Diet-Induced Aortic Valve Disease in Mice Haploinsufficient for the Notch Pathway Effector RBPJK/CSL

Meritxell Nus, Donal MacGrogan, Beatriz Martínez-Poveda, Yolanda Benito, Jesús C. Casanova, Francisco Fernández-Avilaés, Javier Bermejo, José Luis de la Pompa

Objective—Calcific aortic valve disease is similar to atherosclerosis in that both diseases result from chronic inflammation and endothelial dysfunction. Haploinsufficient NOTCH1 mutations have been associated to calcific aortic disease and a bicuspid aortic valve. We investigated whether mice with genetic inactivation of the Notch signaling pathway are prone to develop valve disease when exposed to a predisposing diet.

Methods and Results—Using Doppler echocardiography, histology, immunohistochemistry, quantitative gene expression analysis, and cell culture assays, we examined the effect of a hypercholesterolemic diet supplemented with vitamin D on mice heterozygous for null mutations in the Notch1 receptor or the effector transcription factor gene RBPJK. After 16 weeks on the hyperlipidemic diet, calcific aortic disease was detected in heterozygous RBPJK mice. Analysis of valve leaflets revealed macrophage infiltration, enhanced collagen deposition, proosteogenic protein expression, and calcification. Haploinsufficient null Notch1 mice displayed milder histopathologic changes and did not develop any significant hemodynamic disturbance. Valvular disease correlated with reduced expression of the Notch target gene Hey1 in valves of RBPJK heterozygous mice fed the hyperlipidemic diet. Consistent with the in vivo data, Notch signaling inhibition in porcine valve interstitial cells led to downregulation of HEY1 transcription, activation of osteogenic markers, and increased calcified nodule formation.

Conclusion—We show that Notch signaling disruption via RBPJK heterozygous inactivation results in aortic valve disease. Notch1 heterozygous mice do not show functional impairment, suggesting that additional Notch receptors may be involved in aortic valve homeostasis and disease. Our data establish a genetic mouse model of calcific aortic valve disease and may help to identify a patient population with reduced valvular NOTCH signaling at risk for developing this disease. (Arterioscler Thromb Vasc Biol. 2011;31:1580-1588.)

Key Words: calcification ■ diet ■ heart valves ■ morphogenesis ■ signal transduction

Calcific aortic valve disease (CAVD) is a growing health problem in Western countries. Currently, more than 2% of people over the age of 65 and 4% over the age of 85 experience this condition, and the prevalence of this pathology is rising because the population is aging. In fact both diseases share common risk factors, such as age, smoking, hypertension, and hypercholesterolemia, involving common molecular mechanisms.

The Notch pathway is a local cellular signaling system that regulates cell fate determination, differentiation, and tissue patterning, and dysregulated Notch activity has pathological consequences. Notch genes encode a group of transmembrane receptors (Notch1 to Notch4 in mammals), with a large extracellular region and an intracellular domain involved in nuclear signaling. Interaction of Notch receptors with their cognate membrane-bound ligands (Delta-like and Jagged/Serrate) activates Notch receptors and results in nuclear translocation of Notch intracellular domain. In the nucleus, the Notch intracellular domain associates with the RBPJK/CSL effector transcription factor and recruits coactivator molecules such as MAML1 to activate the expression of target genes.

Notch signaling is required for endocardial differentiation and cardiac valve formation in the mouse. During early valve development, Notch1 activity is detected in the endocardium overlying the presumptive valve territory and persists in the valve endocardium throughout development, suggesting that Notch is important for cardiac valve morphogenesis and remodeling. Inactivating mutations in the human NOTCH1 gene have been identified in patients with severe early CAVD, usually in the context of a bicuspid aortic valve, suggesting that Notch1 haploinsufficiency causes a genetic predisposition to both bicuspid aortic valve and CAVD.
We hypothesized that mice with reduced Notch signaling activity might readily develop CAVD when subjected to a hypercholesterolemic diet. To test this, we carried out parallel studies on mice with targeted mutations for either RBPJK or Notch1. RBPJK is the main nuclear Notch effector, and mutation of RBPJK leads to more severe and systemic phenotypes than are caused by mutation of any single Notch receptor.10

We fed RBPJK and Notch1 heterozygous mutant mice a hypercholesterolemic diet supplemented with vitamin D (HCVD diet). Only RBPJK and not Notch1 heterozygous mice showed worsened hemodynamic parameters under these conditions, suggesting that additional Notch receptor(s) that we find to be expressed in valve tissue, may play an essential redundant function in valve homeostasis. To gain a mechanistic insight into the role of Notch in CAVD, we cultured porcine valve interstitial cells in calcifying media and inhibited Notch signaling in various manners. Molecular analyses revealed that Notch inhibition caused a reduction in Hey1 expression and activated a proosteogenic gene program that led to valve cell calcification.

