Notch signaling in the developing cardiovascular system

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Niessen K, Karsan A. Notch signaling in the developing cardiovascular system. Am J Physiol Cell Physiol 293: C1–C11, 2007. First published March 21, 2007; doi:10.1152/ajpcell.00415.2006.—The Notch proteins encompass a family of transmembrane receptors that have been highly conserved through evolution as mediators of cell fate. Recent findings have demonstrated a critical role of Notch in the developing cardiovascular system. Notch signaling has been implicated in the endothelial-to-mesenchymal transition during development of the heart valves, in arterial-venous differentiation, and in remodeling of the primitive vascular plexus. Mutations of Notch pathway components in humans are associated with congenital defects of the cardiovascular system such as Alagille syndrome, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and bicuspid aortic valves. This article focuses on the role of the Notch pathway in the developing cardiovascular system and congenital human cardiovascular diseases.

Cardiac development; endothelial-mesenchymal transformation; vasculogenesis; angiogenesis

NOTCH PATHWAY

In mammals, the Notch receptor family consists of four type I transmembrane receptors (Notch1 to Notch4) that regulate cell fate decisions through cell-cell interaction (27). Notch receptors are translated as a large (∼300 kDa) precursor protein comprising an extracellular, a transmembrane, and an intracellular domain that undergoes processing in the trans-Golgi network by Furin (76). Furin cleavage occurs at the S1 cleavage site and generates an extracellular fragment (NotchEC) and an extracellular-transmembrane-intracellular fragment (NotchTM) that is expressed on the cell surface as a noncovalently linked heterodimer stabilized by a Ca²⁺ ion (5) (Fig. 1).

The extracellular domain of Notch receptors is comprised of 29–36 multiple epidermal growth factor (EGF)-like repeats, depending on the specific Notch receptor, and 3 lin-12/Notch (LNR) motifs. The EGF-like motifs are responsible for ligand interaction, while LNR motifs are responsible for preventing receptor activation in the absence of receptor-ligand engagement (35, 101, 104, 113). The intracellular domain of Notch receptors is comprised of a recombination signal binding protein-1 for Jκ (RBP-Jκ)–associated molecule (RAM) domain, seven cdc10/ankyrin repeats, of which only the COOH-terminal six assume the proper ankyrin fold, and a transactivation domain (TAD) present in Notch1, Notch2, and Notch3. In addition, there are two nuclear localization signals, a glutamine-rich stretch, and a PEST domain (2, 21, 69, 95, 139). The RAM domain is involved in potentiating Notch signaling through interaction with the transcription factor CSL [C promoter binding factor-1 (CBF1), suppressor of hairless, Lag-1] (also known as RBP-Jκ and CBF1) (123). The ankyrin repeats are involved in protein-protein interactions including interaction with CSL; however, the seventh ankyrin repeat in cooperation with the TAD domain recruits transcriptional activators such as mastermind-like (MAML) and the histone acetyltransferase (HAT) complex (69, 124). The PEST domain is involved in regulating protein half-life of the Notch receptors (31, 92).

In mammals, there are five Notch ligands, Jagged1, Jagged2 (also called Serrate1 and Serrate2), Delta-like1 (Dll1), Dll3, and Dll4, collectively referred to as the DSL (Delta/Serrate/Lag-2) family (Fig. 1). DSL proteins are themselves type I transmembrane proteins, with an extracellular domain comprised of 7–16 EGF-like repeats and a DSL domain, which is unique to Notch ligands. Jagged1 and Jagged2 have an additional cysteine-rich domain and a von Willebrand factor type C domain in the extracellular region (27).

The EGF-like repeats are thought to stabilize receptor-ligand interaction, while the DSL domain is responsible for Notch receptor activation through interaction with EGF-like repeats 11 and 12 of the Notch receptors (104). The cysteine-rich domain of Jagged ligands are thought to control Notch receptor binding specificity, while the von Willebrand factor type C domain is thought to be involved in ligand dimerization (27). The intracellular regions of the DSL ligands are relatively short and contain a PDZ domain in Jagged1 and Dll1 thought to be involved in activating downstream signaling through mechanisms that are not well understood currently (1, 118).

