1. Introduction

Calcific aortic valve disease represents the predominant pathology of tricuspid (trileaflet) and bicuspid aortic valves in developed countries (Ladich et al., 2011). Accounting for approximately half of anatomically isolated aortic stenosis and 25 percent of patients with aortic regurgitation (Roberts, 1970), calcific bicuspid aortic valves requiring surgical intervention present at least two decades earlier than the tricuspid counterpart (Ward, 2000). Mechanisms important in cardiac and organ development — notably, the Notch pathway — have emerged as central players recapitulated and reused during the pathogenesis of calcific aortic valve disease, and support also a common etiology for bicuspid aortic valve and aortic valve calcification (Garg et al., 2005) (Table 1). Active engagement of inflammatory, remodeling, neovascularization and osteogenic (Aikawa et al., 2007a; Aikawa et al., 2007b; Miller et al., 2010; Rajamannan et al., 2003) pathways has conceptually replaced ‘degeneration’ in calcific aortic valve disease pathogenesis and progression (Dweck et al., 2012). Moreover, these pathways invoke similar mechanisms during cardiac morphogenesis. Dysregulated Notch activity has also been reported in vascular inflammation, macrophage activation (Fung et al., 2007), cardiometabolic disorder, and vascular and aortic valve calcification (Fukuda et al., 2012). Preclinical studies suggest that specific blockade of Notch ligand–receptor signaling potently suppresses vascular calcification and calcific aortic valve disease (Fukuda et al., 2012). In this chapter, we review the mechanisms of Notch signaling, aortic valve dysmorphology pertinent to accelerated valve calcification, and discuss the pathways involving Notch that lead to aortic valve calcification and disease.
Table 1. Major components of the Notch1-Hey-BMP2 axis and their actions in cardiac and aortic valve development, and in aortic valve calcification. BMP, bone morphogenetic protein. DLL4, Delta-like 4. JAG1, Jagged1.

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Table 1. Major components of the Notch1-Hey-BMP2 axis and their actions in cardiac and aortic valve development, and in aortic valve calcification. BMP, bone morphogenetic protein. DLL4, Delta-like 4. JAG1, Jagged1.

2. Notch signaling

The human Notch receptor family comprises four members, Notch1 through Notch4, expressed as transmembrane molecules on the cell surface of neighboring cells that enable canonical signaling in a contact-dependent manner (Bray, 2006; Kopan and Ilagan, 2009). Canonical Notch signaling describes the ‘classic’ interaction between membrane-bound receptors and ligands expressed on the surface of neighboring (signaling and receiving) cells, whereas non-canonical signaling encompasses a diverse group of structurally unrelated ligands that contribute to the pleiotropic effect of Notch signaling (Kopan and Ilagan, 2009). In mammals, five members of the Delta-Serrate-LAG-2 (DSL) family have the capacity to activate or modify canonical Notch signaling — Delta-like 1 (Dll1), Dll3, Dll4, Jagged1, and Jagged2. Interaction between Notch receptor and ligand is tightly controlled, and the signaling outcome is determined by the receptor:ligand ratio (Artavanis-Tsakonas and Muskavitch, 2010; Gibert and Simpson, 2003; Heitzler and Simpson, 1991; Wilkinson et al., 1994) that critically determines asymmetry in cell fate and development of neighboring cells. This interaction between receptor and ligand can be modified posttranslationally through Notch glycosylation by lunatic, manic and radical glycosyltransferases (Bray, 2006). The receptor:ligand ratio is dependent on the differential expression of competing ligands on neighboring cells in trans, as opposed to cis interaction through which receptor and ligand expressed on the same cell can also modulate Notch signaling. The complexity of receptor–ligand interaction is further increased by the requirement of heterodimerization of the receptor (Kopan and Ilagan, 2009). Canonical interaction between Notch receptor and ligand leads to two sequential cleavage events at site 2 (S2) and S3. S2 is a ‘permissive’ extracellular juxtamembrane cleavage by a disintegrin and metalloprotease 17 (ADAM17, known also as tumor necrosis factor-α converting enzyme/TACE) and/or ADAM10 (Artavanis-Tsakonas and Muskavitch, 2010;
Bray, 2006), whereas S3 is executed by γ-secretase, a protease with many substrates (McCarthy et al., 2009; Wakabayashi and De Strooper, 2008). S1 cleavage is carried out by a furin-like convertase occurring posttranslationally in the trans-Golgi apparatus before translocation of the nascent Notch receptor to the cell surface (Bray, 2006; Kopan and Ilagan, 2009). Following S3 cleavage, the Notch intracellular domain is liberated and enters the nucleus to form a transcriptional activation complex with the transcriptional factor RBP-Jκ, and the transcriptional coactivator Mastermind to promote target gene transcription (Bray, 2006; Kopan and Ilagan, 2009). Targets indicative of Notch activity include the basic-helix-loop-helix genes of the hairy and enhancer of split (HES) and the hairy-related (HRT or Hey) family (Bray, 2006; Kopan and Ilagan, 2009).

