Original article

Notch1 represses osteogenic pathways in aortic valve cells

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A B S T R A C T

Calcific aortic stenosis is the third leading cause of adult heart disease and the most common form of acquired valvular disease in developed countries. However, the molecular pathways leading to calcification are poorly understood. We reported two families in which heterozygous mutations in NOTCH1 caused bicuspid aortic valve and severe aortic valve calcification. NOTCH1 is part of a highly conserved signaling pathway involved in cell fate decisions, cell differentiation, and cardiac valve formation. In this study, we examined the mechanism by which NOTCH1 represses aortic valve calcification. Heterozygous Notch1-null (Notch1+/-) mice had greater than fivefold more aortic valve calcification than age- and sex-matched wildtype littermates. Inhibition of Notch signaling in cultured sheep aortic valve interstitial cells (AVICs) also increased calcification more than fivefold and resulted in gene expression typical of osteoblasts. We found that Notch1 normally represses the gene encoding bone morphogenic protein 2 (Bmp2) in murine aortic valves in vivo and in aortic valve cells in vitro. siRNA-mediated knockdown of Bmp2 blocked the calcification induced by Notch inhibition in AVICs. These findings suggest that Notch1 signaling in aortic valve cells represses osteoblast-like calcification pathways mediated by Bmp2.

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1. Introduction

Aortic valve calcification/stenosis is the third leading cause of adult heart disease [1] and the most common form of acquired valvular disease in developed countries [2]. The risk factor most closely linked to calcific aortic stenosis is bicuspid aortic valve, present in 1–2% of the population [2]. Increased age is also associated, as 2–4% of individuals over age 65 years have calcific aortic valve stenosis. Other risk factors are male gender, smoking, and hypertension [2]. Calcific aortic stenosis due to hyperlipidemia has been studied in mice [3]. However, there is no genetic mouse model of aortic valve calcification, and the molecular mechanisms of valvular calcification are largely unknown. Examination of human calcified aortic valve tissue reveals that several pathologic pathways are involved, including myofibroblast differentiation into osteoblast-like cells, [4] apoptosis of aortic valve mesenchyme, [5] and infiltration by inflammatory cells [6-8].

We previously reported two families in which heterozygous frame-shift mutations in NOTCH1 were associated with early, severe calcification of the aortic valve with 100% penetrance [9]. Most family members also had bicuspid aortic valves. Other heterozygous NOTCH1 mutations were subsequently associated with aortic valve calcification and aortic aneurysms [10,11].

Notch1 is a member of the Notch family of cell surface receptors—large proteins with multiple epidermal growth factor-like repeats, an intracellular domain consisting of ankyrin repeats, and a transmembrane domain. The Notch receptors, Notch1-4, are activated by the Delta or Jagged families of ligands. Upon activation, the Notch intracellular domain (NICD) is cleaved by a metalloprotease and a presenilin, resulting in translocation to the nucleus, where it binds to Suppressor of Hairless, a DNA binding protein also known as Hairy/Enhancer of Split. Notch activation results in gene expression typical of osteoblasts. We found that Bmp2 binds to Suppressor of Hairless, a DNA binding protein also known as Hairy/Enhancer of Split (Hes) and Hairy-related transcription factor (Hrt/Hey) families [13]. Hrt1, Hrt2, and Hrt3 are enriched in the cardiovascular system [14] and are direct downstream targets of Notch signaling, which is important for cell fate determination and organogenesis.

Notch1 has been implicated in multiple developmental processes, especially cardiovascular [15], bone [16,17], and blood cell [18] development. It is critical for the endothelial-to-mesenchymal transformation that contributes to heart valves [19] and is highly expressed in the valve mesenchyme and endocardium [9]. However, efforts to understand the role of Notch1 in adult tissues have been hampered because Notch1 homozygous-null mice die from vascular defects at embryonic day 9.5 [20].

In this study, we sought to determine whether Notch1+/- mice were predisposed to increased osteoblast gene expression in the aortic valve as they aged. Additionally, we investigated which cell types within the aortic valve leaflet require Notch signaling to repress osteoblast gene expression. Finally, we explored the mechanism by
which Notch1 represses calcification, focusing on Bmp2, a potent pro-osteogenic factor associated with aortic valve calcification [21,22].