Methods

Mice

We used Notch111 and RBPJK10 targeted mutant mice that had been bred into a CD1 background for at least 20 generations. All animals had trileaflet aortic valves except for 1 Notch1KO/+ mouse, which presented a bicuspid valve and was removed from the study. Eight to 10-week-old heterozygous mutant mice for Notch1 (n=14) and RBP (n=14) mice and their wild-type (WT) littermate counterparts (n=28) were assigned to 2 treatment groups (n=7 per group) for a 16-week period: (1) control chow (CC) diet, and (2) an HCVD diet. Food intake and body weight were monitored regularly.

Diet

See Supplemental Methods.

Plasma Cholesterol Levels

See Supplemental Methods.

Echocardiography

Echocardiograms were recorded with a 14-MHz linear probe on a Vivid-7 ultrasound scanner (General Electric Healthcare) by a single experienced sonographer (Y.B.), fully blinded to the animal study group. Conscious sedation was achieved with low-dose intraperitoneal ketamine (80 mg/kg). The left ventricular (LV) end-systolic and end-diastolic diameters and septum and posterior wall thickness were measured using the two-dimensional guided M-mode. Ejection fraction (EF) and fractional shortening (FS) were computed from these measurements.12 B-mode and color-Doppler guided continuous-wave Doppler was used to record the maximal transvalvular jet velocity. Specifically, to avoid Doppler misalignment, coaxial interrogation of the aortic flow was ensured by the operator, and all the measurements were obtained using an angle of interrogation less than 30°. To correct for flow dependence we computed an EF velocity ratio (EFVR =EF (%)/maximal aortic velocity [m/s]) as an additional indicator of disease severity.13 This index was preferred as a flow-corrected index of valve sclerosis because obtaining robust measurements of LV outflow tract size is very difficult in mice, as is accurately positioning the left ventricular outflow tract pulsed-wave Doppler sample volume.

Histology, In Situ Hybridization, and Immunohistochemistry

See Supplemental Methods.

Porcine Aortic Valve Interstitial Cell Culture and Short Hairpin RNA Lentiviral Transduction

See Supplemental Methods.

Quantitative and Semiquantitative Polymerase Chain Reaction

See Supplemental Methods.

Statistics

All data are presented as means±SD. A 1-way ANOVA (means model) was used to estimate a set of planned contrasts. The control of the false discovery rate,14 ie, the expected proportion of false-positives among all significant tests, was used to take into account the multiplicity of the tests. We used 2-way analysis of variance (ANOVA) to study the effect of diet and genotype. For comparisons between groups, the least significant difference (LSD) test was used for post hoc testing. Spearman correlation coefficients (R) were used to evaluate the relationship between continuous variables. All statistical analysis was performed using the statistical package SPSS version 17.0 (SPSS Inc, Chicago, IL) and the open source statistical scripting language R. Probability (P) levels below 0.05 were considered significant.

Results

Valvular and Ventricular Functions Are Impaired in RBPJK Mutants

The WT and RBPKO/+ animals had similar baseline readings of transvalvular maximum velocity and EFVR (P>0.05). After 4 months on HCVD diet, RBPKO/+ mice had higher maximum velocity and lower EFVR values than WT animals (P=0.05 and 0.01, respectively). Remarkably, the aortic valve maximum velocity was significantly higher in RBPKO/+ mice fed the HCVD diet than WT or RBPKO/+ mice on the CC diet (increased 20%, from 1 to 1.20±0.09 m/s; P<0.05; Figure 1A and 1E). Moreover, EFVR was lower in RBPKO/+ mice fed the HCVD diet (decreased 45%, from 70 to 46±17) than in RBPKO/+ mice fed the CC diet or in WT mice fed the HCVD diet (Figure 1D). No significant dietary effect was found on echocardiography measurements in Notch1KO/+ animals (Supplemental Figure I).

RBPKO/+ and WT animals had similar baseline FS and EF values (P>0.05). At 4 months, RBPKO/+ mice had significantly lower FS and EF values than WT (P<0.05, Figure 1B and 1C), suggesting an important genotype effect. Moreover, these values were significantly lower in RBPKO/+ mice fed the HCVD diet (P<0.05). RBPKO/+ mice, but not their WT littermates, showed a significant correlation between transvalvular velocity and levels of total cholesterol (R=0.77; P<0.01) and low-density lipoprotein cholesterol (R=0.82; P<0.001) (Supplemental Figure IIA and IIB), suggesting that the adverse hemodynamic parameters were associated with hypercholesterolemia in RBPKO/+ mice. Thus, RBPKO/+ mice fed the HCVD diet developed a functional impairment in both aortic valve and left ventricle.

