Review

〈Basic Science: Molecular Genetics〉

Notch1 Signaling and Aortic Valve Disease: From Human Genetics to Mouse Models

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Congenital heart disease (CHD) is the most common type of birth defect. Malformations involving the cardiac outflow tract and semilunar valves account for greater than 50% of CHD cases and are largely due to bicuspid aortic valve (BAV), which has a population prevalence of approximately 1%. Mutations in *NOTCH1* were linked to BAV and aortic valve calcification in humans, consistent with the expression of Notch1 in the developing and adult aortic valves. With the use of cellular and murine model systems, we have begun to elucidate the molecular mechanisms by which deficiency in Notch1 signaling results in BAV and aortic valve calcification. *In vitro*, loss of Notch signaling has been shown to contribute to aortic valve calcification via multiple pathways, including Runx2, Sox9, and Bmp2, and appear to be responsive to endothelial nitric oxide signaling during the calcific process. We previously reported a highly penetrant model of dysplastic aortic valves with aortopathy in *Notch1* haploinsufficient adult mice backcrossed into a *Nos3-null* background. Analysis of *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ compound mutant embryos has demonstrated a spectrum of congenital anomalies involving both the left and right ventricular outflow tract; these phenotypes are the result of loss of Notch1 in endothelial and endothelial-derived cells. These congenital cardiac phenotypes are strikingly similar to the recently published *NOTCH1* mutations in families with malformations in both left and right ventricular outflow tracts. Thoracic aortic aneurysms are also associated with BAV in humans; echocardiographic analysis of *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ adult mice revealed an early evidence of ascending aortic aortopathy. In summary, discovery of *NOTCH1* mutations as a cause of aortic valve disease in humans has been supported by the development of cellular and mouse models, which are now beginning to shed light on the underlying mechanisms of BAV and its associated diseases of calcification and aortopathy.

Keywords: congenital heart disease, genetics, bicuspid aortic valve, aortic valve calcification, *NOTCH1*

Introduction

Each year, an estimated eight million infants are born with birth defects,[1)] the most common of which are malformations of the heart. In fact, the reported incidence of congenital heart disease (CHD) has ranged from 6 to 19 per 1000 live births.[2)] Although CHD in children has been addressed by significant improvements in medical and surgical management, it remains a leading cause of mortality in infants less than one year of age.[3)] Advances have led to a growing population of adult survivors with CHD, but significant long-term morbidity and increased risk of having a child with CHD remain common.[4)] Accordingly, it is becoming increasingly important to identify the etiology of CHD, which has remained elusive in the majority of cases. Environmental or non-genetic factors, including infectious agents and teratogens, have long been recognized, but these appear to play a causative role only in a minority of CHD cases.

In light of the tremendous technological advances in human genome analysis, the contribution of genetic etiologic factors in CHD has been an active area of investigation over the past decade. Numerous etiologic and contributing genes to CHD have been identified using

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traditional linkage analysis and candidate gene sequencing approaches; more recently, array-based methodologies or whole-exome/genome sequencing are used.[5)]

Bicuspid aortic valve (BAV), which is characterized by fusion or improper separation of the two leaflets of a normal tricuspid aortic valve, is the most common type of congenital cardiac malformation, with 1 to 2% prevalence.[6)] BAV is often asymptomatic during childhood, but may eventually lead to valve dysfunction with stenosis or regurgitation due to improper opening or closing, respectively. In patients with BAV, significant morbidity is often observed during adulthood because of its association with aortic valve calcification and ascending aortic aneurysm. With valve calcification, the aortic valve opening is narrowed due to mineralization and subsequent stiffness.[7)] This narrowing restricts the aortic valve from opening fully and obstructs blood flow from the heart to the rest of the body through the aorta.

Although the precise etiology of BAV is not completely clear, extensive family-based linkage and association studies have demonstrated a strong genetic component. *NOTCH1* has been identified as a genetic contributor to the development of BAV and its associated calcific aortic valve disease (CAVD).[8)] In this review, we discussed how detection of *NOTCH1* mutations in affected family members with aortic valve disease has led to important insights into the mechanisms of valve calcification. We described a novel mouse model of *Notch1* mutation associated with BAV along with a spectrum of cardiac outflow tract malformations and aortopathy.

