The Long Non-Coding HOTAIR Is Modulated by Cyclic Stretch and WNT/β-CATENIN in Human Aortic Valve Cells and Is a Novel Repressor of Calcification Genes

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Abstract

Aortic valve calcification is a significant and serious clinical problem for which there are no effective medical treatments. Individuals born with bicuspid aortic valves, 1–2% of the population, are at the highest risk of developing aortic valve calcification. Aortic valve calcification involves increased expression of calcification and inflammatory genes. Bicuspid aortic valve leaflets experience increased biomechanical strain as compared to normal tricuspid aortic valves. The molecular pathogenesis involved in the calcification of BAVs are not well understood, especially the molecular response to mechanical stretch. HOTAIR is a long non-coding RNA (lncRNA) that has been implicated with cancer but has not been studied in cardiac disease. We have found that HOTAIR levels are decreased in BAVs and in human aortic interstitial cells (AVICs) exposed to cyclic stretch. Reducing HOTAIR levels via siRNA in AVICs results in increased expression of calcification genes. Our data suggest that β-CATENIN is a stretch responsive signaling pathway that represses HOTAIR. This is the first report demonstrating that HOTAIR is mechanoresponsive and repressed by WNT β-CATENIN signaling. These findings provide novel evidence that HOTAIR is involved in aortic valve calcification.

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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 (BAV) [2,3,4,5] since 64% of calcified aortic valves have BAV morphology [6]. While the role of biomechanical stretch is important to a number of different cell types in the body, it is especially important in the cardiovascular system since cells in the heart and arteries are exposed to stretch every time the heart beats. Calcification of the bicuspid aortic valve is an example of a cardiac disease in which increased biomechanical stretch is associated with disease. Individuals born with bicuspid aortic valve, which occurs in 1–2% of the population [2,4,5], have two aortic valve leaflets instead of the typical three leaflets. BAV leaflets experience increased stretch as compared to normal tricuspid aortic valves (TAVs) with every heartbeat [7,8]. As a result of the role of biomechanical stretch in BAVs, we sought to examine the molecular responses of human aortic valve interstitial cells (AVICs) upon exposure to cyclic stretch.

While it is clear that osteogenic [9,10,11] and inflammatory [12,13,14,15,16,17,18] pathways are involved in aortic valve calcification, the mechanism by which these pathways are activated in BAVs has not been elucidated. We have previously found that microRNAs, a class of short non-coding RNAs, altered in calcified BAVs can modulate calcification related genes [19]. As a result of our previous work [8,20], we were interested in identifying stretch responsive non-coding RNAs that would increase expression of calcification associated genes in aortic valve cells. We focused our efforts on examining the role of HOTAIR in AVIC response to stretch given that HOTAIR has been shown to play roles in human disease [21,22,23,24,25]. However, HOTAIR has not been reported as having a role in cardiac disease. HOTAIR, a 2.2 kb long non-coding RNA (lncRNA), was initially described as modulating HOX gene expression [26]. HOTAIR has been found to down regulate target genes by helping recruit the Polycomb Repression Complex 2 (PRC2) to target genes [21,26,27]. PRC2, which includes EZH2 and SUZ12, trimethylates H3 lysine K27 (H3K27me3) resulting in epigenetic silencing.

In this report we demonstrated that human AVICs exposed to stretch have lower levels of HOTAIR. BAV leaflets have a trend towards lower HOTAIR levels. Targeting HOTAIR via a siRNA
resulted in higher expression of two osteogenic genes, ALPL [9,28,29] and BMP2 [10,30], that are required for calcification of AVICs [11,31]. Additionally, microarray analysis of HOTAIR siRNA treated AVICs showed activation of additional calcification related genes and pathways. Since the WNT/β-CATENIN signaling pathway has been implicated in aortic valve calcification [32,33,34,35] and other calcification [36,37,38,39,40] processes, we sought to determine if WNT/β-CATENIN was involved in this novel modulation of HOTAIR. Specifically, β-CATENIN was found at higher levels in calcified aortic valves [32]. Cyclic stretch increased the expression of β-CATENIN and β-CATENIN target genes, thereby implicating WNT/β-CATENIN signaling in the AVIC response to biomechanical stimuli. Treatment with WNT agonist results in increased ALPL and BMP2 expression while repressing HOTAIR. These findings suggest that HOTAIR plays a role in repressing calcification-associated genes. A potential regulatory mechanism for HOTAIR repression in stretched AVICs would involved increased β-CATENIN signaling in turn repressing HOTAIR.