RBPJK Heterozygous Mice Display Aortic Valve Fibrosis and Inflammation

Histological analysis at 16 weeks indicated that leaflet thickness, as determined by quantifying valvar cuspal area, was
Figure 1. RBPKO heterozygous (RBPKO/+) mice fed an HCVD diet develop aortic valve disease. A to D, Aortic valve maximum velocity (A), FS (B), EF (C), and EFVR (D) examined by echocardiography of WT and RBPKO/+ mice fed the CC or HCVD diet for 16 weeks. One-way ANOVA and LSD post hoc analyses were used to compare differences between groups. *P<0.05 vs CC. E, Transvalvular aortic velocities in WT mice fed the CC diet (left) and RBPKO/+ mice fed the HCVD diet (right) by continuous-wave Doppler imaging.

unaffected by the CC diet in WT or RBPKO/+ mice (Figure 2A, 2C, and 2M). Cusps were thicker (*P<0.05) in WT, Notch1 KO/+ and RBPKO/+ animals fed the HCVD diet (Figure 2D, 2E, 2F, and 2M). Leaflets were also thicker in Notch1KO/+ mice fed the CC diet (Figure 2B and 2M) compared with WT littermates (*P<0.05; Figure 2A and 2M). However, the diet did not have any significant effect on leaflet thickness of Notch1KO/+ mice (Figure 2B, 2E, and 2M). The most significant increase was found in RBPKO/+ mice fed the HCVD diet (*P<0.01; Figure 2F and 2M). Collagen distribution in the leaflets of WT, Notch1KO/+, or RBPKO/+ mice fed the CC diet appeared to be restricted to a thin fibrosa layer (Figure 2A, 2B, 2C, and 2M; green-blue staining). In contrast, collagen in animals fed the HCVD diet extended all over the valve area most extensively in RBPKO/+ mice (Figure 2D, 2E, 2F, and 2M).

To detect macrophage infiltration, we stained aortic valve sections with anti-Mac3 antibody. Mac3 staining was absent in the valve leaflets of mice fed the CC diet (Figure 2G, 2H, 2I, and 2N), but weak staining was seen at the base of the leaflets in WT mice fed the HCVD diet (Figure 2J and 2N). Macrophage infiltration was detected in the leaflets of Notch1KO/+ and RBPKO/+ mice fed the HCVD diet, mostly at the leaflet base fading toward the leaflet tips (Figure 2K, 2L, and 2N). Thus, macrophage infiltration was increased in Notch1KO/+ valve leaflets and more so in the RBPKO/+ leaflets.

To corroborate that fibrosis was indeed taking place in the valves of hyperlipidemic mice, we examined transforming growth factor-β1 (Tgfβ1) and the activated phosphorylated (p) form of its intracellular effector, Smad2. Immunostaining of Tgfβ1 or p-Smad2 was negligible in WT or RBPKO/+ mice fed the CC diet (Supplemental Figure IIIA, IIIC, IIIG, IIII, IIM, and IIIN). Mild staining of both Tgfβ1 and p-Smad2 was found in Notch1KO/+ animals fed the CC diet (Supplemental Figure IIIB, IIIE, IIIII, IIM, and IIIN) and WT and Notch1KO/+ animals fed the HCVD diet (Supplemental Figure IIID, IIIE, IIIII, IIM, and IIIN). The most intense staining of Tgfβ1 or p-Smad 2 was seen in RBPKO/+ mice fed the HCVD diet (Supplemental Figure IIIF, IIIJ, IIIK, IIIM, and IIIN). Thus Notch downregulates inflammatory and fibrosis markers expressed during extracellular matrix remodeling taking place in CAVD.

Notch Signaling Abrogation Activates a Proosteogenic Program in Hypercholesterolemic Animals

To determine whether the stenotic valve cells had converted into osteoblast-like cells, we examined the expression of the osteogenic transcription factors p-Smad1/5/8, the intracellular effector of bone morphogenic protein 2 (Bmp2) in its activated form, and the master regulator Runx2. Immunofluorescence for p-Smad1/5/8 or Runx2 was negligible in WT and RBPKO/+ mice fed the CC diet (Figure 3A, 3C, 3G, 3I, 3M, and 3N), p-Smad1/5/8 staining was increased in WT mice fed the HCVD diet (Figure 3D and 3M), whereas Runx2 was found on the leaflet base extending toward the leaflet tips (Figure 3J and 3N). These expression patterns were similar to those observed in valves of HCVD-fed Notch1KO/+ mice (Figure 3E, 3K, 3M, and 3N), which also showed relatively high levels of p-Smad1/5/8 and Runx2 expression when fed the CC diet (Figure 3B, 3H, 3M, and 3N). Interestingly, the RBPKO/+ mice fed the HCVD diet exhibited intense p-Smad1/5/8 and Runx2 immunostainings over the whole leaflet area (Figure 3F, 3L, 3M, and 3N).

