NOTCH Signaling in Aortic Valve Development and Calcific Aortic Valve Disease

Yidong Wang¹*, Yuan Fang¹, Pengfei Lu², Bingruo Wu² and Bin Zhou³,⁴*

¹The Institute of Cardiovascular Sciences, School of Basic Medical Sciences, Xi’an Jiaotong University Health Science Center, Xi’an, China, ²Department of Genetics, Albert Einstein College of Medicine, Bronx, NY, United States, ³Departments of Genetics, Pediatrics (Pediatric Genetic Medicine), and Medicine (Cardiology), The Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, Bronx, NY, United States, ⁴The Einstein Institute for Aging Research, Albert Einstein College of Medicine, Bronx, NY, United States

NOTCH intercellular signaling mediates the communications between adjacent cells involved in multiple biological processes essential for tissue morphogenesis and homeostasis. The NOTCH1 mutations are the first identified human genetic variants that cause congenital bicuspid aortic valve (BAV) and calcific aortic valve disease (CAVD). Genetic variants affecting other genes in the NOTCH signaling pathway may also contribute to the development of BAV and the pathogenesis of CAVD. While CAVD occurs commonly in the elderly population with tri-leaflet aortic valve, patients with BAV have a high risk of developing CAVD at a young age. This observation indicates an important role of NOTCH signaling in the postnatal homeostasis of the aortic valve, in addition to its prenatal functions during aortic valve development. Over the last decade, animal studies, especially with the mouse models, have revealed detailed information in the developmental etiology of congenital aortic valve defects. In this review, we will discuss the molecular and cellular aspects of aortic valve development and examine the embryonic pathogenesis of BAV. We will focus our discussions on the NOTCH signaling during the endocardial-to-mesenchymal transformation (EMT) and the post-EMT remodeling of the aortic valve. We will further examine the involvement of the NOTCH mutations in the postnatal development of CAVD. We will emphasize the deleterious impact of the embryonic valve defects on the homeostatic mechanisms of the adult aortic valve for the purpose of identifying the potential therapeutic targets for disease intervention.

Keywords: NOTCH, aortic valve development, calcific aortic valve disease, EMT, bicuspid aortic valve

INTRODUCTION

The aortic valve ensures the unidirectional flow through the heart to deliver the oxygenated blood and nutrients to the body through cyclic pumping (1, 2). Over an average lifetime, the heart valves open and close over a billion times (2). The constant mechanical stress causes wear and tear of the heart valves that may lead to calcific aortic valve disease (CAVD) when the valve homeostatic mechanism is exhausted by repeated damages (3, 4). CAVD is the third leading cause of heart disease, and its incidence increases with age (5–12). Aortic valve replacement remains the only
effective treatment option available for CAVD, as currently no medicine is able to prevent the disease progression (8, 9). More than half of the patients with severe CAVD who need aortic valve replacement have a bicuspid aortic valve (BAV), a congenital defect affecting ∼2% of the general population (9, 13, 14). These patients develop CAVD decades earlier than those who have a tri-leaflet aortic valve (1). These observations support that BAV-associated CAVD has a developmental origin, and its postnatal stenotic progression by calcification is triggered by external stimuli as a second hit after the primary structural defect in utero (9). In support of this view of the pathogenesis of CAVD, the developmental defect of the aortic valve can cause abnormal flow dynamics and thereby predispose the valve to endothelial dysfunction and inflammatory activation, leading to earlier calcification in adulthood (9, 15–18). Therefore, better understanding of molecular mechanisms regulating aortic valve development, as well as postnatal aortic valve homeostasis, is required for the development of new therapeutic strategies for CAVD.

During embryogenesis, aortic valve leaflets are made up of valve endocardial cells (VECs) and valve interstitial cells (VICs), with VICs arising from VECs, cardiac neural crest cells (NCCs), or the second heart field (SHF) cardiomyocytes (19–25). Aortic valve development consists of multiple morphological and cellular events that are spatiotemporally coordinated by complex molecular signal networks (1). In the past two decades, researchers have made significant advance in understanding these developmental events and underlying molecular signaling mechanisms by using the experimental approaches including human genetics, animal models, and in vitro cell systems. Among the important discoveries, the NOTCH signaling is recognized as an essential molecular regulator of aortic valve development, which plays critical roles at multiple phases of valvulogenesis (26–31). Furthermore, the NOTCH1 mutations are the first genetic variants identified for congenital BAV and acquired CAVD in human (32). Therefore, we will focus our review on the findings of the roles of NOTCH signaling in aortic valve development and embryonic pathogenesis of BAV, as well as their implications in the postnatal valve homeostasis and CAVD.