2. Material and methods

2.1. Calcification in wildtype and Notch1+/− mice

After weaning, male Notch1+/− mice (a gift from Dr. Tom Gridley) [20] and wildtype controls (both on a C57BL/6 background) were fed a Western diet (TD.01064; Harlan Teklad, Madison, WI). At 10 months of age, the mice were euthanized by CO2 inhalation and cervical dislocation, and the hearts were harvested and fixed in paraformaldehyde. The aortic valves were embedded in paraffin, sectioned transversely, and stained with von Kossa stain and alkaline phosphatase to identify calcification. Valves from four wildtype and six Notch1+/− mice were examined. Calcified areas were measured with ImageJ software (NIH). Calcification in the leaflets was expressed as a percentage based upon the ratio of the calcified area/total area of the valve.

2.2. Sheep AVICs

AVICs were harvested from adult sheep as described [5]. Cells from passages three to eight were treated with N-[N-(3,5-difuoro-phenacyl-l-alanyl)-S-phenylglycine t-butyl ester (DAPT) (Calbiochem, San Diego, CA) for 3 weeks; control cells were treated with vehicle only (dimethyl sulfoxide [DMSO]). The medium with DAPT or DMSO added was changed every 4 days. The cells were washed three times with cold phosphate-buffered saline and stained with 2% Alizarin Red in water for 1 h. Staining was quantified from seven samples for each condition with ImagePro software. von Kossa staining was performed according to standard protocols. Alkaline phosphatase activity staining was performed with a Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich, St. Louis, MO).

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Aortic valve leaflets were dissected from three adult Notch1+/− mice and three age- and sex-matched littermate controls in RNAlater (Ambion, Austin, TX). RNA was isolated with Trizol (Invitrogen, Carlsbad, CA). cDNA was generated with the Superscript III kit (Invitrogen) and random hexamer primers. Quantitative RT-PCR was performed with Taqman primers for Bmp2 and Gapdh (Applied Biosystems, Foster City, CA). ΔΔCT values were calculated; Gapdh served as the control.

For examination of Runx2 and Bmp2 levels in sheep AVICs, RNA was isolated and cDNA generated as described above from AVICs treated with DMSO or DAPT for 2 weeks. Quantitative RT-PCR was performed with the mouse Taqman primer for Runx2 and custom Taqman sheep-specific Bmp2, and Gapdh primers (control) were designed using sequence from the GoSH database. At least three different samples were studied for each condition. ΔΔCT values were calculated.

For examination of Bmp2 levels in cultured aortic valve endocardial and RAW264.7 cells, RNA was isolated and cDNA generated as described above. The cells were harvested after 2 days of treatment with DAPT or DMSO. qRT-PCR was performed with Taqman primers as described above.

2.4. Western blotting

Protein extracts from sheep AVICs treated with DAPT or DMSO were collected in Laemmli buffer. Samples were run on gradient gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes. The blots were probed with Bmp2-specific antibody (sc-6895; Santa Cruz Biotechnology, Santa Cruz, CA) and then stripped and re-probed with Gapdh-specific antibody for normalization. The blots were also probed with osteopontin-specific antibody, MPIIIB10(1), or alkaline phosphatase antibody, B4-78 (Developmental Studies Hybridoma Bank, Iowa City, IA).

2.5. siRNA

Notch1 siRNA (H-007771-00; Dharmacon, Lafayette, CO) was used to target Notch1 in vitro. Fully confluent sheep AVICs were transfected with 100 pM siRNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. As a transfection control and to determine transfection efficiency, cells were transfected with control siRNA (Block-It, Invitrogen) labeled with Alexa Red. The cells were transfected with siRNA every 4 days. siRNA (GAGAAAGCGCGCA-AGCAGAUU; Dharmacon) against sheep Bmp2 was designed with sequence from the GoSH database. To demonstrate the specificity of the Bmp2 siRNA, Western blot analysis was performed with Bmp2- and Bmp4-specific antibodies (sc-6896; Santa Cruz Biotechnology).

The cells were transfected with siRNA and treated with 100 μM DAPT every 4 days. After 3 weeks of treatment, the cells were stained with Alizarin Red as described above. Five samples for each condition were examined.

Fig. 1. Increased aortic valve calcification in 10-month-old male Notch1+/− mice fed a Western diet. (A and B) von Kossa staining shows increased calcification of the aortic valve in Notch1+/− mice. (C and D) Higher magnification views of the boxed areas in (A) and (B). The arrowhead indicates representative area of staining. (E) Quantification of calcification in Notch1+/− (n = 6) or wildtype (WT) (n = 4) valves (2.53% ± 0.64% vs. 0.47% ± 0.16%; *P=0.03).
2.6. Culturing of aortic valve endocardial cells

The aortic valve leaflets from adult mice were dissected and placed in wells coated with 0.1% gelatin in a small amount of medium (20% fetal bovine serum, 40% Ham's medium, 40% Dulbecco's modified Eagle medium, 100 mg/ml endothelial growth supplement factor, and 100 units/ml heparin). Over several days, endocardial cells migrated out of the explant. No beating cells were seen. The endocardial identity of the
2.7. Culturing of RAW264.7 cells

The macrophage cell line RAW264.7 was purchased from ATCC (Manassas, VA) and were cultured according to standard protocols.