Functionality of Notch signaling components is highly context-dependent and conventionally requires cell-to-cell contact to specify cell fate, differentiation, growth, proliferation, survival and apoptosis (Bray, 2006; Fiuza and Arias, 2007; Guruharsha et al., 2012). Interaction between Notch receptor and ligand on adjacent cells results in asymmetric signal transduction, leading to potentially divergent cell fate decision, phenotypic development and growth (Bray, 2006; Kopan and Ilagan, 2009).

3. Congenital aortic valve disease

3.1. Notch dysfunction in aortic valve anomalies and other congenital heart diseases

Congenital aortic valve anomalies frequently associate with other abnormalities in neighboring structures, including the aortic root (e.g. dilatation, aneurysm), aorta (e.g. coarctation of aorta), ventricular outflow tract (e.g. septal defect, transposition of great vessels), and/or coronary arteries (e.g. coronary anomalies) (Perloff, 2003; Ward, 2000). The association of anomalies is due in part to the complexity and critical function of the endocardial cushion, and its formation during cardiac valve and septum development (Camenisch et al., 2010).

The tight regulation of Notch signaling during murine cardiac morphogenesis, particularly of the cardiac outflow tract and semilunar (aortic and pulmonary) valves, have been recently reviewed in detail by de la Pompa and Epstein (de la Pompa and Epstein, 2012). The evolutionarily conserved nature of Notch across mammalian species is generally recognized to be applicable to human. The highly coordinated action of Notch in progenitor cell proliferation and differentiation is instrumental during development. Earliest signs of cardiac morphogenesis occur with formation of the cardiac crescent by midline fusion of first and second heart fields that feature expression of Notch1, Dll4 and Jagged1 in the primitive endocardium (de la Pompa and Epstein, 2012; del Monte et al., 2007; Duarte et al., 2004). Continuing cell proliferation and development leads to the generation of the heart tube, consisting of an outer myocardial layer, middle cardiac jelly of extracellular matrix, and an inner endocardial endothelium (Camenisch et al., 2010; de la Pompa and Epstein, 2012). Demarcation of boundary and tissue layers is marked by expression of Jagged1 limited to the myocardial layer, and Dll4, Notch1, Notch2 and Notch4 in the endocardium. The heart tube gradually undergoes a complex morphologic change with a rightward bend, converting the anterior-posterior
polarity of the heart tube into a right-left (R-) loop. As the looped heart further develops, the valve territories of the atrioventricular canal (AVC) and outflow tract (OFT) are demarcated. The AVC and OFT cushions become the sites for formation of the mitral and aortic valves, respectively, in the left ventricle and the tricuspid and pulmonary valves in the right ventricle (Person et al., 2005). Contribution of endocardium-derived mesenchyme to the development of AVC and OFT valve primordia diverge as the neural crest contribute additionally to the development of the OFT valve primordium (de la Pompa and Epstein, 2012; Zhang et al., 2010). At this stage of development, Jagged1 expression is present in the endocardium and chamber myocardium, whereas expression of Dll4 and Notch1 localizes to the valve and atrial endocardium. Here, Notch coordinates cardiac patterning through regulation of the Notch-Hey-Bmp2 axis (MacGrogan et al., 2011). Bmp2, or bone morphogenetic protein 2, is responsible for AVC specification together with Tbx2/3, members of the T-box transcription factor family with crucial roles in cardiac development (de la Pompa and Epstein, 2012; Ma et al., 2005). Tbx2 is repressible by Tbx20, which has regulatory function in ion channel expression (Shen et al., 2011). Importantly, TBX20 nonsense and missense germ line mutations result in complex septal, chamber and valvular anomalies in human (Kirk et al., 2007). Tbx transcription factors carry strong activation and repression domains and, especially Tbx20, interact with other important cardiac developmental factors including Nkx2.5, Gata4, Gata5 and Tbx5 (Brown et al., 2005; Combs and Yutzey, 2009; Kirk et al., 2007; Plageman and Yutzey, 2005; Stennard et al., 2003). Targeted disruption of Gata5 has been demonstrated to associate with the development of bicuspid aortic valve in the mouse (Laforest et al., 2011), and one study on patients with bicuspid aortic valve found that approximately 4% had rare non-synonymous mutations within the GATA5 transcriptional activation domains (Padang et al., 2012). A functional connection between gata5 and notch1 was reported in a zebrafish study of endoderm formation (Kikuchi et al., 2004), and those findings may potentially be generalized to human, given the evolutionarily conserved nature of the Notch pathway (Artavanis-Tsakonas and Muskavitch, 2010).

