Notch1 Mutation Leads to Valvular Calcification Through Enhanced Myofibroblast Mechanotransduction


Objective—Calcific aortic valve disease (CAVD) is a significant cardiovascular disorder, and controversy exists as to whether it is primarily a dystrophic or osteogenic process in vivo. In this study, we sought to clarify the mechanism of CAVD by assessing a genetic mutation, Notch1 heterozygosity, which leads to CAVD with 100% penetrance in humans.

Approach and Results—Murine immortalized Notch1+/− aortic valve interstitial cells (AVICs) were isolated and expanded in vitro. Molecular signaling of wild-type and Notch1+/− AVICs were compared to identify changes in pathways that have been linked to CAVD—transforming growth factor-β1/bone morphogenetic protein, mitogen-activated protein kinase, and phosphoinositide 3-kinase/protein kinase B—and assessed for calcification potential. Additionally, AVIC mechanobiology was studied in a physiologically relevant, dynamic mechanical environment (10% cyclic strain) to investigate differences in responses between the cell types. We found that Notch1+/− AVICs resembled a myofibroblast-like phenotype expressing higher amounts of cadherin-11, a known mediator of dystrophic calcification, and decreased Runx2, a known osteogenic marker. We determined that cadherin-11 expression is regulated by Akt activity, and inhibition of Akt phosphorylation significantly reduced cadherin-11 expression. Moreover, in the presence of cyclic strain, Notch1+/− AVICs exhibited significantly upregulated phosphorylation of Akt at Ser473 and smooth muscle α-actin expression, indicative of a fully activated myofibroblast. Finally, these Notch1-mediated alterations led to enhanced dystrophic calcific nodule formation.

Conclusions—This study presents novel insights in our understanding of Notch1-mediated CAVD by demonstrating that the mutation leads to AVICs that are fully activated myofibroblasts, resulting in dystrophic, but not osteogenic, calcification. (Arterioscler Thromb Vasc Biol. 2015;35:1597-1605. DOI: 10.1161/ATVBAHA.114.305095.)

Key Words: aortic valve ▪ cadherin-11 ▪ calcification ▪ Notch1

Calcific aortic valve disease (CAVD) is thought to occur in 2 forms, dystrophic and osteogenic, and is believed to be mediated by aortic valve interstitial cells (AVICs).4–6 Dystrophic calcification is more prevalent, found in 83% of valve explants, and involves cell-death–mediated calcification through the nucleation of Ca and P.3–5,7 Dystrophic calcification proceeds as quiescent AVICs differentiate into activated myofibroblasts, which is a complex process regulated by a host of signaling pathways, including transforming growth factor-β1 (TGF-β1), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling.8–12 Hallmarks for myofibroblast-like AVICs include enhanced smooth muscle α-actin (αSMA) and cadherin-11 expression that confer increased contractility and strong intercellular connections, respectively.4,13 The combinatorial effect of these proteins leads to increased intercellular tension and eventual cell-death–mediated calcification.3 Alternatively, osteogenic calcification is found in 13% of calcified valves and is generated through the active secretion of osteoid matrix via osteoblast-like AVICs.6,7 The differentiation to osteoblast-like AVICs is induced by the treatment of potent osteogenic media and is thought to be mediated by bone morphogenetic protein (BMP) signaling, which leads to the expression of the transcriptional activator Runx2.5,14 However, the role of dystrophic and osteogenic processes in the progression of CAVD in vivo is poorly understood. Genetic factors that promote CAVD has provided new physiologically relevant areas of investigation that may further the understanding of CAVD pathogenesis.15,16 Specifically, the Notch1 mutation affects the intrinsic signaling of the cells, predisposing them for CAVD and creating a clinically relevant system to study CAVD.

Notch is a highly conserved cell–cell signaling pathway that plays a crucial role in proper cardiac development and remodeling.17–19 On ligand binding, Notch receptors undergo proteolytic cleavage via γ-secretase and release of the Notch intracellular domain, which translocates to the nucleus to affect cell maintenance, proliferation, and apoptosis.20,21 Mutations in...
Notch can lead to a spectrum of congenital heart defects, such as cardiomyopathy, tetralogy of Fallot, and valvular malformations. In addition to developmental abnormalities, dysregulated Notch function plays a major role in cardiac disease initiation and progression. Specifically in the aortic valve, mutations in Notch1 lead to CAVD with 100% penetrance in humans. Furthermore, valvular calcification observed in Notch1 haploinsufficient patients is more severe, suggesting a direct role of Notch1 signaling in the calcification process.

