Endocardial Notch Signaling in Cardiac Development and Disease

Guillermo Luxán,* Gaetano D’Amato,* Donal MacGrogan,* José Luis de la Pompa

Abstract: The Notch signaling pathway is an ancient and highly conserved signaling pathway that controls cell fate specification and tissue patterning in the embryo and in the adult. Region-specific endocardial Notch activity regulates heart morphogenesis through the interaction with multiple myocardial-, epicardial-, and neural crest–derived signals. Mutations in NOTCH signaling elements cause congenital heart disease in humans and mice, demonstrating its essential role in cardiac development. Studies in model systems have provided mechanistic understanding of Notch function in cardiac development, congenital heart disease, and heart regeneration. Notch patterns the embryonic endocardium into prospective territories for valve and chamber formation, and later regulates the signaling processes leading to outflow tract and valve morphogenesis and ventricular trabecular compaction. Alterations in NOTCH signaling in the endocardium result in congenital structural malformations that can lead to disease in the neonate and adult heart. (Circ Res. 2016;118:e1-e18. DOI: 10.1161/CIRCRESAHA.115.305350)

Key Words: cardiomyopathies ■ endocardium ■ heart valves ■ heart ventricles ■ Notch signaling

The assembly and function of a complex organ such as the heart requires the precise coordination of multiple distinct tissues. The endocardium is a specialized endothelial tissue lining the interior of the heart, closely juxtaposed to the myocardium and in direct continuity with the vascular endothelium. Among the 3 tissue layers forming the mature adult heart (endocardium, myocardium, and epicardium), comparatively less attention has been paid to the endocardium, as it was primarily thought as a physical barrier serving to regulate vascular permeability between blood and subjacent cardiac muscle. However, the importance of the endocardium as a crucial regulator of adult cardiac function derives essentially from its ability to adjust heart pumping to hemodynamic and hormonal demands by modulating cardiac muscle contractile performance and rhythmicity.1 Moreover, recent data demonstrate that the mechanical properties of the aortic valve cusps (ie, stiffness) are regulated by the endothelium.2

The endocardium also plays crucial roles during heart development because it provides patterning signals for myocardial trabeculation3 and compaction,4 contributes to coronary vessel formation,5 and in conjunction with regionalized signals from the myocardium, cells from specific regions of the endocardium will undergo epithelial–mesenchymal transition (EMT) to form the endocardial cushions (ECs). The EC will form several important structures within the heart, including the valve leaflets and the membranous walls of the ventricular and atrial chambers.6,7 In addition, endothelial-derived cells and cells of neural crest origin function in the EC to separate the common outflow tract (OFT) into the pulmonary artery and aorta.8 In keeping with these multiple requirements, it is evident that localized signaling cross talk must take place early to establish a tight functional coupling between the endocardium and the myocardium and ensure normal cardiac growth and homeostasis throughout life.

In this review, we focus on the role of Notch in the endocardium in the context of signaling interactions with the other cardiac tissues to coordinate heart morphogenesis. First, we explain briefly the elements of the Notch signaling pathway, their expression during cardiac development, and the endocardial (and coronary endothelial) activity of the pathway. Second, we describe the developmental origins of the endocardium and its relationship to cardiac and other endothelial lineages, and go on to examine key cardiogenic processes that require cell autonomous and non–cell-autonomous endocardial Notch signaling: patterning of the early embryonic endocardium into prospective territories for valves and ventricular chambers; and the later processes of trabecular compaction and morphogenesis of the OFT and valves. Finally, we provide examples of how Notch dysfunction in the endocardium results in cardiac structural malformations that can lead to
induces conformational changes leading to exposure of the NICD, which contains a transcriptional activation domain (Figure 1). Notch proteins are processed in the Golgi by various glycosyl transferases, including those of the Fringe family (Figure 1). Modified Notch is then targeted to the cell surface as a heterodimer held together by noncovalent interactions. Once in the membrane, the Notch extracellular domain is available to interact with membrane-bound ligands of the Delta (Dll1-4 in mammals) or Jagged (Jag1 and Jag2) families expressed by neighboring cells, and cell–cell contact is required for signaling (Figure 1). Whether Notch ligand–receptor interactions occur through monomeric or dimeric membrane complexes remains the subject of intense debate. \(^{11-13}\) Productive ligand–receptor interaction depends on the activity in the signaling cell of E3 ubiquitin ligases such as mind bomb-1 (Mib1), which ubiquitylates the ligand in its cytoplasmic C-terminal tail, an event crucial for its endocytosis and effective Notch signaling. \(^{14}\) Ligand endocytosis generates mechanical force to pull on Notch, \(^{15,16}\) which in turn induces conformational changes leading to exposure of the S2 site of the receptor recognized by ADAM (A Disintegrin And Metalloprotease-containing) proteins (Figure 1). \(^{17,18}\) The remaining Notch fragment becomes susceptible to cleavage by \(\gamma\)-secretase (at the S3 site)\(^{19,20}\) resulting in release of NICD, which translocates to the nucleus of the signal-receiving cell. \(^{13}\) When released from the cell membrane, NICD can function as a transcription factor. \(^{21}\) In the nucleus, NICD binds directly to the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag-1; Figure 1). \(^{22}\) NICD–CSL binding displaces corepressors and histone deacetylases that repress target genes in the absence of Notch signaling and allows recruitment of the transcriptional coactivator mastermind-like. Formation of the CSL–NICD–mastermind-like ternary complex allows recruitment of additional coactivators to activate transcription of target genes, typically those encoding repressors such as basic-helix-loop-helix transcription factors of the Hes and Hey families. \(^{23}\) Nevertheless, the spectrum of direct Notch targets is large and tissue specific, including Snail1, \(^{24,25}\) p21, \(^{26}\) c-Myc, \(^{27}\) EphrinB2, and Nrarp \(^{28}\) (Figure 1).

### Cardiac Development and Endocardial Notch Activity

The heart develops in the mouse embryo at around E7.5 from bilateral precardiac mesoderm cells that form the cardiac crescent (Figure 2A). \(^{2}\) The crescent contains 2 populations of precardiac cells: the first and second heart fields (FHF and SHF; Figure 2A) that include progenitors of the first cardiac tissues, the myocardium and endocardium. \(^{29}\) The crescent fuses at the midline forming a primitive heart tube consisting of an inner endocardial layer and an outer myocardial layer separated by an extracellular matrix termed cardiac jelly (Figure 2A and 2B). The heart tube grows at both ends by addition of SHF progenitors into its anterior (arterial) and posterior (venous) poles and simultaneously undergoes rightward looping morphogenesis \(^{30}\) (Figure 2C and 2D), changing the initial anterior–posterior polarity into right–left patterning. The FHF will give rise to the left ventricle (LV) and other parts of the heart except the OFT, whereas the SHF gives rise to the OFT myocardium and other parts of the heart except for the LV (Figure 2C and 2D). At E9.5, uneven growth within the heart tube results in ballooning of the chambers and establishment of the trabecular network \(^{31}\) (Figure 2D). The endocardium lines the lumen of the cardiac chambers, and through an EMT process forms the cardiac cushion mesenchyme in the atrioventricular canal (AVC) and proximal OFT (Figure 2D). At E9.5, a third tissue layer covering the myocardium develops from the proepicardium, a mass of coelomic progenitors located at the venous pole of the embryonic heart (Figure 2D). Proepicardium cells attach to and spread over the myocardium to form the primitive epicardial epithelium. The epicardium gives rise to a population of epicardium-derived cells through EMT, which invade the heart and differentiate into various cell types (see below). From E10.5 onward, the OFT is remodeled from a single OFT vessel, which circulates blood into the 3 main aortic arch arteries (AAAs) to join 2 dorsal aortae that distribute the blood throughout the embryo. Neural crest emanating from the dorsal neural tube invades the AAAs to reach the OFT (Figure 2E) and differentiates into smooth muscle cells.