To our knowledge, this is the first evidence that HOTAIR is mecanoresponsive and repressed by WNT/β-CATENIN signaling. The linkages of a lncRNA to aortic valve disease and to biomechanical response pathways are novel findings. These data support the hypothesis that AVICs exposed to increased biomechanical stressors can activate calcification pathways associated with aortic valve calcification.

Ethics Statement

The bicuspid aortic valves were collected under a protocol approved by the IRB at University Clinic of Schleswig-Holstein, Campus Luebeck, Luebeck, Germany. Written consent was obtained under this protocol. The control valves were obtained from organ donors whose hearts could not be used for transplantation. Written consent for the use of tissue for research was obtained from the next of kin by Lifesharing, a donate life organization (www.lifersharing.org). The UCSD IRB deemed this specific project an IRB exempt protocol given that the tissue was from a deceased individual and the research team obtained no donor identifiers.

Experimental Procedures

Human diseased bicuspid valves were collected under an IRB approved protocol [19]. The control valves were obtained from organ donors whose hearts could not be used for transplantation. RNA was isolated from the valve leaflets using Trizol (15596, Invitrogen). HOTAIR and Aortic Valve Calcification Genes

Exposing Human Aortic Valve Interstitial Cells (AVICs) to Cyclic Stretch

Human aortic valve leaflets were harvested from organ donors whose hearts could not be transplanted under an IRB exempt protocol. Human AVICs were cultured according to standard protocols [41,42]. Cells were grown on Collagen-1 coated Bioflex plates (BF-3001C Flexcell International). AVICs were exposed to cyclic stretch of 14% at 1 Hz using a Flexcell FX-5000 Tension system (Flexcell International) or static condition, the control condition, for 24 h at the same time on Bioflex plates.

RNA Isolation

RNA was isolated using RNAeasy columns (74104, Qiagen) or Trizol (15596, Invitrogen) according to the manufacturer’s protocol.

qPCR

cDNA was prepared using Superscript III (Invitrogen). qRT-PCR was performed using TaqMan primers (Applied Biosystems) for ALPL and BMP2 and SYBR Green primers for HOTAIR. ∆∆CT values were calculated; GAPDH was used as the endogenous control. Student t-test was used to determine statistical significance. Standard error of the mean was presented as the error bars in the graphs representing the qPCR data.

HOTAIR siRNA Treatment

AVICs were transfected with either HOTAIR siRNA [21] or scramble using lipofectamine [11,19] for 96 h.

DNA Microarray Experiments

Biotinylated cRNA was prepared using the Illumina RNA Amplification Kit, Catalog #1L1791 (Ambion, Inc., Austin, TX) according to the manufacturer’s directions starting with 250 ng total RNA. For microarray analysis, the Illumina HumanHT-12 v4 Expression BeadChip was used (Illumina, San Diego).

Hybridization of labeled cRNA to the BeadChip, and washing and scanning were performed according to the Illumina BeadStation 500x manual. Essentially the amplified, biotin-labeled mouse cRNA samples were resuspended in a solution of Hyb E1 buffer (Illumina) and 25% (v/v) formamide at a final concentration of

Figure 1. HOTAIR levels are decreased in BAVs and stretched AVICs. A. qPCR demonstrates that BAV leaflets have 69.3% lower levels of HOTAIR as compared to normal aortic valve leaflets (p = 0.08) n = 4. B. Cyclic stretch reduced HOTAIR levels by 67% (p<0.05) n = 3.