Recent investigations of the role of Notch1 deficiency in CAVD have been variable. Acharya et al demonstrated through chemical inhibition that Notch1 has an inhibitory role on the development of CAVD. Further, Nigam et al showed that Notch1 signaling specifically affects osteogenic pathways in AVICs, preventing the progression of osteogenic calcification. Conversely, Zeng et al recently indicated that Notch1 in fact promotes osteogenic calcification in human AVICs. These disparate findings highlight the need for further studies to elucidate the pathological alterations caused by Notch1 mutation. One major limitation of studies evaluating the effect of the Notch1 mutation is lack of a consistent method to recapitulate the effects of the mutation in vitro. Many studies use N-[N-(3,5-difluorophenacetyl-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), a chemical inhibitor of γ-secretase, to mimic Notch1 haploinsufficiency; however, DAPT has potential off-target effects. Therefore, isolation and study of AVICs from Notch1+/− mice represents a more accurate approach to evaluate the Notch1 mutation in vitro.

In this study, we isolated Notch1 mutant AVICs from Notch1+/− Immortomice for the first time. Using these cells, we found that Notch1+/− AVICs display reduced αSMA and Runx2, but notably increased cadherin-11 expression. We determined that upregulated cadherin-11 expression is mediated by enhanced protein kinase B (Akt) phosphorylation and activity in the mutant cells. Further, the inhibition of Akt phosphorylation led to a dramatic decrease in cadherin-11. The involvement of Notch signaling in cadherin-11 expression is
a novel finding directly linking Notch1 heterozygosity with a hallmark of CAVD. Finally, Notch1+/− AVICs were shown to be hypersensitive to mechanical strain, resulting in significant increases in αSMA expression and formation of calcific nodules. These findings reveal that CAVD arising from the Notch1+/− mutation proceed through increased cell–cell tension via cadherin-11, which in a dynamic environment leads to differentiation of AVICs into myofibroblasts that then undergo dystrophic calcification.

Materials and Methods

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

Results

Notch1 Mutation Is Characterized by Enhanced Calcification, Increased Mechanical Stiffness, and Alterations to Cadherin-11 and Runx2 Expression

Tissue level characterization demonstrated that Notch1+/− valves have enhanced calcification, elevated cadherin-11, and increased mechanical stiffness. Von Kossa staining of the valve leaflets revealed higher uptake of stain in the Notch1+/− animals (Figure 1A(a,b)). Accumulation of Von Kossa stain was evenly distributed throughout the tissue, and no punctate stain was observed in the leaflets. Immunohistochemistry analysis of valve tissue revealed enhanced cadherin-11 staining in the Notch1+/− leaflets (Figure 1A(c,d)). Further, biomechanical analysis of leaflet tissue via atomic force microscopy indicated that Notch1+/− valve leaflets are significantly stiffer than wild-type (WT) leaflets (Figure 1B). WT Immorto and Notch1+/− Immorto genotype was confirmed with polymerase chain reaction (Figure 1C). Protein level analysis confirmed that Notch1+/− AVICs have reduced Notch intracellular domain cleavage (Figure 1D; representative Western blot images for all data are in the online-only Data Supplement).

Notch1+/− AVICs also express less αSMA and Runx2 but significantly more cadherin-11 (Figure 1E). On Jagged1 stimulation, αSMA expression was unchanged (Figure 1F), cadherin-11 expression was reduced (Figure 1G), and Runx2 expression was increased (Figure 1H).

Notch1+/− AVICs Have Dysregulated MAPK and PI3K/Akt Signaling

We evaluated the effect of TGF-β1 and BMP2 stimulation on important signaling pathways, such as TGF-β/BMP, MAPK, and PI3K/Akt, which all have been shown to be involved in the calcification process.4–6,8,10 Notch1+/− AVICs have higher basal levels of Smad3 and Smad1/5/8 phosphorylation, but TGF-β1 and BMP2 treatment led to insignificant phosphorylation differences between WT and mutant AVICs (Figure 2A and 2B). Erk1/2 and p38, signaling molecules of the MAPK pathway, had significantly decreased levels of phosphorylation in the Notch1+/− AVICs (Figure 2C and 2D). Conversely, Notch1+/− AVICs displayed significantly increased Akt phosphorylation at both Thr308 and Ser473 with minimal change with TGF-β1 and BMP2 stimulation (Figure 2E and 2F).

