Heart Valve Development
Endothelial Cell Signaling and Differentiation

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Abstract—During the past decade, single gene disruption in mice and large-scale mutagenesis screens in zebrafish have elucidated many fundamental genetic pathways that govern early heart patterning and differentiation. Specifically, a number of genes have been revealed serendipitously to play important and selective roles in cardiac valve development. These initially surprising results have now converged on a finite number of signaling pathways that regulate endothelial proliferation and differentiation in developing and postnatal heart valves. This review highlights the roles of the most well-established ligands and signaling pathways, including VEGF, NFATc1, Notch, Wnt/β-catenin, BMP/TGF-β, ErbB, and NF1/Ras. Based on the interactions among and relative timing of these pathways, a signaling network model for heart valve development is proposed. (Circ Res. 2004;95:459-470.)

Key Words: heart development ■ heart valves ■ valvular heart disease ■ NFAT ■ VEGF ■ TGF-β

Cardiovascular malformations are the most common congenital anomaly, occurring in four to six infants for every 1000 live births.1 Defects in cardiac valves and associated structures are the most common subtype, accounting for 25% to 30% of all cardiovascular malformations.2 A number of congenital valve defects occur as part of well-defined clinical syndromes, including Down syndrome (numerous cardiac cushion defects), LEOPARD syndrome (pulmonic stenosis), chromosome 22 microdeletion syndromes (truncus arteriosus), Holt-Oram, and Noonan syndrome (pulmonic stenosis). In some cases, including Holt-Oram and Noonan syndrome, the genetic defect has now been convincingly identified.3,4 In approximately 25% of cases, however, defects in cardiac cushion development occur separate from any defined syndrome or genetic cause.5

In adults, valvular heart disease remains a major cause of morbidity and mortality; approximately 82,000 valve replacements are performed each year in the USA.6 Possible therapeutic approaches for these adult heart valve diseases include promoting endogenous repair pathways or using autologous progenitor cells for tissue engineered heart valves.7 An increased molecular understanding of the processes controlling heart valve development and remodeling will continue to suggest new therapeutic modalities.8

This review is divided into three parts. First, an anatomic description of heart valve development provides orientation for the molecular events that occur during formation of the heart valves. Second, the major cell signaling pathways implicated in heart valve development are reviewed, with an emphasis on the key role played by endothelial cell proliferation and differentiation. Third, we suggest a model that integrates these major signaling pathways into a signaling network for control of cardiac cushion formation and remodeling.

Anatomic Overview of Heart Valve Development
The heart tube is composed of an outer layer of myocardium and an inner lining of endocardial cells, separated by an
extensive extracellular matrix (ECM) referred to as the cardiac jelly. After rightward looping of the heart, the cardiac jelly overlying the future atrioventricular canal (AVC) and outflow tract (OT) expands into swellings known as cardiac cushions. The formation of the cardiac cushions is a complex event characterized by endothelial-mesenchymal transdifferentiation (EMT) of a subset of endothelial cells that are specified in the cushion-forming regions to delaminate and invade the cardiac jelly, where they subsequently proliferate and complete their differentiation into mesenchymal cells (Figure 1). In a poorly understood process, the cushions protrude from the underlying myocardium, forming thin, tapered leaflets with a single endothelial cell layer and a central matrix comprised of collagen, elastin, and glycosaminoglycans. These delamination and remodeling events depend on further cell differentiation, apoptosis, and ECM remodeling. The final AVC (mitral and tricuspid) valves are derived entirely from endocardial cushion tissue. The end stages in development of the OT (aortic and pulmonic) valves are more controversial. A population of neural crest cells derived from the branchial arches migrates to the distal OT and is required for aortopulmonary septation. Recently, cell lineage analysis of neonatal Tie2-Cre × Rosa26R mice demonstrated the endothelial origin of resident cells throughout the leaflets of aortic and pulmonic valves, as well as the AVC valves.

Signaling Pathways and Proteins Implicated in Heart Valve Development

One of the most informative tools for studying EMT has been an ex vivo cushion explant system, whereby myocardium and endocardium of the developing cushion are explanted onto a type I collagen gel. Cells explanted with this technique recapitulate EMT, allowing quantitation of the extent of EMT after experimental manipulation. This system established key steps governing EMT, including the role of soluble factors within the ECM, the requirement of myocardium in the induction of EMT, and that endocardium from the cushion-forming region is uniquely capable of responding to a myocardial-derived differentiation signal.

During the past decade, tissue explant studies have been complemented by an increasing number of mouse single gene “knockout” experiments that resulted serendipitously in specific disruption (either hypoplasia or hyperplasia) of cardiac cushion formation. These signaling pathways are now converging on a select few signaling “modules”; preliminary results have suggested temporal ordering of the signaling pathways, as well as interactions between signaling modules. Dozens of gene disruptions have now been shown to alter valve phenotypes; this review discusses only the best characterized pathways implicated in endothelial cell proliferation and differentiation within the developing cardiac cushions. Although the emphasis is on endothelium, it should be noted that the overall process of EMT and eventual leaflet formation is dependent on the interaction among myocardium, extracellular matrix, and endothelium.

VEGF: Regulation of Endothelial Cell Proliferation During Valve Development

Numerous lines of research have implicated VEGF in the control of endocardial cushion development (Figure 2). VEGF is a pleiotropic factor that regulates cell proliferation, vascular permeability, chemotaxis, and survival in endothelial cells and vasculogenesis and angiogenesis in the developing embryo. VEGF signals through VEGF-R complexes on the endothelial cell surface involving VEGF-R1/flt-1, VEGF-R2/KDR, and neuropilin-1. The downstream mediators of VEGF-R signaling are an active area of investigation, but appear to include inositol 1,4,5-phosphate/diacylglycerol, and extracellular-related kinase (ERK)/mitogen-activated proliferation kinase (MAPK) pathways, and in valve endothelial cells, NFATc1.