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Hybridization was allowed to proceed at 55°C, for 18 hours after which, the bead array matrix was washed for 10 minutes with 1X High temperature buffer (Illumina), followed by a subsequent 10 minute wash in Wash E1BC buffer. The arrays were then washed with 100% ethanol for 10 min to strip off any remaining adhesive on the chip. A 2-minute E1BC wash was performed to remove residual ethanol. The arrays were blocked for 5 minutes with 1% (w/v) casein-PBS, (Pierce). The array signal was developed via 10-minute incubation with Streptavidin-Cy3 at a final concentration of 1 mg/mL solution of (GE Healthcare) in 1% casein-PBS blocking solution. The Expression BeadChip was washed a final

25 ng/µL. 1.5 µg of each cRNA were hybridized. Hybridization was allowed to proceed at 55°C, for 18 hours after which, the bead array matrix was washed for 10 minutes with 1X High temperature buffer (Illumina), followed by a subsequent 10 minute wash in Wash E1BC buffer. The arrays were then washed with 100% ethanol for 10 min to strip off any remaining adhesive on the chip. A 2-minute E1BC wash was performed to remove residual ethanol. The arrays were blocked for 5 minutes with 1% (w/v) casein-PBS, (Pierce). The array signal was developed via 10-minute incubation with Streptavidin-Cy3 at a final concentration of 1 µg/mL solution of (GE Healthcare) in 1% casein-PBS blocking solution. The Expression BeadChip was washed a final

Figure 2. AVICs exposed to cyclic stretch or HOTAIR siRNA have increased expression of ALPL and BMP2. A and B. Human AVICs exposed to 14% stretch for 24 h have increased ALPL and BMP2 mRNA levels, 50% and 77% respectively, as compared to static controls. N = 6. C and D. AVICs transfected with HOTAIR siRNA have more ALPL and BMP2, 6.06 fold, and 66% increase respectively, as compared to AVICs transfected with scramble control. N = 3. # p<0.005, ‘ p<0.0001. doi:10.1371/journal.pone.0096577.g002

Table 1. Calcification Genes upregulated in HOTAIR siRNA microarray dataset.

| Gene Symbol | Gene Name                                      | Fold Change |
|-------------|------------------------------------------------|-------------|
| BMP1        | BONE MORPHOGENIC PROTEIN 1                     | 1.31        |
| BMP4        | BONE MORPHOGENIC PROTEIN 4                     | 1.36        |
| BMP6        | BONE MORPHOGENIC PROTEIN 6                     | 1.81        |
| BMPR2       | BONE MORPHOGENIC PROTEIN RECEPTOR, TYPE II     | 1.34        |
| COL1A1      | COLLAGEN, TYPE 1, ALPHA 1                      | 2.03        |
| EDN1        | ENDOTHELIN 1                                   | 1.69        |
| MMP2        | MATRIX METALLOPEPTIDASE 2                      | 1.28        |
| MMP10       | MATRIX METALLOPEPTIDASE 10                     | 1.24        |
| MMP12       | MATRIX METALLOPEPTIDASE 12                     | 1.46        |
| POSTN       | PERIOSTIN                                      | 1.8         |

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time in Wash E1BC buffer for five minutes and subsequently dried via centrifugation for 4 minutes at a setting of 275 rcf.

The arrays were scanned on the Illumina BeadArray Reader, a confocal-type imaging system with 532 (cye3) nm laser illuminations. Image analysis and data extraction was carried out as in accordance with Illumina specifications. Preliminary data analysis and QC was carried out using the GenomeStudio software (Illumina). All array data has been deposited in the EBI ArrayExpress Database (E-MTAB-2409).

![Figure 3. WNT/β-CATENIN signaling modulates HOTAIR, ALPL, and BMP2 levels.](image)

**A.** AVICs exposed to cyclic stretch have 39% increased β-CATENIN mRNA levels as compared to static controls. N = 6. **B.** Cyclin D1 (CCND1) levels were examined in stretched AVICs since it is a known WNT/β-CATENIN target gene. Stretched AVICs have 91% increased CCND1 expression as compared to static controls. N = 4. **C.** Treatment with WNT agonist is sufficient to repress HOTAIR by 50% as compared to DMSO treated controls. N = 3. **D and E.** AVICs treated with WNT agonist have increased ALPL and BMP2, 87% and 9.5 fold respectively. N = 3. *p<0.05, %p<0.0005, ′p<0.0001.