Notch Signaling Pathway

Notch encodes a family of transmembrane receptors that are part of an evolutionarily conserved signaling pathway that controls the cellular response to intrinsic or extrinsic cues, which are necessary for developmental programs. This pathway plays a crucial role on cell fate decisions and regulates cellular differentiation, proliferation, and apoptosis.[9–11)] The Notch receptors are single-span transmembrane proteins, which are matured by cleavage in the Golgi apparatus and are transported to the cell membrane. Signaling is initiated by a membrane-bound ligand from neighboring cells upon binding to the Notch extracellular domain. This receptor–ligand interaction induces a series of proteolytic cleavages and results in the release of the Notch intracellular domain, which translocates to the cell nucleus and regulates downstream gene expression.[12)] A family of four Notch receptors (NOTCH1 to NOTCH4) has been reported in mammals; members of the Delta-like (DLL1, DLL3, DLL4) and Jagged (JAG1, JAG2) families serve as ligands for Notch signaling receptors.[10–12)] Among these Notch family members, *NOTCH1*, *NOTCH2*, *NOTCH3*, and *JAGGED1* have been found to harbor mutations that could be associated with cardiovascular disease. Here, we will focus on *NOTCH1* and aortic valve disease.

NOTCH1 Mutations and Aortic Valve Disease

Genetic linkage analysis of a family with multiple generations identified a novel *NOTCH1* mutation in the affected family members who had aortic valve malformations, calcification, and aortopathy.[8)] Among the 11 family members affected, 7 developed aortic valve stenosis secondary to calcification, including 4 individuals who required surgical valve replacement; 6 of these 11 affected family members also had BAV. A genome-wide screening of the available family members revealed linkage to a single locus on chromosome 9q34 and further sequencing identified a mutation in the causative gene, *NOTCH1*.[8)] A C-to-T transition at genomic position 3322 of the *NOTCH1* gene was identified in these individuals; this mutation predicts a premature stop codon in the extracellular domain (N-terminal) at amino acid position 1108. All affected family members who were clinically evaluated had this heterozygous mutation, suggesting an autosomal-dominant inheritance with complete penetrance of this disease phenotype. In addition, evaluation of a smaller family with three individuals affected with BAV, aortic valve calcification, and aortopathy detected a single base pair deletion at position 4515 in *NOTCH1*, which predicted a novel frameshift mutation (H1505del). Subsequent studies on smaller unrelated families have also found missense *NOTCH1* mutations in sporadic cases of BAV.[13, 14)] Additional work has identified inherited missense *NOTCH1* mutations in BAV and other malformations affecting the left side of the heart; these findings were supplemented by *in vitro* mechanistic studies that indicated improper Notch1 maturation as the cause of deregulated Notch signaling.[15, 16)] Together, these reports implied a genotype–phenotype correlation between haploinsufficiency of *NOTCH1* and BAV.

Mouse models are frequently used to better understand the *in vivo* functional effects of disease-causing

gene mutations in humans. In a study on mice harboring a null allele for *Notch1*, *Notch1* heterozygous mice were found to be phenotypically normal, whereas mice homozygous for the *Notch1*-null allele before embryonic day 10.5 (E10.5) demonstrated embryonic lethality due to vascular defects prior to cardiac valvulogenesis.[17, 18] In addition, homozygous mutations in *Jagged1* and *Delta-like 1* in mice were shown to cause embryonic death around E10.5 and manifested with defects in remodeling of the embryonic and yolk sac vasculature.[19, 20] Therefore, the Notch1 signaling components are an absolute requirement during early and late embryonic development.[21] During development, expression of Notch1 has been previously demonstrated not only in the endocardium, but also in the outflow tract cushions; these findings were consistent with the valve phenotypes seen in the affected family members of one study.[8] In addition, Notch1 mRNA transcripts were found to be present in the adult murine aortic valve leaflet endothelium, as well as in the interstitium.[22] Altogether, these observations suggested that Notch1 signaling is important for aortic valve formation and may play a role in the normal function of the adult aortic valve.

