs of normal and stenotic valves following LPS treatment. AVICs of normal and stenotic valves were treated with LPS (200 ng/ml) for 8-24 hours. The levels of Runx2 protein in AVICs of normal and stenotic valves following LPS treatment were examined with immunoblotting. A representative immunoblot of 3 independent experiments shows that Runx2 protein levels are increased following LPS treatment, but are not different in AVICs of normal and stenotic valves.

Figure III. Inhibition of Notch1 with DAPT attenuates Notch1 cleavage induced by TLR4 stimulation in AVICs of stenotic valves. AVICs of stenotic valves were treated with LPS (200 ng/ml) in the presence and absence of γ-secretase inhibitor DAPT (50 µM) for 8-24 hours. Levels of NICD1 were examined with immunoblotting. A representative immunoblot of 3 independent experiments shows that DAPT attenuates NICD1 generation induced by LPS treatment.
**Figure IV.** Inhibition of Notch1 decreases NF-κB DNA-binding activity following TLR4 stimulation. AVICs of stenotic valves were treated with LPS (200 ng/ml) for 4 hours in the presence or absence of γ-secretase inhibitor DAPT (50 µM). NF-κB p65 DNA-binding activity was analyzed using an assay kit. DAPT attenuates NF-κB DNA-binding activity in AVICs of stenotic valves following LPS stimulation. n=4; *P<0.05 vs. corresponding control; ‡P<0.05 vs. LPS alone.

**Figure V.** Activation of Notch1 with Jagged1 augments NF-κB DNA-binding activity following TLR4 stimulation. AVICs of normal valves were treated with LPS (200 ng/ml) for 4 hours in the presence or absence of Notch1 ligand Jagged1 (5 µg/ml). NF-κB p65 DNA-binding activity was analyzed using an assay kit. Jagged1 enhances NF-κB p65 DNA-binding activity in normal cells exposed to LPS. n=4; *P<0.05 vs. untreated control; ‡P<0.05 vs. LPS alone.
Figure VI. Inhibition of both ERK1/2 and NF-κB simultaneously has an effect comparable to that of inhibition of NF-κB alone. AVICs of stenotic valves were treated with PD98059 (25 µM), SN50 (100 µg/ml), or both 1 hour prior to LPS (200 ng/ml) stimulation for 24 hours. Levels of BMP-2 and ALP were examined with immunoblotting. A representative immunoblot of 3 independent experiments shows that inhibition of both ERK1/2 and NF-κB simultaneously has a comparable effect to that of inhibition of NF-κB alone in suppressing BMP-2 and ALP expression.