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Analysis of Microarray Data

1) **Normalization of microarray data.** Expression level data from the Illumina Bead Studio software were normalized using a quantile normalization [43]. Probes whose expression level exceeds a threshold value in at least one sample are called detected. The threshold value was found by inspection from the distribution plots of (log) expression levels.

2) **Sorting the probes according to significance.** Detected probes were sorted according to their q-value, which is the smallest false discovery rate (FDR) at which the gene is called significant.
FDR was the expected fraction of false positive tests among significant tests [36]. We evaluated FDR using Significance Analysis of Microarrays (SAM) and its implementation in the official statistical package samr [37]. In order to not be unduly impressed by accidentally small variances, we set the percentile of standard deviation values used for the exchangeability factor $s_0$ in the test statistic to 75.

3) Statistical analysis of pathways and gene ontology terms. Each gene ontology term or a pathway was treated simply as a set of genes. The probe list, sorted by q-value in ascending order, was translated into Entrez gene ID’s and parsed so that whenever several different probes represent the same gene, only the highest-ranking probe was kept for further analysis. The sorted list of genes was subjected to a non-parametric variant of the Gene Set Enrichment Analysis (GSEA) [38], in which the p-value of a gene set of size n was defined as follows: Let us denote the k-th highest rank in gene set as $r_k$, and define $p_k$ as the probability that out of n randomly chosen ranks (without replacement) the k-th highest is not smaller than $r_k$. The p-value of the gene set was defined as $\min_k [p_k]$. It was designed to detect overrepresented gene sets at the top of the list. Unlike the Kolmogorov-Smirnov statistic used in GSEA, it would not detect underrepresented or other, pathologically distributed, gene sets. Finding the p-value of a gene set of size n required calculation of n rank-order values $p_k$, however, there was no need to adjust the p-values for multiple testing as the rank-order tests are highly statistically dependent. We performed a Bonferroni adjustment of gene set p-values for the number of gene sets tested, even though there were often several gene sets with overlapping gene content (and therefore are statistically dependent), which was partly due to the design of the gene ontology database and partly because genes tended to be involved in multiple processes. We reported only gene sets with adjusted p-values $\leq 0.01$.

**WNT Agonist Treatment**

To examine the role of $\beta$-CATENIN signaling in AVICs, AVICs were treated with 10 $\mu$M of WNT agonist (2-Amino-4-[(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine] [44] (Calbiochem) or DMSO (control) for 48 h.

**Results**

HOTAIR Levels are Decreased in BAVs and Stretched AVICs

Since we had previously implicated microRNAs, small non-coding RNAs, to play a role in calcification of bicuspid aortic valves [19], we sought to examine whether lncRNAs are involved in modulating aortic valve calcification. HOTAIR was examined as a candidate lncRNA since it has a role in human disease [21,22,23,24,25]. To examine if HOTAIR levels are altered in diseased BAVs, HOTAIR qPCR was performed on BAV and control TAV leaflets. The BAVs have 69% less HOTAIR ($p = 0.08$) (Figure 1A). Since BAV leaflets experience increased strain, we examined if cyclic stretch was sufficient to modulate HOTAIR levels in AVICs. qPCR demonstrated that AVICs exposed to stretch have 67% decrease in HOTAIR levels ($p < 0.05$) (Figure 1B).

Cyclic Stretch Increases Expression of Calcification Related Genes

We had previously demonstrated that bicuspid aortic valve leaflets experience increased strain as compared to tricuspid aortic valves [8]; therefore, we sought to examine if cyclic stretch was sufficient to increased expression of $\textit{ALPL}$ and $\textit{BMP2}$, two genes that are important in the calcification process. To model the increased strain experienced by AVICs in bicuspid aortic valves, we exposed human AVICs to cyclic stretch at 1 Hz for 24 h. In AVICs exposed to 14% cyclic stretch for 24 h had significantly increased expression of $\textit{ALPL}$ and $\textit{BMP2}$ as compared to static controls (Figure 2A and B). While $\textit{ALPL}$ has previously been found to be stretch responsive in AVICs [45,46], $\textit{BMP2}$ had not previously been reported to be stretch responsive in AVICs.