MORPHOGENESIS OF THE MAMMALIAN HEART VALVE

The mammalian heart has four valves including atrioventricular (AV) valves and semilunar valves. The AV valves consist of mitral and tricuspid valve, which connects the left or right atrium to the left or right ventricle, and control the blood flow from the atria entering the ventricles (1). The semilunar valves include the aortic and pulmonary valve, which direct the blood flow from the left or right ventricle into the systemic or pulmonary circulation through the aorta or pulmonary artery (1). In all the heart valves, the mature valve leaflets are made up of the surface VECs and the core VICs, which are embedded in the well-organized extracellular matrix (ECM) (4, 8).

During mouse embryogenesis and at embryonic day 7.5 (E7.5), a subset of mesodermal cells located at the anterior part of the primitive streak migrates to the splanchnic mesoderm and forms the cardiac crescent consisting of the first heart field (FHF) and SHF (23–25, 33, 34). At E8.5, the FHF-derived progenitors fuse at the midline of the embryo and become the primitive heart tube, which comprises an outer layer myocardium, an inner layer endocardium, and a sandwiched ECM-rich space, named cardiac jelly (35, 36). The primitive heart tube elongates by adding cells from the SHF to its both ends while simultaneously undergoing rightward looping to form an S-shape primitive heart consisting of the common atrium, AV canal (AVC), common ventricle, and outflow tract (OFT) (37–40). Around E9.5, heart valve development starts with the formation of endocardial cushions within the AVC and OFT regions through endocardial-to-mesenchymal transformation (EMT) (25, 41–43) (Figure 1). EMT is regulated by molecular signals from both myocardium and endocardium. In response to the bone morphogenetic protein (BMP)/transforming growth factor β (TGF-β) signals from the myocardium, a subset of VECs begins to lose their surface markers of endothelial cells and gain the characteristics of mesenchymal cells (44–47). The transformed VECs then migrate into the cardiac jelly and proliferate to form the VICs of endocardial cushions (47). The cellularized endocardial cushions function as valve primordia, which are subjected to complex remodeling through spatiotemporally regulated proliferation and apoptosis of VICs, as well as tightly controlled synthesis and organization of ECM (41, 42). The remodeling process continues until birth when the cellularized endocardial cushions eventually become the mature heart valves with thin leaflets.

Lineage tracing studies show that the majority of VICs in AV valves are from the endocardial precursor cells through EMT (20). In contrast, VICs in the semilunar valves are from three distinct populations including VECs, NCCs, and the SHF-derived cardiomyocytes (20). Regarding the aortic valve, genetic lineage tracing of NCCs and VECs using Wnt1Cre and Tie2Cre, respectively, shows that VECs make the major contribution to the primordia of two coronary leaflets of the aortic valve, whereas NCCs have a minor contribution (20). In contrast, the SHF progenitors, known to contribute to the OFT myocardium and which are essential for the OFT development, have been recently shown to be the major cellular origin of the intercalated cushion mesenchyme, which gives rise to the non-coronary leaflet of the aortic valve (19, 21, 22, 48–51). Functional studies in animal models further confirm the lineage tracing results and link the signal dysregulations in these three progenitors to various congenital aortic valve defects, that is, different BAV subtypes (21, 22, 49, 50, 52).