NOTCH RECEPTOR-LIGAND ACTIVATION

Notch receptor-ligand interaction is thought to result in a conformational change of the Notch extracellular domain exposing a motif that is recognized and cleaved by the metalloprotease tumor necrosis factor-α converting enzyme TACE/
ADAM17 at the S2 cleavage site (7). S2 cleavage occurs just extracellular to the plasma membrane releasing the extracellular Notch fragment. S2 cleavage is followed by two intramembranous cleavage steps mediated by the \( \gamma \)-secretase complex comprising presenilin1, presenilin2, Pen-2, Aph-1, and nicastrin (15, 29, 46, 121). The \( \gamma \)-secretase complex has been shown to cleave Notch receptors at two distinct sites, the S4 site between Ala1731-Ala1732 and the S3 site at the conserved Val1744 in the mouse Notch1 protein (94, 116). \( \gamma \)-Secretase cleavage ultimately releases the intracellular domain of Notch (NotchICD), which subsequently translocates to the nucleus to effect gene transcription (15, 94) (Fig. 2).

The most thoroughly investigated downstream target of Notch signaling is the CSL transcription factor. In the absence of NotchICD, CSL recruits either the silencing mediator of retinoid and thyroid hormone receptors (SMRT)/nuclear receptor corepressor (NcoR)/histone deacetylase (HDAC)-1 or the CBF1 interacting corepressor (CIR)/HDAC2/Sin3A-associated protein, 30-kDa (SAP30) complex to negatively regulate target genes.

Fig. 2. The Notch signaling pathway. Notch receptors undergo processing in the trans-Golgi network by Furin and are expressed on the cell surface as a noncovalently linked heterodimer stabilized by a calcium ion. Receptor-ligand interaction results in 3 additional cleavage events that result in the release of the intracellular region of the Notch receptor (NotchICD). The ectodomain of the Notch receptor and the ligand are thought to be endocytosed by the signaling cell. NotchICD then translocates to the nucleus, where it binds and converts CSL from a transcriptional repressor to a transcriptional activator of the Hes and Hey family of genes. Notch signaling is negatively regulated by hyperphosphorylation of nuclear NotchICD by the nuclear kinase CycC:CDK8, which is recruited to NotchICD by the Notch pathway coactivator MAML.
gene expression (45, 59). NotchICD binding to CSL in the nucleus converts CSL from a transcriptional repressor to a transcriptional activator. CSL has been shown to bind preferentially to a consensus sequence defined by (C/T)(A/G)TG(A/G/T)GA(A/G/T) (98). However, most of the identified direct CSL target genes have a less permissive (C/T)(A/G)TG(A/G) binding sequence. The hairy enhancer of split (HES) and hairy/ enhancer of split-related with YRPW motif (Hey, also called HESR, CHF, Hrt) families of basic helix-loop-helix transcription factors are the most thoroughly investigated direct Notch-CSL targets (13, 48). Other direct targets include Cyclin D1, p21, glial fibrillary acidic protein (GFAP), Nodal, Myc, PTEN, Ephrin B2, and smooth muscle α-actin (SMA) (34, 36, 50, 62, 65, 90, 102, 106, 132).

The exact composition of transcriptional activators and the temporal sequence of recruitment to the CSL-NotchICD complex remain to be elucidated. However, it has been shown that the NotchICD complex includes Ski interacting protein (SKIP), a protein that can interact with CSL, Notch, or SMRT but promotes NotchICD-CSL interactions over CSL-SMRT interaction (74, 138). Additionally, the coactivator MAML binds the NotchICD-CSL complex but does not bind either NotchICD or CSL independently (86, 87, 96). MAML itself has transactivation potential and therefore, in combination with the NotchICD TAD domain, activates gene transcription (30). The p300-HAT complex is also recruited to NotchICD, which in cooperation with MAML is necessary for activation of gene transcription (30) (Fig. 2).

To negatively regulate NotchICD activity, MAML also recruits the nuclear kinase CycC:CDK8, which hyperphosphorylates the TAD and PEST domain of NotchICD (31). Phosphorylation of NotchICD at conserved serine residues in the PEST domain by CycC:CDK8 recruits the ubiquitin ligase Fbw7/Sel7, resulting in ubiquitin-mediated proteasome degradation (31). Negative regulation of Notch signaling also occurs by GSK-3β via phosphorylation of NotchICD, and ubiquitination of NotchICD by the E3-ubiquitin ligases Itch and c-Cbl targets NotchICD for degradation (24, 28, 52, 99).