HOTAIR siRNA Activates Expression of Gene Related to Aortic Valve Calcification

Since HOTAIR levels were decreased by stretch and trended lower in bicuspid aortic valve leaflets, we examined if decreasing HOTAIR levels in AVICs resulted in alterations in $\textit{ALPL}$ and $\textit{BMP2}$ expression by using a previously published and validated...
siRNA [21]. ALPL and BMP2 were identified as candidate HOTAIR target genes based upon examination of the supplemental data from Gupta et al [21]. qPCR demonstrated that AVICs transfected with HOTAIR siRNA had significantly increased expression of ALPL and BMP2 (Figure 2C and D).

In order to examine if HOTAIR modulates additional genes and pathways associated with aortic valve calcification in an unbiased manner, we performed gene expression profiling comparing RNA from AVICs transfected with HOTAIR siRNA or scramble control. A number of aortic valve calcification associated genes were upregulated in HOTAIR siRNA samples (Table 1). Additionally, gene ontology (GO) terms such as inflammatory response (3.15×10−6), cytokine response (6.36×10−6), leukocyte migration (1.3×10−4), and ossification (2.05×10−2) were modulated by HOTAIR siRNA.

The full list of genes altered ≥1.2 fold with a p-value ≤0.05 is provided in Dataset S1. The biologic and molecular function GO terms modulated by HOTAIR siRNA are provided in Datasets S2 and S3.

WNT Signaling is Sufficient to Repress HOTAIR

Since WNT/β-CATENIN signaling has been implicated in aortic valve calcification [32,33,34,35], we examined if WNT/β-CATENIN signaling plays a role in the stretch modulation of ALPL, BMP2, and HOTAIR. We performed qPCR for β-CATENIN and the β-CATENIN target gene CYCLIND1 (CCND1) in stretched and static AVICs. We found that stretch increases expression of β-CATENIN and CCND1 (Figure 3A and B).

To examine if WNT was sufficient to repress HOTAIR, we performed HOTAIR qPCR on RNA from the WNT agonist treated AVICs and found that WNT agonist treatment resulted in 50% less HOTAIR (Figure 3C). The β-CATENIN signaling mediated reduction in HOTAIR levels were also seen in AVICs treated with BIO, a GSK3β inhibitor that increases β-CATENIN signaling (Figure S1).

To examine if increased WNT/β-CATENIN signaling was sufficient to activate ALPL and BMP2 in AVICs, we treated AVICs with a WNT agonist for 48 h and found that these AVICs had increased ALPL and BMP2 mRNA levels as compared to controls (Figure 3D and E).

Discussion

In this report, we demonstrate novel regulatory mechanisms for the lncRNA, HOTAIR, and implicate for the first time that HOTAIR plays a role in heart disease. We show that HOTAIR levels appear to be decreased in diseased BAVs as compared to TAVs. AVICs exposed to cyclic stretch have decreased HOTAIR levels. These data are the first report of biomechanical regulation of a lncRNA. Based upon our qPCR and microarray data, we demonstrate that HOTAIR represses ALPL and BMP2 along with additional calcification and inflammatory genes. Stretch is sufficient to activate Wnt/β-CATENIN signaling in AVICs. Increasing Wnt signaling in AVICs results in increased calcification gene expression and decreased HOTAIR levels. As the result of these findings, we propose the model (Figure 4) that biomechanical stretch activation of calcification pathways in BAVs involves modulation of Wnt/β-CATENIN, HOTAIR, and epigenetic regulation of calcification related genes.

Our data finds that BAV leaflets tend to have lower HOTAIR levels as compared to normal TAVs. Since others and we have found that BAV leaflets experience increased strain [7,8], our data that AVICs exposed to cyclic stretch have decreased HOTAIR are consistent with the biomechanical mechanism of BAV calcification. In addition to stretch-mediated repression of HOTAIR that appears to occur in BAVs, it is possible that SNPs and mutations in the HOTAIR locus could be associated with aortic valve calcification. Since currently very little is known about SNPs associated with aortic valve calcification [47], it is not possible answer this question with the current SNP data. Additionally, further study is required to examine if perturbation of HOTAIR is sufficient to induce aortic valve calcification in vivo.