To confirm this phenotype, we examined Osterix and Osteopontin, which are regulated by Runx2 and Bmp2, respectively. Weak background staining was found in aortic valve leaflets of WT and RBPKO/+ mice fed the CC diet (Supplemental Figure IVA, IVC, IVG, IVI, IVM, and IVN). Osterix staining was found in Notch1KO/+ valves (Supplemental Figure IVB and IVM), whereas Osteopontin was absent (Supplemental Figure IVH and IVN). In WT mice, the HCVD diet induced Osterix and Osteopontin staining in the aortic valve attachments (Supplemental Figure IVD and IVN). The aortic leaflets of Notch1KO/+ mice fed with HCVD diet displayed mild staining of either marker (Supplemental Figure IVF, IVK, IVM, and IVN), whereas RBPKO/+ mice displayed intense immunostaining of both markers at the leaflet base extending toward the cusps (Supplemental Figure IVF, IVL, IVM, and IVN). Thus, osteogenic markers were expressed widely in Notch1KO/+ and RBPKO/+ animals.
fed the HCVD diet and were more conspicuously present in RBPKO/+ mice.

Osteogenesis Is Associated With Valve Leaflet Calcification in Notch Mutants

To examine the presence of calcification in the valve cusps, we used Von Kossa staining. The presence of melanocytes in AV and aortic valves may confound the interpretation of Von Kossa staining and has been considered an issue previously in C57BL/6 mice; however, this is not the case in CD1 mice, where melanocytes do not accumulate, and this is an advantage of using this outbred strain. No calcification was observed in the aortic valve leaflets of WT mice fed the CC diet (Figure 4A and 4G) or in RBPKO/+ mice fed the CC diet (Figure 4C and 4G). Weak staining (~0.5% of leaflet area) was detected in WT mice fed the HCVD diet (Figure 4D and 4G) and Notch1KO/+ mice fed the CC diet (Figure 4B and 4G). Moderate staining (~1% of leaflet area) was found in Notch1KO/+ mice fed the HCVD diet (Figure 4E and 4G). The most extensive staining was found in RBPKO/+ mice fed the HCVD diet, covering ~2.5% of the valve area (Figure 4F) (P<0.05). These data indicated that calcification was more extensive in RBPKO/+ mice fed the HCVD diet.

Widespread Notch Receptor Expression in Murine Aortic Valve Leaflets

As the phenotype of RBPKO/+ was more severe than that of Notch1KO/+ mice, we examined whether in addition to Notch1, other Notch receptors were also expressed in valve tissue. Notch1 mRNA was detected in the endothelium and interstitial cells of the aortic valve leaflets of WT CD1 mice (Supplemental Figure VA and VB). Notch2 mRNA was detected in the interstitial cell compartment in WT (Supplemental Figure VC and VD). Notch3 protein was not detected in valve leaflets, despite being found in aortic ring cells (Supplemental Figure VE and VF). Notch4 expression was restricted to the endothelium of the aortic valve (Supplemental Figure VG and VH).
NOTCH Signaling Inhibition Downregulates HEY1 mRNA and Enhances Calcification in Porcine Valve Interstitial Cell Culture

To gain mechanistic insight into CAVD in RBPKO/+ mice, we used porcine aortic valve interstitial cells (PAVIC) cultured in calcifying medium and inhibited Notch signaling using the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl Ester (DAPT). After 3 weeks in calcifying medium, alizarin red staining revealed sparse calcification foci in control cells. In contrast, the DAPT-treated PAVIC exhibited a 2-fold increase in number of large calcification nodules (Figure 5A and 5B; P<0.05). Alizarin red staining revealed few calcification foci in control cultures and increased calcification in DAPT-treated PAVIC (Figure 5A). Reverse transcription–polymerase chain reaction analysis of Notch target genes showed that Hey1 expression was reduced, whereas Hes1 and Hey2 remained unchanged in DAPT-treated cells compared with control cultures (Figure 5C). Concomitantly, the osteogenic markers BMP2, Runx2, alkaline phosphatase (ALP), and osteocalcin (BGLAP/OTC) were also upregulated (Figure 5C). These data suggest that Notch represses calcification in PAVIC by maintaining/activating Hey1 expression.

