Notch Signaling in Cardiac Development
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Abstract—The Notch signaling pathway has been demonstrated to play a critical role during mammalian cardiac development based on recent findings from gene-targeted mice. In addition, mutations in the Notch signaling pathway have been associated with human congenital heart defects such as Alagille syndrome, bicuspid aortic valve disease, calcification of the heart valves, and ventricular septal defects. Recently, it was demonstrated that Notch activation in the endocardium regulates ventricular myocardial development and that the Notch downstream target genes Hey1 and Hey2 are required for the establishment of the atrioventricular canal myocardial boundary. The Notch pathway has previously been implicated in regulating endothelial-to-mesenchymal transition during development of the heart valves, and recent reports further dissect the role of individual Notch downstream target genes during this process. In addition, a role for the Notch pathway during cardiac neural crest cell development has been identified, which provides a potential mechanism for the findings seen in Alagille syndrome. This review focuses on recently reported findings that elucidate mechanisms regulated by the Notch pathway during ventricular, atrioventricular canal, and outflow tract development. (Circ Res. 2008;102:1169-1181.)

Key Words: Notch ■ cardiac development ■ endothelial–mesenchymal transformation ■ vasculogenesis ■ angiogenesis

Congenital heart defects represent the most common congenital defect in newborns, occurring in ~1% of live births.1 The heart is the first organ to develop during embryogenesis and plays a central role during both development and adulthood, because it is required for pumping nutrients and oxygen to all organs. The embryonic circulatory system is composed of vascular, cardiac, and hematopoietic components that arise from mesodermal origins during gastrulation (reviewed elsewhere2). Formation of the cardiac mesoderm is a highly regulated process that requires inductive and repressive signals emanating from the ectoderm, endoderm, and notochord at various stages of development (reviewed elsewhere3).

Before a morphologically identifiable heart the cardiac mesoderm forms a series of cardiac progenitor cell fields, which include the primary and secondary heart fields and the cardiac crescent (reviewed elsewhere4). When the cardiac crescent fuses along the embryonic midline, at embryonic day (E)8.0 in the mouse, it forms the first morphologically identifiable heart structure called the primitive heart tube, comprised of an outer myocardial and an inner endocardial cell layer (reviewed elsewhere5). In addition to the cardiac mesoderm of the primary heart field, cells from the secondary heart field (starting at E8.5) and the neural crest (starting at E10.0) migrate into the outflow region of the heart.4,5 The secondary heart field gives rise to the myocardium of the
The Notch Pathway

In mammals, the Notch pathway consists of 4 type I transmembrane receptors (Notch1 to Notch4) and 5 type I transmembrane ligands, Jagged1, Jagged2, Delta-like (Dll)1, Dll3, and Dll4, collectively referred to as the DSL (Delta/Serrate/Lag-2) family. Notch receptors are translated as large prepro-protein comprising extracellular, transmembrane, and intracellular domains, each consisting of numerous protein modification and protein interaction motifs. Under certain conditions, a Notch receptor is capable of being expressed on the cell surface as a large unprocessed protein, but, more frequently, Notch proteins are processed in the Golgi apparatus by Furin and expressed on the cell surface as a large unprocessed protein, which can then interact with the extracellular region of another Notch receptor on a neighboring cell. This interaction results in cleavage events; the first cleavage event is mediated by TACE (S2 cleavage) and is followed by 2 cleavage events (S3 and S4) mediated by the γ-secretase complex. Cleavage of the Notch receptor results in the release of the Notch intracellular domain (NotchICD) and its subsequent translocation to the nucleus, where it binds and converts CSL from a transcriptional repressor to a transcriptional activator.

Figure 1. The Notch pathway. The Notch signaling pathway is initiated by interaction of a ligand-expressing signaling cell and a receptor-expressing receiving cell. Notch ligands (Delta-like 1/3/4 and Jagged1/2) are transmembrane ligands that contain multiple epidermal growth factor-like (EGF) repeats and a Delta/Serrate/Lag2 (DSL) motif in the extracellular domain and several different motifs in the intracellular domain, depending on the specific ligand. Notch receptors (Notch1 to -4) are heterodimeric transmembrane receptors that contain multiple EGF-like repeats and 3 Lin12/Notch (LNR) motifs in the extracellular domain. The Notch intracellular domain contains an RBP-Jκ-associated molecule (RAM) motif, 7 ankyrin (ANK) motifs, 2 nuclear localization signals, and a transactivation domain (TAD). Receptor–ligand interaction results in 3 cleavage events; the first cleavage event is mediated by TACE (S2 cleavage) and is followed by 2 cleavage events (S3 and S4) mediated by the γ-secretase complex. Cleavage of the Notch receptor results in the release of the Notch intracellular domain (NotchICD) and its subsequent translocation to the nucleus, where it binds and converts CSL from a transcriptional repressor to a transcriptional activator.
Once in the nucleus NotchICD interacts with a transcriptional repressor called CSL (CBF1, suppressor of hairless, Lag-1), also known as RBP-Jκ (recombination signal-binding protein 1 for J-κ) and CBF1 (C promoter–binding factor 1), which results in derepression/activation of Notch-CSL target genes.33 The HES (hairy enhancer of split) and Hey (hairy/ enhancer of split–related with YRPW motif) (also called HESR, CHF, Hrt) families of basic helix–loop–helix transcription factors are the best defined direct Notch-CSL targets.34,35 Other direct targets include CyclinD1, p21, glial fibrillary acidic protein (GFAP), Nodal, Myc, PTEN, EphrinB2, and smooth muscle α-actin (SMA).36–44 In addition to Notch-CSL interaction, there is evidence that Notch participates in signaling independent of CSL (reviewed elsewhere45,46). Notch signaling has been reviewed in greater detail elsewhere and earlier in this series.15,47–49