In addition to the Notch interacting proteins discussed above, Deltex binds the ankyrin repeats of NotchICD and positively regulates Notch signaling, independent of CSL. The exact mechanism is unclear but involves targeting NotchICD to the late endosome where it accumulates (44, 51, 79). Other posttranslational events also affect various aspects of Notch signaling, including receptor and ligand stability (99) and receptor-ligand specificity (42), and have been extensively reviewed previously (9, 37, 39, 57, 88).

CARDIAC DEVELOPMENT

Early Cardiac Progenitors

The heart is the first functional organ to form during vertebrate development. Cells fated to form the heart are first identifiable based on expression of cardiac markers in the anterior third of the primitive streak (not including the node) during gastrulation (embryonic day 7.0; E7.0) (Fig. 3). The transcriptional coactivator CITED2 is expressed by primitive streak cells that will go on to become heart, and represents one of the earliest known markers of cardiac progenitors (114). The prospective heart precursors undergo epithelial-to-mesenchymal transition (EMT) and migrate bilaterally from the primitive streak forming the left and right heart fields, also referred to as the primary heart field. Formation of the primary heart field is dependent on expression of the basic helix-loop-helix (bHLH) transcription factors Mesp1 and Mesp2 (61). Mesp1 and Mesp2 double-deficient embryos have a specific defect in the development of the cardiac and anterior-cephalic mesoderm (61). With the use of Mesp1- and Mesp2-deficient embryonic stem cells in chimera embryo studies, it was shown that Mesp1 and Mesp2 are redundantly required for EMT and
migration of cardiac and anterior-cephalic mesoderm from the primitive streak (61). Lineage analysis of the Mesp1-derived cells demonstrated that the majority of cells in the myocardium and endocardium are derived from Mesp1 expressing mesoderm (111, 112).

The endoderm underling the primary heart field plays an important role in specifying the cardiogenic phenotype. Using transplantation studies of mesoderm from a noncardiogenic source transplanted into the cardiogenic field, Inagaki et al. (47) demonstrated that noncardiogenic mesoderm can be reprogrammed to the cardiac fate. This reprogramming was further shown to be in part due to secreted factors from the endoderm, such as members of the bone morphogenetic protein (BMP) family, sonic hedgehog, fibroblast growth factor (FGF)-8, and Crescent. In addition to the endoderm, the ectoderm secretes Wnt inhibitors that are required for induction of a cardiogenic fate (reviewed in Ref. 6).

Even at this early stage in heart development, cell fate analysis reveals that compartmentalization of heart chambers has occurred. Myocardial atrial and ventricular precursors are present in the posterior region and ventricular progenitors in the anterior region of the heart field (105). The most frequently used primary heart field markers are Nkx2.5 (homolog of Drosophila Tinman), BMP2, Tbx20, GATA4, GATA5, and GATA6. Although these markers are expressed in the primary heart field, the exact boundaries of the primary heart field are not precisely delineated by the expression of these genes. The heart field extends both laterally and medially of Nkx2.5 and BMP2 expression, while BMP2 expression extends posterior and anterior to the heart field (105).

The cardiac crescent is formed when the right and left heart fields undergo anterior-medial migration and subsequently fuse at the anterior end (Fig. 3). Initially, all cells of the cardiac crescent have cardiomyogenic potential, but signals from the prospective myocardium and neurogenic tissue subdivide the cardiac crescent into ventral myogenic and dorsolateral nonmyogenic domains (100). The ventral myogenic domain of the cardiac crescent gives rise to the myocardium of the heart tube, while the dorsolateral nonmyogenic domain gives rise to the mesocardial and pericardial roof cells (100). Studies in Xenopus laevis have demonstrated that the Notch ligand Serrate1 (Jagged1) but not Delta1 or Delta2 is expressed in the dorsolateral nonmyogenic domain, and the dorsal-most region of the myogenic domain expresses both Serrate1 and Notch1 in an overlapping pattern (107). Activation of the Notch pathway by Serrate1 reinforces Serrate1 expression in the dorsolateral region and suppresses the myogenic potential of cells residing in this region (107). Although Notch activation suppresses myogenic potential, expression of primary heart field markers Nkx2.5 and GATA4 is unaffected (107). In addition, CSL-deficient mouse embryos develop a primitive heart tube, and heart field specification appears normal (93, 119). These findings suggest that Notch signaling controls cardiac cell fate downstream of heart field specification.