To further support these data, we transduced PAVIC cultures with MAML1 and RBPJk short hairpin RNAs (shRNAs). After 2 weeks of culture in calcifying medium, the cultures transduced with control lentiviral vector showed very sparse calcification areas (Figure 5D and 5E), whereas PAVIC transduced with shRNA against MAML1 or RBPJk showed a marked increase in area of calcification (Figure 5D and 5E). Reverse transcription–polymerase chain reaction analysis of these cultures indicated a clear reduction in MAML1 and RBPJk transcription (Figure 5F). These data demonstrated that Notch signaling attenuation caused calcification in PAVIC.
dysfunction highlighting the additive effect of predisposing genotype and dietary (environmental) factors in cardiac valve disease. Notch signaling is essential for ventricular chamber development, and these observations are consistent with a critical role for Notch in maintaining ventricular homeostasis in adulthood.

**Notch Mutant Valve Tissue Displays Hallmarks of CAVD**

Epidemiological data and evidence from hyperlipidemic animal models have indicated that CAVD is an atherosclerotic-like process involving endothelial inflammation, fibrosis, and calcification. In our murine models, we used a hyperlipidemic, vitamin D–supplemented diet to drive valvular inflammation, fibrosis, and calcification processes over a 16-week period.

Demonstration of the expression of Notch pathway elements in macrophages within atherosclerotic plaques has led to the suggestion that Notch signaling mediates inflammatory responses and promotes inflammation. Thus, Delta4 has been shown to trigger Notch proteolysis and macrophage activation in culture, whereas Notch3 knockdown during macrophage differentiation decreased the transcription of proinflammatory genes implicated in atherosclerosis. Unexpectedly, we found more conspicuous evidence for inflammation in both RBPKO/+ and Notch1KO/+ mouse models (detected by Mac-3 staining), suggesting that reduced Notch dosage promotes the inflammatory response. This proinflammatory effect may be partially explained by increased sensitivity of endothelial cells to lipid-induced damage in Notch mutants. For example, it has been shown that impaired Notch4 activity in graft endothelium of transplant atherosclerosis results in vascular cell adhesion molecule-1 expression and endothelial cell apoptosis. The increased inflammation observed in our mutants might represent the outcome of diminished Notch function both in macrophages and endothelium, with the effect that the endothelial phenotype is dominant.

The extracellular matrix layering the aortic valve cusp in mouse is organized into collagen in the fibrosa (or arterial aspect of the cusp), elastic fibers in the ventricularis (ventricular aspect), and proteoglycans in the spongiosa. Compared with that of human, it contains relatively less collagen in the fibrosa and elastin in the ventricularis, whereas valve interstitial cells density is qualitatively increased throughout. Mouse undergo age-associated degeneration that primarily affects the annulus, similar to degeneration in humans, but involves proteoglycan accumulation instead of the collagen seen in humans. We suspect that the more pronounced valve cusp thickening in the HCVD diet-fed RBPKO/+ mice and, to a lesser degree, Notch1 KO/+ mice was associated with increased fibrosis, as it correlated well with the observation of enhanced TGFβ1 signaling (through p-Smad2) in valve leaflets. TGFβ1 is a potent stimulator of collagen-producing cardiac fibroblasts and when chronically overexpressed causes tissue fibrosis and organ dysfunction. Excessive mesenchymalization and extracellular matrix deposition have been shown to negatively affect valve mechanics by causing stiffening, loss of flexibility, and restricted leaflet motion. In

**Discussion**

We have established a genetic model of CAVD by feeding RBPJk heterozygous mice a hypercholesterolemic diet. Under similar experimental conditions, Notch1 heterozygous mice developed adverse histopathology but no significant hemodynamic disturbance, consistent with the expression of Notch2, Notch3, and Notch4 in the valves, any of which might be important for aortic valve homeostasis. These results imply that mutations in other NOTCH receptors might be found in CAVD observed in sporadic and familial cases where NOTCH1 is not mutated.

**RBPJk Heterozygous Mutant Mice Develop Aortic Valve Disease**

We show that the RBPKO/+ mice fed an HCVD diet for 4 months develop CAVD. Compared with WT animals, indexes of valvular dysfunction increased during follow-up in RBPKO/+ mice, highlighting an adverse interaction between diet and genotype in these mice. Importantly, the transvalvular maximum velocity in RBPKO/+ mice was higher than in controls, despite a parallel reduction of LV systolic function indexes. These findings were confirmed by the EFVR, which is a flow-corrected index. Moreover, RBPKO/+ mice had LV...
addition, abnormal extracellular matrix mineralization can be the trigger for valve calcification and stenosis.31

Finally, calcification constitutes the end stage process and chief indicator of disease in patients.32 In the RBPJK+/− (and to a lesser extent, Notch1KO/+ ) valve leaflets, increased Bmp2 signaling (via p-Smad1/5/8) and upregulation of Runx2, Osterix, and Osteopontin were consistent with the activation of an osteogenic-like gene program seen in CAVD patient valves33 and animal models.24 The calcification detected in RBPJK+/− mice was mild compared with humans with CAVD. This may indicate that their CD1 atherosclerosis-resistant background, these mice are naturally resistant to calcification or that the process of leaflet mineralization requires longer follow-up. Nevertheless, calcification in RBPJK+/− mice was associated with significant hemodynamic dysfunction, suggesting that other factors, such as fibrosis and leaflet thickening (ie, sclerosis), could contribute to the worsened hemodynamics seen in this model.

