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Notch and cardiac outflow tract development

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Abstract

Congenital heart disease represents the most common form of human birth defect, occurring in nearly 1 in 100 live births. An increasing number of patients with these defects are surviving infancy. Approximately one-third of congenital heart defects involve malformations of the outflow tract. Related defects are found in isolation and as part of common human syndromes. Our laboratory has investigated mechanisms of cardiac morphogenesis with particular attention to outflow tract formation. During cardiogenesis, neural crest cells interact with second heart field myocardium and endocardial cushion mesenchyme. Our recent work demonstrates that Jagged1/Notch signaling within the second heart field initiates a signaling cascade involving Fgf8, Bmp4, and downstream effectors that modulate outflow tract development and aortic arch artery patterning. Hence, complex tissue–tissue interactions and integration of multiple pathways converge to orchestrate proper patterning of the outflow region. The role of Notch signaling in adult cardiac homeostasis and disease is an area of active investigation.

Keywords

congenital heart disease; Notch; outflow tract; neural crest; second heart field

Introduction

One of the many challenges in understanding cardiac morphogenesis and, by extension, congenital heart disease, is identifying the cues and signaling pathways controlling differentiation of cardiac precursor cells into the diverse cell populations that form the mature heart. Formation of this complex organ requires interactions between multiple cell populations, and understanding the developmental mechanisms underlying these interactions will give further insight into development and disease. One of the important subsets of congenital heart defects are those that involve malformations of the outflow tract of the heart, including the aorta, pulmonary arteries, aortic arch, and ductus arteriosus. The outflow tract is formed during development through interactions between cells from within the heart itself, such as the endocardial cells and cardiomyocytes, as well as migrating cells arising from the cardiac neural crest. This review will focus on the signaling pathways and interactions between cell populations that underlie morphogenesis of the outflow tract, with particular attention to the role of the Notch signaling pathway.

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Conflicts of interest

The authors declare no conflicts of interest.

Neural crest ablation models have demonstrated a critical role for cardiac neural crest cells in proper outflow tract formation.^{1,2} The cardiovascular phenotypes that result from neural crest ablation include persistent truncus arteriosus (PTA), which is the result of absence of outflow tract septation, overriding aorta, and double-outlet right ventricle. Overriding aorta describes a phenotype in which the aorta misaligns over the ventricular septum with an accompanying ventricular septal defect (VSD). Hence, blood entering the aorta comes from both right and left ventricles. It also describes a spectrum of disease that includes double-outlet right ventricle (DORV), an anomaly where the majority of the blood entering the aorta comes from the right ventricle. Also included in this spectrum is tetralogy of Fallot (TOF), a common congenital heart disorder that has four major components: valvular or subvalvular pulmonary stenosis, a VSD, a misaligned aorta, and right ventricular hypertrophy.

Congenital defects can be found either in isolation, or as part of human syndromes. Amongst the more common human syndromes involving outflow tract defects is DiGeorge syndrome, commonly associated with micro- or macro-deletions on chromosome 22q11, usually including deletion of *TBX1*, which has been strongly implicated in the etiology of this disorder.³ Relevant to the focus of this review on Notch signaling, Alagille syndrome is a human disorder involving outflow tract cardiac defects. This syndrome is characterized by a spectrum of anomalies including congenital heart defects, such as peripheral pulmonary arterial stenosis, aortic constriction, semilunar valve defects, and tetralogy of Fallot, as well as impaired differentiation of intrahepatic bile ducts, skeletal defects, eye abnormalities, and kidney anomalies. Human mutations in Alagille syndrome have been identified in components of the Notch signaling pathway including *NOTCH2* and *JAGGED1*, a ligand of the Notch receptor.⁴

Outflow tract formation

As mentioned above, the cardiac outflow tract is formed by a complex interplay of processes between several different cell types both from within the looped heart itself, as well as populations of invading extracardiac cells. Endothelial-to-mesenchymal transformation (EMT) contributes to the formation of the outflow tract cushions, which are the primordia of the semilunar valves. Second heart field cells populate the endocardial cushions, although whether they migrate into the endocardial cushion or enter as a result of EMT from the endocardium is unclear.⁵ Neural crest cells migrate into the endocardial cushions of the outflow tract, where their precise role in valve formation remains to be fully elucidated.⁵

The outflow septum, which divides the systemic circulation from the pulmonary circulation, begins as a shelf-like structure distal to the outflow tract. The shelf forms between the fourth aortic arch artery (eventual aorta) and sixth aortic arch artery (eventual pulmonary artery) and it grows into the distal outflow tract towards the proximal outflow tract. Neural crest cells are important for this septation process, and for patterning of the great vessels, and eventually will give rise to some of the smooth muscle cells within the arterial wall.⁶ The shelf grows proximally, where it comes into continuity with the bulging outflow tract cushions. Cells from the second heart field form the smooth muscle cells near the base of the aorta and pulmonary artery and populate regions adjacent to the more distal neural crest-derived smooth muscle cells within the walls of the great arteries. After septation of the outflow tract, the outflow endocardial cushions further remodel into the mature aortic and pulmonary valves.