### Nonstandard Abbreviations and Acronyms

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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AAA</td>
<td>aortic arch arteries</td>
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<td>epithelial–mesenchymal transition</td>
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<td>GOF</td>
<td>gain-of-function</td>
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<td>LV</td>
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<td>LVNC</td>
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</tr>
<tr>
<td>NICD</td>
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<td>SHF</td>
<td>second heart field</td>
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<td>semilunar valve</td>
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<td>ventricular septal defect</td>
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<td>WT</td>
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### First Heart Field (FHF)

The FHF will give rise to the left ventricle (LV) and other parts of the heart except the OFT, whereas the SHF gives rise to the OFT myocardium and other parts of the heart except for the LV (Figure 2C and 2D). At E9.5, uneven growth within the heart tube results in ballooning of the chambers and establishment of the trabecular network (Figure 2D). The endocardium lines the lumen of the cardiac chambers, and through an EMT process forms the cardiac cushion mesenchyme in the atrioventricular canal (AVC) and proximal OFT (Figure 2D). At E9.5, a third tissue layer covering the myocardium develops from the proepicardium, a mass of coelomic progenitors located at the venous pole of the embryonic heart (Figure 2D). Proepicardium cells attach to and spread over the myocardium to form the primitive epicardial epithelium. The epicardium gives rise to a population of epicardium-derived cells through EMT, which invade the heart and differentiate into various cell types (see below). From E10.5 onward, the OFT is remodeled from a single OFT vessel, which circulates blood into the 3 main aortic arch arteries (AAAs) to join 2 dorsal aortae that distribute the blood throughout the embryo. Neural crest emanating from the dorsal neural tube invades the AAAs to reach the OFT (Figure 2E) and differentiates into smooth muscle cells.
covering the AAAs endothelium (Figure 2E). Progressively, the AAAs remodel to form the mature aortic arch and major branches (Figure 2E). Neural crest cells also invade the OFT and contribute mesenchyme to the OFT septum and SLV cusps (Figure 2F). In the mature 4-chambered heart, the myocardium forms the contractile tissue of the ventricular walls, the epicardium contributes to a subset of coronary endothelial cells, coronary smooth muscle cells, part of the AV valves, and the cardiac fibroblasts, and the endocardium gives rise to the cardiac valves and coronary vessel endothelium (Figure 2F and 2G). Defective endocardial Notch signaling affects cardiac valve and chamber development, leading to diseases such as bicuspid aortic valve (BAV) and LV noncompaction (LVNC) cardiomyopathy (Figure 2H and 2I). Notch signaling is also involved in epicardium and coronary vessel formation, a topic beyond the scope of this review that has been comprehensively discussed.34–36

The Notch pathway is active early in cardiac development.7 At E9.5, the Jag1 ligand is expressed in the endocardium delineating the presumptive valve territory of the AVC...
Figure 2. Key events in cardiac development. A, At human embryonic day 14 (hE14) or mouse E7.0. In the gastrulating embryo cardiac progenitors (purple) migrate into the anterior half of the primitive streak to reach the head folds. At E7.5, progenitor cell populations of the first and second heart fields (FHF; purple; SHF; yellow) have fused in the midline of the embryo. At E8.0, the heart tube stage, the approximate contributions of FHF and SHF are shown. B, The heart tube has an outer myocardial layer (light brown) and an inner endocardial endothelium (red) separated by cardiac jelly (gray). C and D, At E9.0, the heart is elongated and looping rightward (C). At E9.5, formation of the atrioventricular canal (AVC) separates the 2 chambers’ territories, the developing atria and ventricles (C). Formation of the valve primordia occurs around E10, together with ventricular chamber development that begins with trabeculæ formation in the developing left and right ventricles, and epicardial formation from the proepicardium, located in the venous pole of the heart (D). E, Outflow tract (OFT) and aortic arch arteries (AAA) remodeling is dependent on the cardiac neural crest (CNC) cells. The OFT region of the heart initially exists as a single outflow vessel, which circulates blood into three major pairs of AAAs to join 2 paired dorsal aortae that distribute the blood throughout the embryo. Left, Neural crest cells migrate from the dorsal neural tube, surrounding the aortic arch arteries to the cardiac outflow (Continued)
Figure 2. Continued. Middle, The neural crest cells that invest the endothelial tubes of the AAAs differentiate into vascular smooth muscle cells. Right, the AAAs remodel to form the mature aortic arch, with neural crest–derived vascular smooth muscle cells contributing to most of the aortic arch and its major branches. F, Contribution of neural crest (purple) and epicardial (blue)–derived cells (NCDC and EPDC) to the cardiac valves. NCDCs migrate through the pharyngeal arches and into the OFT to initiate the reorganization of the OFT and formation of semilunar valves. EPDCs contribute to the formation of the coronary arteries, the interstitial cells in the myocardium, and the AV valves. In a top view of the septated heart, the relative contributions of NCDCs and EPDCs to cardiac valve leaflets and cusps are shown. G, E13.5–birth. The 4 chambers and valves are shown. The epicardium is shown in gray, and the coronary vessels in red and blue. H, Cushion fusion between E11.5 and E12.5 is a critical step in valve morphogenesis. Aberrant fusion events can lead to bicuspid aortic valve (BAV). R–NC and R–LC fusions might have different causes. I, Scheme depicting the thick ventricular walls of the normal adult heart and the thin and trabeculated walls of a noncompacted heart. In all panels, the ventral aspect of the heart is shown (anterior to the top). A indicates atria; la, left atrium; lv, left ventricle; LVNC, left ventricular noncompaction; mv, mitral valve; pv, pulmonary valve; rv, right ventricle; and tv, tricuspid valve.

and throughout the myocardium (Figure 3A–3A′ and 3D), whereasDll4 is expressed in the endocardium (Figure 3B–3B′ and 3D). Mib1 is expressed in both endocardium and myocardium and has the potential to ubiquitylate both ligands. At this stage, Notch1 activity is relatively uniform in the AVC and the OFT endocardium but restricted to the endocardium at the base of the developing trabeculae in the ventricles (Figure 3C–3C′ and 3D). At E12.5, Jag1 is expressed in the myocardium around the semilunar valves (SLVs) and in the endocardium (Figure 3E and 3H) where Notch is active (Figure 3G and 3H). Dll4 expression in valve endocardium is greatly diminished when compared with E9.5 (Figure 3F and 3H). In the chambers, myocardial Jag1 expression is maintained (Figure 3I and 3L), Dll4 expression is attenuated (Figure 3F and 3H), and Notch activity remains widespread in the endocardium (Figure 3K and 3L). Once the general cardiac pattern is established, substantial cellular proliferation, tissue morphogenesis, and terminal differentiation of the different cardiac structures occur, and the heart becomes fully functional at birth (Figure 2G). In late gestation, Jag1