MOLECULAR SIGNALS UNDERLYING EARLY HEART VALVE MORPHOGENESIS

The rightward looping of the primitive heart tube leads to the formation of morphologically distinct atria, AVC, ventricles, and the OFT (36). In addition, the myocardium of these regions is already molecularly and functionally different (47, 53). The myocardium at AVC and OFT provide the crucial molecular signals that induce VECs to undergo EMT (41,
Biochemical and genetic studies in chick and mouse embryos have identified BMP/TGF-β signaling as a key inducer of EMT (54). Early studies using cultured chicken AVC tissues in a collagen gel assay have demonstrated that TGF-β2 and TGF-β3 are the main cytokines that induce VECs to undergo EMT (55, 56). However, studies in mice have found that only TGF-β2 is required for the initiation of EMT by the AVC explants using the similar collagen gel assay (56), and the Tgfβ2 or Tgfβ3 null mice have no obvious defects in the EMT process and endocardial cushions (57). In contrast, BMP2 and BMP4 are expressed exclusively by the AVC and OFT myocardium, respectively, during early valve development (58–61). Myocardial-specific disruption of Bmp2 in mice blocks EMT and results in endocardial cushion defects at AVC, suggesting BMP2 is a crucial inducer of EMT (58). Molecular analysis of Bmp2 knockout embryos by RNA in situ hybridization has revealed that loss of Bmp2 leads to decreased expression of Tgfβ2, indicating TGF-β2 acts downstream of BMP2 during EMT (58). BMP2 also promotes the expression of Snai1 and its nuclear stability through inhibiting GSK3β (62). Furthermore, T-box transcriptional factor Tbx2 is another downstream target of BMP2 and is expressed in the myocardium of AVC and OFT, but absent in the chamber myocardium (63). Molecular analysis has shown that the BMP2-TBX2 signaling axis is essential for specifying the AVC myocardium by repressing the chamber myocardium gene program (54, 63). Consistently, loss of Tbx2 in mice results in EMT and endocardial cushion defects at AVC and OFT, whereas overexpression of Tbx2 induces ectopic expression of Tgfβ2 in the chamber myocardium and ectopic EMT by the chamber endocardial cells (63, 64). Together, the findings of these studies demonstrate local myocardial signals in AVC and OFT that dedicate the cell fate of VECs during EMT to cellularize the endocardial cushions as early valve primordia.

### THE NOTCH SIGNALING PATHWAY

In addition to the aforementioned molecular signals from the AVC and OFT myocardium, others and we have studied the functions of NOTCH signaling in heart valve development. The NOTCH signaling pathway is highly conserved across species from zebrasises to humans (26, 27, 65). It provides a major intercellular signaling mechanism that mediates the crosstalk between adjacent cells. The NOTCH pathway consists of the NOTCH ligands and receptors that are both single-pass transmembrane proteins at the cell surface (Figure 2). Mammals have four NOTCH receptors (NOTCH1-4) and five ligands that belong to two families: Serrate/Jagged families (JAG1,2) and Delta (DLL1,3,4) (26, 27, 65). A NOTCH receptor has a large extracellular matrix (ECM) arrangement. The AVC and OFT cushions eventually give rise to the atrioventricular valves (mitral and tricuspid valve) and semilunar valves (aortic and pulmonary valve), respectively. VECs, valve endocardial cells.

![Figure 1](image128x549 to 468x710)
The NOTCH signaling plays critical roles in early cardiomyocyte differentiation, ventricular trabeculation, cardiac valve formation, OFT development, and angiogenesis, as well as cardiovascular homeostasis. In the cardiovascular system, the NOTCH signaling is associated with congenital heart disease and several adult cardiovascular diseases including atherosclerosis, hypertension, and CAVD.

**NOTCH SIGNALING IN EMT AND ENDOCARDIAL CUSHION FORMATION**

The NOTCH signaling in the endocardium is critical for the initiation of EMT and endocardial cushion formation. Several NOTCH pathway genes (Dll4, Jag1, Notch1, Notch4, Hey1, and Hey2) are expressed in the endocardium within AVC and OFT regions (i.e., VECs) between E9.0 and E11.5 when EMT takes place. Genetic disruption of Notch1 or Rbpj severely blocks EMT and results in hypocellular endocardial cushions in mice. A similar phenotype was observed in mice with deletion of Jag1 using either Cdh5Cre or Nfatc1Cre driver, suggesting Jag1 is a key ligand inducing the NOTCH signaling during EMT and endocardial cushion formation. In contrast, a recent study using Tie2Cre driver demonstrated thatDll4 but not Jag1 is the key ligand in this process. This discrepancy might be due to different genetic backgrounds and/or Cre drivers used in these studies. In addition, the endothelial specific disruption of Mib1, an upstream regulator of the NOTCH signaling through modulating the ligand activity, recapitulated the EMT defect observed in the Notch1 mutant mice. Together, these studies demonstrated the role of Jag1 in EMT and endocardial cushion formation.