Endocardial precursors are identifiable at the cardiac crescent stage as a population of Flk1+/TAL1+/positive cells distributed throughout the cardiac crescent (18). Lineage analysis of Mesp1-derived mesoderm reveals that the endocardium is derived from the same mesoderm as the myocardium, thereby demonstrating that the endocardium and myocardium are of the same origin (111, 112). During subsequent folding of the embryo, the cardiac crescent fuses into a linear tube-like structure that starts beating at E8 in the mouse and at ~3 wk of gestation in humans (117) (Fig. 3).

In addition to the primary heart field, the existence of a secondary heart field has been identified in both the developing chick and mouse heart. The secondary heart field is located in the splanchnic mesoderm that underlies the floor of the caudal pharynx and expresses many of the same markers as the primary heart field such as Nkx2.5 and GATA4, but also expresses unique makers such as FGF-10 and Nkx3.1 (60, 115). The extent of the contribution of cells from the secondary heart field to the adult heart is not fully understood. In the chick, the secondary heart field generates the smooth muscle cells of the conus and truncus only (14, 127). In the mouse, cells from the secondary heart field migrate into the arterial pole between E8.25 and E10.5, and, in addition to the smooth muscle cells of the conus and truncus, a population of the myocardial cells of the right ventricle are also generated from the secondary heart field (60) (Fig. 3, green).

Heart Looping

Fusion of the cardiac crescent along the midline results in the formation of the primitive heart tube. The primitive heart tube is composed of an outer myocardial cell layer and an inner endocardial cell layer separated by a layer of extracellular matrix called the cardiac jelly. As heart development progresses, the linear heart undergoes several morphological changes that align and fuse the chambers of the heart. Mouse embryos that are null for CSL, or Notch1-deficient embryos which also express a Notch2-hypomorphic mutation, show randomized heart looping and axial rotation defects (65, 103). Additionally, embryos deficient for the Notch ligand Dll1 display randomized heart looping and axial rotation (65, 97). However, this phenotype is not reproduced in Notch1-deficient or Notch2-deficient embryos (38, 65). Defects in heart looping in Dll1-deficient embryos were found to be due to loss or misexpression of Nodal, Lefty2, and Pitx2, which are part of the evolutionarily conserved signaling cascade that controls left-right morphogenesis. Krebs et al. (65) found that, in Dll1-deficient embryos, Nodal and Lefty2 expression was absent from the left lateral plate mesoderm (LPM), and Pitx2 expression was randomized in the LPM or not expressed at all (65). However, other investigators found that Nodal and Lefty2 expression was absent from only 50 and 25% of Dll1-deficient embryos, respectively. In the remaining embryos, Nodal and Lefty2 were expressed in the left, right, or both LPM (97). The specific reasons for the discrepancy between these two groups of investigators remain to be elucidated, but is likely due to the difference in genetic backgrounds of the two Dll1-deficient strains. Nodal expression was further shown to be dependent on two CSL binding sites in a node-specific enhancer located −9.5 to −8.7 kb 5’ of the nodal gene (65). In addition, ectopic activation of Notch signaling in early zebrafish embryos results in Nodal and Pitx2 expression in the right LPM and ultimately left-right patterning defects (103).

Endothelial-to-Mesenchymal Transformation

Beginning at E9.0 in the mouse, localized swellings of the cardiac jelly appear in the atrioventricular (AV) canal and cardiac outflow tract (OFT), forming the superior and inferior
cardiac cushions. Cardiac cushions are acellular swellings of extracellular matrix protein secreted by the myocardium (22). At E9.5, endocardial cells of the AV canal and OFT are activated by signals emanating from the myocardium and by interendocardial signaling pathways to undergo endothelial-to-mesenchymal transition. Endothelial-to-mesenchymal transition is a specific form of EMT required for mesenchymal cell formation from endocardial cells during cardiac cushion development. It is a critical process whereby endocardial cells undergo phenotypic and morphological alterations, resulting in loss of apical-basolateral polarity and disruption of intercellular junctions, and acquire the ability to degrade the basement membrane and migrate away from the confines of the endothelial sheet to invade the underlying cardiac jelly. As development progresses, the mesenchymal cells undergo proliferation, resulting in fusion of the cardiac cushions within the lumen of the heart tube forming the initial septa. Further remodeling of the cardiac cushion results in the formation of thin protruding leaflets comprised of endocardial cells and extracellular matrix protein (ECM) that go on to develop into the heart valves. In the AV canal, EMT-derived cells are the sole contributor to the mitral and tricuspid valves, while in the OFT, EMT- and neural crest-derived cells contribute to the aortic and pulmonary valves.