AV Canal Development

As briefly discussed in the introduction, cells fated to become heart tissue (the cardiac mesoderm) are specified during the early stages of mesoderm formation in the primitive streak, at a stage before a morphologically identifiable heart or heart chambers. Specification of the cardiac mesoderm is largely independent of the Notch signaling pathway, as demonstrated by the presence of a primitive heart tube in Notch-deficient mouse embryos.50 It also should be noted that although the primitive heart tube develops normally in Notch-deficient embryos, signaling through Notch1 and Notch2 and the Notch ligand Dll1 is required for determination of the embryonic left–right axis and, therefore, proper looping of the heart tube.38,51 In addition, several reports have demonstrated that Notch signaling is involved in repressing the myogenic potential in a subset of cardiac mesoderm cells.52–54 These results suggest Notch signaling acts after the specification of the cardiac mesoderm and formation of the primitive heart tube during the initial stages of cardiac development.

The AV canal cushion, which is the first structure that regulates the flow of blood between the atria and ventricles, gives rise to the tricuspid and mitral valves and part of the AV septum.13 The AV canal is comprised of an outer myocardial and inner endocardial layers separated by a thin layer of extracellular matrix protein called the cardiac jelly. Commencing at E9.0, localized swellings of the cardiac jelly in the AV canal form the superior and inferior cardiac cushions, comprised of extracellular matrix proteins and glycosaminoglycans secreted by the myocardium.55

One of the important processes during AV canal development is the specification of the boundary between the functionally and molecularly distinct AV canal myocardium and the chamber myocardium (Figure 2). The AV canal myocardium does not acquire a high gap-junction density and conductivity as the atrial and ventricular myocardium, or trabeculation as the ventricular myocardium.56 In both the chick and the mouse, the AV canal uniquely expresses both the bone morphogenetic protein 2 (BMP2) ligand and the T-box 2 (Tbx2) transcription factor.57,58 Cardiac-specific deficiency of BMP2 results in AV canal defects, whereas Tbx2 deficiency results in expression of chamber specific myocardial genes in the AV canal.58,59 A series of experiments has further demonstrated that Tbx2 is a downstream target of the BMP2 signaling pathway.59,60 Although BMP2 and Tbx2 have been shown to be required for AV canal development, until recently what restricts BMP2 to the AV canal was not known.

Previous studies have revealed that both loss- and gain-of-function of the Notch pathway results in defects in AV canal development. A Notch2-hypomorphic allele, Jagged1-deficient, Hey2-deficient, Hey1/2-deficient, Hey1/L-deficient, CSL-deficient, and Notch1-overexpressing mice all display defects in some aspect of AV canal development.50,61–68 However, because of the severity of defects in these mutants and the fact that heart defects can be secondary to defects in other embryonic tissues, it has been difficult to ascertain the role of the Notch pathway in AV canal myocardial development. Recently, 2 reports investigated the downstream mo-
lecular mechanisms responsible for the defects observed in the Notch pathway mutants. Both reports demonstrated that the Notch target genes Hey1 and Hey2 are critically involved in restricting BMP2 and Tbx2 expression to the AV canal.69,70 However, there are several important differences between the 2 studies, which are discussed below.

The first study was mainly conducted in the developing chick heart; which only has 2 of the 4 mammalian Notch receptor homologs (Notch1 and Notch2). Notch2 was found to be the only Notch receptor expressed in the myocardium during chick cardiac development.69 The Notch ligand Jagged1 was found to be initially expressed throughout the heart tube myocardium, but, as the AV canal developed, Jagged1 expression was lost from the AV canal myocardium.69 In contrast, Jagged2 was found to be solely expressed in the atrial myocardium in an overlapping pattern with Jagged1.69 The Notch target gene Hey1 was found to be expressed in both the ventricle and atrial myocardium, whereas Hey2 was expressed exclusively in the ventricular myocardium.69 The expression patterns of Notch pathway components suggested that a signaling axis comprising Jagged1/2, Notch2, and Hey1/2 may be involved in regulating AV canal boundary formation. To test this hypothesis the Notch pathway was activated by electroporation of a constitutively active Notch2 (Notch2ICD) or CSL (CSL-vp16) construct directly into the linear heart tube, at a stage before AV canal development. Following AV canal development misexpression of the constitutively active Notch components in the AV canal myocardium, using ex vivo cultures, resulted in reduced BMP2 expression.69 Furthermore, overexpression of Hey1 or Hey2, by the same method, reduced BMP2 expression in the AV canal myocardium, suggesting Notch signaling through induction of the Hey genes represses BMP2 expression.69 When the Notch-activated (Notch2ICD) hearts were further analyzed for Hey1 and Hey2 expression, it was found that only Hey1 expression was induced, suggesting Notch2 regulates Hey1 but not Hey2 expression during heart development.69 Interestingly, neither Notch2ICD nor Hey1/2 misexpression induced the expression of atrial or ventricular chamber specific genes in the AV canal, suggesting that the specification of the chamber myocardium phenotype is not initiated by Notch but rather that Notch signaling represses AV canal myocardial phenotype. These results explain how the chamber myocardium represses BMP2 or Tbx2 expression; however, it does not explain why expression of Jagged1/2 and the Hey gene are excluded from the chick AV canal. One hypothesis tested is that BMP2 signaling or Tbx2 directly, negatively regulates expression of Notch pathway genes in the AV canal. To investigate this possibility, Tbx2 was misexpressed in the ventricular myocardium using the same electroporation method. Tbx2 misexpression resulted in reduced expression of both Hey1 and Hey2, suggesting that a negative regulatory loop exists between Tbx2 and Hey1/2 expression.69 Further analysis of Tbx2 misexpressing hearts failed to reveal an effect on Jagged1 expression, suggesting that Tbx2 regulates the Notch pathway downstream of receptor–ligand activation.69 However, Notch2 expression or activation level was not assessed in the Tbx2-misexpressing hearts; thus, a direct effect on Notch2 activation cannot be ruled out. This report further demonstrated that in Hey2-deficient mice there is an increased expression zone of BMP2 in the developing heart, supporting the findings in the chick that Notch via the Hey genes regulates AV boundary formation by regulating the BMP2/Tbx2 pathway.69