Cardiac valve formation begins with myocardial cells signaling to endocardial cells in the AVC and OFT cushions to undergo epithelial-mesenchymal transformation (transition) (EMT) (de la Pompa and Epstein, 2012). Coordinated by Notch and RBP-Jκ (del Monte et al., 2007; Timmerman et al., 2004), Bmp2 instructs cushion endocardial cells to invade the extracellular matrix and become the cushion mesenchyme (Hinton and Yutzey, 2011), and acting via Snail1/2, the Notch–Hey–Bmp2/4 axis promotes EMT and subsequent completion of valve tissue development (MacGrogan et al., 2011) (Figure 1). Interference with Notch signaling results in abnormal development of the aortic valve and cardiac outflow tract as demonstrated in animal studies (de la Pompa and Epstein, 2012; Garg et al., 2005; Mohamed et al., 2006; van den Akker et al., 2012). As discussed below, BMP2 also mediates aortic valve calcification.

Bicuspid aortic valve represents one of the most common anomalies of the heart or vessels (Roberts, 1970; Roberts et al., 2012; Ward, 2000), and its association with other anomalies is well recognized. For instance, ~10% of relatives of patients with hypoplastic left heart syndrome have bicuspid aortic valve (Loffredo et al., 2004), and aortic abnormalities such as coarctation of aorta and interrupted aortic arch are present in 20–85% (Presbitero et al., 1987;
Stewart et al., 1993) and ~27% (Roberts et al., 2012) of cases, respectively. Individuals with bicuspid aortic valve consistently have dilatation of the ascending aorta (Hahn et al., 1992). As a common variation noted by several investigators (Higgins and Wexler, 1975; Hutchins et al., 1978), a higher incidence of left coronary arterial system dominance (defined by the presence of the posterior descending artery arising from the left circumflex artery, as opposed to the right coronary artery) is observed in patients with bicuspid aortic valve. The phenotypic heterogeneity and overlap suggest common developmental mechanisms and gene networks that closely interact; the extent of the interactions may vary depending on the penetrance of the mutation(s), effect size of the variants, and the interaction between genes and signaling pathway.

In a study of two unrelated families, one of which included five generations, Garg and colleagues observed mutations in \textit{NOTCH1} that segregated with aortic valve disease, particularly with bicuspid aortic valve and aortic valve calcification; but also, to a lesser extent, with tetralogy of Fallot, ventricular septal defect, mitral atresia, double-outlet right ventricle, or hypoplastic left ventricle (Garg et al., 2005). \textit{NOTCH1} is located on chromosome 9q34.3 and encodes the 2,556-amino acid transmembrane Notch1 receptor. Affected members of one of the families analyzed had autosomal dominant inheritance of a point mutation (R1108X) resulting from a C-to-T transition of nucleotide 3322. Another unrelated family analyzed had
a single base pair deletion leading to a frameshift mutation (H1505del) at position 4515. These mutations produced truncated transcripts that are believed to undergo nonsense-mediated decay, supporting haploinsufficiency of NOTCH1 in the pathogenesis of congenital heart disease (Garg et al., 2005). Of note, despite the high propensity to development of bicuspid aortic valve and other cardiac anomalies in individuals with the NOTCH1 mutation (R1108X) (Garg et al., 2005), aortic valve calcification was present even in a minority of family members with the mutation who did not have bicuspid or dysmorphic aortic valves, suggesting that the penetrance of the NOTCH1 mutation is variable (or the effects compensated for by another Notch receptor or other mechanisms), and that maldistribution of mechanical stress alone can not explain accelerated valve calcification in these individuals.