Fgf8 and outflow tract formation

Several molecules have been implicated in outflow tract development, primarily through the use of gene knockout technology. The molecular pathways connecting the functions of these molecules are only just beginning to be understood. One important signaling molecule is Fgf8, a soluble protein expressed in the cardiac crescent and in multiple tissues throughout development including pharyngeal endoderm and ectoderm, and in the second heart field.^{7,8}

Fgf8 is a member of the fibroblast growth factor (Fgf) family, and has proven to be a critical molecule during early embryogenesis, as homozygous null mice die during gastrulation.⁹ Therefore, closer examination of Fgf8 signaling during development was undertaken through the development of various hypomorphic and conditional alleles. Global Fgf8 hypomorphs demonstrate complex out-flow tract defects including PTA and DORV, as well as abnormal arch artery patterning.^{10–12} Similar defects of outflow tract development were seen when Fgf8 was deleted in the Tbx1 expression domain.^{12,13}

Two research groups have addressed the cell-type-specific role and temporal requirement for Fgf8 in outflow tract development. Loss of Fgf8 in the second heart field resulted in outflow tract abnormalities including DORV and PTA, revealing a cell-autonomous role for Fgf8 within the second heart field. Fgf8 deletion in a broader cardiac expression domain using the Nkx2.5-Cre line resulted in severely truncated heart tubes, and deletion within early mesodermal precursors resulted in abnormal cardiogenesis. In contrast, deletion with a cTnT-Cre line, which is expressed at a later time point, did not result in outflow tract abnormalities.^{14,15} Therefore, Fgf8 signaling appears to be critically important for early cardiogenesis, and less crucial for outflow tract formation in those cells that have already committed to the cardiac lineage.

Although an important role for Fgf8 signaling during cardiogenesis had been well characterized, its target tissues and downstream effectors were not well understood until recently. Given that Fgf8 is a soluble factor, could it be responsible for sending a signal from the second heart field to the neural crest and/or endothelium? Two well-designed studies addressed this question, and demonstrated that Fgf8 from the second heart field signals in an autocrine loop to the second heart field itself to mediate appropriate outflow tract morphogenesis. Unexpectedly, outflow tract remodeling is not dependent on Fgf receptor signaling within the neural crest or endothelium. In contrast, loss of both Fgfr 1 and 2, or loss of an adaptor protein FRS2 α which should be used by Fgf receptors, in second heart field precursors (using either Islet1-Cre or Mef2c-AHF-Cre) resulted in outflow tract abnormalities nearly identical to those seen in Fgf8 cardiac-specific mutants described earlier.^{16,17} These results indicate that Fgf8 is not directly signaling to endothelium or to neural crest to coordinate cardiogenesis. Rather, these data suggest that Fgf8 signaling within the second heart field may result in expression of another soluble factor that subsequently affects neural crest and/or endothelium to orchestrate outflow tract morphogenesis.

A candidate for such an effector of Fgf signaling may be the bone morphogenic class of proteins, of which Bmp4 is one of the most highly expressed members within the developing outflow tract.¹⁸ Cardiac-specific deletion of Fgf8 results in a downregulation of Bmp4 in the developing out-flow tract, and the consequence of cardiac-specific deletion of Bmp4 is PTA.^{15–17,19,20}

In order to address the target tissues of Bmp signaling, various groups have deleted components of the Bmp receptors, including Alk2 and Alk3, in neural crest and other tissues. Conditional deletion of these Bmp receptors in neural crest cells produces phenotypes strikingly similar to the Fgf8 mutants, including PTA and abnormal aortic arch artery patterning.^{18,21} The role of Bmp signaling to the neural crest is further supported by Smad4 conditional deletion in this cell population. Smad4 is a common Smad functioning downstream of activated Bmp receptors, and its deletion in neural crest produces PTA and hypocellular outflow tract cushions.²²

Similar to its importance in the neural crest, Bmp signaling to the outflow tract endocardium may also be important. Both the Alk2 and Alk3 receptors were found to be critically important in the endothelium for AV cushion development, and their deletion resulted in abnormal

cellularization and defective EMT of the AV cushions.^{23–25} However, closer examination of the role of Alk2/3 in the endocardium of the outflow tract must be undertaken to fully characterize the importance of Bmp4 signaling in this region of the heart.

