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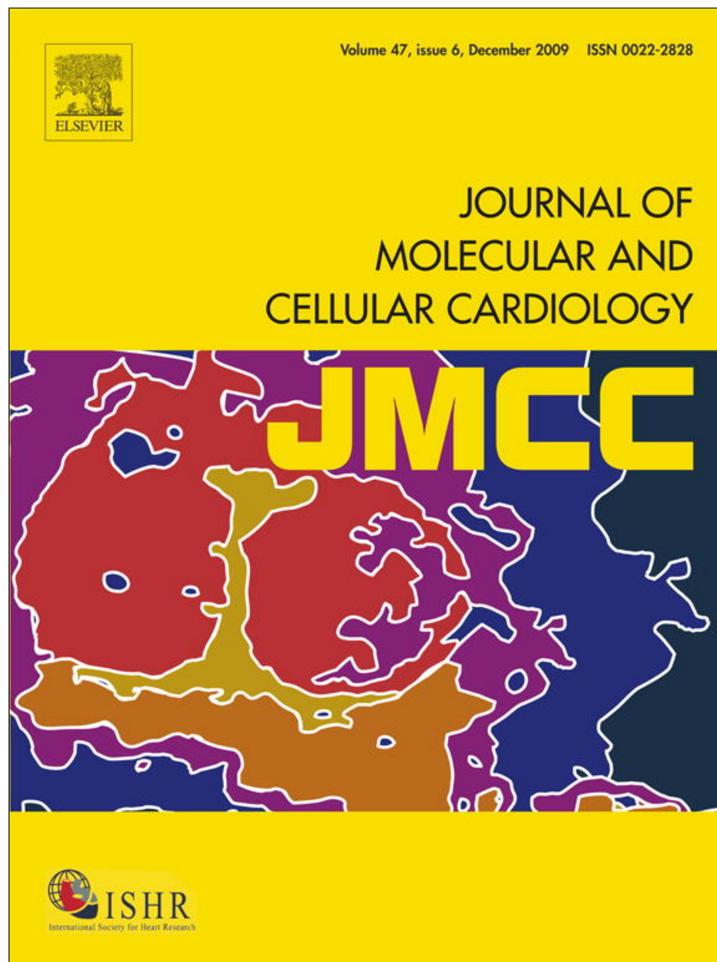

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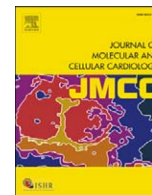

Original article

Notch1 represses osteogenic pathways in aortic valve cells

Vishal Nigam [a,1], Deepak Srivastava [a,b,*]

[a] Gladstone Institute of Cardiovascular Disease and Departments of Pediatrics, University of California, San Francisco, CA 94158, USA
[b] Biochemistry and Biophysics, University of California, San Francisco, CA 94158, USA

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ABSTRACT

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 transactivation domain. The Notch signaling pathway has been highly conserved throughout evolution [12,13]. 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 recombination signaling protein-J (Rbpj-κ). This complex activates the expression of transcriptional repressors, including the 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

* Corresponding author. Gladstone Institute of Cardiovascular Disease, 1650 Owens Street, San Francisco, CA 94158, USA. Tel.: +1 415 734 2716; fax: +1 415 355 0141.
E-mail addresses: vnigam@ucsd.edu (V. Nigam), dsrivastava@gladstone.ucsf.edu (D. Srivastava).
[1] University of California, San Diego, 9500 Gilman Drive, MC 0984, La Solla, CA 92093-0984, USA.

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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 CO_2 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-difluorophenacetyl-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 and placed 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 (www.itb.cnr.it/gosh/) [23] and International Sheep Genomics Consortium (www.sheephapmap.org) databases. 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 (l-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 (GAGAAAAGCGGCA-AGCAAAUU; 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.