Mutations or abnormal copy number variants in the gene (JAG1) encoding Jagged1, a Notch ligand, on chromosome 20p12 can cause a range of cardiovascular anomalies (McElhinney et al., 2002; Oda et al., 1997). However, the distribution and manifestations of cardiovascular anomalies, including the frequency of bicuspid aortic valve and calcific aortic valve disease, differ considerably between the JAG1 and NOTCH1 mutations (Garg et al., 2005; McElhinney et al., 2002). Although JAG1 mutation is well recognized as a primary cause of Alagille syndrome, familial as well as ‘sporadic’ tetralogy of Fallot, among other anomalies, has been reported (Eldadah et al., 2001; Greenway et al., 2009). Tetralogy of Fallot is a syndrome that comprises ventricular septal defect, pulmonary stenosis, right ventricular hypertrophy and an overriding aorta, in association with aortic regurgitation in ~6% of patients (Abraham et al., 1979). Mutations in JAG1 have been identified in 60–75% of individuals with Alagille syndrome (Colliton et al., 2001; Li et al., 1997; Oda et al., 1997; Spinner et al., 2001), a condition characterized by cholestatic jaundice due to biliary tree anomalies, skeletal deformities, systemic vascular malformations and aneurysms (Kamath et al., 2004), and a high frequency of right-sided cardiovascular anomalies (62% of 200 patients) (McElhinney et al., 2002). In patients with left-sided anomalies (22 of 200 individuals (McElhinney et al., 2002)), a comparison of those with (n = 17) and without (n = 5) JAG1 mutation did not reveal an obvious trend favoring the distribution nor preponderance of valvular aortic stenosis, supravalvular aortic stenosis, aortic coarctation, or bicuspid aortic stenosis without stenosis (McElhinney et al., 2002). Those findings suggest that aortic valve disease, such as bicuspid aortic valve and at least moderate-severe aortic stenosis, is relatively uncommon (<5%) in patients with Alagille syndrome (McElhinney et al., 2002), and implies that JAG1 mutation per se does not predispose to aortic valve calcification in human, as evidenced by the paucity of left-sided abnormalities. Interestingly, although previous mouse studies have reported high lethality associated with endothelial-specific deletion of Jag1 (Benedito et al., 2009; High et al., 2008), one recent study demonstrated a high frequency of cardiac, great vessel, coronary, and valve defects resembling features of tetralogy of Fallot in human; and in animals, chondrogenic nodules and calcification were observed in the aortic valve (5 of 10 transgenic animals versus 0 of 10 controls) (Hofmann et al., 2012). The authors of the study postulated that murine Jag1 was essential to morphogenesis of the interventricular septum and cardiac valves, and particularly, in valve remodeling postnatally through modulation of extracellular matrix (Hofmann et al., 2012).
The complexity of gene-phenotype effects in human is highlighted by variable penetrance of JAG1 mutation (e.g. G274D missense mutation) and phenotypic expression, as demonstrated by differences in the degree of glycosylation, protein trafficking and cell-surface protein expression given the same mutation (Lu et al., 2003). This heterogeneity is reminiscent of the variable effects of NOTCH1 in the pathogenesis of bicuspid aortic valve and other cardiovascular anomalies (Garg et al., 2005), and epigenetic factors such as intracardiac fluid forces may be important contributors that couple with transcription factors to affect cardiogenesis and valve development (Hove et al., 2003; Lee et al., 2006; Vermot et al., 2009).