**ENDOCARDIAL CUSHION FORMATION**

The activation of the NOTCH signaling regulates multiple cellular functions including fate specification, differentiation, proliferation, apoptosis, and migration during embryogenesis and in adulthood. Studies using animal models have led to the identification of many critical roles of the NOTCH signaling in coordinating organ development and maintaining tissue homeostasis. In the cardiovascular system, the NOTCH signaling plays critical roles in early cardiomyocyte differentiation, ventricular trabeculation, cardiac valve formation, OFT development, and angiogenesis, as well as cardiovascular homeostasis. The dysregulation of the NOTCH signaling is associated with congenital heart disease and several adult cardiovascular diseases including atherosclerosis, hypertension, and CAVD. Next, we will focus our discussion on the roles of the NOTCH signaling in aortic valve development and CAVD.
signaling in the SHF derivatives also critically controls EMT in OFT through interaction with the FGF8-BMP4 signaling (95).

In addition to its VEC autonomous function, the NOTCH1 signaling in VECs also interacts with the signals from the myocardium to regulate EMT during endocardial cushion formation (59, 62). It is noted that while ventricular endocardial cells with ectopic activation of the NOTCH1 signaling acquire an EMT gene program, they are unable to migrate away from the cultured ventricular explants, but invasion can be triggered after additional BMP2 treatment (62). These observations suggest a synergic function between endocardial NOTCH and myocardial BMP signaling in promoting EMT. On the other hand, loss of myocardial Bmp2 inhibits the expression of Notch1 in VECs and EMT (58), whereas inhibition of the NOTCH signaling attenuates the invasive phenotype induced by BMP2 in cultured ventricular explants (62). To reveal the endocardial specific functions of the NOTCH1 signaling, we deleted Notch1 in the endocardial cells of the developing mouse heart by using an Nfatc1 Cre driver (59). In contrast to the germline Notch1 null mutants, which have severe overall vascular defects that affect early cardiac morphogenesis (76, 80), our Notch1 conditional knockout embryos develop normal vascular networks, thereby allowing us to specifically dissect the endocardial-specific roles of NOTCH1 signaling in cardiac development (59). Our studies confirmed that deletion of Notch1 in the endocardial cells impairs EMT and results in hypocellular endocardial cushions. Furthermore, we show that endocardial NOTCH1 is required for maintaining the myocardial Bmp2 expression through WNT4-mediated paracrine regulation (59). Our studies demonstrate a NOTCH–WNT–BMP signal axis as a key crosstalk mechanism between endocardium and myocardium essential for EMT and early heart valve development.

As discussed above, mouse embryos with conditional knockout of myocardial Bmp2 exhibit decreased expression of Notch1 in the endocardial cells (58). This observation further supports a crosstalk between myocardial BMP and endocardial NOTCH signaling during EMT and endocardial cushion formation. Interestingly, the expression pattern of Hey1 and Hey2, two well-known targets of the NOTCH signaling, is complementary to Bmp2 (and Tbx2) (96–98). Deletion of Hey1 and Hey2 simultaneously results in the expansion of AVC myocardium evidenced by the extended expression of Bmp2 to the atrial myocardium (97, 98). While Hey1 and Hey2 are normally expressed by the atrial and ventricular chamber myocardium, respectively, and absent in the AVC myocardium, their chamber expression can be suppressed by the ectopic expression of Tbx2 (97, 98). In contrast, overexpression of NICD, Hey1, or Hey2 in the chicken heart inhibits the expression of Bmp2 in AVC (62). These findings support that this negative feedback loop between the endocardial NOTCH and myocardial BMP signaling defines the regional gene program in the chamber and non-chamber myocardium, which regulates the cell fates of VECs during EMT and early heart valve formation (98, 99).