Upregulated Cadherin-11 Expression in Notch1+/− AVICs Is Enhanced Akt Activity

Because Erk1/2, p38, and Akt signaling were abnormal in Notch1+/− cells, we sought to determine their role in cadherin-11 expression by inhibiting their activity. We treated WT and Notch1+/− AVICs with a MEK1/2 inhibitor (U0126), which prevents Erk1/2 phosphorylation, a p38 inhibitor, and an Akt inhibitor. U0126 and p38 inhibitor treatment did not significantly affect cadherin-11 expression in either cell type; however, Akt inhibition led to near complete abrogation of cadherin-11 expression (Figure 3A). Because of the dramatic

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**Figure 2.** Notch1+/− aortic valve interstitial cells (AVICs) have dysregulated mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling. Transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein 2 (BMP2) stimulation leads to comparable increases in Smad3 and Smad1/5/8 phosphorylation in both WT and Notch1-deficient cells (A, B). MAPK signaling is dysregulated with significant decreases in Erk1/2 and p38 phosphorylation in the mutant cells (C, D). Additionally, PI3K/Akt signaling is enhanced in the mutant cells with increased Akt phosphorylation at both Thr308 and Ser473 (E, F). * indicates significant difference (P<0.05) vs WT control and # indicates significant difference (P<0.05) vs Notch1+/− control. NT indicates no treatment.
response caused by Akt inhibition, we further evaluated the PI3K/Akt pathway. Akt phosphorylation was expectedly inhibited by the Akt inhibitor (Figure 3B and 3C), and glyco-
gen synthase kinase 3β (GSK-3β) phosphorylation increased with Akt inhibition (Figure 3D). The role of GSK-3β activity in cadherin-11 expression was assessed with lithium chloride treatment, a GSK-3β inhibitor, which caused a significant reduction in GSK-3β activity (as indicated by an increase in phosphorylation) and cadherin-11 (Figure 3E and 3F); however, inhibition of GSK-3β did not normalize the increased cadherin-11 in the Notch1+/− cells.

**Deficient Notch1 Signaling Leads to Hypersensitivity to Mechanical Strain and Myofibroblast Activation**

Strain-induced activation of Notch1 signaling is reduced in the Notch1+/− AVICs as evidenced by a decrease in Notch intracellular domain cleavage (Figure 4A). With the application 10% cyclic mechanical strain, which approximates in vivo diastolic loading, αSMA expression is dramatically increased, cadherin-11 expression remains unchanged, and Runx2 expression is decreased (Figure 4B–4D). Notably, unstrained Notch1+/− AVICs have less αSMA than WT but exceed the WT cells when strained. WT and Notch1+/− AVICs both have increased phosphorylation of Erk1/2 and p38 under stretch (Figure 4E and 4F). Similarly, Akt phosphorylation at Ser473 was increased with mechanical strain in both cell types. As with αSMA, phosphorylation of Akt Ser473 underwent larger increases because of strain in Notch1+/− AVICs (Figure 4G). Akt phosphorylation at Thr308 (data not shown) and GSK-3β phosphorylation were not significantly affected because of mechanical strain (Figure 4H). Finally, the inhibition of Akt phosphorylation did not affect αSMA expression in unstrained cultures; however, Akt inhibition abrogated the strain-dependent increase in αSMA expression (Figure 4I).

**Notch1+/− AVICs Have Active Cadherin-11 Engagement and Calcify Through a Dystrophic Pathway**

Immunostaining revealed that Notch1+/− AVICs have significantly more cadherin-11; however, WT cells have more αSMA than mutant cells (Figure 5A and 5B). When treated with TGF-β1, however, both WT and Notch1+/− AVICs revealed increases in αSMA and cadherin-11 (Figure 5C and 5D). Calcific nodule formation was assessed in a physiologically relevant strain (10%) system as previously described. Notch1+/− AVICs formed significantly more calcific nodules than WT cells with and without TGF-β1; however, TGF-β1 treatment dramatically increased the number of nodules formed (Figure 5E). Apoptosis and necrosis stains were conducted to describe nodule viability. Annexin V and propidium iodide stains revealed significant uptake of propidium iodide in the nodule center and

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**Figure 3.** Upregulated cadherin-11 expression in Notch1+/− aortic valve interstitial cells (AVICs) is mediated by enhanced protein kinase B (Akt) activity. Cadherin-11 expression is significantly reduced by the Akt inhibitor in both wild-type (WT) and Notch1+/− AVICs (A). The inhibition of Akt leads to a significant decrease in Akt phosphorylation at Thr308 and Ser473 (B, C). Further, Akt inhibition leads to decreased glycogen synthase kinase 3β (GSK-3β) activity as evidenced by an increase in GSK-3β phosphorylation (D). Lithium chloride (LiCl) treatment reduces GSK-3β activity (E) and leads to a modest but significant decrease in cadherin-11 expression (F). * indicates significant difference (P<0.05) vs WT control and # indicates significant difference (P<0.05) vs Notch1+/− control.
faint Annexin V stain on the periphery of the nodule characteristic of dystrophic calcification (Figure 5F, 5G, 5I, and 5J). Calcific nodules from both genotypes were intensely stained via Alizarin red, a calcification stain (Figure 5H and 5K).