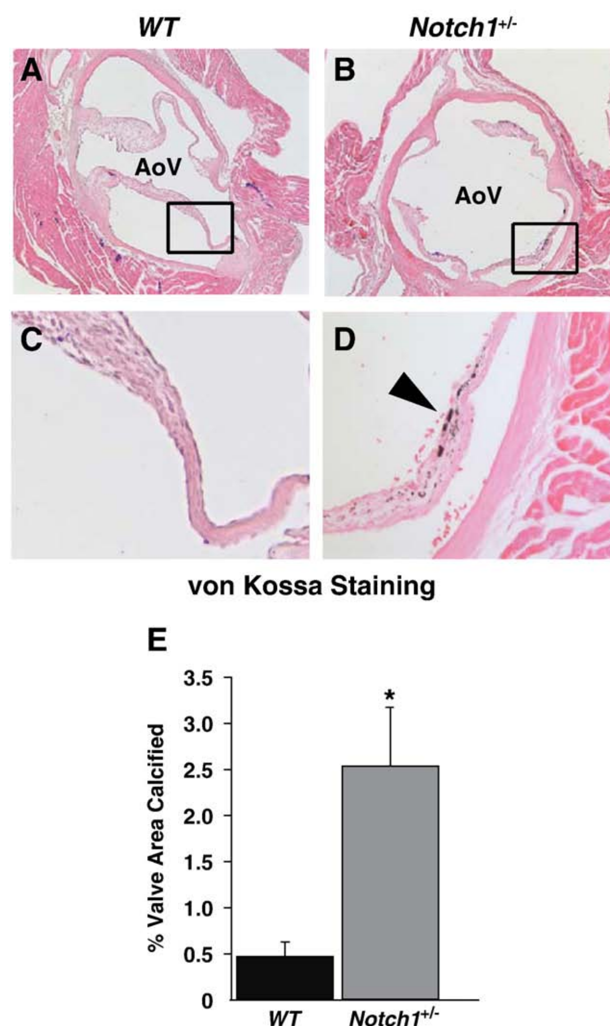

Fig. 1. Increased aortic valve calcification in 10-month-old male *Notch1*$^{+/-}$ mice fed a Western diet. (A and B) von Kossa staining shows increased calcification of the aortic valve in *Notch1*$^{+/-}$ mice. (C and D) Higher magnification views of the boxed areas in (A) and (B). The arrowhead indicates representative area of staining. (E) Quantification of calcification in Notch1$^{+/-}$ ($n = 6$) or wildtype (WT) ($n = 4$) valves (2.5% ± 0.64% vs. 0.47% ± 0.16%; *$P = 0.03$).

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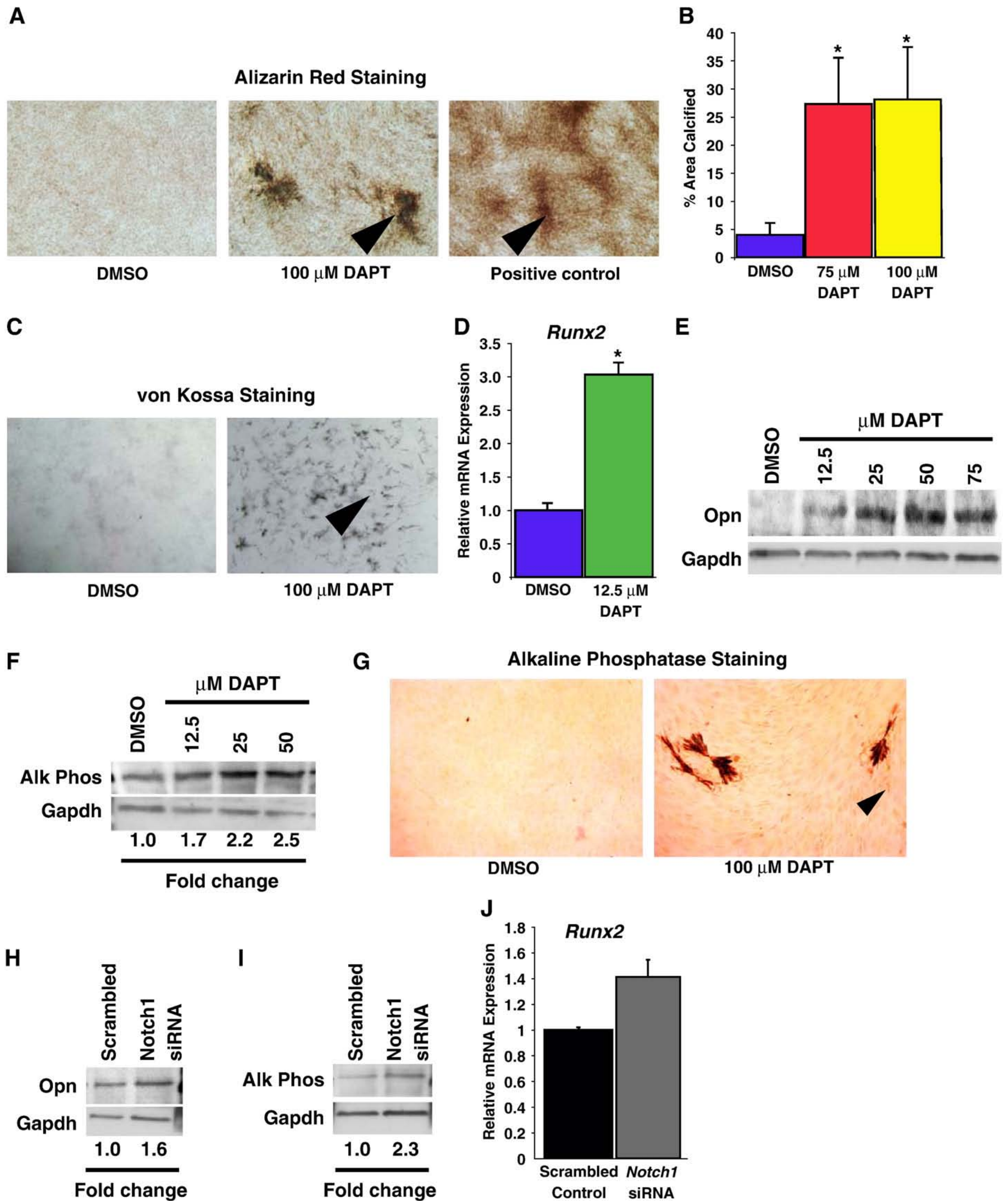
2.6. Culturing of aortic valve endocardial cells