**NOTCH SIGNALING IN AORTIC VALVE REMODELING AND EMBRYONIC PATHOGENESIS OF BAV**

After EMT, the cellularized endocardial cushions undergo a complex remodeling process and eventually give rise to the valve leaflets and septa, which divide the heart into four chambers in mammals (Figure 3B). The remodeling process is coordinated by multiple cell populations, including VECs and VICs of different
embryonic origins in VECs, NCCs, and SHF. Signals from these cells precisely control the spatiotemporal cell proliferation and apoptosis, as well as dynamic ECM production and organization, which together ensure the proper transformation from the blunted bulky valve primordia into the elongated thin valve leaflets (1, 100). Alterations of these signals can cause congenital heart defects. For example, mutations in the NOTCH pathway genes have been identified to be associated with congenital heart defects. In particular, the NOTCH1 mutations are among the first genetic variants identified for familial BAV in non-syndromic autosomal-dominant human patients (32). Different NOTCH1 mutations have been observed in BAV patients by several groups (101–104). Furthermore, mutations in Jag1 or Notch2 cause Alagille syndrome (AGS), which has defects in multiple organs including the heart valves, and mutations in Jag1 or Notch1 have also been identified for a subset of tetralogy of Fallot patients (81–83).

Gene expression studies with RNA in situ hybridization and immunostaining have found that Notch1 and Notch4 are expressed in the endothelial cells, whereas Notch2 and Notch3 are present in the smooth muscle cells of the aorta and the pulmonary artery. Jag1 is broadly expressed in the endothelium, the NCC-derived mesenchyme, and the OFT myocardium (79, 95). Inhibition of NOTCH signaling by overexpressing a dominant-negative version of MAML (MNMAML) specifically in the cardiac NCC lineage using the Pax3Cre or Wnt1Cre driver in mice causes defects in aortic arch patterning and ventricular septum and pulmonary artery stenosis, which reminisce many OFT defects seen in AGS (79, 95). Analysis of the mutant mouse embryos indicates that loss of NOTCH in the cardiac NCC lineage impairs the differentiation of NCC precursors into smooth muscle cells (79), whereas it has no effect on cell proliferation or migration. In addition, the deletion of Jag1 or the inhibition of NOTCH in the SHF lineage results in defects in the aortic arch and ventricular septum, as well as thickened semilunar valve leaflets where the valve defect is attributed to the decreased cell apoptosis and disorganized ECM (21). Similar defects are present in the mice with the endothelial specific deletion of Jag1 (93). Furthermore, genetic interactions between the NOTCH pathway genes and other vascular essential genes are observed in the mouse models of cardiac OFT malformations including BAV. For instance, the study of the compound heterozygous mice for Notch1 and Nos3 has suggested that genetic interactions between the NOTCH and endothelial nitric oxide synthase (eNOS) signaling pathways are essential for aortic valve remodeling (105).

Pan-endothelial deletion of NOTCH pathway genes using Tie1Cre or Tie2Cre as a driver in mice causes severe vascular defects that result in embryonic lethality at the middle gestation (80), excluding the investigation of the functions and mechanisms of NOTCH signaling in regulation of aortic valve remodeling after EMT. In addition, Cre drivers including the endocardial specific Cre Nfatc1Cre that mediate gene deletion before EMT disrupt genes in VECs and their daughter VICs, thereby complicating the phenotypic interpretation and molecular signaling identification specific to the post-EMT valve remodeling. To overcome these limitations and specifically study the roles of molecular signals in VECs during aortic valve remodeling, we have developed a new Cre driver termed Nfatc1enCre, in which the expression of Cre is under the control of an Nfatc1 gene enhancer (106, 107). Functional validation shows that the Nfatc1enCre mice activate the LacZ reporter gene expression specifically in VECs, but not in VICs, beginning around E11.0 (106). We have then generated mouse models with VEC-specific deletion of Notch1 or Rbpj. In contrast to the early lethal phenotype observed in the pan-endothelial Notch1 or Rbpj knockout mice, our VEC-specific Notch1 mutant mice can survive into adulthood, thereby allowing us to study the role of NOTCH1 signaling in valve remodeling. We have found by echocardiography that the adult VEC Notch1 conditional knockout mice display increased peak velocity across the aortic valve, indicating a phenotype of aortic valve stenosis (52). Histological and pathological analyses have further indicated that the aortic valves of the mutant mice are thickened, fibrotic, and calcified. In the most severe cases, the cartilage-like structures are present in the leaflets and aortic roots (52). Significantly, some of the mutant mice have developed BAV due to the fusion of the two coronary leaflets (52). These findings demonstrate that the VEC-specific Notch1 knockout mice recapitulate the common BAV seen in CAVD patients associated with the NOTCH1 mutations. Thus, the new mouse model offers an excellent opportunity to study the developing mechanism of BAV caused by the NOTCH1 mutations.