Discussion

There are 4 notable findings in this study: (1) Notch1 mutation leads to AVICs with a myofibroblast-like phenotype as evidenced by increased cadherin-11 expression, (2) upregulated cadherin-11 expression is mediated by increased Akt activity, (3) Notch1+/− AVICs become fully activated myofibroblasts in the presence of mechanical strain, and (4) activated Notch1+/− AVICs lead to enhanced dystrophic calcification in vitro.

Previous studies evaluating the role of Notch1 mutation in CAVD have been variable and have primarily focused on Notch1 signaling in osteogenic calcification. However, our data suggests that Notch1+/− AVICs suppress osteogenic differentiation and follow a myofibroblast-like phenotype. We found that Notch1+/− AVICs display decreased Runx2 expression (Figure 1E), which is essential for osteogenic differentiation. Moreover, the activation of Notch1 via Jagged1 ligand binding significantly increased the expression of Runx2 in WT cells (Figure 1H), suggesting that the activation of Notch1 signaling could in fact induce osteogenic differentiation. This is consistent with a recent report highlighting the role of Notch1 in promoting osteogenic responses in human AVICs. Conversely, several studies have indicated that canonical Notch1 signaling plays a protective role by preventing the transcription of Runx2 mRNA and subsequent osteogenic differentiation. However, in these systems, Notch1 deficiency was simulated through DAPT treatment, which affects all Notch signaling and may have unknown off-target effects that confound the data, and changes in mRNA level do not necessarily translate to changes in protein expression, which we show here. Interestingly, Notch signaling has also been implicated in the regulation of myofibroblast differentiation. Sassoli et al demonstrated that reduced Notch1 signaling lead to myofibroblast differentiation and αSMA expression in cardiac fibroblasts. Further, Fan et al showed that this phenotypic shift was associated with a reduction in Notch receptor and ligand expression. Here, we show that Notch1-deficient AVICs exhibit myofibroblast-like properties that promote dystrophic CAVD.

The transition of fibroblasts to myofibroblasts is a complex process that often occurs after tissue injury. The myofibroblast is crucial to the tissue repair process, but the persistence of this phenotype can lead to pathological remodeling of the ECM. Many factors influence this transition from fibroblasts to myofibroblasts, including inflammatory signals and the mechanical environment. Myofibroblast differentiation occurs through 2 stages, first to an intermediate cell phenotype, the protomyofibroblast, and then to the fully activated...
myofibroblast. Protomyofibroblasts possess myofibroblast-like properties with changes in focal adhesions proteins and stress fibers, but they do not express αSMA. When in the presence of TGF-β1 and mechanical stretch, protomyofibroblasts become differentiated myofibroblasts characterized by αSMA and cadherin-11. In CA VD, myofibroblasts are thought to contribute directly to the thickening and stiffening of stenotic valves, as well as the dystrophic calcification observed in diseased tissue explants. We demonstrated in a previous study that the presence of cadherin-11 coupled with increased contractility via αSMA leads to apoptosis and subsequent cell aggregation and calcification in AVICs. Additionally, we found that human leaflets excised from patients with CA VD were highly enriched in cadherin-11 that was colocalized with αSMA in areas of significant calcification. In the present study, we found that reduced Notch1 signaling leads to significant alterations to myofibroblast markers, as well as a significant increase in tissue stiffness, which has been shown to promote myofibroblast differentiation and dystrophic calcification in AVICs. We also found that human leaflets excised from patients with CA VD were highly enriched in cadherin-11 that was colocalized with αSMA in areas of significant calcification. In the present study, we found that reduced Notch1 signaling leads to significant alterations to myofibroblast markers, as well as a significant increase in tissue stiffness, which has been shown to promote myofibroblast differentiation and dystrophic calcification in AVICs. The expression of the myofibroblast marker cadherin-11 was dramatically increased in Notch1−/− AVICs (Figure 1A and 1E). Activation of Notch1 signaling reduced cadherin-11 (Figure 1G), suggesting that the increase in cadherin-11 expression was because of Notch1-deficient signaling. Surprisingly, the myofibroblast marker αSMA was reduced in the mutant cells, and this reduction was not affected by Notch1 activation, suggesting that these changes are indirectly affected by dysregulated Notch1 signaling. Taken together, Notch1−/− AVICs represent a phenotype that resembles an intermediary myofibroblast expressing enhanced cadherin-11, reduced αSMA, and decreased osteogenic activity.