Figure 3. Endocardial Notch activity during cardiac development. A–D, In the E9.5 heart, the ligands Jag1 and Dll4 are expressed in the atrioventricular canal (AVC) endocardium (A, A′, B, B′, and D) and in chamber myocardium and endocardium, respectively (A, A′, B, B′, and D), whereas the Notch1 receptor is active in the endocardium (C–C′, and D). E–H, At E12.5, Jag1 is strongly expressed in the endocardium lining the outflow tract endocardial cushions (E and H), whereas Dll4 expression is reduced when compared with E9.5 (F and H), and endocardial N1ICD expression persists (G and H). I and L, At E12.5, Jag1 is strongly expressed in trabecular myocardium (I, arrowhead, L) and weakly in the compact myocardium, whereas Dll4 is weakly expressed in endocardium (J, arrowhead, L) and N1ICD is expressed throughout chamber endocardium (K, arrowhead, L). M–P, At E14.5, Jag1 is expressed in trabecular myocardium (M, arrowhead, P), Dll4 in the coronary vessels (N and P), and N1ICD in chamber endocardium and coronary vessels endothelium (O and P). A indicates atria; AVC, atrioventricular canal; cv, cardiovascular; la, left atrium; lv, left ventricle; and rv, right ventricle.
is expressed in smooth muscle tissue around the valves (not shown), and in chamber myocardium (Figure 3M and 3P), Dll4 in developing coronary vessels (Figure 3N and 3P). and Notch1 activity persists in valve and chamber endocardium (Figure 3O and 3P). Defective NOTCH signaling has been shown to cause various cardiac pathologies including Alagille syndrome (AGS), BAV and LVNC (Figure 2H), and LVNC (Figure 2I). We suggest that these pathologies result from the disruption of the NOTCH-mediated communication between the endocardium and the surrounding cardiac tissues.

**Origin of the Endocardium**

The timing of endocardial specification and whether the endocardium is derived from progenitors that lack myocardial potential is controversial. Data from chick and zebrafish models involving retroviral single-cell tagging and tracking studies suggest that specification of endocardial and myocardial cells occurs before their ingress through the primitive streak during gastrulation, perhaps as early as the blastula stage and significantly before their migration to the heart field. Another prevailing view inferred mainly from mouse, embryonic stem cell models, and cell lineage-mapping studies contended that endocardial and myocardial cells in mammals are derived from a common multipotent progenitor within the cardiac mesoderm still present at the cardiac crescent stage. Moreover, although it was clear that the OFT endocardium and myocardium both derived from a common Flk1+ progenitor cells with progressively restricted lineage differentiation at different time points during gastrulation. Notch1 progenitors give rise to the FHF at the early primitive streak stage (E6.25) and then to the SHF progenitors at late primitive streak stage (E7.25) with the expression of Mesp1 in the 2 populations overlapping at E6.75. FHF progenitors are unipotent and give rise to either cardiomyocytes or endothelial cells, whereas SHF progenitors are either unipotent or bipotent consistent with a temporal differentiation delay between FHF and SHF during the progressive restriction of fate potential by the cardiogenic mesoderm progenitors. Therefore, the endocardium is essentially specified at gastrulation in mice although some of the endocardium in the OFT and RV may originate from multipotent SHF precursors not yet committed at the cardiac crescent stage. In summary, the endocardium is a specialized endothelial subpopulation originated by vasculogenesis from progenitor cells in the cardiac crescent. One important issue is that the endocardium expresses specific markers that are not expressed in endothelial cells. For example, the transcription factor, NFATc1, expressed in the nucleus of endocardial cells since the beginning of endocardial differentiation is not required for early endocardial specification but is necessary for later valve morphogenesis.

**Endocardial Patterning**

Notch patterns the early endocardium, which expresses several Notch elements and whose interaction with the myocardium is critical for cardiac development. Initial functional analysis was performed on mice with systemic Notch signaling inactivation (RBPJκ or Notch1 targeted mutants) in which the expression of early valve and chamber endocardial markers was disrupted, suggesting impaired endocardial differentiation at a time when myocardial patterning was unaffected. Gain-of-function (GOF) experiments, in contrast, show that ectopic N1ICD expression throughout the endocardium extends the uniform N1ICD distribution observed in prospective valve tissue, thus expanding valve patterning to ventricular endocardium. A key feature of Notch signaling in the midgestation mouse heart (E9.5–10.5) is that endocardial cells express the ubiquitin ligase Mib1 and both the Dll4 ligand and the Notch1 receptor and thus have the potential to send and receive signals. This is true for the presumptive valve territory (AVC and OFT) and for the early ventricle. Notch inactivation in the endocardium results in decreased ligand expression. Thus, RBPJκ and Notch1 mutants show reduced Dll4 expression in the endocardium, whereas Notch GOF expands Dll4 expression throughout this tissue, suggesting the existence of a Notch-dependent positive feedback loop regulating Dll4 expression in the embryonic endocardium. These results imply that Notch-active endocardial cells behave as a developmental field and are specified together as valve or chamber endocardium. This situation contrasts with findings in the central nervous system and other tissues. In the following sections, we will describe how endocardial Notch activity patterns the various cardiac territories.