Notch and outflow tract development

Notch is a highly conserved pathway with important functions throughout various stages of development of disease. The five ligands (3 Delta and 2 Serrate proteins) and the four Notch receptors are transmembrane proteins with multiple epidermal growth factor (EGF)-like repeats. Upon ligand binding to Notch receptor, the receptor undergoes a series of cleavage events that release the Notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus, NICD is able to regulate transcription through its interactions with Mastermind-like protein (MAML) and with recombination signal binding protein for immunoglobulin J-kappa region (RBP-J). The most well-described direct transcriptional targets of Notch are the *Hrt* and *Hes* family of genes.²⁶ There are several other direct Notch targets, including *c-Myc*, and many more as yet undiscovered direct targets of Notch are likely to exist.²⁷

The function of Notch signaling can be divided into three broad categories: lateral inhibition, lineage determination, and boundary formation.²⁸ Notch signaling is extremely sensitive to temporal and spatial cues. This was first demonstrated in elegant studies involving neurogenesis, in which it was shown that early Notch signaling initially dictates the number of cells that can acquire a neurogenic fate, while later it influences the lineage decision of neural versus glial cell fate.²⁹

The Notch signaling pathway has been shown to be required within neural crest precursors for proper patterning of the outflow tract region. Inactivation of Notch signaling within neural crest cells using a dominant-negative form of MAML, which has been shown to be a pan-Notch inhibitor, results in congenital heart defects including pulmonary artery stenosis and aortic arch patterning defects that were associated with defective smooth muscle formation.³⁰ At least part of the phenotype resulting from loss of Notch signaling in the neural crest may be attributed to loss of Notch2 signaling, since Notch2 inactivation in neural crest produces small-caliber aortas and pulmonary arteries due to defects in the smooth muscle.³¹ The ligand involved in this process appears to be endothelial Jagged1, which signals to Notch on neural crest cells to induce vascular smooth muscle differentiation.³² Human mutations in both *JAGGED1* and *NOTCH2* have been found to result in Alagille syndrome, giving further credence to the importance of this signaling pathway in proper formation of the outflow tract.⁴

In another study, which again implicates the potential importance of Notch signaling in the outflow tract region in human disease, *NOTCH1* mutations were reported in patients with aortic stenosis. Aortic stenosis resulting from calcification of the aortic valve is a common disease in adults, and in children aortic stenosis may possibly result in failure of the left ventricle to develop properly. The incidence of aortic stenosis increases with age in adults, and the incidence is also increased in the 2% of the population that has a bicuspid aortic valve. *NOTCH1* haploinsufficiency is associated with aortic valve disease including early calcification and bicuspid aortic valve disease. The proposed mechanism for this is an early induction of Runx2 through the *HRT* genes.³³

Of interest, related to the role of Notch in valvular formation, global Notch1 and RBP-J mutants have hypocellular endocardial cushions and defective EMT. Consistent with this, the expression of Snail and Slug, mediators of EMT, is downregulated in these mutants.^{34,35} Complementary studies demonstrate that Jagged1 ligand stimulation of endothelial cells is sufficient to induce EMT.³⁵ Expression analysis suggests that Notch1 and Delta-like 4 are active in endothelium, but further analysis of lineage requirements using conditional alleles

will be required to determine whether this defect is a result of the loss of Notch1 or RBP-J signaling within the endocardium.³⁴

These previous studies give tantalizing clues into a potential pathway by which second heart field precursors are communicating to neural crest and endothelium to direct outflow tract formation. Recent work from our laboratory further advances this model, and implicates Notch signaling as a key mediator of this process. Either deletion of the Notch ligand Jagged1, or inhibition of Notch signaling using dominant negative MAML in the second heart field resulted in outflow tract abnormalities including PTA, DORV, and aortic arch artery patterning defects (Figure 1). Of interest, inhibition of Notch signaling in the second heart field also affected the development of neighboring tissues. We observed faulty migration of cardiac neural crest cells and defective EMT within the outflow tract cushions. Moreover, our data show that Notch is a critical mediator of Fgf8 signaling in the second heart field. The defective EMT was rescued in an *ex vivo* assay by the addition of recombinant Fgf8.³⁶