3.2. Aortic valve dysmorphology, bicuspid aortic valve and calcification

Anomalies of the aortic valve can be classified based on size, shape, the number of valve leaflets, cuspal inequality, nature of commissures (e.g. unicomissural, acquired fusion), and location of a false raphé if present (Perloff, 2003; Ward, 2000). Unicuspid, quadricuspid and six-cuspid aortic valves occur rarely (Perloff, 2003), and associated mutations have not been reported, unlike bicuspid aortic valves resulting from impaired Notch1 signaling (Garg et al., 2005). Unicuspid and bicuspid aortic valves often prematurely develop valve calcification at least two decades earlier than their normal trileaflet counterpart (Pachulski and Chan, 1993). Although maldistribution of mechanical stress contributes to the fibrocalcific process, additional factors apart from biomechanical forces including inflammatory and profibrotic processes direct the differentiation of valve fibroblasts into myofibroblasts and osteoblasts that promote osteogenesis (Dweck et al., 2012; Rajamannan et al., 2003).

Maldistribution of shear stress on valve cusps is thought to promote calcification of the aortic valve seen in unicuspid, bicuspid, and tricuspid aortic valve with cuspal inequality (Perloff, 2003). Bicuspid aortic valve is found in 1–2% of the general population in the United States, with a slight male predominance reported in some studies (Roberts et al., 2012; Ward, 2000). Maldistribution of diastolic force among valve cusps and sinus attachment is thought also to promote ascending aortic dilatation or aneurysm (Burks et al., 1998; Perloff, 2003; Roberts, 1970). However, it remains unclear whether these aortic manifestations are genetically determined or represent a byproduct of mechanical stress, given that aortic dilatation is indistinct among regurgitant, stenotic and functionally normal bicuspid aortic valves (Hahn et al., 1992). Emerging evidence supports increased proteolytic activity in the aortic valve and adjacent areas including the aorta that may enhance the remodeling processes (Aikawa et al., 2007b).

Valvular calcification in the early stages causes aortic sclerosis, which predicts increased risks for cardiovascular morbidity and mortality (Otto et al., 1999). As the process progresses, the aortic valve orifice narrows while the valve anatomy and function become gradually distorted to produce valvular aortic stenosis with or without regurgitation, myocardial hypertrophic response, myocardial fibrosis, heart failure, and hemodynamic instability (Dweck et al., 2012). In recent years, the concept of degeneration in the pathogenesis of calcific aortic valve disease has been superseded by that of phenotypic modulation recapitulating embryonic development, angiogenesis, acquired and innate immune activation, wound healing and bone formation (Hakuno et al., 2009).
4. Acquired aortic valve disease

4.1. Aortic valve calcification and systemic inflammation

Aortic valve sclerosis has been estimated to affect at least 20% of adults over 65 years of age in the general population (Lindroos et al., 1993; Stewart et al., 1997). Calcific aortic valve disease represents a continuum of maladapted calcification in the aortic valve arising from active inflammatory and oxidative processes (Kaden et al., 2004; New and Aikawa, 2011; Towler, 2008), as well as a shift in the valve interstitial phenotype from chondrogenic to osteogenic. Early calcification of the aortic valve leads to increased valve leaflet thickness and stiffness in a condition termed aortic valve sclerosis (Otto et al., 1999). Continuation of the inflammatory process propagates angioneogenesis and biomineralization, leading to formation of calcium nodules that distort valve geometry and function, culminating in outflow-limiting aortic stenosis with or without regurgitation (Dweck et al., 2012; Rajamannan et al., 2011). Conditions that promote systemic inflammation, such as atherosclerosis, dyslipidemia and diabetes mellitus, have been shown to exacerbate the development of calcific aortic valve disease (Rajamannan et al., 2011). While statins may stabilize atheromatous plaques, reduce vascular calcification and clinical adverse outcomes, they have unfortunately not been shown to benefit calcific aortic valve disease in disease progression or patient outcomes (Chan et al., 2010; Cowell et al., 2005; Rossebo et al., 2008).