**Early Valve Development**

Early valve development begins with the formation of the primitive valve primordium by a process of regionalized EMT that takes place initially in the AVC and later in the OFT endocardium to produce cushion mesenchyme (Figure 4A and 4B). Notch pathway genes are expressed in prospective valve endocardium. Targeted inactivation of Notch1 (or RBPJκ) results in severely hypoplastic ECs at E9.5 because of impaired EMT (Figure 4C). Transmission electron microscopy analysis revealed that although E9.5 RBPJκ-deficient AVC cells show features of activated premigratory endocardium, they remain in close association, maintain adherens junctions, and fail to detach from each other and migrate individually to invade the cardiac jelly. The proposed mechanism is that N1ICD-RBPJκ directly activates the EMT drivers Snail1/2, whose expression is severely reduced in RBPJκ mutant AVC and OFT endocardium. Loss of Snail expression prevents downregulation of vascular endothelial cadherin (Cdh5)–mediated endocardial cell adhesion and EMT is blocked (Figure 4D). These in vivo observations were confirmed by the defective EMT of AVC and OFT explants from Notch1 or RBPJκ mutants or wild-type (WT) explants treated with a Notch signaling inhibitor, cultured in vitro on collagen gels, indicating that Notch is essential for this process. Functional analysis has revealed the requirement of the transcription factor Hey2 or the synergistic effect of Hey1 and Hey2
Figure 4. Developmental processes in cardiac valve development. A, Diagram of an E9.5 heart. B, Detail of the boxed area in A highlighting the outflow tract (OFT) and atrioventricular canal (AVC) endocardial cushions where epithelial–mesenchymal transition (EMT) takes place and valves form. Myocardium, brown; endocardium, red; extracellular matrix, gray. During EMT, the AVC cushion tissue is invaded by endocardial-derived mesenchyme (light blue), whereas the OFT is invaded by migratory neural crest (NC) mesenchyme (purple) and endocardial-derived mesenchyme (light blue). The interaction between NC-derived and endocardial mesenchyme is crucial for OFT valve morphogenesis. C, Cross section of a WT E9.5 heart (left) showing the 4 developing chambers. The arrow points to a forming trabecula and the arrowhead to mesenchyme cells in the AVC; in the Notch1 mutant heart (middle), trabeculae are poorly developed (arrow) and the AVC is devoid of mesenchyme (asterisks). Ectopic N1ICD expression in the endocardium of E9.5 R26N1ICD;Tie2-Cre embryos (right) causes impaired trabeculation (arrow), and mesenchyme cells distribute along a large AVC area. D, Signaling circuitry regulating formation of the valve primordia via EMT. Cartoon depicting the E9.5 OFT and AVC. Myocardium, brown; endocardium, red. AVC myocardial Bmp2 is required for Tgfβ2, Notch1, Snail1, Snail2, and Twist1 expression. Mib1-Dll4(Jag1)-Notch1 signaling occurring in a field of OFT or AVC endocardial cells leads to Snail1/2 activation and Bmp2 repression in endocardium via Hey proteins. Snail1/2 in turn repress Cdh5 so that EMT occurs. Endocardial Notch activity leads to Wnt4 expression that in turn promotes myocardial Bmp2 expression and EMT. E, Pathway establishing chamber vs valve domains in the developing heart. Myocardial Hey1 and Hey2 (green) confine Bmp2 expression to prospective valve myocardium (purple). Bmp2/Smad signaling together with Gata4 drive valve-specific gene expression. Endocardial Notch signaling (red) activates Hey1–HeyL, which repress Bmp2 in the endocardium. F, Top, Semilunar (SL) valves morphogenesis. From E13.5 onward, once mesenchyme cells occupy the endocardial cushion, cusps (Continued)
Figure 4 Continued. Excavation is driven by a solid endothelial ingrowth of the free edges of the valve cusps on their arterial face, producing an epithelial ridge between the emerging cusps and the arterial wall. The ridge eventually becomes luminated to yield the sinus of Valsalva. Bottom. AV valves morphogenesis. The mural leaflets of both AVVs are supported by AV myocardium at their ventricular side. As the primordial leaflets distend into the lumen, thin strands of elongated muscle remain attached to the valve leaflet and programmed cell death yields a mobile leaflet and remnants that contribute to the chordae tendineae and papillary muscles. Myocardium is depicted in brown, proteoglycans in pink, and mesenchyme in gray. la indicates left atrium, lv, left ventricle; ra, right atrial; rv, right ventricle; and WT, wild-type.

abrogation or Hey1 and HeyL in valve development. The endocardial expression of these genes responds to Notch signaling manipulation, suggesting that they are Notch targets in this tissue (Figure 4D).

In complementary Notch1 GOF studies, constitutive Notch1 activity in the endocardium enables ectopic, noninvasive EMT of ventricular endocardial cells, conferring valvular features to an otherwise nonvalvular, EMT-refractory ventricular endocardium. Notch GOF studies also revealed the integration of endocardial Notch and myocardial Bmp2 signaling to promote EMT: Notch is required for Snail expression and Bmp2 signaling regulates Snail activation, suggesting that both pathways converge to activate cardiac EMT. Recent studies with conditional loss-of-function (LOF) alleles have confirmed previous findings and have added complexity to the model, suggesting that endocardial Notch signaling induces the expression of Wnt4, which subsequently acts as a paracrine factor to upregulate Bmp2 expression in the adjacent AVC myocardium to signal EMT (Figure 4D). Jag1 has also been suggested to be required for EMT as Jag1+/flox;VE-Cadherin-Cre mice show hypocellular EC in vivo and in vitro. Because the majority of Jag1+/flox;VE-Cadherin-Cre mice survive to adulthood, the absolute requirement of Jag1 in valve primordium formation is debated. Our current data (MacGrogan et al, in preparation) suggest that Mik1 acting in the endocardium ubiquititates Dll4 (and perhaps Jag1) in signaling cells so that productive ligand–receptor interaction occurs, and the Notch1 receptor is activated in neighboring endocardial cells leading to downstream events leading to EMT both in AVC and OFT (Figure 4D).

Notch and Hey transcription factors regulate Bmp2 expression to pattern valve versus chamber territories in the heart. Thus, data from Notch GOF and LOF together with Hey1 and Hey2 GOF and LOF studies indicate that Bmp2 expression is confined to the AVC myocardium by Hey1 and Hey2 both of which expressed in the chambers. In the endocardium, Notch1-dependent Hey1, Hey2, and HeyL are differentially expressed in the AVC chambers and repress Bmp2. Ectopic N1ICD expression in endocardium expands AVC patterning to ventricular endocardium but does not affect Hey1, Hey2, or Bmp2 myocardial expression. Ectopic N1ICD expression in the myocardium leads to expansion of Hey1 and loss of Bmp2 expression, and chambers’ markers expand to the AVC. In both N1ICD GOF models, Bmp2 is repressed in the endocardium, similar to the WT situation. Systemic abrogation of Notch signaling leads to downregulation of Hey genes and ectopic endocardial Bmp2 expression, indicating that Notch represses Bmp2 in the endocardium via Hey proteins (Figure 4E). The complexity of the signaling and transcription factor network regulating AVC-specific gene expression and patterning is suggested by recent work showing that the Gata4 transcription factor together with Bmp2/Smad signaling activates AVC-specific enhancers, leading to H3K27 acetylation and subsequent AVC-myocardium–specific gene expression (Figure 4E). AVC patterning is also essential for delay of electric impulses between atria and ventricles, and defective AVC maturation can lead to arrhythmias such as AV re-entry tachycardia, AV nodal block, and ventricular pre-excitation. Wnt and Notch signaling converge to regulate AVC maturation and electric programming upstream of Tbx3.

OFT Development and Remodeling

The OFT at the arterial pole of the heart is a transient structure that connects the embryonic ventricles to the aortic sac. The OFT is elongated by addition of SHF progenitors and subsequently as the circulatory system transits from a single to a double pathway; the OFT rotates clockwise and the aorta and pulmonary orifices reposision above the left and right ventricles, respectively. These morphogenetic movements depend on the coordinated interactions between multiple cell types originating from within and outside the heart: the endocardium, endothelial-derived mesenchyme, SHF-derived cardiac and smooth muscle progenitors, and cardiac neural crest (CNC).

Deficient SHF contribution to the OFT leads to a spectrum of malformations such as double outlet right ventricle and over-riding aorta, which reflect a continuum of incorrect alignment defects. These defects closely resemble those seen in humans with AGS, a disorder caused by JAG1 or NOTCH2 mutations and characterized by right-sided OFT defects and disorders in the skeletal, ocular, renal, and hepatic organ systems. JAG1 LOF mutations or reduced copy number of JAG1 and NOTCH1 have also been identified in nonsyndromic tetralogy of Fallot, which has 4 major components: valvular or subvalvular pulmonary stenosis, ventricular septal defect (VSD), over-riding aorta, and RV hypertrophy. Systemic Jag1 deletion in mice results in embryonic lethality at E10.5, whereas heterozygous Jag1 mice exhibit eye defects but none of the other characteristic AGS phenotypes. However, mice double heterozygous for the Jag1 null allele and a Notch2 hypomorphic allele exhibit characteristic AGS abnormalities including heart developmental defects such as hypoplastic RV and over-riding aorta. Accordingly, homozygous systemic deletion of PSI which encodes for the γ-secretase complex member Presenilin 1, or combined Hey1;HeyL or Hes1 deletion, all display characteristic right OFT defects although none of these genes have been linked to congenital heart disease (CHD) in humans to date (Table).