The second study was conducted in the developing mouse heart. It has previously been demonstrated that Hey1 is predominately expressed in the atrial myocardium, whereas Hey2 is predominately expressed in the subcompact layer of the ventricular myocardium.71 In the mouse, Notch2 and Jagged1 are not expressed in the ventricular myocardium as they are in the chick.62,72–74 Jagged1 is expressed in the atrial myocardium; however, expression of any Notch receptors or ligands other than Jagged1 has not been demonstrated in the chamber myocardium.62,72–74 Gene-targeting studies have revealed that Hey1 or HeyL alone are not required for heart development.61,66 Although the phenotype of Hey2-deficient mice is variable, these mice have high mortality in the first weeks after birth because of cardiovascular defects including ventricular septal defects, pulmonic stenosis, AV canal valve irregularities, and cardiac hypertrophy.66,75,76 Furthermore, Hey1 and Hey2 double-deficient embryos die at E9.5 as a result of severe heart defects including a thin ventricular myocardium and lack of arterial differentiation, suggesting compensation between Hey family members.66 When Hey1-deficient mouse embryos were analyzed for BMP2 expression, it was found that BMP2 expression extended from the AV canal into the atrial myocardium, whereas in wild-type embryos BMP2 expression was only detected in the AV canal myocardium.70 When BMP2 expression was analyzed in Hey2-deficient or Hey1/2 double-deficient embryos an increase in BMP2 expression was observed, albeit not in a chamber specific manner.70 Similar to the results obtain in the Hey1/2-misexpressing chick hearts, when Hey1 or Hey2 was overexpressed in all cells of the cardiac lineage in the mouse, there was a reduction in BMP2 and Tbx2 expression in the AV canal myocardium.70 Furthermore, in Hey1-misexpressing embryos, the AV canal is reduced in size; however, a cardiac cushion still forms and EMT occurs.70 In Hey2-misexpressing embryos, no AV canal forms and the ventricle is continuous with the atria.70 Enforced expression of the Hey genes was accomplished using a binary Cre-loxP transgenic model in which Hey1/2 was ectopically expressed in Mespl-derived cells.77 Mespl (also called mesoderm posterior 1) is a basic helix–loop–helix–type transcription factor expressed in the cardiac mesoderm starting at E7.0 and marks both the myocardial and endocardial lineages of the heart.77,78

In comparison to the first study, when the regulation of Hey genes was investigated 2 important differences were reported. When Tbx2 was misexpressed throughout the mouse heart, using the same Mespl-Cre binary transgenic model, expression of Hey1 or Hey2 was unaffected, suggesting that a negative-feedback loop between Tbx2 and the Hey genes does not exist in the mouse.70 One possible explanation for this discrepancy is that the misexpression of Tbx2 was not accompanied by a necessary cofactor required for repression of the Hey genes in the mouse. A further difference between these 2 reports involves the regulation of the Hey genes...
themselves. When Notch2-deficient or constitutively active Notch2 (Notch2ICD) overexpressing embryos were analyzed for the expression of the Hey genes no obvious alteration in Hey gene expression was evident, suggesting that Hey gene expression in the heart is independent of Notch2 signaling in the mouse.70 One possible explanation for this observation is that in the mouse, induction of Hey2 and repression of Tbx2 in the ventricular myocardium may involve the Tbx20 transcription factor.79 Nevertheless, similar experiments done with overexpression of constitutively active Notch1 (Notch1ICD) in the mouse did result in the induction of Hey1 and Hey2 expression and the repression of BMP2 expression in the AV canal myocardium.68 However, in the Notch1ICD-overexpressing embryos, the AV myocardium had undergone trabeculation raising the possibility that the AV canal myocardium had differentiated into ventricular myocardium (which expresses Hey2 but not BMP2). Taken together, these reports suggest that the expression of the Hey genes in the atrial and ventricular myocardium creates a boundary between the AV canal and chamber myocardium. However, whether or not the Notch pathway regulates the Hey genes or whether a negative regulatory loop exists between Tbx2 and the Hey genes remains to be resolved and may represent species-specific mechanisms.