Cadherin-11 is a type II classical cadherin that plays significant roles in normal development and many diseases, such as inflammatory disorders and cancer. First observed in osteoblasts, cadherin-11 has now been associated with a variety of fibrotic diseases, such as pulmonary fibrosis, dermal fibrosis, and CA VD. Cadherin-11 is a unique intercellular junction from a biomechanical perspective because it resists 2-fold higher forces than typical cadherins, such as N-cadherin, thus creating tighter, stronger junctions. These unique characteristics are essential to the progression of dystrophic calcification in CA VD, as we showed in a previous study that knockdown of cadherin-11 prevents calcification of porcine AVICs, even when exposed to TGF-β1. We identified a functional role of cadherin-11 in CA VD; however, the regulation of cadherin-11 expression is unknown in AVICs. In the present study, cadherin-11 expression is dramatically increased in Notch1-deficient AVICs; therefore, we examined downstream mediators from Notch1 that may alter cadherin-11 expression. MAPK and PI3K/Akt signaling were highly dysregulated in Notch1−/− AVICs with reduced p38 and Erk1/2 phosphorylation, but significantly increased Akt phosphorylation at both Ser473 and Thr308 (Figure 2D–2F). Using inhibitors for these pathways, we found that p38 and Erk1/2 inhibition did not significantly affect cadherin-11 levels, but the inhibition of Akt dramatically abrogated cadherin-11 expression (Figure 3A). Akt is a central node in the PI3K/Akt pathway and is responsible for a plethora of cellular processes, including cell survival,
proliferation, metabolism, and migration.43 When PI3K is activated, it converts phosphatidylinositol (3–5)-bisphosphate to phosphatidylinositol (3–5)-trisphosphate which recruits Akt to the plasma membrane, leading to its phosphorylation via phosphoinositide-dependent kinase 1 and mammalian target of rapamycin complex 2 at Thr308 and Ser473, respectively.44 Activated Akt phosphorylates a wide variety of substrates mediating multiple cellular events. Interestingly, GSK-3β, a downstream substrate of Akt has been implicated in cadherin-11 expression in prostate and breast cancer cells with the inactivation of GSK-3β, leading to a reduction in cadherin-11 mRNA and protein levels.45 We therefore assessed the role of GSK-3β in our cells by inhibiting its activity via lithium chloride treatment. We found that lithium chloride led to a significant decrease in GSK-3β activity and a significant reduction in cadherin-11 expression; however, the decrease in cadherin-11 expression was modest when compared with the decrease observed with inhibition of Akt (Figure 3F).

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AVICs reside in a highly dynamic environment and undergo constant cyclic mechanical strain in vivo. Further, AVIC mechanobiology is directly influenced by mechanical strain, and we and others have previously shown that increased magnitude of strain plays a significant role in the initiation and progression of CAVD.3,46–48 We hypothesized that Notch1+/− AVICs may have alterations in their mechanotransductive response to strain that would lead to early disease and CAVD observed in Notch1 haploinsufficient patients. Our data show that Notch1 signaling is activated by mechanical strain, and this activation is decreased in Notch1+/− AVICs (Figure 4A). We hypothesized that this decrease in mechanically induced Notch intracellular domain cleavage in the mutant cells may lead to deleterious changes that contribute to CAVD. Our data reveal that mechanical strain led to increased αSMA expression with no change in cadherin-11 expression and a drastic reduction in Runx2 expression (Figure 4B–4D).

Notably, Notch1+/− AVICs had higher αSMA expression than WT cells under strain. Analysis of signaling proteins revealed an exaggerated Akt Ser473 phosphorylation response to mechanical strain in Notch1+/− AVICs (Figure 4G). Further, this increase in αSMA was abrogated with Akt inhibition (Figure 4I). Interestingly, Wang et al connected Akt Ser473 phosphorylation with αSMA expression by demonstrating that stiffer substrates activate Akt Ser473 and push the cell toward a myofibroblast phenotype.10 These findings along with the increased stiffness observed in Notch1+/− aortic valves suggest an in vivo environment that would promote myofibroblast activation. Akt signaling has been shown to be regulated by Notch1; however, their relationship is not well understood.49–51 In our system, Notch1+/− AVICs have increased Akt phosphorylation, suggesting that Notch1 activation decreases Akt phosphorylation. It is possible that Notch1 plays a protective role in preventing myofibroblast differentiation by modulating Akt Ser473 activity. The loss of this regulatory mechanism in the Notch1+/− cells allows for an unfettered increase in phosphorylation of Akt Ser473 and subsequent αSMA upregulation.
In the presence of mechanical strain, Notch1+/− AVICs become fully activated myofibroblasts as evidenced by the presence of both αSMA and cadherin-11. Interestingly, the presence of mechanical strain also decreased Runx2 expression (Figure 4D), further supporting the notion of Notch1+/− AVICs being pushed toward a myofibroblast phenotype. WT cells, however, did not exhibit significant changes to cadherin-11 expression because of mechanical strain and, therefore, do not represent a fully activated myofibroblast. We hypothesized that the presence of cadherin-11 in the Notch1+/− AVICs would lead to the development of more severe calcification. We subjected WT and mutant cells to a dynamic calcific nodule assay as previously described and found significantly more calcific nodules generated by the mutant cells with and without TGF-β1 stimulation (Figure 5E). As expected, TGF-β1 treatment led to the development of significantly more calcific nodules in both cell types. Immunostaining images revealed that TGF-β1 led to the full activation of WT cells and enhanced αSMA and cadherin-11 expression in the Notch1+/− AVICs (Figure 5A–5D). Furthermore, these nodules stained positively for cell death and resembled dystrophic calcific nodules as previously described (Figure 5G and 5J). These findings reveal a role of cadherin-11 for modulating calcification severity and provide evidence that enhanced cadherin-11 contributes to the development of CAVD in Notch1 haplosufficient patients.