OFT morphogenesis is also dependent on a second source of extra cardiac cells derived from the CNC and required for OFT septation, formation of the arterial valves (also called semilunar [SLVs] because of the shape of their leaflets, which are also called cusps) and remodeling of the AAAs (Figure 2E). Deficient CNC investment into the OFT
Table. Mutations in Notch Pathway Elements Affecting Heart Development in Humans and Mice

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<td>Homozygous lethal (E10.5)</td>
<td>3</td>
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<tr>
<td>Notch1flx/flx;Nfatc1pan-Cre</td>
<td>Hypoplastic EC</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
<td>Homozygous lethal (E12.5)</td>
<td>62</td>
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<tr>
<td>Notch2+−/−</td>
<td>Hypomorphic allele, AGS phenotype in Jag1+/dDSL; Notch2+/del1 double heterozygous</td>
<td>n.d.</td>
<td>NOTCH2</td>
<td>AGS</td>
<td>Hi</td>
<td>68, 73, 76, 77</td>
</tr>
<tr>
<td>Notch2flx/flx;Tie2-Cre</td>
<td>Myocardial hypoplasia and reduced trabeculation</td>
<td>n.d.</td>
<td>NOTCH2</td>
<td>n/a</td>
<td>Homozygous lethal (E10.5)</td>
<td>68, 76, 77</td>
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<tr>
<td>Notch2flx/flx;Pax3-Cre</td>
<td>Narrow aortas and pulmonary arteries</td>
<td>n.d.</td>
<td>…</td>
<td>AGS (PAS)</td>
<td>…</td>
<td>78</td>
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<tr>
<td>Notch2flx/flx;Tag1-Cre</td>
<td>Narrow aortas and pulmonary arteries</td>
<td>n.d.</td>
<td>…</td>
<td>AGS (PAS)</td>
<td>…</td>
<td>78</td>
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<tr>
<td>RBPJk−/−</td>
<td>Defective cardiac looping, hypoplastic EC and impaired trabeculation</td>
<td>Yes</td>
<td>RBPJK</td>
<td>n/a</td>
<td>Homozygous lethal (E10.5)</td>
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<td>RbpJk−/−</td>
<td>Aortic valve sclerosis, stenosis</td>
<td>Yes</td>
<td>…</td>
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<td>RBPJkflx/flx;Tie2-Cre</td>
<td>Impaired trabeculation</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
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<tr>
<td>RPJkflx/flx;Dermo1-Cre</td>
<td>Ventricular septal defect</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
<td>…</td>
<td>80</td>
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<tr>
<td>Dil4−−</td>
<td>Reduced atrial and ventricular chambers, defective trabeculation</td>
<td>Yes</td>
<td>DLL4</td>
<td>n/a</td>
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<tr>
<td>Jag1−−</td>
<td>Pericardial edema</td>
<td>n.d.</td>
<td>JAG1</td>
<td>AGS (heterozygous mutations)</td>
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<td>Jag1−−;Notch2−−</td>
<td>Perinatal lethality</td>
<td>n.d.</td>
<td>JAG1;NOTCH2</td>
<td>AGS</td>
<td>Hi</td>
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<td>Jag1flx/flx;Cdh5-Cre</td>
<td>TOF, valve calcification</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
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<td>63</td>
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<tr>
<td>Jag1flx/flx;Nfatc1-Cre</td>
<td>Hypoplastic EC</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
<td>…</td>
<td>62</td>
</tr>
<tr>
<td>Jag1flx/flx;Mef2c-AHF-Cre</td>
<td>AA abnormalities, VSD, ASD</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
<td>…</td>
<td>83</td>
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<tr>
<td>Jag1flx/flx;Islet1-Cre</td>
<td>AA abnormalities, VSD, ASD</td>
<td>Yes</td>
<td>…</td>
<td>n/a</td>
<td>…</td>
<td>83</td>
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<tr>
<td>Mib1flx/flx;cTnT-cre</td>
<td>LVNC</td>
<td>Yes</td>
<td>MIB1</td>
<td>LVNC</td>
<td>Hi</td>
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<tr>
<td>Psen1−−</td>
<td>VSD, DORV, PAS</td>
<td>Yes</td>
<td>PSEN1, PSEN2</td>
<td>Dilated cardiomyopathy</td>
<td>…</td>
<td>74, 84, 85</td>
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<tr>
<td>Psen1−−;Psen2−−</td>
<td>Defective cardiac looping, Homozygous lethal (E10.5)</td>
<td>n.d.</td>
<td>PSEN1, PSEN2</td>
<td>n/a</td>
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<td>86</td>
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<tr>
<td>Hey2−−</td>
<td>VSD, ASD, PAS, TOF, TVA, AVV dysfunction</td>
<td>Yes</td>
<td>HEY2</td>
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<td>56, 58, 87, 88</td>
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<tr>
<td>Hey1−−;Hey2−−</td>
<td>Defective cardiac looping, hypoplastic EC and impaired trabeculation, homozygous postnatal lethality</td>
<td>Yes</td>
<td>HEY1, HEY2</td>
<td>n/a</td>
<td>…</td>
<td>57, 89</td>
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<tr>
<td>Hey1−−;Hey3−−</td>
<td>VSD, AV defects</td>
<td>Yes</td>
<td>HEY1, HEY3</td>
<td>n/a</td>
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<td>59</td>
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<tr>
<td>Hes1−−</td>
<td>VSD and OA Homozygous perinatal lethality</td>
<td>Yes</td>
<td>HES1</td>
<td>n/a</td>
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<tr>
<td>DNMAML;Pax3-Cre</td>
<td>OFT, AA abnormalities, VSD, postnatal lethality</td>
<td>…</td>
<td>MAML</td>
<td>n/a</td>
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<td>91</td>
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<tr>
<td>DNMAML;Wnt1-Cre</td>
<td>OFT, AA abnormalities, postnatal lethality</td>
<td>…</td>
<td>…</td>
<td>n/a</td>
<td>…</td>
<td>91</td>
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(Continued)
results in persistent truncus arteriosus because of lack of mesenchymal contribution to the aortic-pulmonary septum and a perimembranous VSD, which results from the inappropriate growth and fusion of the conotruncal ridges of the outlet septum and ECs of the inlet septum. Moreover, CNCs play essential roles in positioning the cushions and patterning the valve cusps within the developing outflow cushions. CNCs affect the deployment of SHF progenitors and are involved in cross talk with other pharyngeal mesoderm progenitors, including endothelial cells, as they approach the distal heart tube. Normally CNCs come in close contact with SHF cells as they migrate into the OFT, and positional cues are exchanged by cell–cell interactions. Conditional inhibition of Notch signaling using a dominant-negative form of the transcriptional coactivator mastermind (Figure 1) induced with the CNC driver lines resulted in double AA abnormalities, VSD, TVA, neonatal lethality. Likewise, deletion of the Jag1 ligand or Notch signaling inhibition by dominant-negative mastermind-like using either Pax3-Cre or Mef2c-AHF-Cre driver lines resulted in a variety of defects that appear with variable penetrance: pulmonary artery stenosis, VSD, atrial septum defect, and OFT misalignment resulting in a variety of de-