Studies exploring Notch signaling beyond congenital disorders and developmental biology identified Dll4 in macrophage-mediated inflammation (Fung et al., 2007). Recently, Fukuda and colleagues demonstrated that blockade of Dll4-Notch signaling using anti-Dll4 monoclonal antibody decreased BMP2, a central regulator of osteogenesis and bone mineralization (Fukuda et al., 2012), in line with other studies showing reduced aortic valve calcification with BMP2 knockdown by siRNA (Nigam and Srivastava, 2009), and the proinflammatory cytokine, TNF-α, accelerated BMP2-mediated calcification of human aortic valve interstitial cells from patients with calcific aortic valve stenosis (Yu et al., 2011). BMP2 mediates aortic valve calcification via Runx2 (Osf2/Cbfa1), a transcriptional activator of osteoblast development or gene expression (Ducy et al., 1997; Kaden et al., 2004; Mohler et al., 2001), and is suppressible by activation of Notch1 via Hey (HRT) (Acharya et al., 2011; Nigam and Srivastava, 2009). Moreover, the marked attenuation of aortic valvular calcification and stenosis through the blockade of angiogenesis-promoting Dll4 in a mouse model of hypercholesterolemia (Figure 2) also supports the current theory that angioneogenesis is a crucial stage in the natural history of calcific aortic valve disease (Dweck et al., 2012), recapitulating cardiogenesis and valve development (de la Pompa and Epstein, 2012; van den Akker et al., 2012). Thus, Dll4 critically bridges inflammation and angioneogenesis to osteogenesis in calcific aortic valve disease (Fukuda et al., 2012). These effects are probably independent of Notch 1 (Nus et al., 2011), since activation of the receptor presumably leads to inhibition of valve calcification (Acharya et al., 2011), whereas evidence on the benefits of Dll4 blockade (i.e. interruption of Dll4–Notch signaling) suggests that a Notch receptor other than Notch1, when activated, potentiates the development and progression of valve calcification. A shift in the Notch receptor:ligand ratio
and/or the DLL:Jagged (Notch ligands) ratio may plausibly alter the cell-to-cell signalling strength and modality *in cis* and/or *in trans*, thus, modifying the final functional outcome. Much work remains to be done to fully delineate the mechanisms through which anti-Dll4 antibody exert inhibitory effects on inflammation and calcification.

**Figure 2.** *Ex vivo* mapping using fluorescence reflectance imaging to grossly visualize the biomineralization of the hearts and vessels of atherosclerosis-prone (low-density lipoprotein receptor-deficient, *Ldlr*^−/−^) animals fed a hypercholesterolemic diet, and independently treated with IgG isotype control or anti-Dll4 monoclonal antibody (Dll4 Ab). 750-nm CLIO750 nanoparticles were used to image macrophages, and 680-nm VisEn OsteoSense680 was used for the detection of osteogenic activity (top and bottom rows). Decreased osteogenic activity in the anti-Dll4 monoclonal antibody treated specimen is visualized using alkaline phosphatase (ALP) staining (middle row). Adapted from Fukuda and colleagues (Fukuda et al., 2012).
5. Clinical implications

Calcific aortic valve disease in individuals with severe aortic stenosis can progress quickly after presentation with symptoms, usually portending limited short-term survival (Turina et al., 1987). Clinical trials on medical therapy including statins have found little benefit and utility in forestalling disease progression, with no demonstrated impact on survival. Since the evidence suggests that inflammatory cells, particularly macrophages, play a crucial role in calcification, anti-inflammatory therapies may prevent development of arterial and valvular calcification. We and others have demonstrated that lipid lowering reduces inflammation (Aikawa et al., 1998; Aikawa et al., 2001; Chu et al., 2012; Libby and Aikawa, 2002; Libby et al., 2011). However, clinical trials (e.g. SALTIRE, SEAS, etc.) have failed to demonstrate that lipid lowering attenuates development of aortic stenosis. Preclinical findings suggest that macrophage accumulation precedes calcific changes in arteries and valves while lesions with advanced calcification are often unassociated with macrophages (Aikawa et al., 2007a; Aikawa et al., 2007b). This may suggest that anti-inflammatory therapies need to be initiated early (Aikawa and Otto, 2012), and thus clinical trials involving patients who had been diagnosed with aortic stenosis due to advanced calcification did not show substantial benefits of lipid lowering therapy. To establish more effective therapies, it is crucial to better understand the complex mechanisms for aortic valve calcification. To identify individuals with subclinical aortic valve calcification and those with high probability or propensity of developing severe aortic valvular stenosis, methods for early detection of calcific changes (e.g., molecular imaging, biomarkers) need to be developed. National Institutes of Health of the United States of America has formed the Working Group of Calcific Aortic Valve Disease to facilitate basic research on this devastating global health threat and initiated federal funding (Rajamannan et al., 2011).

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