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



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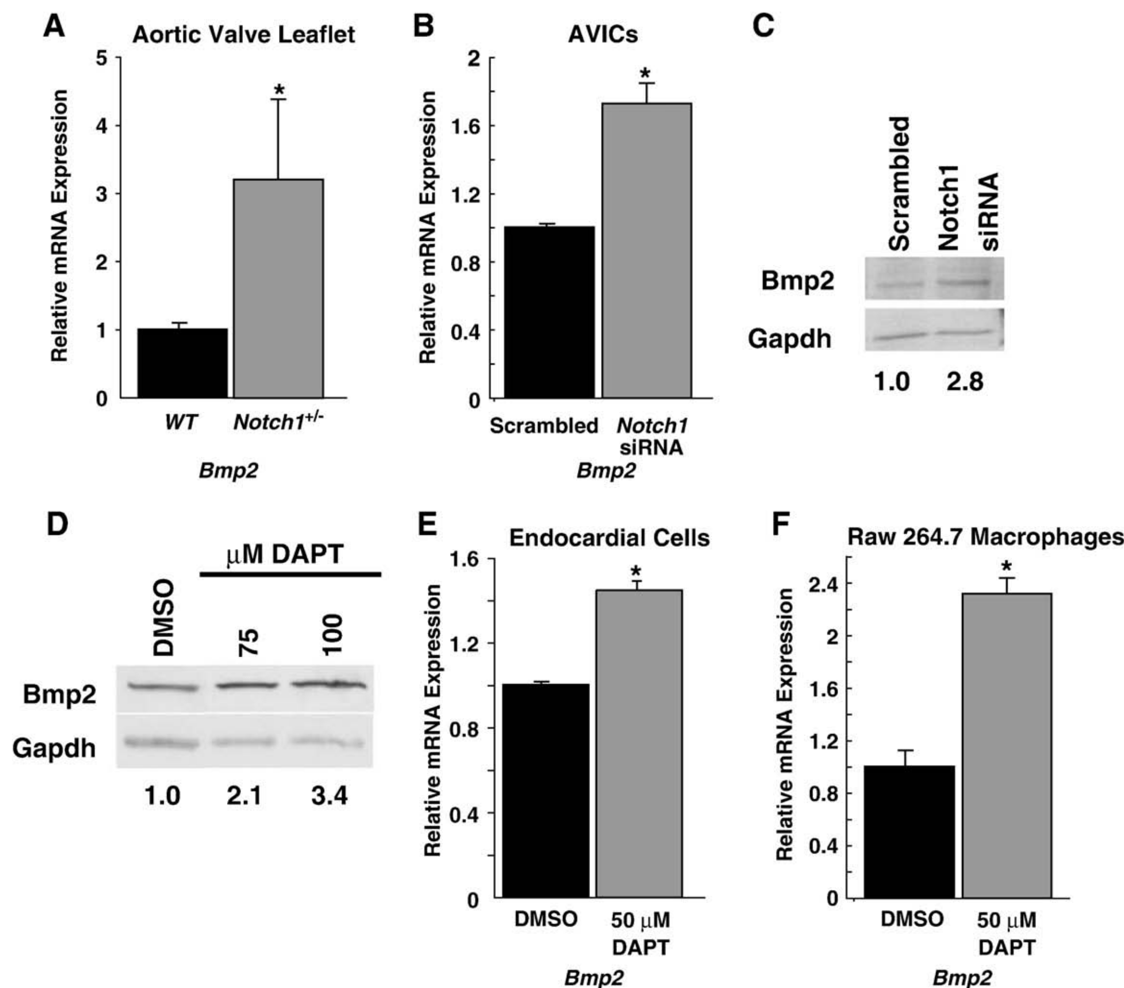