Following proper AV canal boundary formation, a subset of endocardial cells lining the AV canal are activated by signals emanating from the myocardium and interendocardial signaling pathways to undergo EMT.13 Notch1, Notch2, Notch4, Jagged1, and Dll4 are all expressed in the AV canal endocardium.51,74,80 In addition, the Notch downstream target genes Hey1, Hey2, and HeyL are also expressed in the AV canal endocardium from the onset of EMT.61 Analysis of active Notch1 (Notch1ICD) levels reveal high levels of Notch1 activation in the cardiac cushion endocardium, whereas Notch1 activity is not found in the mesenchyme or myocardium.81 Zebrafish injected with constitutively active Notch1ICD or mice overexpressing Notch1ICD show hypercellular AV canals and enlarged AV valves, suggesting an increase in endocardial cushion EMT secondary to ectopic Notch activation.68,82 In addition, both Notch1-deficient and CSL-deficient embryos show significant reduction in AV canal EMT, as determined using an ex vivo AV canal explant assay which provides a measure of the degree of EMT taking place.82 Analysis of the Hey2-deficient and Hey1/L double-deficient embryos also revealed a defect in AV canal EMT using the ex vivo AV canal explant assay.61 Furthermore, when Hey1/L double-deficient hearts were analyzed in vivo fewer cells were observed in the cardiac cushions.81 Further analysis of the EMT defect observed in Hey2- or Hey1/L-deficient AV canal explants revealed similar numbers of migrating cells; however, the migrating cells did not adopt a mesenchymal morphology.81,82 These data suggest that the initial events of EMT occur normally in Hey2-deficient and Hey1/L double-deficient embryos, but the migrating cells fail to successfully undergo complete mesenchymal transformation.

In contrast Notch1 deficiency results in a defect in the induction of EMT, with very few migrating cells, which lack mesenchymal morphology.61,82 Thus, Notch may regulate the initial event of EMT and the acquisition of a mesenchymal morphology though different downstream target genes. Further analysis of Notch mutant embryos revealed 2 possible mechanisms for the defects described. In the Notch1-deficient, Hey2-deficient, and Hey1/L-deficient embryos, the EMT defect was accompanied by decreased matrix metalloproteinase 2 (MMP2) expression.63 MMP2, in addition to other MMPs, is an important regulator of EMT and is required for the migration and invasion of EMT-generated cells into the cardiac cushion.83

The second mechanism by which Notch mediates EMT involves the negative regulation of vascular endothelial (VE)-cadherin expression in the AV canal endocardium. VE-cadherin is an endothelial-specific adherens junction protein that is necessary for maintaining endothelial integrity.84 The ability of Notch to regulate VE-cadherin expression involves the induction of the Snail transcriptional repressor, a well-known regulator of adherens junction protein expression during development and disease.82 Activation of Notch signaling in endothelial cell lines or blocking Notch signaling during heart development results in the induction or the repression of Snail expression, respectively.85 In contrast, our laboratory has observed that Notch activation results in the induction of Slug, a close Snail family member but not Snail expression in human endothelial cells.85 Similar to Snail, Slug represses VE-cadherin and also other endothelial markers such as CD31 and Tie2.85 We find that Notch is involved in regulating Snail expression indirectly by synergizing with TGFβ signals, thereby potentially explaining the decreased Snail expression observed in Notch-deficient embryos.85 Furthermore, Slug deficiency in mice results in AV canal cardiac cushion EMT defects, which is compensated for by a relative increase in Snail expression.85 These data suggest the mechanism by which Slug and Snail function are similar and involve the negative regulation of VE-cadherin expression and initiation EMT. Thus, although Slug and Snail may be functionally equivalent, they are uniquely regulated during cardiac cushion development. These findings lead to a model where Notch signaling via Snail family members initiates EMT by repressing expression of VE-cadherin and other endothelial markers, which is not affected by the Hey genes, whereas the Hey genes regulate the acquisition of a mesenchymal phenotype.

Vascular endothelial growth factor (VEGF) is a key regulator of EMT in the AV canal. VEGF is expressed throughout the endocardium before EMT (E9.5); however, at the onset of EMT VEGF expression becomes restricted to a subpopulation of endocardial cells and by E10.5, it is predominantly expressed in the AV canal myocardium.86 Enforced expression of VEGF165 in myocardial cells of the AV canal before cardiac cushion development results in excessive endocardial cell proliferation, decreased EMT, and delayed cardiac cushion fusion.87,88 These data suggest that VEGF is a negative regulator of EMT and promotes an endocardial phenotype. It has also been shown that activation of the Notch pathway induces EMT and suppresses the VEGF pathway by down-regulating expression of VEGFR2.89 However, VEGF165 has been shown to upregulate expression of Notch receptors and ligands in endothelial cells from various vascular beds.90,91 In
Figure 3. AV canal EMT. EMT is initiated in the AV canal at E9.5 in the mouse. The AV canal myocardium expresses soluble factors such as BMP2 and TGFβ1 that induce EMT in the endocardium by inducing genes such as Snail. At the same time, the AV canal endocardium expresses the Notch receptors (1/2/4) and the Notch ligands Jagged1 and Dll4. Activation of the Notch pathway is necessary for induction of EMT in the AV canal. Notch receptor and ligand expression is regulated by the VEGF pathway; however, VEGF signaling must be inhibited before EMT, which is accomplished by the Notch target gene Hey2. In addition the Hey genes are involved in regulating an invasive mesenchymal morphology, partially by regulating MMP2 expression. The Notch pathway also regulates induction of EMT through the regulation of Snail expression, in cooperation with the TGFβ pathway, and by directly regulating Slug expression. Slug and Snail converge on the VE-cadherin promoter and negatively regulate endocardial phenotype. These data suggest that within the endocardium EMT would be inhibited in cells that have active VEGF signaling. In cells in which Notch and TGFβ signaling is activated, the VEGF pathway is inhibited and EMT is initiated.