BAV affects ∼2% of general populations and is the most common congenital heart malformation (2, 13, 100, 108, 109). It is associated with a high incidence of CAVD and other cardiac complications including aortic dilation, aneurism, and dissection, which can lead to sudden death (8, 9, 110). A BAV may occur isolated or present in a complex syndrome such as Turner, DiGeorge, or Shone syndrome (111, 112). BAV can be caused by the fusion of two of three leaflets or the absence of any one leaflet and the fusion of the right and left leaflet (R–L) is the most common type present in ∼70% of all the BAV patients (100). Studies using pig (113), hamster (114–117), and mice models (52, 118) have demonstrated that BAV arises from abnormal aortic valve development. As discussed previously, the NOTCH1 mutations are associated with the common R–L type of BAV in humans (32). In our mouse studies, we find that the VEC-specific deletion of Notch1 results in BAV due to the fusion of the left and right coronary leaflet during the early post-EMT remodeling of the aortic valve. At the cellular level, loss of Notch1 in VEC causes increased proliferation but decreased apoptosis of the VICs (Figure 3B). In contrast, overactivation of NOTCH in VECs results in decreased proliferation and increased apoptosis of VICs, leading to the hypoplastic valves (52). At the molecular level, we show that NOTCH1 expressed in VECs promotes the expression of Tnfa, which subsequently induces the apoptosis of VICs. Of note, similar aortic valve remodeling defect has been found by another study using the same Cre driver to delete Jag1, Notch1, or Rbpj, indicating that the JAG1–NOTCH1 signaling in VECs is required for aortic valve remodeling (78). In this study, loss of NOTCH1 signaling in VECs leads to increased proliferation of VICs accompanied by decreased expression of Hbegf, which is a negative regulator of proliferation through restricting
BMP signaling (119). Further molecular studies demonstrate that Hbegf is a direct NOTCH target gene in VECs for the proliferation of VICs, as the supplement of soluble HBEFG to the cultured OFT explants from the jag1 mutant embryos can rescue the hyperproliferative phenotype (78). These findings support a dual function of the NOTCH1-regulated signaling from VECs in the apoptosis and proliferation of VICs through tumor necrosis factor α and HBEFG, respectively (Figure 3B). These findings are also consistent with the fact that VICs in front of a growing leaflet are highly proliferative, whereas those on the rear side are more likely apoptotic (52). Thus, the NOTCH1 signaling is a critical determinant that controls the balanced proliferation and apoptosis of VICs, which sculpts the endocardial cushions into the thin valve leaflets.

NOTCH SIGNALING IN AORTIC VALVE HOMEOSTASIS AND CALCIFICATION

The aortic valve leaflets, unique tissue microarchitecture with endurant and flexible characteristics essential for a lifelong cyclic function, consist of three layers including fibrosa, spongiosa, and ventricularis (9). The fibrosa layer is located at the aortic side and made up of collagen-rich matrix, VICs, and VECs facing the aortic wall. The spongiosa is the middle layer containing VICs embedded in glycosaminoglycan-rich matrix. The ventricularis layer is rich in elastin fibers and consists of VICs and surface VECs facing the left ventricle (4). VECs on the surface of a leaflet form a barrier that regulate the valve permeability, the adhesion of inflammatory cells, and paracrine or signaling including mechanosensory signals from VECs to VICs (9). VICs are the major cell population in a leaflet, producing ECM and controlling the ECM organization during valve remodeling. In the adult valve, VICs are quiescent under the physiological condition but become activated upon the stimulation by molecular and mechanical signals including the high blood pressure, altered hemodynamics, and inflammatory molecules (9). The activated VICs then undergo transdifferentiation into synthetic myofibroblast and produce many ECM proteins, resulting in pathological ECM remodeling, which makes the valve prone to calcification (5–8).