There are ongoing efforts to elucidate the transcriptional networks operating during cardiac EMT (http://www.mouseatlas.org/morgen/content). However, both loss- and gain-of-function studies have revealed a critical role of the Notch pathway in regulating EMT during heart development. In the mouse, Notch1, Notch4, and Dll4 are expressed in the AV canal and OFT endocardium at the onset of EMT. Furthermore, both Notch1-deficient and CSL-deficient embryos have significantly reduced EMT in the AV canal, as determined using an ex vivo AV canal explant assay, which provides a measure of the degree of EMT taking place (125). Analysis of Notch1-deficient and CSL-deficient embryonic hearts reveals reduced expression of TGF-β2 and its receptors (125), which are critical initiators of EMT during heart development. However, by use of the AV canal explant assay, exogenous TGF-β2 or TGF-β3 was not sufficient to rescue the defect in EMT in Notch1-deficient and CSL-deficient embryos (125). These results suggest that, although Notch signaling is important for expression of TGF-β2 pathway components, it is not clear whether Notch is downstream of, upstream of, or synergistically required for TGF-β-dependent EMT during heart development. A caveat of interpreting data from the Notch1 and CSL mutant embryos is the significant delay in embryonic development observed in these mutants. The delay is particularly apparent in the heart, and therefore EMT might be significantly delayed but not impaired. In fact, in AV canal explant assays, a very small population of α-smooth muscle actin (SMA)-positive cells is detectable, suggesting that Notch1-deficient and CSL-deficient embryos are capable of initiating cardiac EMT at a reduced level (125). Supporting a role for the Notch pathway in EMT of the AV canal, it was shown that zebrafish injected with constitutively active Notch1 developed hypercellular AV canals and enlarged AV valves as a result of increased EMT (125).

Notch1-deficient embryos also display defects in ventricular trabeculation because of a decrease in myocardial proliferation during early development (36). Notch1 activity is found to be highest in the trabecular endocardium, where it controls the expression of NRG1 and BMP10, which are responsible for myocardial differentiation and proliferation, respectively (36). In addition, constitutive expression of Notch1ICD in Mesp1-derived cells results in abnormal heart morphogenesis (131). In Mesp1-derived cells, the Notch pathway is activated in the cardiogenic mesoderm and all subsequent cells of the endocardial and myocardial lineage (111, 112, 131). The most severe defects induced by ectopic Notch activation in this model were defects in myocardial trabecular development, the appearance of a cell mass in the AV canal, and right-shifted interventricular septum (131). Myocardial trabeculae were observed in the compact myocardial layer and the AV canal region, two regions where trabecular myocardium is usually absent (131). In addition, in vitro experiments have clearly demonstrated that activation of the Notch pathway in endothelial cells results in EMT (91). This study also showed that Notch-mediated EMT was cell autonomous and independent of TGF-β signaling, supporting the importance of the role of Notch signaling during EMT in heart development (91).

The Notch target gene Hey2 is initially expressed in ventricles precursors of the heart tube and later becomes restricted to the compact myocardial layer of the ventricles. Hey2 is also highly expressed at the onset of EMT in the AV canal endocardium (128). However, expression of Hey2 is not sustained in the mesenchymal cells of the AV canal, suggesting Notch activity is required for initiation of EMT but not for maintaining a mesenchymal phenotype. Early in heart development, Hey1 is expressed in the lateral aspect of the cardiac crescent, which gives rise to the sinus venosus and atria. Later, Hey1 becomes restricted to the atrial myocardium. HeyL is not expressed during heart development, and levels of Hey1 and Hey2 in the heart gradually decline after birth. The mechanism by which Hey1 and Hey2 become differentially expressed remains to be resolved, since both genes are direct targets of Notch activation. In the atrium, there is high expression of Jagged1, which could potentially regulate Hey1 expression (78, 126). However, Notch receptors or ligands are not observed in the compact myocardial layer, suggesting that, in this context, Hey2 may be regulated by a pathway other than Notch (126).