Ventricular Development

Ventricular development is critical for adult cardiac function as the ventricles provide the main contractile force of the heart. The ventricles consist of several types of myocardium that develop at distinct points in ventricular development (reviewed elsewhere98). The trabecular myocardium represents folded sheets of highly organized cardiomyocytes lined by endocardium that lies on the inner surface of the ventricles. The trabecular myocardium starts to develop at E9.0, following heart looping and requires signals from both the myocardium itself and the overlying endocardium.43 During later stages of development, the sheets of trabecular myocardium undergo further remodeling into the papillary muscles, the cardiac conduction system, and the interventricular septum (reviewed elsewhere98). A second type of myocardium is the compact myocardium that underlies the epicardium. The compact myocardium initially forms a layer that is 1 to 2 cell layer thick but undergoes thickening because of proliferation and to a large extent compaction of the trabecular myocardium (reviewed elsewhere98). The compact myocardium forms the thick muscular wall of the ventricles, is thicker in the left ventricle, and undergoes further growth postnatally (reviewed elsewhere98).

The NRG1-ErbB2/4, EphrinB2/EphB4, and BMP10 signaling pathways are known to be required for ventricular trabecular formation. Neuregulin 1 (NRG1) is a paracrine ligand secreted by the ventricular endocardium that results in dimerization of ErbB2 and ErbB4 in the trabecular myocardium. NRG1-, ErbB2-, or EphB4 deficiency all result in embryonic lethality and defects in trabecular development.94–96 Bone morphogenetic protein 10 (BMP10) is a secreted ligand of the BMP pathway that is expressed in the trabecular myocardium and activates the BMP pathway through phosphorylation of the Smad1/5/8 proteins.97 BMP10-deficient animals die during embryogenesis, and loss of BMP10 is associated with decreased ventricular trabecular proliferation.97 EphrinB2 and Ephrin receptor B4 (EphB4) comprise a cognate ligand–receptor pair that is expressed in the ventricular endocardium. Either EphB4 deficiency or EphrinB2 deficiency results in embryonic lethality with a failure of myocardial trabecular formation.98

A recent report by Grego-Bessa et al investigated the ventricular defects observed in Notch pathway mutant mice. During normal trabecular development, there is close association between the ventricular endocardium and the developing trabecular myocardium. However, in Notch signaling–deficient embryos, this close association is not properly formed and the myocardium becomes thickened into a sheet and does not adopt the complex folded sheets of typical trabecular myocardium.43 At this point in ventricular development, active Notch1 (Notch1ICD), Dll4, and Dll1 expression corresponds with the developing ventricular endocardium.43 Furthermore, during the initial stages of trabecular formation active Notch1 expression is highest in the endocardial cells at the base of the developing trabecular sheet.43 Further analysis of trabecular defects in Notch-deficient embryos, using endothelial-specific Notch1-targeted or CSL-targeted mice, demonstrated that active Notch signaling is required in the ventricular endocardium for proper trabecular development.43 In the Notch signaling–deficient embryos the expression of myocardial differentiation markers (α-cardiac actin, SMA, and Irx4) are unaffected.43 However, the expression of endocardial cell markers (Irx5) and trabecular myocardial markers (HOP, Irx3, and PEG1) are severely reduced, suggesting that Notch signaling regulates trabecular development subsequent to myocardial specification.43 Analysis of BMP10, NRG1, and EphrinB2 expression in the Notch
signaling–deficient embryos revealed a decrease in both the expression and activation of these key pathways.\(^43\)

To investigate the mechanism by which Notch signaling regulates BMP10, NRG1, and EphrinB2 expression, a detailed analysis loss-of-function mutants for these genes was conducted. Analysis of NRG1 expression in EphrinB2-deficient and BMP10-deficient embryos revealed that NRG1 expression is dependent on active EphrinB2 signaling.\(^43\) EphrinB2 expression was unaffected by NRG1 deficiency or BMP10 deficiency, and the regulation of EphrinB2 expression was attributable to direct regulation of its promoter by Notch1 and CSL.\(^43\) BMP10 expression was unaffected by either EphrinB2 deficiency or NRG1 deficiency, suggesting that BMP10 expression is dependent on an unknown paracrine factor induced by Notch signaling in the ventricular endocardium.\(^43\) NRG1 has previously been shown to regulate trabeculae differentiation and the in vivo culture of CSL-deficient embryos with exogenous BMP10 rescued the differentiation potential of CSL-deficient cardiomyocytes, as demonstrated by increased expression of the differentiation marker PEG1.\(^43\) In addition, BMP10 has been previously shown to regulate trabeculae proliferation and the in vivo culture of CSL-deficient embryos with exogenous BMP10 rescued the proliferation potential of CSL-deficient cardiomyocytes, as demonstrated by increased 5-bromodeoxyuridine incorporation.\(^43\) These data suggest that Notch activation regulates trabecular development by regulating 2 processes: induction of EphrinB2 and NRG1 expression, resulting in trabecular differentiation and induction of BMP10, resulting in proliferation of trabecular cardiomyocytes.

In a complementary study, constitutive expression of Notch1ICD in all cardiac cells resulted in defects in myocardial trabecular development, the appearance of a cell mass in the AV canal and right-shifted interventricular septum.\(^68\) In this report, myocardial trabeculae were observed in the compact myocardial layer and the AV canal region, 2 regions where trabecular myocardium is usually absent.\(^68\) As Notch pathway components are not expressed in the ventricular myocardium, the defects in myocardial development could suggest that the ectopic expression of Notch1ICD in the endocardium results in increased NRG1 and BMP10 expression which, in turn, results in excessive myocardial differentiation and proliferation.\(^43\) Another possibility is that the Notch pathway components are expressed at levels below detection in the myocardium but still at a biologically functional level and that overexpression of Notch1ICD highlights a new direct role for the Notch pathway in the myocardium.