In this study, we demonstrate that Notch1+/− AVICs become fully activated myofibroblasts (increased αSMA and cadherin-11 expression), which leads to dystrophic calcification in a dynamic mechanical environment. The effect of Notch1-deficient signaling is manifest in enhanced Akt activity and the loss of the regulation of strain-induced Akt Ser 473 activation, resulting in enhanced cadherin-11 and αSMA, respectively (Figure 6). However, the loss of Notch1 signaling affects other relevant pathways that may contribute substantially to the regulation of AVIC phenotype. Here, we observed significant alterations to 3 pathways that have been implicated in myofibroblast differentiation and the development of valvular disease: TGF-β/BMP, MAPK, and PI3K/Akt. Significant cross-talk has been observed between these pathways; however, the interplay between these signaling components is not well understood. It is beyond the scope of this study to elucidate the intricate interactions and convergence between these pathways; however, our data does present novel information regarding myofibroblast differentiation and creates new directions for CAVD research.

The goal of elucidating CAVD mechanism is to identify significant contributors to disease that are targetable through pharmacological treatment. The findings in this report are significant because they provide potentially paradigm shifting evidence that Notch1 mutation leads to dystrophic, but not osteogenic, CAVD. Furthermore, the clear and distinct role of increased cadherin-11 in the Notch1+/− AVICs is significant because it has been demonstrated as a critical component of valvular calcification, and inhibition of cadherin-11 function may be a promising approach for treating CAVD. Additionally, the discovery of the role of PI3K/Akt signaling in αSMA and cadherin-11 expression presents many targetable proteins, such as phosphoinositide-dependent kinase 1 and mammalian target of rapamycin complex 2, to be further explored.

Sources of Funding
This work was supported by the National Institute of Health (HL094707 and HL115103). J. Chen and M.K. Sewell-Loftin were supported by Predoctoral Fellowships from the American Heart Association (11PRE7990023 and 12PRE12070154).

Disclosures
None.

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Notch1+/− leads to myofibroblast differentiation of valve cells, which results in dystrophic, but not osteogenic, CAVD. In essence, the Notch1 signaling regulates Notch1 in aortic valve disease. J Mol Cell Cardiol 2013;53:1580–1590. doi: 10.1016/ATVBHA.112.300912.


Notch1 Mutation Leads to Valvular Calcification Through Enhanced Myofibroblast Mechanotransduction


Arterioscler Thromb Vasc Biol. 2015;35:1597-1605; originally published online May 28, 2015; doi: 10.1161/ATVBAHA.114.305095

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figure I: Representative Western blots that correspond to Figure 1 in the manuscript
Supplemental Figure II: Representative Western blots that correspond to Figure 2A,B in the manuscript
Supplemental Figure III: Representative Western blots that correspond to Figure 2C,D in the manuscript
Supplemental Figure IV: Representative Western blots that correspond to Figure 2E,F in the manuscript
Supplemental Figure V: Representative Western blots that correspond to Figure 3A in the manuscript
Supplemental Figure VI: Representative Western blots that correspond to Figure 3B,C in the manuscript
Supplemental Figure VII: Representative Western blots that correspond to Figure 3D,E in the manuscript.
Supplemental Figure VIII:
Representative Western blots that correspond to Figure 3F in the manuscript
Supplemental Figure IX: Representative Western blots that correspond to Figure 4B in the manuscript
### Supplemental Figure X: Representative Western blots that correspond to Figure 4C,D in the manuscript

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Supplemental Figure XI: Representative Western blots that correspond to Figure 4E,F in the manuscript
Supplemental Figure XII: Representative Western blots that correspond to Figure 4G,H in the manuscript
**Supplemental Figure XIII:** Representative Western blots that correspond to Figure 4I in the manuscript
MATERIALS AND METHODS

Overview
WT and Notch1 +/- murine AVICs were isolated from WT and Notch1 +/- Immortomice and expanded in culture. WT and mutant cells were assessed for calcification potential, protein expression alterations, cell signaling dysfunction, and response to mechanical strain. All experiments were conducted on BioFlex Pronectin plates unless otherwise indicated.