Several studies have also revealed a critical role of Hey2 during heart development. The phenotypes of Hey2-null mice are variable, but 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 (17, 25, 63). Gene targeting studies have revealed that Hey1 or HeyL alone is not required for heart development. However, Hey1 and Hey2 double-deficient embryos die at E9.5 because of severe heart defects, including missing ventricular trabeculae and lack of arterial differentiation (25). In addition, combined loss of both Hey1 and HeyL results in a significant reduction in endocardial EMT, similar to the Notch1- or CSL-deficient embryos (26). Known expression patterns of Notch pathway component have been summarized in Table 1.
Defects in Vascular Remodeling

Expression of Notch pathway components during murine heart development

<table>
<thead>
<tr>
<th>Notch Pathway Component</th>
<th>Expression Pattern</th>
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<tbody>
<tr>
<td>Notch1</td>
<td>Expressed in the cardiac crescent (E7.5). At E8.0 to E11.5 expression is limited to the entire endocardium and highly expressed in the AV canal and outflow tract endocardium (124, 132).</td>
</tr>
<tr>
<td>Notch2</td>
<td>Expressed in the AV canal endocardium (E12.5) and the outflow tract (E11.5 and 14.5). Expressed in atrial and ventricular myocardium (E13.5) (76, 80, 82).</td>
</tr>
<tr>
<td>Notch3</td>
<td>Expressed in the cardiac crescent (E7.5) but not detected after heart tube formation (E8.0) (132).</td>
</tr>
<tr>
<td>Notch4</td>
<td>Expressed in the endocardium (E10.5) (90).</td>
</tr>
<tr>
<td>Jagged1</td>
<td>Expressed in the AV canal and outflow tract endocardium and atrial myocardium (E10.5-E12.5) (77).</td>
</tr>
<tr>
<td>Jagged2</td>
<td>Expression has not been analyzed</td>
</tr>
<tr>
<td>DI11</td>
<td>Not expressed in the heart (4)</td>
</tr>
<tr>
<td>DI13</td>
<td>Expression has not been analyzed</td>
</tr>
<tr>
<td>DI14</td>
<td>Expressed in the cardiac crescent (E8.0) and the endocardium from E8.5 onward. Expression is further restricted to the ventricular endocardium after E11.5 (3, 19).</td>
</tr>
<tr>
<td>Hey1</td>
<td>Expressed in the lateral portion of the heart tube (E8.5) and the endocardium and septum transversum (E9.5). Expressed exclusively in the atrial myocardium at E10.5 (71, 124).</td>
</tr>
<tr>
<td>Hey2</td>
<td>Expressed in the anterior portion of the heart tube (E8.5) and the AV canal and OFT endocardium (E11.0). Highly expressed in the subcompact ventricular myocardium (E10.5) (12, 71).</td>
</tr>
<tr>
<td>HeyL</td>
<td>It has been reported that HeyL is not expressed in the embryonic heart (72), but combined targeting of Hey1 and HeyL results in cardiovascular defects related to EMT (26).</td>
</tr>
</tbody>
</table>

AV, atrioventricular; E, embryonic day; OFT, outflow track; EMT, epithelial-to-mesenchymal transition.

ARTERIALVENOUSSPECIFICATION

Early Vascular Progenitors

Vascularogenesis is the de novo development of endothelial cells from mesodermal progenitors called angioblasts, while angiogenesis is the formation of new vessels from preexisting vessels. All embryonic mesoderm, with the exception of the prechordal plate, has angioblast potential (89). Angioblast differentiation in situ forms a primary vascular plexus that undergoes extensive remodeling into arteries, veins, and capillaries. The three types of vascular structures are morphologically, functionally, and molecularly distinct. At the molecular level, artery-specific factors include Ephrin-B2, CD44, Neuroglin-1, Hey1, and Hey2, while vein-specific factors include EphB4, COUP-TFI, and Lefty1 (11, 25, 135).

Expression of Notch Pathway Components

Notch pathway components are initially expressed throughout the developing cardiovascular system but become mainly restricted to cells that will acquire an arterial fate later in development. At E9.5, Notch1, Notch4, and DI14 are expressed throughout the primitive vascular plexus. By E13.5, Notch1, Notch2, Notch4, DI14, Jagged1, and Jagged2 expression has become mainly restricted to the arterial endothelium (126). Notch1 and DI14 are also expressed in the capillary endothelium. In contrast, artery smooth muscle cells express low levels of Notch1 and high levels of Notch3 and Jagged1 (126). Notch4 is also expressed in the cardiac vein, human umbilical endothelial cells, and capillary endothelium. Expression patterns of Notch receptors and ligands in the vasculature have been extensively reviewed in Ref. 49. When injured, arteries induce expression of various Notch receptors and ligands (75).