2.8. Statistical analysis

The statistical significance of differences between groups was determined with the unpaired t test. P ≤ 0.05 was considered significant.

3. Results

3.1 Notch1+/− mice have increased aortic valve calcification

To determine if Notch1+/− mice were predisposed to any increase in aortic valve calcification, we fed male Notch1+/− and wildtype littermates a Western diet for 10 months. Ten months was chosen because no significant calcification is present in wildtype mice at this age yet we considered it may be long enough for evaluation of age-dependent calcification in Notch1+/− mice. Mice were sacrificed, and aortic valve leaflets assessed by transverse sections. All of the mice had tri-leaflet valves. However, the calcification valve area was over fivefold greater in Notch1+/− mice than in wildtype littermates (P < 0.03) as shown in Fig. 3.
measured by von Kossa staining (Fig. 1). Since valve pigmentation can mimic von Kossa staining, adjacent sections were also assayed for alkaline phosphatase activity, which demonstrated the specificity of the von Kossa staining for calcification (Supplemental Fig. 1). Thus, decreased Notch1 signaling increased aortic valve calcification in mice on a Western diet. We did not find evidence of aortic valve stenosis in mice as observed in humans, suggesting a less penetrant phenotype in mice.

### 3.2. Inhibition of Notch signaling in sheep AVICs causes calcification and activates the osteoblast-like phenotype

Because cultured AVICs have been used to model cell-autonomous calcification of aortic valves [5,24], we tested whether disruption of Notch activity in AVICs caused calcification. Primary cultures of sheep AVICs were incubated with the γ-secretase inhibitor DAPT, which prevents cleavage of the NICD and blocks Notch signaling [19]. After 3 weeks, the DAPT-treated AVICs had over fivefold more calcification than control cells (P<0.03; n=7) (Figs. 2A and B). Similar results were obtained with porcine AVICs (data not shown), von Kossa staining of DAPT-treated AVICs demonstrated that increased calcification resulted from Notch inhibition (Fig. 2C). Since human aortic valve calcification is associated with the activation of osteoblast-like gene expression [4], we examined the DAPT treated sheep AVICs for changes in Runx2, osteopontin (Opn), and alkaline phosphatase levels. qRT-PCR analysis demonstrated that sheep AVICs treated with as little as 12.5 μM DAPT had threefold higher expression of Runx2, a master transcriptional regulator of osteoblast fate [25], than DMSO-treated control cells (P<0.002; n=3) (Fig. 2D). Opn and alkaline phosphatase levels increased in a dose-dependent manner with increasing concentrations of DAPT (Figs. 2E and F). Cells treated with DAPT also had increased alkaline phosphatase activity (Fig. 2G). Because DAPT can affect other pathways, we tested the specificity of its effects on Notch signaling by knocking down Notch1 expression with a previously validated Notch1 siRNA [26]. Although Notch1 antibodies are not sensitive enough to detect endogenous Notch1 protein in AVICs, AVICs treated with Notch1 siRNA for 2 weeks had increased Opn and alkaline phosphatase protein levels (Figs. 2H, I) as well as Runx2 expression (Fig. 2J), similar to the effects observed with DAPT. These results suggest that Notch1 signaling within AVICs normally represses osteoblast-like gene expression and calcification.

#### 3.3. Decreased Notch signaling increases Bmp2 expression in vivo and in vitro

Bmp2 is a potent pro-osteogenic factor that is associated with aortic valve calcification in clinical specimens [21,22]. Recently, Notch2 was shown to repress Bmp2 expression in embryonic chicken hearts [27]. To test the hypothesis that Notch1 prevents aortic valve calcification by repressing pro-osteogenic factors, we examined Bmp2 expression in aortic valve leaflets from Notch1-/- mice and sex-matched wildtype littersmates. As shown by qRT-PCR, Bmp2 expression was more than threefold greater in the leaflets from Notch1-/- mice (n=3, P<0.02) (Fig. 3A). We also investigated whether Notch signaling repressed Bmp2 in vitro by treating AVICs with Notch1 siRNA or DAPT and measuring Bmp2 levels. Knockdown of Notch1 in AVICs resulted in increased Bmp2 mRNA and protein levels (Figs. 3B and C). Furthermore, chemical inhibition of Notch in sheep AVICs increased Bmp2 protein levels (Fig. 3D). To determine whether Notch inhibited Bmp2 in other cell types in the aortic valve leaflet, we treated cultured primary aortic valve endocardial cells and RAW264.7 cells, a macrophage cell line, with DAPT. qRT-PCR demonstrated increased Bmp2 levels in both cell types (Figs. 3E, F). Thus, decreased Notch signaling increases Bmp2 levels in aortic valve leaflets in vivo and in aortic valve cells in vitro.