As the compact myocardium develops because of proliferation and the compaction of the trabecular myocardium, the Notch target gene Hey2 largely becomes restricted to the compact myocardial layer of the ventricles. However, Hey2 is additionally expressed in the interventricular septum and the endocardial cells of the cardiac cushions.\(^99,100\) As discussed earlier, Hey2 plays an important role in regulating AV canal boundary formation by repressing expression of BMP2 and Tbx2; however, several studies have also revealed a critical role of Hey2 in regulating ventricular development.

Recently, 2 reports investigated the role of Hey2 in ventricular myocardial development.\(^101,102\) As discussed above, Notch signaling regulates Hey1 but not Hey2 expression in the chick chamber myocardium, and Hey2 expression is independent of Notch signaling in the mouse myocardium. Using 3 conditional knockout strategies Xin et al demonstrated that cardiomyocyte–specific deletion of Hey2 results in impaired cardiac contractility and a malformed right ventricle.\(^102\) Cardiomyocyte and smooth muscle cell–specific deletion of Hey2 results in impaired cardiac contractility, malformed right ventricle, and ventricular septal defects.\(^102\) In contrast, endothelial–specific deletion of Hey2 has no apparent effect on heart development.\(^102\) When Hey2-deficient mice were further analyzed, both reports demonstrated increased expression of the atrial markers (ANF, Tbx5, Mlc1a, Mlc2a) in the left ventricle.\(^101,102\) In addition Xin and colleagues demonstrated an increase in sarcolin (Sln) in the left ventricle, a known inducer of cardiac contractility defects and heart failure.\(^102,103\) These data suggest that Hey2 maintains compact ventricular myocardial phenotype and represses atrial phenotype. Interestingly, increased expression of atrial markers were only identified in the left ventricle, suggesting that the left and right ventricles have unique transcriptional programs. This is possibly attributable to the fact that the left ventricle is specified by the primary heart field, whereas the right ventricle is specified by the secondary heart field (reviewed elsewhere).\(^104\)

The 2 reports above did differ in one important conclusion.\(^101,102\) Xin and colleagues demonstrated that when Hey2 is overexpressed throughout the heart, there is a repression of the atrial genes (Sln, Mlc1a, and Mlc2a) in the atria.\(^102\) In contrast, Koibuchi and Chin reported that overexpression of Hey2 throughout the heart did not affect expression of the atrial markers (ANF, Tbx5, Cx40, Mlc1a, or Mlc2a) in the atria.\(^101\) However, Koibuchi and Chin did observe that the misexpressed atrial genes (ANF, Tbx5, Cx40, Mlc1a, or Mlc2a) in the left ventricle of Hey2-deficient embryos are repressed by reintroduction of Hey2.\(^101\) The mechanism by which Hey2 represses atrial phenotype may involve 2 routes. Hey2 itself is a repressor and overexpression of Hey2 may result in repression of atrial genes. However, another mechanism has been reported that involves the GATA family of genes. GATA4 and GATA6 are expressed throughout the ventricular myocardium and are potent inducers of atrial and trabecular myocardial gene expression (eg, ANF).\(^105\) In the compact myocardium, however, GATA activity needs to be blocked for proper maturation and Hey2 has been shown to negatively regulate GATA activity by repressing GATA4 and GATA6 promoter activity and by physically interacting with GATA4 and GATA6 to block the induction of ANF expression.\(^106\) Together, these data suggest that the default myocardial phenotype of the developing heart is atrial and that Hey2 is responsible for repressing the atrial phenotype in the compact ventricular myocardium, allowing proper ventricular development. The signaling cascades involved in ventricular development are summarized in Figure 4.

**OFT Development**

The OFT is an embryonic structure that connects the ventricles to the ascending aorta and pulmonary arteries, and gives...
the trabecular myocardium and results in differentiation of cardiomyocytes (increased PEG1 expression). Also within the endocardium, Notch induces expression of an unknown secreter factor which induces BMP10 expression in the trabecular myocardium. Activation of the BMP10 pathway results in increased cell proliferation, possibly by repressing p57 expression. Following trabecular myocardial development, the compact myocardium is formed in the left and right ventricles. The compact myocardium expresses high levels of Hey2. Hey2 is necessary for repression atrial and trabecular myocardial gene expression in the left ventricle. This may occur by 2 mechanisms, first Hey2 may directly repress atrial and trabecular gene expression (ANF, Cx40) and Hey2 can block GATA4/6 expression (inducers of atrial/trabecular phenotype) and function within the compact myocardium.

Figure 4. Trabecular and compact myocardial development. Trabecular myocardial development is initiated around E9.0 in the mouse, and is characterized by the formation of complex folded myocardial outgrowths on the inner surface of the ventricles that are lined by endocardium. Notch activation is the highest in the endocardial cells at the base of the developing trabecular myocardium. Notch activation in the endocardium results in the expression of EphrinB2 and activation of the EphrinB2/Eph4 pathway, which results in upregulation of NRG1. NRG1 is a secreted ligand that activates ErbB2/4 in response to the aortic and pulmonary valves, the aortic arch, and the OFT septum (reviewed elsewhere). The OFT is formed by cells generated by the secondary heart field, the neural crest, and the cardiac mesoderm (endocardium) (reviewed elsewhere). Whereas the secondary heart field gives rise to the OFT myocardium, neural crest–derived cells migrate into the OFT cushions, which separate the OFT into aorta and pulmonary artery, and differentiate into the vascular smooth muscle layer of the aortic arch. Two important processes during cardiac neural crest cell development are the initiation of EMT in the neural tube, allowing for migration of the cardiac neural crest cells to the heart, and the differentiation of the migratory cardiac neural crest cells into specific cell types. Although neural crest cells migrate into the OFT cardiac cushions, their importance to valve formation has not been established. Lineage analysis has suggested that the majority of cells within aortic and pulmonary valves are of endocardial origin. In addition, numerous studies have suggested that the BMP pathway is critically required for neural cell migration into the OFT cushions and for differentiation of neural crest cells to smooth muscle cells of the aortic arch arteries. Furthermore, in neural crest–specific Alk2-deficient embryos, the OFT cardiac cushions are hypocellular because of defects in neural crest cell migration, although the aortic valves develop normally. In the Alk2-deficient embryos the proximal (close to the right ventricle), OFT septum is missing, which results in truncus arteriosus. These data suggest that EMT in the OFT is sufficient for proper valve formation and that cardiac neural crest cells are required for septal and aortic arch development. However, it is also possible that the septal defects are attributable to increased hemodynamic forces resulting from defects in aortic arch artery development.