Defects in Vascular Remodeling

Notch1-deficient mice die before E10.5 because of severe defects in cardiovascular development (122). In Notch1-deficient embryos, the yolk sac and cerebral primary vascular plexus are formed, but subsequent remodeling of the vascular plexus is defective. In addition, intersomitic vessels are highly disorganized, and the dorsal aorta and cardinal vein are formed but are significantly reduced in size (67). These results suggest that Notch1 is not required for angioblast differentiation but is required for remodeling of the vascular plexus. Notch4-deficient mice are viable and fertile; however, Notch1/Notch4 double-deficient mice have vascular defects similar to but more severe than Notch1-deficient animals (67). This suggests a partial redundancy between Notch1 and Notch4 in the developing vasculature. Notch2-deficient mice die at E11.5 because of a variety of defects in the cardiovascular and renal systems (38, 82). Notch3-deficient mice, in contrast, are viable without any obvious defects in cardiovascular development (68). Vascular remodeling defects are also seen in DI14- and Jagged1-deficient mice, resulting in death at E9.5 and E11.5, respectively (32, 66, 134). Expression of constitutively active Notch4 driven by the flk1 promoter in endothelial cells during development results in yolk sac remodeling defects and disorganized embryonic vessel development.

Molecular Patterning of Arterial Venous Specification

Arterial and venous endothelial progenitors have been shown to be specified before vessel assembly or blood flow (41). Recent studies have revealed a signaling cascade for arterial specification that involves sequential activation of Sonic Hedgehog (Shh), vascular endothelial growth factor (VEGF), and Notch (71). In zebrafish, Shh deficiency results in a loss of arterial specification and decreased VEGF expression (71). In contrast, Shh activation in zebrafish results in upregulation of VEGF and ectopic arterial specification (as determined by expression of EphrinB2) of vessels otherwise destined to become veins (71). Activation of the Notch pathway in mature vessel endothelium, via Tie2-driven inducible expression of Notch4ICD, results in expression of EphrinB2 and increased smooth muscle layers resulting in arterialization of the venous vessels (8). In this model, overexpression of Notch4ICD results in death of adult animals within weeks of expression. Interestingly, the effects of Notch4ICD expression are reversible on reversal of Notch4ICD expression (8). In mice, Hey1/ Hey2 or Notch1/Notch4 double-deficient and
Dil4-deficient embryos display defects in arterial specification to various degrees (25, 67). Studies in zebrafish have provided significant insight into the role of the Notch pathway in arterial-venous specification. Enforced expression of Notch5, which is similar to mammalian Notch3 by sequence, in the endothelium results in reduced venous expression offlt-4, a venous marker (70). Conversely, blocking Notch signaling in the dorsal aorta, using dominant-negative CSL, results in loss of arterial expression of EphrinB2 (70).

Notch target genes have also been shown to play a role in arterial-venous specification. A mutant of gridlock, the only Hey family member expressed in the zebrafish vasculature, shows defects in aortic assembly resembling coarctation of the aorta (137). Antisense-directed knockdown of gridlock suppresses arterial marker expression and expands contiguous regions of the vein during embryonic vascular development (136). However, overexpression of gridlock acts to repress venous fate rather than promote arterial specification (136). The role of gridlock is controversial, as others have suggested that Notch mediates arterial specification independently of gridlock (70). In addition, VEGF stimulation was shown to upregulate Notch1 and Dil4 expression exclusively in arterial endothelial cells. VEGF treatment of Shh-deficient but not Notch-deficient embryos is capable of rescuing arterial EphrinB2 expression.

In venous vessels, expression of the orphan nuclear receptor COUP-TFII was shown to regulate the expression of Notch1 and Jagged1 in arterial-venous specification (135). Loss of COUP-TFII resulted in ectopic venous expression of Notch1 and Jagged1, resulting in arterialization of the venous vessels (135). In contrast, ectopic expression of COUP-TFII in the endothelium, via the Tie2 promoter, resulted in reduced Jagged1 expression and arterial expression of EphB4, a venous marker. Together these findings suggest that COUP-TFII is upstream of Notch signaling in arterial-venous specification, and that Notch signaling represses venous differentiation. Thus the current paradigm depicts a signaling cascade for arterial differentiation where Shh is upstream of VEGF and VEGF is upstream of Notch signaling.