#### 3.4. Bmp2 is necessary for Notch-related calcification

To determine whether the increase in Bmp2 in AVICs was required for calcification in the setting of decreased Notch signaling, we inhibited Notch signaling in sheep AVICs with DAPT in the presence of Bmp2 siRNA or control siRNA for 3 weeks. Bmp2 siRNA specifically and efficiently knocked down expression of Bmp2 but did not affect Bmp4 levels (Fig. 4A). As before, DAPT (with control siRNA) increased calcification in AVICs more than fivefold vs. DMSO controls. Strikingly, knockdown of Bmp2 lowered calcification to control levels, suggesting that the DAPT-induced increase in Bmp2 was necessary for calcification in AVICs (Fig. 4B) (P<0.03). This finding indicates that Notch repression of Bmp2 is an important mechanism by which Notch1 normally represses calcification in aortic valve cells.

### 4. Discussion

This study shows that decreased Notch1 signaling predisposes to increased aortic valve calcification in mice and that inhibition of Notch1 in cultured aortic valve cells also induces calcification. In vivo and in vitro, Notch1 signaling repressed valvular Bmp2 expression, and de-repression of Bmp2 was involved in calcification induced by Notch1 inhibition. Thus, Notch1 signaling appears to prevent aortic valve calcification in part by repressing Bmp2 expression within the valve (Fig. 5).

Genetic studies have provided compelling evidence that NOTCH1 mutations contribute to valve disease in humans [9], and rare variants may also contribute to disease in some settings [10,11,28]. Our in vivo and in vitro results suggest that Notch signaling may repress a default
functions of Notch1 in leaflet morphology during development and in repressing calcification postnatally. This observation is consistent with the presence of aortic valve calcification in several human subjects heterozygous for NOTCH1 who had tricuspid aortic valve leaflets.

Our findings suggest that the interplay between Notch1 and Bmp2 has a key role in calcification within aortic valve cells. We showed that Notch1 can repress Bmp2, although it is unknown whether this effect is mediated directly through the Bmp2 enhancer or indirectly. Another pro-calcification pathway involves Runx2, a central regulator of osteoblast development [25]. Runx2 expression is increased by treatment with Bmp2 [29]. Notch1 attenuates the ability of Runx2 to activate the Osteocalcin enhancer as a result of a physical interaction between Runx2 and the Hrt repressors [9]. Thus, Notch1 may prevent aortic valve calcification by repressing not only Bmp2 expression but also the activity of osteogenic genes downstream of Bmp2, such as Runx2.

Our observations raise the interesting possibility that valve calcification in humans with NOTCH1 mutations is a result of a cell fate switch of valve mesenchymal cells into an osteoblast-like lineage. This possibility is supported by evidence that Notch1 represses osteoblast differentiation [16,30] and that Bmp2 and Runx2 promote osteoblast commitment. Although Bmp2 was necessary for Notch1-related calcification, additional pathways may be involved. For example, several Wnt family members [31] and β-catenin [32] are involved in cardiovascular calcification, and Notch1 represses β-catenin/Wnt activity [16,33,34]. Since Notch1 [35–39], Bmp2 [38], and Wnts [40] have key roles in cell proliferation and lineage choices, the interplay between these signals is likely involved in regulation of a wide range of cell fate decisions.

Our findings also have several intriguing clinical implications. In conjunction with chemical inhibition of Notch in AVICs, Notch1+/fl mice could provide insights into the early molecular pathogenesis of aortic valve calcification. Future studies of calcification in AVICs derived from induced pluripotent stem cells [41,42] made from patients with NOTCH1 mutations may also reveal early mechanisms and allow formal testing of the cell fate switch hypothesis. As further clinical genotypic/phenotypic data are obtained, it may be possible to link specific mutations with distinct clinical prognoses. Finally, the finding that inhibiting Notch signaling with a γ-secretase inhibitor in vitro increases aortic valve calcification raises the question of whether patients treated with γ-secretase inhibitors for Alzheimer’s disease [43] should be monitored for aortic valve calcification.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2009.08.008.
References