Notch1 +/- Mice and Genotyping
On a CD1 background, mice carrying the Notch1 tm1Con allele were crossed to “immortomouse” (Charles River, 237 HO, 238 HE) to obtain the Notch1 +/- Immorto genotype. Mouse lines were outcrossed onto CD1 and maintained as sibling matings. Genotyping was performed by polymerase chain reaction analysis using the primer pairs (f: GATATCGTGG TGCATACCTCTCTG; r: GTGGTC TAGGATGCTTGGGTCTAG) for the Notch1 WT allele, (f: GATA TCGTGTTGCATACCCCTCCTG; r: GTCAGTTTCATAGCCTGAAGAACG) for the Notch1 null allele, (f: CCTCTGAGCTATTCCAGAAGTAGTG; r: TTAGAGCTTTAATCTCTGTAGG TAG) for the Immorto allele. All breeding and experimental procedures were done with prior approval by the Vanderbilt Institutional Animal Care and Use Committee.

Atomic Force Microscopy Analysis
Murine aortic valve leaflets from wild type and Notch1 +/- mutants were processed as previously described for micromechanical measurement with AFM. Briefly, 12 month old mice were sacrificed and whole hearts were excised into cold PBS. Samples were flash frozen without fixation in OCT and cryosectioned at 10 μm. Sections were prepared for AFM analysis by rinsing out the OCT in PBS, blocking with 10% FBS for 30 min. Slides were washed in PBS 3X, rinsed in ddH2O, and immediately subjected to AFM analysis (Bruker, Santa Barbara, CA). Scanning was completed on multiple 30 μm X 30 μm areas using borosilicate glass particle tips with a nominal diameter of 3 μm and spring constant of 0.03 N/m. The tip was calibrated to a 2.5 MPa poly(dimethysiloxane) standard prior to sample analysis. For each animal, a minimum of two scans on each of three sections were analyzed and a median modulus value was calculated for each scan. Median values were aggregated to create average modulus for the samples for statistical comparisons.

Von Kossa Staining
For calcification analysis, slides with sections adjacent to those analyzed via AFM were stained using the von Kossa protocol. Samples were fixed using 10% neutral buffered formalin prior to incubation with silver nitrate. After dehydration and mounting, samples were imaged using a Nikon Eclipse E800 microscope (Nikon Inc., Melville NY) with a Spot RT3 camera (Spot Imaging Solutions, Sterling Heights, MI).

Immunohistochemistry
Unfixed frozen 10 μm sections adjacent to those analyzed via AFM and von Kossa were stained for cadherin-11. Briefly, samples were fixed for 15 min in 4% paraformaldehyde, blocked for 1 h using 10% FBS, incubated with anti-cadherin 11 antibody at 1:100 (Cell Signaling, Boston, MA) in 1% FBS for 2 h at room temperature, washed with PBS + 0.01% Tween, incubated with 1:300 αSMA conjugated to Cy3 (Sigma, St. Louis, MO) and 1:300 Alexa-Flour 647 for 1 h at room temperature. Slides were mounted using ProLong Gold Anti-Fade reagent with DAPI (Invitrogen, Grand Island, NY) and imaged using Olympus BX53.
WT and Notch1+/− AVIC Isolation and Culture

WT and Notch1+/− AVICs were isolated and expanded in vitro for the first time. WT and Notch1+/− Immortomice (4-6 weeks) were euthanized, and aortic valve leaflets were excised. The tissue was digested in a 600 U/mL collagenase solution (Worthington Biochemical Corp, Lakewood, NJ) for 1 h at room temperature. Leaflets were carefully pipetted onto 0.1% gelatin coated TCPS and allowed to adhere for 2 days in immortalized media (10% FBS, 1% penicillin/streptomycin antibiotic, 10 U/mL INF-Y) at 33°C and 5% CO2. Tissue cultures were monitored as AVICs migrated onto the tissue culture polystyrene. Each animal was able to generate one unique cell line, and cells were characterized via morphological assessment and confirmation of the presence of αSMA. Cells were expanded and cryopreserved for experimentation. Prior to experimentation, cells were transitioned at 37°C and 5% CO2 overnight. Four independent cell lines for WT and Notch1+/− mice were used in this study. This murine AVIC system represents a strong in vitro model for studying Notch1 deficiency; however, cell signaling molecules could vary from human specimen.