Notch1 and Aortic Valve Calcification

CAVD is a late-onset cardiac phenotype that affects approximately 2 to 3% of the population by 65 years of age.[23] In this condition, calcium depositions are found on the aortic valve cusps and can eventually cause stiffness and narrowing of the valve, resulting in reduced blood flow through the aortic valve. Aortic valve calcification has been reported to be not a degenerative process, but an active regulated cellular process that leads to an osteoblast-like phenotype.[24] In healthy individuals, valve cusps are composed of extracellular matrix (ECM) interspersed with valve interstitial cells (VIC) encapsulated by a single layer of valve endothelial cells (VEC). ECM provides the biomechanical forces to withstand the oscillating hemodynamic environment around the valve during each cardiac cycle.[25] VICs are quiescent and regulate the ECM turnover in healthy valves, but become activated in calcific conditions by expressing genes that are important for bone formation, including Runx2, suggesting osteoblast-like differentiation.[8, 26] In healthy valves, the endothelium functions as a physical barrier and communicates with the underlying VICs. Conditions leading to calcification, including ECM degradation, aberrant matrix deposition and fibrosis, inflammatory cell infiltration, lipid accumulation, and neoangiogenesis, have been reported in the setting of valve injury.[27] Published reports suggested that calcification involves the coordinated actions of VECs, VICs, circulating inflammatory and immune cells, and bone marrow-derived cells. Although the molecular signaling pathways through which VECs communicate with VICs are not well understood, the role of Notch1 was speculated from the results of the above-mentioned studies, which showed that affected individuals with *NOTCH1* mutations also developed calcification.[8]

Loss of NOTCH1 expression has been demonstrated in close proximity to calcific nodules in human aortic valves.[22] An *in vitro* calcification model using porcine aortic valve interstitial cells (pAVICs) previously showed that inhibition of Notch1 activity resulted in accelerated calcification and that addition of Sox9 was able to prevent calcification.[28, 29] Expression of *Sox9*, a transcription factor involved in the regulation of the genes for chondrogenesis, has been shown to correlate with the Notch target gene *Hes1* and the other Notch pathway components.[28] Two independent studies demonstrated that Notch1 signaling directly regulated Sox9 expression through an Sox9 promoter binding site and that Sox9 participated in Notch1-induced cell motility, cell invasion, and loss of E-cadherin expression.[28, 30] In addition, Notch1 can regulate the expression of the transcription factor, Runx2, which is important for osteoblastic differentiation and skeletal morphogenesis.[8, 26] *In vitro* overexpression of *Notch1* was shown to upregulate the Notch signaling downstream targets, *Hey1* and *Hey2*, and to subsequently repress Runx2 through physical interaction independent of histone deacetylase activity.[8] On the other hand, *in vivo* studies showed that haploinsufficient *Notch1* mice developed aortic valve calcification with aging and that knockdown of Bmp2, a downstream target of Notch1, prevented the calcification induced by Notch inhibition in *in vitro* studies.[29] A recent study using endothelial cells (ECs) derived from human pluripotent stem cells demonstrated that the *NOTCH1* mutations found in the families with aortic valve disease disrupted the epigenetic architecture, resulting in activation of pro-osteogenic and inflammatory gene networks.[31] The authors of this study identified transcriptional nodes in these gene networks controlled by SOX7,

TCF4 (a Wnt signaling effector), and SMAD1 (a BMP signaling effector) as those that were mostly affected in the *NOTCH1* haploinsufficient model.

All these studies implied that active Notch1 signaling is necessary to suppress the osteogenic and inflammatory gene networks, as well as calcification, in the aortic valve. However, the impaired signaling network involving Notch1 and through which VECs communicate with VICs during calcification remains clear. Studies using co-culture and transwell culture of pAVICs with human umbilical vein endothelial cells demonstrated reduced calcific nodules in presence of ECs and highlighted the role of secretory molecules from ECs in preventing calcification.[32] Nitric oxide (NO) is one of the well-established signaling molecules produced and secreted by ECs. The inhibitory role of NO has been reported in TGF-β1-induced calcification in pAVICs.[33] A separate study on stenotic human aortic valve tissue indicated uncoupling of the nitric oxide synthase (NOS) enzyme due to increased oxidative stress in the calcific region.[34] Our group demonstrated that inhibition of endothelial NOS (Nos3) by L-NAME induced spontaneous calcification of pAVICs with reduced Notch1 nuclear localization.[32] Addition of an NO donor in similar experimental conditions reduced the number of calcific nodules and increased Notch1 nuclear localization. Although these cellular models have suggested that NO signaling regulates Notch1 signaling, the molecular mechanism underlying these observations remains to be elucidated.