During development of the aortic arch and the aortic arch arteries the expression of Notch1 and Notch4 is restricted to the endocardium. In contrast, Notch2 and Notch3 expression is observed in the cardiac neural crest–derived vascular smooth muscle cells surrounding aortic arch arteries. Jagged1 is also expressed by the vascular smooth muscle cells surrounding aortic arch arteries, whereas Jagged2 expression is more diffuse but overlaps with Jagged1 expression. DI1 is expressed by the endocardium of the heart but not in the endothelium of the aortic arch arteries, whereas DI4 is expressed by the endothelium of the aortic arch arteries and the endocardium. DI3 is not expressed in the aortic arch or aortic arch arteries. The Notch target genes Hey1, Hey2, and HeyL are all expressed by the vascular smooth muscle cells surrounding aortic arch arteries, and colocalize with smooth muscle actin expression. The expression pattern of the Notch receptors, ligands, and target genes suggests multiple roles in OFT development, including differentiation of cardiac neural crest cells into vascular smooth muscle cells of the aortic arch arteries. Previous in vitro studies investigating the role of the Notch pathway in smooth muscle cell phenotype have been contradictory, with findings that suggest that Notch can either promote or inhibit smooth muscle cell differentiation. A recent report by High et al specifically investigated the role of Notch signaling during cardiac neural crest cell differentiation in vivo. As stated above, multiple Notch receptors and ligands are expressed in cardiac neural crest–derived cells, making loss-of-function studies difficult. To overcome this problem, a dominant-negative mastermind–like (MAML-DN) construct under the control of a Cre-lox system was used to block Notch signaling in the cardiac neural crest cells. MAML is a Notch coactivator and overexpressing the Notch interacting domain of MAML (MAML-DN) has been demonstrated to block signaling through all of the Notch receptors and ligands. However, whether or not all Notch signaling requires MAML has not been established, and MAML-DN also has the potential to block other MAML-dependent signaling pathways, such as p53 and Mef2C. However, in this study, MAML-DN did not appear to block Mef2C signaling, suggesting that the effects are attributable to inhibition of Notch activity. When Notch signaling was blocked before cardiac neural crest cell differentiation (Pax3-cre, Wnt1-cre), it resulted in pulmonary stenosis and aortic arch patterning defects, reminiscent of Alagille syndrome, which is discussed in detail below. In the MAML-DN embryos, the aortic arch arteries
were characterized by loss of vascular smooth muscle markers (SMA, SM22α) and a disorganized smooth muscle layer.112 However, markers for cardiac neural crest cell migration (plexinA2) demonstrated an equal number of migrating cardiac neural crest cells, suggesting that the defect is subsequent to cardiac neural crest cell specification and migration to the OFT.112 Furthermore, when Notch signaling was blocked following cardiac neural crest cell differentiation into vascular smooth muscle cells (SM22α-cre), no defects in cardiac development were observed. These data suggest that Notch signaling is required following neural crest cell migration but before formation of a vascular smooth muscle layer and that Notch signaling is not required for maintaining smooth muscle cell phenotype in the aortic arch arteries.112 To investigate the stage of cardiac neural crest cell development that Notch signaling is required, High et al112 used a neural tube explant model. In this model, the neural tube of MAML-DN or wild-type explants treated with a γ-secretase inhibitor of Notch signaling were cultured ex vivo, and different stages of cardiac neural crest cell development were assessed. When the Notch pathway was blocked by either method, the induction of vascular smooth muscle cell marker expression failed to occur, suggesting that Notch is involved in the differentiation of cardiac neural crest cells into vascular smooth muscle cells of the aortic arches. However, the downstream targets responsible for Notch-mediated vascular smooth muscle cell differentiation remain unclear.