Fig. 3. Decreased Notch1 signaling increases *Bmp2* expression *in vivo* and *in vitro*. (A) *Bmp2* expression in aortic valve leaflets was more than threefold higher in *Notch1*$^{+/-}$ mice than controls (*P<0.02; n = 3). (B) AVICs treated with *Notch1* siRNA had 73% more *Bmp2* mRNA than cells treated with scrambled siRNA (*P<0.01; n = 3). (C) Bmp2 protein levels were higher in sheep AVICs treated with *Notch1* siRNA than in control cells treated with scrambled siRNA. GAPDH was used as a loading control for Western blots. (D) Bmp2 protein levels were higher in DAPT-treated than control DMSO-treated sheep AVICs. (E) Cultured aortic valve endocardial cells treated with 50 μM DAPT had 45% more *Bmp2* mRNA than DMSO-treated cells (*P<0.03; n = 6). (F) RAW264.7 cells treated with 50 μM DAPT had 130% more *Bmp2* mRNA than DMSO-treated cells (*P<0.02; n = 3).

cells was confirmed by immunohistochemistry with antibodies to platelet/endothelial cell adhesion molecule-1 and Notch1.

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. $P \leq 0.05$ 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 calcific valve area was over fivefold greater in *Notch1*$^{+/-}$ mice than in wildtype littermates (P<0.03) as

Fig. 2. Inhibition of Notch signaling in cultured sheep AVICs with DAPT increases calcification and activates osteoblast-specific genes. (A) Calcification of AVICs treated with DMSO (negative control) or DAPT demonstrated by Alizarin Red staining (red–brown). AVICs treated with β-glycerol phosphate and asorbic acid served as positive controls. Arrowheads indicate representative areas of calcification. (B) Quantification of Alizarin Red staining from seven independent samples for each condition. The percent area of calcification was significantly greater in DAPT-treated cells (75 μM, 27.3 ± 8%; 100 μM, 28.1 ± 9.3%) than in DMSO-treated controls (4.0 ± 2%; *P<0.03 vs. control). (C) von Kossa staining of DAPT-treated AVICs showed more calcification than in DMSO-treated cells. (D) *Runx2* mRNA levels assayed by qPCR were threefold higher in sheep AVICs treated with DAPT than in DMSO-treated controls (*P<0.002 vs. control; n = 3). (E) Osteopontin (Opn) protein levels were higher by Western blot in sheep AVICs treated with DAPT. GAPDH was used as a loading control. (F) Alkaline phosphatase protein levels were higher in DAPT-treated cells than in DMSO-treated controls by Western blot. G, DAPT-treated AVICs had increased alkaline phosphatase activity (red areas, arrowhead). Arrowheads indicate representative areas of alkaline phosphatase activity. (H) AVICs treated with *Notch1* siRNA for 2 weeks had increased osteopontin protein levels by Western blot. (I) Western blot analysis shows increased alkaline phosphatase levels in *Notch1*-treated AVICs. (J) *Runx2* mRNA levels increased > 40% in AVICs treated with *Notch1* siRNA (P<0.06; n = 3).

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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 littermates. 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*.