Notch Signaling Inhibits Valve Calcification

It has been suggested that Notch1 maintains myofibroblast cell fate and blocks osteogenesis in valve leaflets by down-regulating the transcriptional and signaling activities of Runx2/Cbfa133 and Bmp2.34 In agreement with this notion, Runx2 and Bmp2 expression or signaling activity was found to be consistently upregulated in vivo in aortic valves of HCVD-fed RBPJK+/− mice and in vitro, in PAVIC cultures treated with the Notch inhibitor DAPT. Moreover, our study points to Hey1 as a key Notch target gene whose downregulation is associated with osteogenic gene expression and calcification in vivo and in vitro. Hey1 has been shown to be an important negative regulator of cell differentiation/maturation programs by transcriptional repression of tissue-specific target genes (reviewed in35). Downregulation of Hey1 mRNA by small interfering RNA in osteogenic lineage-derived mouse calvarial MC3T3 cells induced matrix mineralization in conjunction with osteogenic gene expression, suggesting that Hey1 acts as a negative regulator of osteoblast maturation.36 This was demonstrated to occur via interaction with Runx2, with the effect that Hey1 partially abrogated Runx2 transcriptional activity.8,36 Notch1 signaling has also been shown to activate Hey1 transcription directly, by virtue of RBPJK consensus binding sites present within the 5′-end promoter region and the first coding exon of the Hey1 gene.37

Our findings suggest a model (shown in Figure 6) in which Notch inhibits the osteoblast and maintains the myofibroblast cell fate in the aortic valve by activating Hey1 directly through RBPJK. Hey1 in turn abrogates the transcriptional activity of Runx2 or other critical regulators of the osteogenic program that we have seen to be upregulated in vivo in the Notch1KO/+ and RBPJK+/− valve leaflets and in vitro in PAVIC cultures treated with DAPT (Figure 5E). Interestingly, Hey1 has been identified as a direct target for BMP-mediated osteogenic differentiation36 and suggests that a crosstalk between BMP and Notch signaling modulates the interaction between Hey1 and other nuclear factors, such as Runx2.

Is There a Notch Receptor Redundancy in Aortic Valve Homeostasis?

Our study lends support to the idea that Notch1 haploinsufficiency causes a less severe aortic valve phenotype in mice
Limitations of the Study

One factor influencing our study has to do with the genetic background of our mice. Our Notch mutant mice (Notch1KO/+ and RBPKO/+), have been bred into a CD1 background that is relatively resistant to atherosclerosis (unpublished data). We did not consider this an issue because we undertook a comparative study examining RBPKO/+ versus Notch1KO/+ and CD1 WT control mice. We used an atherogenic diet containing cholesterol and cholate, often used to study the pathology and genetics of atherosclerosis in inbred mouse strains and supplemented with vitamin D; this diet has been shown to cause CAVD in other settings. However at 4 months, adverse effects affecting all the mice precluded longer follow-up. Most of the hemodynamic changes in the RBPKO/+ mice were quite modest at this stage, despite clear evidence of valvular inflammation, thickening, and osteogenic transformation, but they were nevertheless significant compared with Notch1KO/+ or WT mice. Given that CAVD is a stage-dependent process, we suspect that a longer follow-up would have allowed the development of overt stenosis.

Conclusions

The murine model described herein represents a first approximation of CAVD caused by reduced Notch dosage. Most likely, Notch operates at multiple levels of disease progression, and it will be necessary in future studies to determine what impact tissue-specific effects have on disease overall. Moreover, breeding the mutants into an atherosclerosis-prone background (ie, ApoEKO) will permit longer follow-up studies. From a clinical standpoint, our data suggest the existence of a population of patients at risk and likely to harbor mutations of NOTCH signaling elements other than Notch1. Systematic examination of each Notch component individually should help clarify the full contribution of the Notch pathway to CAVD.

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Disclosures

None.