Alagille Syndrome

Mutations in the Jagged1 locus are associated with 94% of patients with Alagille syndrome (AGS).120 In addition, mutations in the Notch2 locus have been identified in patients with Jagged1-independent AGS.121 AGS is an autosomal dominant disorder most commonly associated with neonatal jaundice and impaired development of intrahepatic bile ducts with additional abnormalities of the eye, heart, kidney, and skeleton, with variable penetrance. The most common cardiovascular defect is peripheral pulmonary stenosis; in addition, 13% of AGS patients have tetralogy of Fallot.122–124 The cardiac AGS phenotype is consistent with the expression pattern of Jagged1 and Notch2 in both the endothelium, the cardiac neural crest cells and the smooth muscle cells of the pulmonary arteries.72,112 Analysis of mutations in the Jagged1 locus have revealed, in some cases, complete loss of the Jagged1 locus and, in other cases, inactivating mutations that lead to a misexpressed or truncated Jagged1 protein.125 However, the type or location of mutation within the Jagged1 locus does not correspond with the differences or extent of phenotypes found in AGS patients.123 In mice, heterozygous mutations in Jagged1 or Notch2 do not reproduce the AGS phenotype.63,73 Jagged1 heterozygote mice display eye defects, whereas Jagged1-null mice die at E10 as a result of vascular defects.65 Notch2 haploinsufficiency in mice results in kidney defects and myocardial hypoplasia, but the phenotype does not resemble findings of cardiovascular and kidney defects in patients with AGS.82,73 However, mice doubly heterozygous for Jagged1-null and Notch2-hypomorphic alleles develop jaundice and impaired development of intrahepatic bile ducts with associated abnormalities of the eye, heart, and kidney, thus reproducing an AGS phenotype.126 In addition, Hey2 deficiency in mice results in cardiovascular defects including tetralogy of Fallot and pulmonary stenosis in a strain-specific manner, suggesting that a signaling axis of Jagged1, Notch2, and Hey2 is involved in AGS.75 The cardiovascular phenotypes in AGS patients include valvular pulmonary stenosis, attributable to narrowing of the pulmonary valve, and pulmonary stenosis of the peripheral pulmonary vasculature.123 The mechanistic basis for valvular pulmonary stenosis remains unclear, although it may reflect the role of Notch in regulating EMT in the cardiac cushion.82,89

As discussed above, in mice neural crest–specific ablation of Notch signaling results in aortic arch branching defects and pulmonary stenosis, although in these mice the site of pulmonary stenosis is not specified but appears to be in multiple sites within the pulmonary vasculature.112 Ablation of neural crest Notch signaling results in a severe defect in the development of the smooth muscle cells of the sixth aortic arch artery in mice, which is the embryonic origin of the pulmonary artery.112 However, whereas the smooth muscle cells of the adult proximal pulmonary trunk (close to right ventricle) are neural crest–derived, the smooth muscle cells of the pulmonary arteries are not.5 It is possible that defects in development of the sixth aortic arch artery are attributable to neural crest–derived cells that contribute to the sixth aortic arch during embryonic development but are replaced by smooth muscle cells of the pulmonary vasculature during remodeling of the pulmonary vasculature during later stages of development. Another possibility is that the peripheral pulmonary stenosis in AGS patients is caused by defects in signaling between the endothelial and smooth muscle layers mediated by the endothelial expression of Jagged1 in the pulmonary vasculature.127

Aortic Valve Disease

In humans, mutations in the Notch1 locus results in a spectrum of heart defects.128 The most prevalent malformations are bicuspid aortic valve disease and calcification of the aortic valve.128 Calcification of the aortic valve is the third leading cause of heart disease in adults, whereas the presence of bicuspid aortic valve is present in 1% to 2% of the population.128 The mechanism by which human Notch1 mutations effect aortic valve development and calcification is poorly understood. However, calcification of the aortic valve is thought to be attributable to endothelial dysfunction. Notch pathway components, and Notch1 in particular, are highly expressed in arterial endothelial and endocardial cells, which correlate with the importance of the Notch pathway in regulating endothelial function in the aortic valve. Using an in vitro system, it was demonstrated that Notch1, Hey1, and Hey2 physically interact with and repress the function of the transcription factor RUNX2.128 RUNX2 has been linked to valvular calcification in both rabbit and mouse where it regulates expression of several osteogenic genes, such as osteopontin and osteocalcin.129 It is not known whether Notch1 mutations found in humans results in lower Hey1 and Hey2 expression in the aortic valve, higher RUNX2 activity, or expression of osteogenic genes that could explain the calcification of the aortic valve. In addition, during aortic
valve development the 3 valve leaflets are remodeled from 3 distinct cardiac cushions. A failure to initiate EMT in 1 of the cardiac cushions or the fusion between 2 of the cardiac cushions could result in a bicuspid aortic valve.\textsuperscript{61,82} In addition, tetralogy of Fallot, an anomaly commonly found in AGS, is commonly associated with a bicuspid aortic valve, suggesting that mutation in several Notch pathway components are associated with bicuspid aortic valve disease.

**Conclusions**

The importance of the Notch pathway during vascular development has been established for several years. Recent studies demonstrate that Notch is also critical for development of the heart in different ways. In ventricular development Notch has 2 roles during trabecular development: first, for differentiation of cardiomyocytes by the induction of NRG1 expression; and second, for proliferation of cardiomyocytes through BMP10. Notch activation is also necessary for AV canal boundary formation and for AV canal EMT. Finally, Notch activation in cardiac neural crest cell development is required for proper formation of the OFT. As noted throughout the review, in each area, important questions remain with regard to the mechanism of Notch action and its crosstalk with other pathways. Thus, elucidating Notch function in the heart will require ongoing investigation.

**Sources of Funding**

Work in the laboratory of A.K. is supported by grants from the Heart and Stroke Foundation of British Columbia and the Yukon, the Canadian Institutes of Health Research, the Canadian Cancer Society, the Stem Cell Network, Genome Canada, and Genome British Columbia. K.N. was supported by a doctoral research award from the Canadian Institutes of Health Research, the Canadian Cancer Society, and the Canadian Cancer Society. A.K. is a senior scholar of the Michael Smith Foundation for Health Research. A.K. is a senior scholar of the Michael Smith Foundation for Health Research.

**Disclosures**

None.

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Notch Signaling in Cardiac Development
Kyle Niessen and Aly Karsan

Circ Res. 2008;102:1169-1181
doi: 10.1161/CIRCRESAHA.108.174318
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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