While our finding that HOTAIR is repressed by biomechanical stretch, we anticipate that additional cardiac lncRNAs will be identified as being modulated by stretch. This belief is based upon the fact that the heart experiences stretch with each heart beat and the growing roles of lncRNAs. Additionally, the mechanisms by which biomechanical stretch regulate cardiac gene expression are especially poorly understood.

As the result of our data that stretch and WNT/β-CATENIN represses HOTAIR along with published data, we propose that cyclic stretch activates expression of ALPL and BMP2 via the release of epigenetic repression. HOTAIR has been shown to recruit PRC2 complexes to trimethylate H3K27 [21,26,27], which silences transcription. Gupta et al identified potential HOTAIR/PRC2 binding sites by overexpressing HOTAIR in breast cancer cells and used antibodies to members of the PRC2 complex and H3K27me3 for ChiP-tiling array experiments [21]. Their published supplemental data demonstrated that increased HOTAIR expression resulted in increased binding of PRC2 complex to the ALPL and BMP2 promoters. As a result of these data, we propose the model (Figure 4) that stretch increased WNT/β-CATENIN signaling which in turn represses HOTAIR, thereby decreasing H3K27 trimethylation at ALPL and BMP2. As a result of this release of epigenetic repression, stretched AVICs have increased expression of ALPL, BMP2, and other calcification related genes.

Supporting Information

Figure S1 Treatment with BIO is sufficient to repress HOTAIR in AVICs. BIO is a potent activator of β-CATENIN signaling. AVICs treated with BIO have 56% less HOTAIR as compared to AVICs treated with meBIO, the negative control for BIO. N = 3. *p<0.05.

Dataset S1 Excel file containing genes altered >1.2 fold with a p<0.05 in the HOTAIR siRNA microarray data. (XLS)

Dataset S2 Excel file contain list of Biologic Process GO terms modulated by HOTAIR siRNA. (XLSX)

Dataset S3 Excel file contain list of Molecular Function GO terms modulated by HOTAIR siRNA. (XLS)

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Author Contributions

Conceived and designed the experiments: KC JD VP VN. Performed the experiments: KC JD VP VN. Analyzed the data: RS GH. Contributed reagents/materials/analysis tools: SM VN. Wrote the paper: VN.
References

1. Association AH (2004) Heart Disease and Stroke Statistics—2004 Update; Association AH, editor. Dallas, Texas.

2. Freeman RV, Otto CM (2005) Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. Circulation 111: 3316–3326.

3. Ward C (2000) Clinical significance of the bicuspid aortic valve. Heart 83: 81–85.

4. Fedak PW, Verma S, David TE, Leask RL, Weisel RD, et al. (2002) Clinical and pathophysiological implications of a bicuspid aortic valve. Circulation 106: 904–909.

5. Beppu S, Suzuki S, Matsuda H, Ohmori F, Nagata S, et al. (1993) Rapidity of progression of aortic stenosis in patients with congenital bicuspid aortic valves. Am J Cardiol 71: 322–327.

6. Davies MJ, Treasure T, Parker DJ (1996) Demographic characteristics of patients undergoing aortic valve replacement for stenotic relation to valve morphology. Heart 75: 174–178.

7. Robincek F, Thubrikar MJ, Cook JW, Fowler B (2004) The congenitally bicuspid aortic valve: how does it function? Why does it fail? Ann Thorac Surg 77: 177–185.

8. Sieto K, Pastuszko P, del Alamo JC, Lasher J, Nigam V (2013) Bicuspid aortic valves experience increased strain as compared to tricuspid aortic valves. World Journal for Pediatric & Congenital heart surgery 4: 362–366.

9. Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, et al. (2003) Human aortic valve calcification is associated with an osteoblast phenotype. Circulation 107: 2181–2184.

10. Mohler ER, 3rd, Chavda MK, Chang AW, Vyvahare N, Levy RJ, et al. (1999) Identification and characterization of calcifying valve cells from human and canine aortic valves. J Heart Valve Dis 8: 254–260.