References

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Supplement Material

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Supplementary Methods

Mouse genotyping

For genotyping, DNA was extracted from tails. All animals were treated according to the Spanish National Institute of Health’s guidelines for animal care and experimentation. The protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). Blood samples were collected from the orbital sinus at baseline and at the end of the study for the measurement of total plasma cholesterol (TC) and HDL and LDL-cholesterol by enzymatic colorimetric assay on an Advia 2400 (Bayer).

Diets

The CC diet was a commercial chow diet for mouse maintenance (2018TG, Harlan, London, UK). The HCVD diet was a modified AIN93M diet with 18% lactalbumin, 7% saturated fat, 1.25% cholesterol and 0.5 sodium cholate (5D4Q, TestDiet, London, UK) supplemented with 5 IU of vitamin D3 (cholecalciferol) (CS515, TestDiet, London, UK).

Plasma cholesterol levels

As expected, animals fed the CC diet showed no significant changes in plasma TC, LDL-cholesterol or HDL-cholesterol, irrespective of genotype. Compared with littermate counterparts fed the CC diet, animals maintained for 16 weeks on the HCVD diet showed an almost two-fold TC increase, caused by a four- to five-fold increase in LDL-C (P<0.05; Supplementary Figure 6A, B and not shown). HDL-cholesterol levels were unchanged in all the mice (Supplementary Figure 6C). The serum cholesterol levels are consistent with previous findings in mice resistant to atherosclerosis. 1, 2

Histology and quantification

At the end of the study (16 weeks), hearts were perfused with PBS, dissected and fixed in 4% paraformaldehyde (PFA) and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, California). Serial cross sections (10 µm) were stored at -80 °C.
Changes in collagen and leaflet morphology were monitored by Masson’s Trichrome staining. Light microscopy images were obtained with an Olympus BX51 microscope and digital color sections were quantified using image analysis software (JmicroVision Software Version 1.2.7, Geneva, Switzerland) by a reader blinded to sample identity. Valve cusp area was measured including proximal (hinge), middle, and distal aspects of the valve cusps. Sequential sections were reviewed and measurements were obtained from representative sections. Four sections were measured and averaged from four mice. Aortic valve leaflet thickness was measured relative to the leaflet thickness in the CC diet fed WT group, which was set at 100%.

Tissue calcification (Von Kossa staining) and positive immunostaining were measured by quantifying the amount of black (calcification), green or red pixeled (immunostaining) areas. Four sections were measured and averaged from four mice for Von Kossa and three sections were measured and averaged from three mice for immunostainings. The relative calcified or immunostained valve area was calculated as the percentage of the total leaflet area.

In situ hybridization

Non-radioactive in situ hybridization in sections was performed on section on PFA-fixed and paraffin-embedded wild-type CD1 adult mouse hearts as described.™ Probes information will be provided upon request.

Antibodies

Inflammation was detected with anti-Mac3 rat monoclonal antibody (SC19991, Santa Cruz Technol, 1:200). Pro-fibrosis markers were detected using: rabbit monoclonal antibody to TGFβ1 (sc-146, Santa Cruz, 1:10) and rabbit monoclonal antibody P-Smad 2 (3108, Cell Signalling, 1:100). The following antibodies were used as markers of pro-osteogenesis: rabbit monoclonal antibody P-Smad 1/5/8 (9511, Cell Signalling, 1:100), goat polyclonal antibody to Osteopontin (ab36125, Abcam, 1:100) and rabbit polyclonal antibody to Osterix (ab22552, Abcam, 1:100) and mouse monoclonal antibody to Runx2 (ab54868, Abcam, 1:50).

Porcine aortic valve interstitial cells (PAVICs) culture

Aortic valve leaflets were excised from pig hearts obtained from the local abattoir. PAVICs were isolated by sequential collagenase digestion as previously described in.™ Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 20% fetal bovine serum, penicillin/streptomycin and L-glutamine. Cultured cells were used for experiments between passages 3 and 6. To induce
osteogenic differentiation of PAVIC, 5 mM β-glycerophosphate and 5 mM calcium chloride were added to complete medium and cells were treated with 50 µM N-[N-(3,5-difuorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) or vehicle (ethanol). The medium was changed every 3-4 days during 21 days. Cells were then washed 2-3 times with cold phosphate-buffered saline, fixed with 4% PFA and stained with 2% Alizarin Red S in water for 1 hour.

**Lentivirus production**

Pseudotyped lentivectors were produced according to 5. Subconfluent HEK293T cells cultured in 10-cm plates with DMEM-Glutamax (Invitrogen), 10% FBS, and antibiotics were transiently cotransfected using Lipofectamine Plus (Invitrogen) with the following plasmids: 5 µg pLKO.1 control vector, lentiviral *MAML1*-shRNA or *RBPJk*-shRNA vectors (respectively TRCN0000003353 and TRCN0000016207 from Mission shRNA library; Sigma-Aldrich), 5 µg packaging plasmid pCMVdR8.74, and 2 µg vesicular stomatitis virus G envelope protein plasmid pMD2G (plasmid 12259; Addgene). Supernatants were collected after 2 days and added together with polybrene (10 µg/ml) to subconfluent PAVICs. After 72h, calcification media was added (DMEM 20% FCS with 5 mM βGP and 5 mM CaCl2). After 2 weeks, cells were stained with Alizarin Red and RNA was extracted.