AVIC Treatment and Analysis

All experiments were conducted on BioFlex Pronectin culture plates unless otherwise stated. These plates were chosen because the stiffness of the BioFlex substrate is approximately on the order of magnitude of the WT and Notch1+/− valve leaflets. In order to analyze signaling changes between WT and mutant AVICs, cells were serum starved for 3 h, treated with 1 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) and 100 ng/ml BMP2 (R&D Systems, Minneapolis, MN) for 30 minutes, and assessed via Western blotting. For inhibition of signaling pathways, cells were similarly serum starved for 3 h and treated with 10 μM U0126, a MEK1/2 inhibitor specific to Erk1/2 phosphorylation, and SB 203580 at 1 μM and 10 μM to inhibit p38 and Akt, respectively, for 30 minutes. AVICs were lysed and analyzed via Western blotting. In order to assess the role of GSK-3β, GSK-3β activity was inhibited by the treatment of 20 μM LiCl (Sigma, St. Louis, MO) for 24 h in full media.

Immobilized Jagged1-Fc

The process of ligand immobilization was performed as previously described. Briefly, tissue culture plates were incubated in a solution of goat anti-human IgG antibody (20 μg/ml) (Sigma, St. Louis, MO) in PBS for 30 minutes at 37°C and then blocked with growth media for 30 minutes. Plates were then incubated with either human IgG (10 μg/ml) (Sigma, St. Louis, MO) or Jag1-Fc (10μg/ml) (R&D Systems, Minneapolis, MN) for 2 h at 37°C. After incubation, plates were washed two times in growth media and then seeded with cells.

Mechanical Strain Analysis and Calcific Nodule Assay

WT and Notch1+/− AVICs were plated on BioFlex Pronectin culture plates (Flexcell International Corporation, Hillsborough, NC) at 5x10⁴ cells/cm² in normal growth media and were given a day to reach confluence. Growth media was replaced with fresh growth media and the cells were then subjected to equibiaxial strain via the Flexcell-4000 Tension System at a strain magnitude of 10% and a frequency of 1 Hz for designated times. For CN assays, WT and mutant cells were seeded on BioFlex plates at 7x10⁴ cells/cm² in normal growth media. Normal growth media was then removed and replaced with growth media supplemented with 1 ng/ml TGF-β1 and then subjected to equibiaxial strain at a strain magnitude of 10% and a frequency of 1 Hz for 24 h. CNs were stained with Alizarin red and counted via standard light microscopy.

Assays for Cell Viability

AVICs were rinsed with PBS and stained with Annexin V conjugated with Alexa fluor 488 (5% solution in Annexin binding buffer; Invitrogen, Grand Island, NY) for 15 minutes to detect apoptotic cells. Propidium Iodide (0.4% solution in Annexin binding buffer; Invitrogen, Grand Island, NY) for 15 minutes to detect apoptotic cells.
Island, NY) was used as a counter-stain for necrotic cells. Apoptosis and necrosis images were taken after 24 h of equibiaxial strain using a fluorescence microscope (Nikon TE300 Inverted Tissue Culture Microscope).

**Immunofluorescence**
AVICs were plated on fibronectin functionalized coverslips and treated with 1 ng/mL TGF-β1 for 24 h. The cells were then fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin for 1 h at room temperature. A primary antibody to cadherin-11 (Cell Signaling, Boston, MA) was added to the coverslips for 3 h at room temperature. After thorough washing in PBS, a fluorescently labeled secondary antibody (Alexa Fluor 488, Invitrogen, Grand Island, NY) was added to the coverslip with a primary antibody to αSMA conjugated with Cy3 (Sigma, St. Louis, MO) for 1 h. The coverslips were then washed and sealed with ProLong Gold antifade reagent (Invitrogen, Grand Island, NY) overnight prior to imaging with a Nikon Eclipse E800 equipped with a Spot RT3 camera.

**Western blotting**
AVICs were washed twice in PBS and lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland) and centrifuged to remove membrane components. Protein quantification was conducted via BCA Protein Assay (Pierce, Rockford, IL), and samples were normalized. Proteins were separated via SDS-Page and transferred onto a nitrocellulose membrane (Li-Cor, Lincoln, NE). Membranes were blocked in milk for 1 h and incubated with primary antibody overnight at 4°C. Membranes were then washed 3X in PBS and incubated for 40 min with secondary antibody followed by 3 washes and imaged via Odyssey CLx (Li-Cor, Lincoln, NE).

**Statistical Analysis**
The data are reported as the mean of all replicates, and error is given as standard error of the mean. Statistical significance between treatments was determined by one-way ANOVA and Holm-Sidak tests.

**REFERENCES FOR DETAILED MATERIALS AND METHODS**