There is no cure for advanced CAVD patients, except for valve replacement. However, because of high risks of the surgery for the elderly patients and postoperative complications with anticoagulant medicines, the surgical treatment is not suitable for all CAVD patients. Previously considered as a passive consequence of aging (7), CAVD is now recognized as an active disease driven by chronic inflammation (120). Molecular signaling pathways underlying inflammation are thus potential targets for the disease diagnosis and therapy. Signal molecules include eNOS (121), renin–angiotensin system (122–124), NOTCH (89, 90), WNT/β-catenin (122), BMP/TGF-β signaling (122), RANK/RANKL pathway, vitamin K–dependent proteins (125), and microRNAs (9, 126). Here we focus on the NOTCH signaling, whereas the other pathways and molecules are reviewed in recent articles (9, 127).

Since the initial identification of two NOTCH1 mutations associated with BAV and CAVD by Garg et al. (32), additional NOTCH1 mutations have been identified in BAV patients by several groups around the world (101, 103, 104). In addition, genome-wide association study has identified several genetic variants in GATA4/5/6 genes associated with BAV patients (128–130), whereas the targeted disruption of Gata5 in mice leads to BAV accompanying impaired NOTCH signaling (128, 131, 132). The studies using animal and tissue culture models have further explored the underlying molecular mechanisms. Studies with RNA in situ hybridization show that murine Notch1 mRNA is abundant in VECs and VICs immediately underneath VECs between E11.5 and E13.5 when the intensive valve remodeling is undergoing (32). A similar expression pattern is observed in the adult aortic valves by using the same method. However, antibody staining shows the NOTCH1 protein expression in VECs, but not VICs (52, 78, 89, 92). By using a transgenic mouse line that reports all NOTCH activities via the consensus RBPJ binding sites, we have shown that the NOTCH signaling is highly activated in VECs but absent in VICs during aortic valve development (52). In contrast, in the adult aortic valves, the NOTCH activities are greatly reduced in VECs, whereas some VICs display the NOTCH signals (52). In addition, a reduction of nuclear NOTCH1 activity is seen in the calcified fibrosa when comparing to the non-calcified regions of the diseased valves (90, 133). Together, the dynamic expression pattern of NOTCH1 in the aortic valve indicates potential dual functions in prenatal aortic valve remodeling vs. postnatal homeostasis.