### Table. Continued

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Phenotype</th>
<th>Defective Endocard. Signaling</th>
<th>Human Gene</th>
<th>Disease</th>
<th>Mechanism of Human Disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMAML;Islet1-Cre</td>
<td>OFT, AA abnormalities, VSD, TVA, neonatal lethality</td>
<td>*</td>
<td>n/a</td>
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<tr>
<td>DNMAML;Met2c-AHF-Cre</td>
<td>OFT, AA abnormalities, VSD, neonatal lethality</td>
<td>*</td>
<td>n/a</td>
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<td>83</td>
</tr>
<tr>
<td>Numbflox/flox;Numbflox/numbflox;Nkx2.5-Cre</td>
<td>OFT septation defect, DORV, AVSD, embryonic lethal (E15.5–18.5)</td>
<td>*</td>
<td>NUMB, NUMBL</td>
<td>n/a</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>N1ICD ME;Mesp1-Cre (NGOF)</td>
<td>Impaired ventricular myocardial differentiation, ectopic trabeculation in AVC Homozygous lethal (E10.5)</td>
<td>*</td>
<td>n/a</td>
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<td>53</td>
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<tr>
<td>R26N1ICD;Tie2-Cre (NGOF)</td>
<td>Expansion of valve features to ventricles, ectopic partial EMT in ventricles Homozygous lethal (E10.5)</td>
<td>Yes</td>
<td>n/a</td>
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<td></td>
<td>51</td>
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<tr>
<td>R26N1ICD;Nkx2.5-Cre (NGOF)</td>
<td>Hypoplastic EC and thin chamber myocardium, defective trabeculation embryonic lethal (E11.0)</td>
<td>Yes</td>
<td>n/a</td>
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<td></td>
<td>51</td>
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<tr>
<td>R26N1ICD;ctnT-Cre (NGOF)</td>
<td>Hypoplastic EC and delayed trabeculation, embryonic lethal (E11.5)</td>
<td>...</td>
<td>n/a</td>
<td></td>
<td></td>
<td>51</td>
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<tr>
<td>Hey1 ME;Mesp1-Cre (HGOF)</td>
<td>Reduced AVC extension, embryonic lethal (E11.5)</td>
<td>Yes</td>
<td>n/a</td>
<td></td>
<td></td>
<td>64</td>
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<tr>
<td>Hey2 ME;Mesp1-Cre (HGOF)</td>
<td>Absent AVC, no EC, defective trabeculation, embryonic lethal (E10)</td>
<td>Yes</td>
<td>n/a</td>
<td></td>
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</tr>
</tbody>
</table>

AA indicates aortic arch; AGS, Alagille syndrome; ASD, atrial septum defect; AVSD, atrioventricular septum defect; AVV, atrioventricular valve; BAV, bicuspid aortic valve; CAVD, calcific aortic valve disease; CAV, calcific aortic valve; CAVD, calcific aortic valve disease; CN, dominant negative; DORV, double outlet right ventricle; EMT, epithelial–mesenchymal transition; EC, endocardial cushion; HI, haploinsufficiency; HGOF, Hey gain-of-function; LVNC, left ventricular noncompaction; MAML, Mastermind-like; Mib1, mind bomb-1; n.a., not-applicable; NEDD, Notch extracellular domain; NGOF, Notch gain-of-function; OA, over-riding aorta; PAS, pulmonary artery stenosis; RVHP, right ventricular hypoplasia; TOF, tetralogy of Fallot; TVA, tricuspid valve atresia; and VSD, ventricular septal defect.

*Hypothetical.

Valve Morphogenesis and Aortic Valve Disease

By the time it reaches its definitive length, the OFT is formed by mesenchyme derived from the endocardium proximally...
(conus) and CNCs distally (truncus). Concurrent with rotation and septation of the OFT, the aortic and pulmonary valves form at either side of the conotruncal boundary by progressive enlargement and fusion of the EC. The outflow cushions excavate gradually from E12.5 to E15.5, to achieve their typical semilunar morphology (Figure 4F). On the inflow side, fusion of ECs at the AV junctions yields a right and left AV. Through a variety of mechanisms (excavation or delamination), the fused cushions eventually yield the leaflets and cords of the tension apparatus. Thus, the chordae tendinae and the fibrous continuity separating the mitral and tricuspid valves in addition to the leaflets arise from EC endothelial cells. These valvulogenic processes are driven by a combination of endocardial gene expression programs and biomechanical forces elicited by evolving hemodynamics, but the precise mechanisms are unclear.

Although the role of Notch signaling during EMT is well documented, Notch function in post-EMT morphogenesis remains uncertain. Nevertheless, in light of the association with BA and calcification, there has been significant interest in elucidating the potential role of Notch1 in EC fusion and remodeling. Strikingly, the expression of N1ICD in ECs and remodeling valve endocardium throughout late developmental stages suggests that the endocardium is the primary site for Notch activity in the ECs post-EMT. Given these considerations a relevant question is what are the mechanism(s) involved in the transition from proliferation and expansion of the ECs to remodeling and elongation of the valves cusps and leaflets? Throughout valve development, endocardial Notch is likely required to interface with other pathways to coordinate valvulogenesis. Several developmental signaling pathways have been identified in the cushions to regulate valve remodeling including members of the Bmp and Egfr pathways. For example, global or endothelial deletion of the heparin-binding epidermal growth factor results in normal development, but excessive enlargement of the conal cushions caused by increased Bmp signaling and proliferation resulting in thickened SLV cusps and AV leaflets (MacGrogan et al, in preparation). We found that Jag1 can transcriptionally upregulate heparin-binding epidermal growth factor, which released in a soluble form associates with the valve extracellular matrix and inhibits mesenchymal cell proliferation through epidermal growth factor receptor. This suggests that endocardial Notch signaling functions during valve morphogenesis to regulate Egfr and Bmp signaling and thus limit valve cushion proliferation before remodeling. Importantly, SLV cusps remodeling may also depend on appropriate tissue–tissue interactions among the SHF and CNC. In addition to the previously described OFT defects, inhibition of Notch in the SHF using the dominant-negative mastermind-like transgene resulted in dysmorphic and thickened SLV cusps and aortic regurgitation caused by diminished mesenchymal apoptosis and increased extracellular matrix deposition. Given that cardiovascular defects in the Notch SHF mutants occur, in part, because of abnormal CNC patterning, CNCs were interpreted to provide instructive signals to orchestrate apoptosis and changes in extracellular matrix production during SLV remodeling.