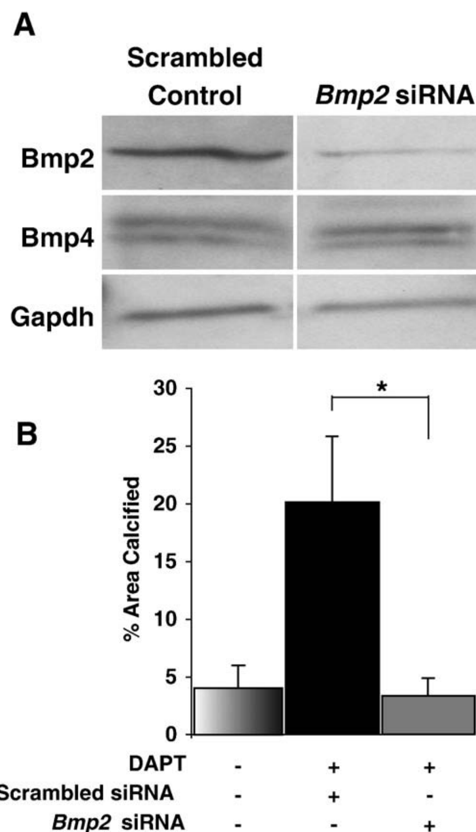

Fig. 4. *Bmp2* expression is necessary for calcification induced by Notch inhibition. (A) Western blot analysis showed that *Bmp2* siRNA specifically knocked down Bmp2 but not Bmp4. (B) Sheep AVICs treated with *Bmp2* siRNA and DAPT for 3 weeks had >80% less calcification than cells treated with control siRNA and DAPT ($3.4\pm1.5\%$ vs. $20.1\pm5.7\%$, $^{*}P=0.02$ vs. control siRNA; $n=5$).

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

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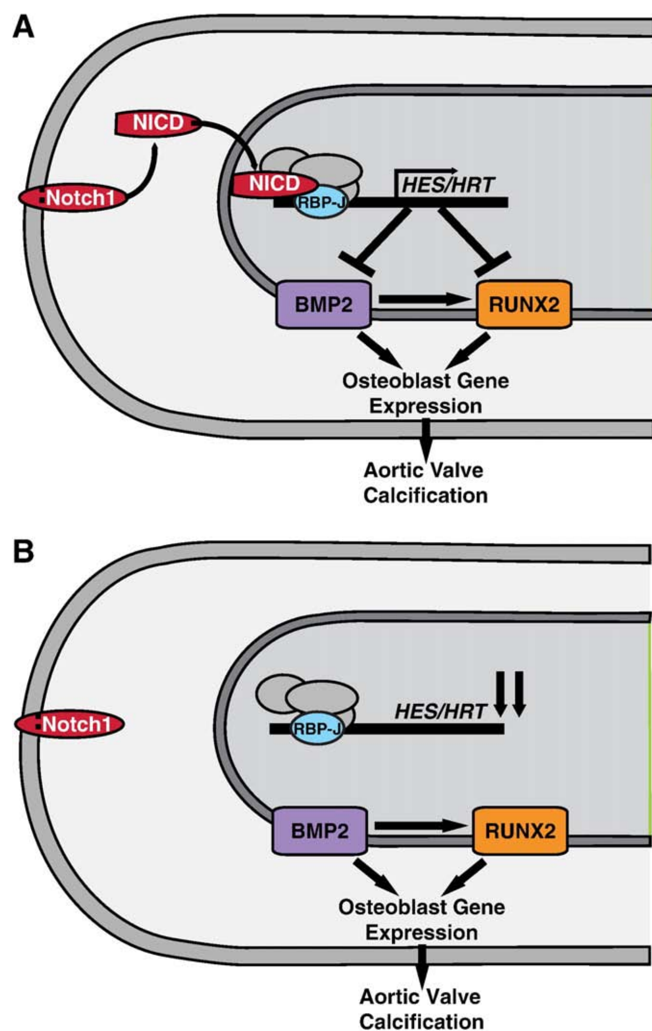