MUTATIONS OF NOTCH PATHWAY COMPONENTS IN HUMAN DISEASE

Aortic Valve Disease

In humans, mutations in the Notch1 locus result in a spectrum of heart defects (33). The most prevalent malformations are bicuspid aortic valve disease and calcification of the aortic valve (33). Calcification of the aortic valve is the third leading cause of heart disease in adults, while the presence of the bicuspid aortic valve is present in 1–2% of the population (43). Mutations in the Notch1 locus result in a premature stop codon in the extracellular domain of Notch1, likely resulting in rapid degradation of mRNA by the nonsense-mediated mRNA decay pathway (33). The resulting defects may be due to haploinsufficiency, or alternatively, the premature stop codon could result in the expression of a truncated Notch1 protein that could function as a dominant-negative mutant. These hypotheses have yet to be tested experimentally. The mechanism by which human Notch1 mutations affect aortic valve calcification is poorly understood. However, calcification of the aortic valve is thought to be due to endothelial dysfunction. As discussed above, Notch pathway components are highly expressed in arterial endothelial cells, which correlates with the importance of the Notch pathway in regulating endothelial function in the aortic valve. With the use of an in vitro system, it was demonstrated that Notch1, Hey1, and Hey2 repress the function of the transcription factor RUNX2 (33). 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 (20). It was further suggested that Notch signaling via upregulation of Hey1 and Hey2 results in Hey1 or Hey2 physically interacting with RUNX2, thereby inhibiting RUNX2 function (33). It is not known whether Notch1 mutations found in humans result in lower Hey1 and Hey2 expression in the aortic valve, thereby allowing higher RUNX2 activity, subsequent expression of osteogenic genes, and calcification of the aortic valve.

Alagille Syndrome

Mutations in the Jagged1 locus are associated with 94% of patients with Alagille syndrome (AGS) (130). In addition, mutations in the Notch2 locus have been identified in patients with Jagged1-independent AGS (84). 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 pulmonic stenosis, and 13% of AGS patients have Tetralogy of Fallot (58, 64, 85). Tetralogy of Fallot is a condition characterized by ventricular septal defect, overriding aorta, infundibular pulmonary stenosis, and often right ventricular hypertrophy. In less frequent cases, AGS has been associated with other defects of the cardiac cushion (23, 85). The cardiac AGS phenotype is consistent with the expression pattern of Jagged1 and Notch receptors in the cardiovascular system. Analyses 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 (120). However, heterozygous mutations in Jagged1 or Notch2 do not reproduce the AGS phenotype in mice (38, 134). Jagged1 heterozygote mice display eye defects, while Jagged1-null mice die at E10 because of vascular defects (134). 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 (38, 81). However, mice doubly heterozygous for a Jagged1-null and Notch2-hypomorph allele develop jaundice and impaired development of intrahepatic bile ducts with associated abnormalities of the eye, heart, and kidney, reproducing an AGS phenotype (83). The reason that human Jagged1 haploinsufficiency results in AGS while in mice there is an additional requirement for Notch2 insufficiency is unknown. Possible explanations include a higher basal expression of Jagged1 in mice, compensating expression of a Notch ligand, or increased avidity of Notch receptor-ligand interaction in the mouse cardiovascular system.

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an autosomal dominant disorder associated with defects in arterial vascular...
Notch signaling plays a crucial role in cardiovascular development and in human vascular disease. In addition, emerging evidence indicates that members of the Hey family of bHLH proteins are critical downstream mediators of Notch signaling in the vasculature. However, a thorough understanding of the downstream targets of Hey proteins and the function of these downstream targets remains to be elucidated. Early studies on functional changes triggered by Notch in vascular cells have been performed, but much remains to be investigated in this area also. Notch signaling appears to integrate with feedback loops of the VEGF, platelet-derived growth factor, Wnt, and BMP pathways, but the molecular mechanisms of how this occurs are still under investigation. Undoubtedly, there will be further examples of vascular diseases that are influenced or perhaps even caused by aberrant signaling in Notch pathway genes as investigators place greater emphasis in these unmined areas of research.

REFERENCES

NOTCH AND CARDIAC DEVELOPMENT


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