Mouse Model of BAV with Associated Calcification and Aortopathy

Based on the above observations regarding endothelial NO and the Notch1 signaling pathways, mice harboring mutations in both endothelial NO synthase (Nos3) and Notch1 were generated. *Notch1*$^{+/-}$ mice did not demonstrate embryonic lethality, but ~65% of neonatal lethality was observed in *Notch1* heterozygous mice with global deletion of *Nos3*.[32, 35] BAV was found in almost 100% of surviving adult (2–4 months of age) *Notch1*$^{+/-}$ mice with an *Nos3*$^{-/-}$ background (*Notch1*$^{+/-}$ *;Nos3*$^{-/-}$); this incidence was significantly greater than the 25 to 30% incidence of BAV in *Nos3*$^{-/-}$ mice.[36] Cumulatively, these *in vivo* experiments indicated a strong genetic interaction between *Nos3* and *Notch1* and showed that *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ adult mice could serve as a murine model for BAV. Detailed characterization of the embryonic hearts of a significant percentage of *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ mice that suffered from neonatal lethality revealed a spectrum of cardiac malformations, including thickened aortic and pulmonary valves, ventricular septal defects, and overriding aorta.[35] Endothelial-specific deletion of *Notch1* using Tie2-Cre also recapitulated the cardiac phenotypes of global *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ mutants. These observations clearly demonstrated the role of endothelial Notch1 in outflow tract remodeling and semilunar valve development.

Although these *in vivo* murine studies utilizing *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ mice demonstrated the importance of endothelial Notch1 in multiple aspects of cardiac outflow tract (OFT) development, the applicability of this model to the phenotype found in humans with *NOTCH1* mutations (i.e., predominantly left-sided cardiac defects) remains questionable.[8] Interestingly, repeat examination of the published data from the family with CHD and *NOTCH1* mutation showed an individual with tetralogy of Fallot (TOF). This finding was consistent with the phenotype observed in the *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ mice embryos. In addition, a patient-based chromosomal microarray study linked *NOTCH1* microdeletion with TOF[37] and *NOTCH1* missense (*G200R)* mutation has been identified in right-sided malformations.[38] Further work was performed on 428 probands with left-sided CHD and identified *NOTCH1* mutations in 14 individuals, 11 of which were associated with familial CHD. Interestingly, among the familial cases, nine had a family member with a right-sided cardiac outflow tract malformation (i.e., TOF and pulmonary atresia).[39]

However, these findings are not surprising, as mutations in *JAGGED1* and *NOTCH2*, which are members of the NOTCH signaling pathway, are associated with right-sided cardiac malformations in the setting of Alagille syndrome. Additionally, we have found that *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ mice manifested with evidence of early ascending aortopathy, which was reported in a subset of family members harboring mutations in *NOTCH1*[40]; however, further work is needed to define the role of Notch signaling in ascending aortopathy. Nevertheless, we surmise that the *Notch1*$^{+/-}$*;Nos3*$^{-/-}$ compound mutant mice could serve as a potential *in vivo* model to define the molecular mechanisms underlying BAV and its associated diseases.

Summary

In this review, we described the advances in our understanding of the role of Notch1 in aortic valve disease, specifically BAV and CAVD. We focused on our initial insights on the role of Notch1 in the process of valve calcification and its regulation by endothelial NO. Future studies will need to define the mechanisms by which endothelial NO activates Notch1 signaling. Additional examination of the downstream targets of Notch1 signaling is needed to discover novel therapeutics for CAVD. Generation of a mouse model that includes the cardiac developmental defects in families with *NOTCH1* mutations will be beneficial for further investigation of the cellular and molecular mechanisms of BAV disease development.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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