Fig. 5. A potential model for repression of aortic valve calcification by *NOTCH1*. (A) Notch is activated by its ligands, Delta or Jagged. The Notch intracellular domain (NICD) is cleaved from the cell surface and translocates to the nucleus, where it binds to its co-activator, recombination signaling protein-J (RBP-J). This complex binds to the promoter of transcriptional repressors such as *HES* or *HRT*, whose protein products may in turn reduce *BMP2* expression, thereby repressing BMP2-induced aortic valve calcification. *NOTCH1* can also repress the transcriptional activity of *RUNX2*, a central transcriptional regulator of osteoblast-specific genes [9]. (B) With decreased NOTCH1 signaling, *BMP2* and *RUNX2* expression is increased resulting in increased aortic valve calcification. Adapted from Yoon et al. [13].

pathway of osteoblast gene expression and calcification. In AVICs, aortic valve endocardial cells, and a macrophage cell line, decreased Notch signaling resulted in increased *Bmp2* levels. Given the current knowledge and our findings in AVICs, these myofibroblast cells may be the cell type that contributes most to the calcification event, although endocardial cells may contribute as well. Further study is required to determine the relative contribution of *Notch1* within specific aortic valve cell types in repressing aortic valve calcification. The murine and cell culture model systems described here may be useful for studying the pathogenesis of aortic valve calcification and for developing novel therapeutics.

Although the murine model of *Notch1* deficiency displayed an increase in valve calcification, it was much milder than the human disease and none of the *Notch1*$^{+/-}$ mice had bicuspid aortic valves. These findings indicate that the human aortic valve is more dose-sensitive to alterations in *NOTCH1* function and that genetic background may also affect phenotypic severity. The presence of calcification in tricuspid aortic valve leaflets suggests independent 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*$^{+/-}$ 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.

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References

[1] Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, et al. Heart disease and stroke statistics-2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2006;113(6):e85–151.

[2] Freeman RV, Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. Circulation 2005;111(24):3316–26.

[3] Weiss RM, Ohashi M, Miller JD, Young SG, Heistad DD. Calcific aortic valve stenosis in old hypercholesterolemic mice. Circulation 2006;114(19):2065–9.

[4] Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, Springett M, et al. Human aortic valve calcification is associated with an osteoblast phenotype. Circulation 2003;107(17):2181–4.

[5] Jian B, Narula N, Li QY, Mohler 3rd ER, Levy RJ. Progression of aortic valve stenosis: TGF-beta1 is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis. Ann Thorac Surg 2003;75(2):457–65 discussion 65-6.

[6] Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation 1994;90(2):844–53.

[7] Olsson M, Dalsgaard CJ, Haegerstrand A, Rosenqvist M, Ryden L, Nilsson J. Accumulation of T lymphocytes and expression of interleukin-2 receptors in nonrheumatic stenotic aortic valves. J Am Coll Cardiol 1994;23(5):1162–70.

[8] Wallby L, Janerot-Sjoberg B, Steffensen T, Broqvist M. T lymphocyte infiltration in non-rheumatic aortic stenosis: a comparative descriptive study between tricuspid and bicuspid aortic valves. Heart 2002;88(4):348–51.

[9] Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, et al. Mutations in NOTCH1 cause aortic valve disease. Nature 2005;437(7056):270–4.

[10] Mohamed SA, Aherrahrou Z, Liptau H, Erasmi AW, Hagemann C, Wrobel S, et al. Novel missense mutations (p.T596M and p.P1797H) in NOTCH1 in patients with bicuspid aortic valve. Biochem Biophys Res Commun 2006;345(4):1460–5.

[11] McKellar SH, Tester DJ, Yagubyan M, Majumdar R, Ackerman MJ, Sundt 3rd TM. Novel NOTCH1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. J Thorac Cardiovasc Surg 2007;134(2):290–6.

[12] Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284(5415):770–6.

[13] Yoon K, Gaiano N. Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci 2005;8(6):709–15.

[14] Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D. HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. Dev Biol 1999;216(1):72–84.

[15] Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, et al. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 2000;14(11): 1343–52.

[16] Sciaudone M, Gazzerro E, Priest L, Delany AM, Canalis E. Notch 1 impairs osteoblastic cell differentiation. Endocrinology 2003;144(12):5631–9.

[17] Shindo K, Kawashima N, Sakamoto K, Yamaguchi A, Umezawa A, Takagi M, et al. Osteogenic differentiation of the mesenchymal progenitor cells, Kusa is suppressed by Notch signaling. Exp Cell Res 2003;290(2):370–80.

[18] Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. Nat Immunol 2004;5(3):247–53.

[19] Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, et al. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev 2004;18(1):99–115.