SLV malformations are common in humans, including BA, which occurs in 0.5% to 3% of the population, and pulmonic valve stenosis in 10% of all CHD in adults. BA is usually asymptomatic at birth but increases the risk of disease later in life, including calcification, stenosis, and aortopathy. BA morphology varies between right and left (R–LC) coronary cusp fusion and noncoronary (R–NC) fusion at a ratio of ≈0.7 to 0.3 (Figure 2H). Insight into the developmental basis of BA was gained from studies of Nos3 null mice and an inbred Syrian hamster strain, suggesting that R–N BA is caused by defective formation of noncoronary cushions, whereas the R–L BA is likely the result of defective OFT septation. To date, only NOTCH1 has been implicated in the cause of non-syndromic BA in human. In a study of 2 unrelated families, Garg et al observed mutations in NOTCH1 that segregated with aortic valve disease, particularly with BA and aortic valve calcification. In addition to BA, other forms of LV OFT obstruction such as coarctation of the aorta and hypoplastic left heart have been related to NOTCH1 polymorphisms and mutations in the extracellular domain of NOTCH1. The mechanism of calcific aortic valve disease was proposed to be haploinsufficiency of NOTCH1 resulting from failure to repress a pro-osteogenic gene program in the valves mediated by Runx2 upregulation through the Hey genes. Recent in vitro modeling of calcific aortic valve disease in endothelial cells derived from human-induced pluripotent stem cells extended these findings to multiple epigenetic and transcriptomic targets affected by NOTCH1 haploinsufficiency. Thus, hemodynamic shear stress, which protects against calcification, activates antiosteogenic gene networks and represses proinflammatory molecules in WT but not in NOTCH1 mutant endothelial cells, consistent with the notion that the effects of shear stress are mediated, in part, by NOTCH1. This study further showed that heterozygous nonsense mutations in NOTCH1 disrupted several epigenetic marks, including H3K27ac at N1ICD-bound enhancers resulting in derepression of latent anti-inflammatory and pro-osteogenic gene networks.

Ventricular Trabeculation

Ventricular chamber development begins in the mouse around E9.5. The morphological landmark of this process is the formation of trabeculae, a mesh of endocardium-lined luminal cardiomyocyte projections that give the embryonic myocardial walls their characteristic spongy appearance. Trabecular cardiomyocytes and endocardium are in close apposition, especially at the base of the forming trabeculae, and communication between both tissue layers regulates oriented trabecular growth and patterning. Trabecular increase cardiac output and permit nutrition and oxygen uptake in the embryonic myocardium before coronary vascularization and failure in trabeculae formation is embryonic lethal. Cells at the tip of the longitudinal axis of the trabeculae are
more differentiated than those at the base. As development proceeds, trabeculae extend radially and form a network while also increasing in length. Progressively, the ventricular myocardium differentiates into 2 distinct layers: the outer, proliferative, compact myocardium and the inner, more differentiated, trabecular myocardium.

Systemic or endocardium-specific Notch mutant embryos show impaired trabeculation, defective trabecular marker expression and reduced ventricular cardiomyocyte proliferation, leading to the formation of an anomalous spongy myocardial wall (Figure 5A and 5B). These findings suggest that endocardial Notch1 signaling is required for proliferation and differentiation of chamber myocardium. Notch signaling abrogation affects the expression of 3 signaling pathways required for trabeculation: Bmp10, Nrg1/ErbB, and EphrinB2. Bmp10 is expressed in trabecular myocardium where it sustains proliferation. Phenotypic rescue of RBPJK mutant embryos after incubation in Bmp10-conditioned medium suggests that in trabecular myocardium, Notch modulates proliferation via Bmp10. Endocardial Nrg1 is crucial for trabeculation via activation of ErbB2-4 receptors expressed on myocardium. Addition of Nrg1 to cultured RBPJK mutant embryos rescues their cardiomyocyte differentiation defect. The EfnB2 ligand and EphB4 receptor signaling system is also required for trabeculation. Endocardial EfnB2/EphB4 expression and activity is impaired in Notch mutants, and EfnB2 is a direct transcriptional target of N1ICD/RBPJK. Studies with targeted mutant embryos indicated that Nrg1 transcription is reduced in EfnB2 mutants (but not vice versa), whereas Bmp10 mutants show normal EfnB2 and Nrg1 expression, suggesting that EfnB2 acts upstream of Nrg1 and that both molecules may act independently of Bmp10 during trabeculation. Recently, the Hand2 transcription factor has been shown to be an important downstream component in the Notch-dependent endocardium–myocardium signaling mechanism active in trabeculation, and a direct regulator of Nrg1. Additional work has shown that during trabeculation the endocardial activity of the peptidyl-prolyl isomerase FKBP12 tightly regulates N1ICD stability. Our current data suggest a model in which Mib1-Dll4-Notch1 signaling in the endocardium mediates an endocardium–myocardium interaction that promotes cardiomyocyte proliferation and differentiation leading to trabeculae formation (Figure 5C).

LV Noncompaction

Formation of the trabecular network is followed by a period of remodeling at E14.5 called compaction, when trabeculae stop growing and thicken radially until they are no longer distinguishable from the myocardial wall. Simultaneously, epicardium-derived cells invade the compact myocardium and, together with ventricular endocardial cells, contribute to the formation of the coronary vasculature that will support the rapid proliferation of the compact myocardium, which becomes the main source of contractile force of the heart. Several pathways are crucial for these developmental processes, in which signals derived from endocardium and myocardium interplay with others derived from coronary blood vessels to coordinate ventricular myocardium patterning, proliferation, trabecular maturation/compaction, and coronary vessel formation.

Defective trabecular compaction can manifest as a cardiomyopathy termed LVNC (OMIM 601493), characterized by the presence of prominent trabeculations separated by deep invaginations in an abnormally thin ventricular wall. LVNC can be a mild disease revealed by a depressed systolic function or can evolve into a serious condition with complications including systemic embolism, malignant arrhythmias, heart failure, and sudden death. LVNC is generally transmitted as an autosomal dominant trait, but the underlying molecular mechanisms are still poorly understood.

Conditional inactivation in the embryonic mouse myocardium of the Mib1 gene, encoding the E3 ubiquitin ligase Mib1 essential for Notch ligand signaling (Figure 1), causes a phenotype strongly reminiscent of LVNC. Thus, E16.5 Mib1flx; cTnT-Cre embryos show a dilated heart with a thin compact myocardium and large, noncompacted trabeculae, protruding toward the ventricular lumen (Figure 5D and 5E). These embryonic features are present in newborn and adult mutant mice as revealed by echocardiographic analysis of 6-month-old mice that also shows reduced ejection fraction and a high ratio of noncompacted to compacted myocardium, diagnostic of LVNC in humans.

Sequencing of the MIB1 gene in a cohort of 100 LVNC patients (48% familial cases) identified 2 mutations (V943F and R530X) in 2 probands. The 2 MIB1 mutations were inherited in an autosomal dominant fashion across various generations, together with LVNC. Image analysis of the hearts of both probands’ fathers as well as those of family members with inherited MIB1 mutations demonstrates the presence of prominent trabeculations in both ventricles and reduced cardiac performance. Importantly, analysis of peripheral blood from these patients reveals significantly below-normal expression of NOTCH1 and its targets DTX1 and GATA3. These data demonstrate that the 2 MIB1 mutations affect NOTCH signaling and segregate with LVNC.