Indeed, the functional studies using mouse models, cultured aortic VICs, and stem cell–derived endothelial cells have collectively revealed the critical roles and mechanisms of NOTCH1 in the regulation of valve calcification. Western diet can induce a 5-fold more calcification in the heterozygous Notch1 null mice (89). Similarly, after 16 weeks of hyperlipidemic diet feeding, the heterozygous Rbpj mice exhibit milder histopathologic features of CAVD including macrophage infiltration, enhanced collagen deposition, pro-osteogenic protein expression, and calcification, but have no obvious hemodynamic changes (91). Endothelial deletion of jag1 results in the formation of calcific nodules in the aortic valve of adult mice (93). We have generated and studied a conditional Notch1 knockout mouse model in which Notch1 was specifically deleted in VECs, and the mutant mice display the typical phenotypes of aortic valve stenosis at a young age of 3 months (52). The mouse CAVD is associated with increased collagen and glycoproteins and calcific nodules. In the most severe case, the cartilage-like structure was seen in the valve leaflet and the annulus (52). Importantly, the mouse CAVD is proceeded with embryonic thickening of the aortic valve leaflets, with a partial penetration of BAV resulting from the fusion of two coronary leaflets resembling the most common form of BAV in humans. In addition, Nos3−/−; Notch1+/− mice develop aortic valve disease, indicating a genetic interaction between Nos3 and Notch1 in calcific progression (134, 135). In line with these in vivo findings, inhibition of NOTCH in cultured aortic VICs from sheep, rat, or pig promotes calcific nodules formation and osteogenic gene expression.
Studies using primary VICs from multiple species and endothelial cells differentiated from the human induced pluripotent stem cells (hiPSCs) of patients with the NOTCH1 mutations have begun to uncover the deep molecular mechanisms underlying the CAVD progression associated with the NOTCH1 haploinsufficiency in humans (Figure 4). The NOTCH1 signaling activates the expression of Hey1 and Hey2, which in turn represses the transcription of Runx2, a key transcriptional regulator of osteoblast cell fate (32). Bmp2 mediates the induction of osteogenic gene expression and calcification by the NOTCH inhibition (89). Genome-wide screening also finds NOTCH1 positively regulates the expression of matrix Gla protein (MGP), which is known to inhibit calcification through sequestering BMPs (136). In cultured aortic VICs, inhibition of the NOTCH signaling results in decreased expression of Sox9, and the loss of Sox9 function is associated with aortic valve calcification (90). Conversely, the supplementation of Sox9 is able to attenuate the calcification resulting from the NOTCH inhibition. These findings suggest Sox9 as another downstream mediator of the NOTCH signaling in the regulation of aortic valve calcification. In a coculture system, nitric oxide (NO) produced by endothelial cells inhibits the calcification of VICs, whereas overexpression of Notch1 can cancel out the calcification of VICs induced by the NO inhibition (134, 137). Recently, Majumdar et al. report that NO mitigates the aortic valve calcification by activating NOTCH signaling through S-nitrosylation of USP9X (137). These findings support interactions between NO and NOTCH1 in aortic valve calcification. In human studies, analysis of the blood samples from 61 patients with aortic stenosis has revealed an association between the NOTCH1 variants and the OPG/RANKL/RANK gene program (138). In addition, modeling CAVD in endothelial cells differentiated from hiPSCs by introduction of the NOTCH1 mutations from patients with BAV and aortic valve stenosis has found that the NOTCH1 haploinsufficiency leads to the alteration of epigenetic architecture, resulting in derepression of latent pro-osteogenic and proinflammatory gene networks (139). Furthermore, a critical role of the telomere shortening in the initiation of calcific program has been reported in the Notch1+/− mice (140). More recently, by using hiPSC technology and machine learning, Theodoris et al. have identified a small molecule XCT790, which is able to correct the gene network changes resulting from the NOTCH1 haploinsufficiency and to prevent the disease progression in a mouse model of CAVD (141). Although multiple observational studies and randomized controlled trials remain needed, XCT790 represents a promising drug for treating CAVD disease in clinical. Together, these current findings reveal a crucial role of NOTCH1 in aortic valve calcification, while the underlying mechanisms remain an area of intensive investigation.

CONCLUSIONS AND PERSPECTIVES

Heart valve development is a precisely controlled morphogenic process involving cell differentiation, proliferation, apoptosis, and migration, as well as ECM organization. Alteration of molecular signals that regulate this process can cause congenital heart valve defects including BAV, which may lead to CAVD with severe clinical consequences. The structural defects predispose the affected valves to calcification. By using human genetics, animal models, and tissue culture systems, clinicians and basic scientists have worked together and demonstrated the important functions of NOTCH1 in regulation of aortic valve development and CAVD.

The basic and translational research on NOTCH signaling in regulation of aortic valve development and CAVD has led to the identification of a small molecule XCT790 that can halt the disease progression in a mouse model of CAVD. This exemplifies a new avenue for effective drug discovery. However, CAVD is a disease affected by many factors, whereas NOTCH haploinsufficiency contributes to only a small subset of the inheritable cases. Future studies will be needed to broaden the research area to (1) identify additional genetic variants that cause BAV and CAVD using unbiased approaches such as next-generation sequencing, (2) decipher deeper mechanisms underlying disease progression in CAVD patients with or without BAV, and (3) reveal the shared mechanisms among
BAV-associated and acquired CAVD that will facilitate the development of a common therapeutics suitable for all patients. It is expected that combination of clinical studies using advanced next-generation sequencing for large patient cohorts and basic researches with hiPSC technology, machine learning, CRISPR-guided gene editing, and single-cell technologies will uncover new BAV and calcific genes, informing pathogenetic mechanisms of CAVD and targeted gene therapeutics for the disease.

**AUTHOR CONTRIBUTIONS**

YW and BZ wrote the manuscript. PL, BW, and YF read and critically edited the manuscript. YF drew the figures. All authors contributed to the article and approved the submitted version.

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