11. Nigam V, Srivastava D (2009) Notch1 represses osteogenic pathways in aortic valve cells. J Mol Cell Cardiol.

12. Kaden JJ, Demple CE, Groholz R, Tran HT, Kilic R, et al. (2003) Interleukin-1 beta promotes matrix metalloproteinase expression and cell proliferation in calcific aortic valve stenosis. Atherosclerosis 176: 205–211.

13. Kaden JJ, Demple CE, Groholz R, Fischer CS, Voce DC, et al. (2005) Inflammatory regulation of extracellular matrix remodeling in calcific aortic valve stenosis. Cardiovasc Pathol 14: 10–17.

14. Naito Y, Tsujino T, Wakabayashi K, Matsumoto M, Ohyama M, et al. (2010) Increased interleukin-10 expression in nonrheumatic aortic valve stenosis. Int J Cardiol 141: 269–273.

15. Edle ME, Shirani J, Wolf P, Brown DL (2000) Matrix metalloproteinase expression in nonrheumatic aortic valve stenosis. Cardiovasc Pathol 9: 281–286.

16. Soini Y, Satta J, Maatta M, Aalto-Harminen H (2001) Expression of MMP2, MMP9, MT1-MMP, TIMP1, and TIMP2 mRNAs in valvular lesions of the heart. J Pathol 194: 225–231.

17. Rajamannan NM (2012) Oxidative-mechanical stress signals stem cell niche mediated Lrp5 osteogenesis in eNOS−/− mice. J Cell Biol 193: 1623–1634.

18. Rajamannan NM, Subramaniam M, Stock SR, Stone NJ, Springett M, et al. (2005) Atorvastatin inhibits calcification and enhances nitric oxide synthase production in the hypercholesterolemic aortic valve. Heart 91: 806–810.

19. Westendorf JJ, Kahlert RA, Schroeder TM (2004) Wnt signaling in osteoblasts and bone diseases. Gene 341: 19–39.

20. Glass DA, 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, et al. (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8: 751–764.

21. Shao J, Cheng SL, Fingerthum JA, Charlton-Kuczynski N, Loewy AP, et al. (2005) Mesp2 promotes cardiovascular calcification by activating paracrine Wnt signals. J Clin Invest 115: 1210–1220.

22. Favermann L, Mkhaylova L, Malmquist J, Nurminskaya M (2008) Extracellular transglutaminase 2 activates beta-catenin signaling in calcifying vascular smooth muscle cells. Circ Res 102: 1552–1557.

23. Cheng SL, Shao JS, Halstead LR, Distelhoust K, Sierra O, et al. (2010) Activation of vascular smooth muscle parathyroid hormone receptor inhibits Wnt/beta-catenin signaling and aortic fibrosis in diabetic arteriosclerosis. Circ Res 107: 271–282.

24. Jiao B, Namula N, Li QY, Mohler ER, 3rd, Levy RJ (2003) 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 75: 457–463; discussion 463–456.

25. Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, et al. (2012) HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene.

26. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, et al. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 129: 1311–1323.

27. Xie Y, Wang X, Story M (2009) Statistical methods of background correction for Illumina BeadArray data. Bioinformatics 25: 751–757.

28. Liu J, Wu X, Mitchell R, Kintner C, Deng S, et al. (2005) A small-molecule agonist of the Wnt signaling pathway. Angew Chem Int Ed Engl 44: 1887–1900.

29. Lehmann S, Walther T, Kempter J, Rastan A, Garbade J, et al. (2009) Mechanical strain and the aortic valve: influence on fibrillasts, extracellular matrix, and potential stenosis. Am J Thorac Surg 88: 1476–1483.

30. Ferdous Z, Jo H, Nerem RM (2011) Differences in valvular and vascular cell phenotypes. Circ Res 107: 2181–2184.

31. Thanassoulis G, Campbell CY, Owens DS, Smith JG, Smith AV, et al. (2013) Genetic associations with valvular calcification and aortic stenosis. The New England journal of medicine 368: 503–512.