In silico modeling suggests that MIB1 functions as a homodimer, in which MIB1 monomers form a head-to-tail homodimer that interact with JAG1 through the Herc2 domain. Moreover, FRET signal measurements and coimmunoprecipitation studies showed that WT MIB1 monomers dimerize and that dimers are also formed between WT and mutant MIB1 monomers or between mutant monomers. Both mutations result in loss of MIB1 WT function. In the case of R530X, this would be because of haploinsufficiency caused by insufficient synthesis of WT MIB1 protein; in the case of V943F, this would be because of a dominant-negative effect of the mutant protein, titrating down the amount of functional WT MIB1 dimers through heterotopic or homotypic interactions. In both cases, loss of MIB1 function leads to disease inherited in an autosomal dominant fashion.

Analysis of chamber-specific markers in Mib1flx; cTnT-cre mutant embryos revealed the expansion of compact myocardium markers to the trabeculae and reduced expression of trabecular markers. Global gene expression analysis confirmed these results and also showed that genes involved in the differentiation of endocardium, trabecular cardiomyocytes, and...
coronary vessels were downregulated in mutant embryos. In this regard, Bmp10 was downregulated in Mib1<sup>lox</sup>;cTnT-cre mutants, similar to the situation in endothelial-specific RBPJk mutants, indicating that endocardial–myocardial Notch signaling is crucial first for trabeculae formation and later for trabecular maturation and compaction. The observation that compact zone markers are expanded to the trabeculae of E15.5 Mib1<sup>lox</sup>;cTnT-cre mutant mice further suggests that trabecular patterning and maturation are impaired. Our working model suggests that myocardial Mib1 activity
enables Jag1-dependent activation of Notch1 in the endocardium, triggering downstream signaling events that sustain normal trabecular patterning, maturation, and compaction over a developmentally regulated time window (Figure 5F). Abrogation of Mib1-dependent signaling in the myocardium disrupts chamber maturation and compaction, resulting in LVNC. These data establish the causal role of NOTCH deregulation in LVNC.

Endocardial Notch Signaling in Zebrafish Heart Regeneration

The adult zebrafish heart exhibits a remarkable capacity to regenerate after ventricular resection or cryoinjury-induced myocardial infarction. The cryoinjury model triggers similar processes to those occurring in the mammalian heart after an ischemic injury: cardiac cell death, inflammatory cell infiltration, and the deposition of a fibrin and collagen-rich scar. The fish heart dissolves this scar, and injured tissue is replaced by new cardiomyocytes generated from dedifferentiation as seen in the resection model.

Examination of Notch expression in the zebrafish heart after cryoinjury shows a rapid upregulation of Notch signaling in the endocardium at the injury site (J. Münch, PhD, et al, unpublished observations, 2015), in agreement with reports of Notch reactivation after cardiac resection. Functional and gene expression analysis in the regenerating heart reveals that endocardial Notch activity is required for not only cardiomyocyte proliferation as reported in the ventricular resection model but also fibrotic tissue deposition and attenuation of the inflammatory response. As regeneration proceeds, the endocardium forms a scaffold that facilitates the entry of newly generated cardiomyocytes into the fibrotic area. Our work underscores the function of endocardial Notch signaling in cardiac regeneration as an important mediator between fibrotic tissue deposition and cardiomyocyte proliferation at the inner injury border (Münch et al, submitted).

Disease Modeling

Mutations in NOTCH signaling components in humans are implicated in CHD. The generation and analysis through the years of a great variety of targeted mutant mice harboring standard and tissue-specific mutations in several Notch pathway components suggest that a large number of these genes may be involved in CHD. This research also illustrates the connection between the fields of developmental biology and pathology. In the simplest situation (Mendelian inheritance) and to understand disease mechanisms, one would wish to find a direct correlation between the alterations caused in humans by mutations in a given gene and the phenotypes of targeted mutant mice for that same gene. This has not been always the case; the reasons for which could be multiple including the existence of as yet unidentified additional conditions/factors involved in the disease (comorbidities), that the disease is genetically complex, that the expressivity of the disease phenotype is influenced by the epigenome, or that the use of inbred strains in experimental studies causes a severe phenotype that is not paralleled in the human situation (or vice versa). For NOTCH, the correspondence between the human and the mouse cardiac disease phenotypes has been satisfactory to date. One illuminating example has been research in AGS, in which the generation of Jag1 targeted mutant mice was initially somewhat disappointing because heterozygous animals failed to exhibit the majority of the phenotypes associated with AGS in humans. However, mice doubly heterozygous for the Jag1 null allele and a Notch2 hypomorphic allele reproduced most of the clinically relevant phenotypes observed in patients with AGS (Table). This finding led to the identification of NOTCH2 mutations in a cohort of JAG1 mutation-negative patients with AGS. Thus, animal model studies shed light into how Notch2 and Jag1 mutations interact to create a more representative mouse model of AGS and provided an explanation of the variable phenotypic expression observed in patients with AGS.

The advent of next-generation sequencing technology coupled with efficient DNA capture has enabled the use of exome sequencing as a new approach to study the genetic basis of human disease that combined with genome-wide association studies, is a powerful tool to identify the basis of complex genetic traits. Genomic editing technologies such as Crispr/Cas9 opens the door for the relatively rapid generation of inactivating mutations in new disease-candidate genes identified by genome-wide association studies and the generation of potentially pathogenic mutations identified by exome sequencing or by any other next-generation sequencing-related approach.

Conclusions

In this review, we have described studies that uncover the role of Notch during cardiac patterning, OFT morphogenesis, and valve and chamber development and disease. These findings are of not only fundamental biological interest but also have important implications for understanding the cause of CHD. Data from several laboratories indicate that (1) the endocardium is a crucial source of signals that play key roles in cardiac development and disease; (2) the Notch pathway is a paradigmatic endocardial-derived signal, which regulates cell fate specification and tissue patterning in the early vertebrate heart to define chamber versus valve domains; (3) endocardial Notch and myocardial Bmp2 converge on Snail to activate EMT, leading to valve primordium formation; (4) endocardial Notch activity modulates in a non–cell autonomous manner various myocardial signals (Tgfβ, Bmp10) required for valve and trabecular development; (5) during OFT and valve morphogenesis Notch is required for myocardial Fgf8 and Bmp4 expression and EMT and mediates the communication between endocardium- and NC-derived mesenchyme that results in the mature valve; (6) Notch influences cellular proliferation, differentiation, and apoptosis during these processes; (7) NOTCH1 mutations cause BAV leading to precocious calcific aortic valve disease; (8) endocardial/endothelial NOTCH1 is required for the activation of antiosteogenic molecules and repression of proinflammatory gene signatures in the adult valve; (9) endocardial Notch is required for ventricular chamber development: during trabeculation, Mib1-Dll4-Notch1 signaling regulates cardiomyocyte proliferation and differentiation, and later Mib1-Jag1-Notch1 signaling promotes trabecular patterning, maturation, and compaction; (10) MIB1 mutations
cause familial LVNC inherited in an autosomal dominant fashion; (11) in the zebrafish regenerating heart, endocardial notch signaling mediates fibrotic tissue deposition and cardiomyocyte proliferation at the inner injury site. An important direction for future work will be to uncover the mechanisms of activation of the different tissue-specific notch elements, both in space and time, and to broaden the studies relating NOTCH signaling alterations to clinical phenotypes.

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Disclosures

None.

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