Further investigation is necessary to understand the temporal and spatial characteristics of these pathways. Is Notch directly or indirectly regulating Fgf8 secretion in the second heart field? Can phenotypes in the Notch and/or Fgf8 mutants be rescued by reinstitution of Bmp4 expression? In light of the recent advances describing migration of epicardial precursor cells into the heart, what role does this cell population play in outflow tract morphogenesis? Finally, there are many examples of outflow tract defects that do not, as of yet, fall neatly into this model. One such example includes our laboratory's work on PlexinD1. Loss of PlexinD1 in the endothelial compartment results in PTA, and further work will be necessary to determine whether this signaling cascade is connected to the aforementioned Notch, Fgf8, and Bmp4 pathways, or quite possibly involves another pathway leading to a common phenotype.^{37,38}

Notch and ventricular maturation

During ventricular maturation, trabecular myocytes are intimately associated with the endocardium, and appropriate cross-talk is necessary for proper ventricular trabeculation. Well-designed studies have recently begun to dissect signaling pathways between adjacent endocardial cells and the trabecular myocardium.³⁹ This work has implicated endocardial Notch as a regulator of both cardiomyocyte proliferation and differentiation. Ephrin B2 is a direct target of Notch, acting upstream of neuregulin-1 to induce cardiomyocyte differentiation. Notch regulates BMP10 signaling in the myocardium to control proliferation independent of ephrin and neuregulin signaling. Endocardial Notch therefore appears to regulate key pathways involved in ventricular maturation, although details of the signaling mechanisms between endocardium and myocardium remain to be fully elucidated.

Notch and postnatal myocardium

Notch has recently been implicated in homeostasis of the postnatal myocardium. Reactivation of Notch1 signaling in neonatal ventricular cardiomyocytes induces cell cycle reentry.^{40,41} Cell cycle progression involves both an RBP-J-dependent induction of cyclin D1, as well as an RBP-J-independent translocation of cyclin D1 to the nucleus.⁴⁰ Interestingly, *in vitro* postnatal day 5 cardiomyocytes respond to Notch activation by activation of a DNA damage checkpoint and subsequent apoptosis. This age-dependent decline in the ability to reenter the cell cycle parallels the decline of endogenous Notch in postnatal cardiomyocytes.^{40,41}

Loss of Notch1 in the differentiated ventricular myocardium does not result in structural anomalies, and these mice reach adulthood and appear grossly normal.⁴² However, hemodynamic overload of mice with ventricular cardiomyocyte-restricted loss of Notch1 results in excessive hypertrophy, fibrosis, and a higher mortality than in control mice.⁴² Consistent with a role for Notch1 in postnatal homeostasis, infarcted hearts injected with a

virus that expresses activated Notch have improved hemodynamic function when compared with controls. Activation of the c-Met and Akt signaling pathways are associated with this prosurvival effect.⁴³ It is not clear whether the cardioprotective role of Notch in hemodynamic overload and infarction models is due to a protective effect on differentiated cardiomyocytes, or whether Notch is maintaining a progenitor pool within the heart that is able to regenerate damaged tissue.

Finally, there is some emerging evidence to suggest that the Notch pathway may be implicated in human dilated cardiomyopathy. The presenilin proteases are required for cleavage and activation of the Notch receptor, among other substrates. Recently, *PSEN1* and *PSEN2* mutations have been found to be associated with dilated cardiomyopathy and heart failure.⁴⁴

Many questions remain regarding the role of Notch in the adult myocardium. Does Notch signaling play a role in the postnatal myocardium of unstressed hearts, or is it important for survival and/or regeneration only under conditions of stress? Does Notch exert a cardioprotective role primarily via proliferative or anti-apoptotic activity, or is maintenance of a progenitor pool required for repair? Are signaling cascades that are active during cardiogenesis later reactivated during postnatal stem cell activation?

In this review, we have attempted to highlight some of the key pathways involved in outflow tract development, including the Notch pathway. The importance of Notch signaling during cardiogenesis and outflow tract development is illustrated by the discovery of genetic mutations in components of this pathway in human disease. Insights derived from the elucidation of Notch activities during cardiac development are likely to be relevant to a further understanding of Notch actions in the adult heart.

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Figure 1.

Aberrant cardiac morphogenesis resulting from inhibition of Notch signaling in the second heart field in late-gestation *Islet1-Cre; dnMAML* embryos. (A) Volume-rendered MicroCT image of a mutant with PTA. There is a single overriding vessel (truncus arteriosus) and a VSD (arrowhead). (B) Another mutant embryo exhibits DORV. The arrowhead points to the aorta (note the coronary vessel originating from the base of this vessel) and the tailed arrow points to the pulmonary artery.