**Reverse-transcription polymerase chain reaction (RT-PCR)**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) after 21 days treatment and 500ng of RNA were reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Primers amplifying predicted exonic sequences were designed using available pig (Sus scrofa) sequence from NCBI and Primer3. 6 Semi-quantitative RT-PCR was performed for 35 cycles with annealing at 55°C according to standard procedures. Primer sequences and references are listed in Suppl. Table I.
**Supplementary Table I.**

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Supplementary Figure legends

Supplementary Figure I. *Notch1* heterozygous (*Notch1KO/+*) mice do not display significant functional changes when fed a HCVD diet. (A) Aortic valve maximum velocity, (B) FS, (C) EF and (D) EFVR examined by echocardiography of WT and *Notch1KO/+* mice fed the CC or HCVD diets for 16 weeks. One-way ANOVA and LSD post-hoc analyses were used to compare differences between groups. No significant differences were found.

Supplementary Figure II. Correlation analysis. Correlation in *RBPKO/+* mice between AV maximum velocity measured by echocardiography and (A) total serum cholesterol and (B) LDL-cholesterol.

Supplementary Figure III. *Notch1KO/+* and *RBPKO/+* mice fed a HCVD diet display strong immunostaining of TGFβ1 and p-Smad2. Detection of the pro-inflammatory markers TGFβ1 (in green) and P-Smad2 (in red) and DAPI (blue) in aortic valves of: WT mice fed the (A, G) CC diet, or (D, J) HCVD diet; *Notch1KO/+* mice fed (B, H) CC diet, or (E, K) HCVD diet; and *RBPKO/+* mice fed the (C, I) CC diet, or (F, L) HCVD diet. Arrows indicate positively immunostained areas. (M, N) Mean data of percentage of positively stained for TGFβ1 and P-Smad2 for all groups (n=3 per group). One-way ANOVA and LSD post-hoc analysis were used to compare differences between groups. Bars with different letters mean significantly different (p<0.05) vs. all other groups (LSD post-hoc analysis). Scale bar, 0.1 mm.

Supplementary Figure IV. Aortic valves of *Notch1KO/+* and *RBPKO/+* mice fed a HCVD diet display strong immunostaining of Osterix and Osteopontin. Detection of the pro-osteogenic marker osterix (green overlapping with DAPI, light blue) and DAPI (blue) in aortic valves of: WT mice fed the (A, G) CC diet, or (D, J) HCVD diet; *Notch1KO/+* mice fed (B, H) CC diet, or (E, K) HCVD diet; and *RBPKO/+* mice fed the (C, I) CC diet, or (F, L) HCVD diet. Arrows indicate positively immunostained areas. (M, N) Mean data of percentage of positively stained for Osterix and Osteopontin for all groups (n=3 per group). One-way ANOVA and LSD post-hoc analysis were used to compare differences between groups. Bars with different letters mean significantly different (p<0.05) vs. all other groups (LSD post-hoc analysis). Scale bar, 0.2 mm.

Supplementary Figure V. Expression of Notch receptors in aortic valve cusps. (A, B) *Notch1*, (C, D) *Notch2* and (G, H) *Notch 4* mRNAs detected in the aortic valve leaflets of WT mice by *in situ* hybridization. (E, F) Immunofluorescence showing
Notch3 expression (green) in aortic ring (F, arrow), and its absence in leaflets. Nuclei are counterstained with DAPI (blue). Scale bar, 0.1 mm in (A, C, E) and 30 µm in (B, D, F).

**Supplementary Figure VI.** HCVD diets increase the total serum cholesterol and LDL-cholesterol in all mice. (A) Total cholesterol, (B) LDL and (C) HDL-cholesterol levels in WT and RBPKO/+ mice at baseline and after 16 weeks on the HCVD diet. Differences between groups and time points were detected by repeated measures ANOVA. *Significantly different from basal levels (P<0.05); Ψ, significantly different from littermates fed the CC diet (P<0.05).

**References**

Supplementary Figure I_Nus et al.
Supplementary Figure II_Nus et al
Supplementary Figure III_Nus et al
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**Supplementary Figure IV_Nus et al.**
Supplementary Figure V_Nus et al.
Supplementary Figure VI_Nus et al.