[20] Swiatek PJ, Lindsell CE, del Amo FF, Weinmaster G, Gridley T. Notch1 is essential for postimplantation development in mice. Genes Dev 1994;8(6):707–19.

[21] Mohler 3rd ER, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS. Bone formation and inflammation in cardiac valves. Circulation 2001;103(11):1522–8.

[22] Kaden JJ, Bickelhaupt S, Grobholz R, Vahl CF, Hagl S, Brueckmann M, et al. Expression of bone sialoprotein and bone morphogenetic protein-2 in calcific aortic stenosis. J Heart Valve Dis 2004;13(4):560–6.

[23] Caprera A, Lazzari B, Stella A, Merelli I, Caetano AR, Mariani P. GoSh: a web-based database for goat and sheep EST sequences. Bioinformatics 2007;23(8): 1043–5.

[24] Osman L, Yacoub MH, Latif N, Amrani M, Chester AH. Role of human valve interstitial cells in valve calcification and their response to atorvastatin. Circulation 2006;114(1 Suppl):I547–52.

[25] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997;89(5):747–54.

[26] Akiyoshi T, Nakamura M, Yanai K, Nagai S, Wada J, Koga K, et al. Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. Gastroenterology 2008;134(1):131–44.

[27] Rutenberg JB, Fischer A, Jia H, Gessler M, Zhong TP, Mercola M. Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors. Development 2006;133(21):4381–90.

[28] McBride KL, Riley MF, Zender GA, Fitzgerald-Butt SM, Towbin JA, Belmont JW, et al. NOTCH1 mutations in individuals with left ventricular outflow tract malformations reduce ligand-induced signaling. Hum Mol Genet 2008 Sept 15;17(18): 2886–93.

[29] de Jong DS, Vaes BL, Dechering KJ, Feijen A, Hendriks JM, Wehrens R, et al. Identification of novel regulators associated with early-phase osteoblast differentiation. J Bone Miner Res 2004;19(6):947–58.

[30] Zanotti S, Smerdel-Ramoya A, Stadmeyer L, Durant D, Radtke F, Canalis E. Notch inhibits osteoblast differentiation and causes osteopenia. Endocrinology 2008;149 (8):3890–9.

[31] Shao JS, Cheng SL, Pingsterhaus JM, Charlton-Kachigian N, Loewy AP, Towler DA. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. J Clin Invest 2005;115(5):1210–20.

[32] Rajamannan NM, Subramaniam M, Caira F, Stock SR, Spelsberg TC. Atorvastatin inhibits hypercholesterolemia-induced calcification in the aortic valves via the Lrp5 receptor pathway. Circulation 2005;112(9 Suppl):I229–34.

[33] Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, et al. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33(3):416–21.

[34] Siveke JT, Lubeseder-Martellato C, Lee M, Mazur PK, Nakhai H, Radtke F, et al. Notch signaling is required for exocrine regeneration after acute pancreatitis. Gastroenterology 2008;134(2):544–55.

[35] Fox V, Gokhale PJ, Walsh JR, Matin M, Jones M, Andrews PW. Cell-cell signaling through NOTCH regulates human embryonic stem cell proliferation. Stem Cells 2008;26(3):715–23.

[36] Chen VC, Stull R, Joo D, Cheng X, Keller G. Notch signaling respecifies the hemangioblast to a cardiac fate. Nat Biotechnol 2008 Oct;26(10):1169–78.

[37] Nemir M, Croquelois A, Pedrazzini T, Radtke F. Induction of cardiogenesis in embryonic stem cells via downregulation of Notch1 signaling. Circ Res 2006;98(12): 1471–8.

[38] Pera MF, Andrade J, Houssami S, Reubinoff B, Trounson A, Stanley EG, et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 2004;117(Pt 7):1269–80.

[39] Ivey KN, Muth A, Arnold J, King FW, Yeh RF, Fish JE, et al. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell 2008;2(3): 219–29.

[40] Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423(6938): 409–14.

[41] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861–72.

[42] Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, NY) 2007;318(5858):1917–20.

[43] Fleisher AS, Raman R, Siemers ER, Becerra L, Clark CM, Dean RA, et al. Phase 2 safety trial targeting amyloid beta production with a gamma-secretase inhibitor in Alzheimer disease. Arch Neurol 2008;65(8):1031–8.