Transgenic mouse and in situ hybridization studies have suggested indirectly that VEGF is a specific mediator of heart valve development. Using a LacZ-tagged VEGF allele, VEGF was found broadly expressed throughout endocardial cells at E9.0, but became restricted at E9.5 to a subset of endocardial cells lining the AVC and OT. Other groups have suggested that VEGF specification to the cushion forming area does not occur until E10.5; the disparate results may reflect differences in methodology. Regardless, this spatiotemporal expression pattern of VEGF was the first indication of a specific role for VEGF in endocardial cushion formation. These VEGF-expressing endothelial cells in the cushion-
forming region may be a unique subpopulation of endothelial cells predetermined to undergo EMT.25 Alternatively, the VEGF-producing cells may induce proliferation and/or increased permeability of adjacent endothelial cells in the developing cardiac cushions, or possibly induce neighboring endothelial cells to undergo EMT.

Further studies have characterized functional consequences of VEGF spatiotemporal specificity during valve development. Three-fold overexpression of VEGF in the cardiovascular system of murine embryos caused death between E12.5-E14.5 because of delayed OT septation, excessive endothelial cell proliferation, and cardiac enlargement.27 Selective myocardial overexpression of VEGF165 during E3.5 to 9.5 resulted in failure of cardiac cushion formation at the AVC and OT.26 These embryos also had multilayered endocardium, suggesting a dysregulation of the differentiation process and overexpression of an endothelial phenotype. These results were confirmed ex vivo using the collagen explant system: addition of exogenous VEGF inhibited EMT in the forming AVC cushions. Together, these findings confirm that VEGF levels must be tightly regulated during normal heart development, and that even moderate increases in VEGF expression can have profound developmental consequences, possibly by inhibiting endothelial cell differentiation and thereby negatively regulating EMT.

**Hypoxia, Hyperglycemia, and VEGF in the Developing Valve**

The proximate regulators that increase VEGF expression in the nascent cardiac cushions have not yet been definitively identified, but hypoxia and hyperglycemia have each been suggested to regulate VEGF expression in developing heart valves (Figure 2).

Epidemiological data suggest a correlation between hypoxia and cardiovascular anomalies, specifically patent ductus arteriosus and atrial septal defects.28 Tissue explant studies have shown that hypoxia decreases cardiac cushion EMT, an effect that was abrogated by addition of soluble VEGF-R1.29 In addition, VEGF expression was increased approximately 10-fold in cardiac cushions under hypoxic conditions. These results suggest that fetal hypoxia may increase VEGF expression in the cushion-forming areas and inhibit EMT. It is tempting to speculate that fetal hypoxia during cushion development could contribute to congenital cardiac defects in the cardiac valves and interatrial septum.

Neonates born to diabetic mothers have an approximately 3-fold increase in congenital heart disease, including approximately 10- to 20-fold increased risk of rare congenital heart defects such as double outlet right ventricle and truncus arteriosus.30 Strict glycemic control during pregnancy can dramatically reduce the risk of congenital defects in children born to diabetic mothers, suggesting that hyperglycemia has a direct teratogenic effect.31 In the developing mouse, hyperglycemia reduces VEGF expression.32 Using a mouse cardiac cushion explant system, it has been shown that elevated glucose inhibits AVC cushion EMT.33 Adding back VEGF165 abrogated the effect of hyperglycemia by allowing normal cushion EMT. Interestingly, cushion endothelial cells exposed to hyperglycemic conditions at E9.5 exhibited persistent CD31 expression, suggesting that these cells were unable to complete the differentiation program from endothelial to mesenchymal phenotype. Parenthetically, cardiac cushions from mice deficient for CD31 were competent to undergo EMT, even in the presence of hyperglycemia.

These results argue that decreased VEGF expression during development inhibits cushion formation, possibly by inhibiting endothelial migration into the cardiac jelly. VEGF expression must be tightly controlled during cardiac cushion formation, as either over- or under-expression of VEGF causes hypoplastic cardiac cushions. The evidence at this point supports a specified developmental pathway for endocardial cushion VEGF signaling, with a superimposed environmental influence that leads to altered VEGF expression, and therefore dysregulation of cardiac valve formation and septation. That is, hypoxia and elevated glucose are likely pathological, rather than physiological, regulators of VEGF expression during cardiac cushion development (Figure 2).

**NFATc1: Transcriptional Regulator of Cardiac Cushion Endocardium**

The NFAT family of proteins consists of five functionally related transcription factors (NFAT1 to NFAT5). With the exception of a distantly related *Drosophila* homologue, the genes encoding NFAT proteins are found only in vertebrate genomes.34 Originally described as an inducible nuclear factor that activated T cell transcription, NFAT (nuclear factor in activated T cells) signaling has since been shown to be crucial for neuronal guidance, skeletal, and cardiac muscle...
hypertrophy, skeletal muscle fiber-type specification, osteoclast differentiation, and cardiac valve development. Mice deficient for expression of NFATc1/NFAT2 (nfatc1<sup>-/-</sup>) die by E13.5 secondary to a specific defect in cardiac cushion formation. The exact observations differed between groups; one reported selective failure of aortic and pulmonic valve development, whereas the other reported defects in all four cardiac valves and their associated septa. During the cushion-forming window, NFATc1 expression is intranuclear and limited to endocardium overlying the nascent cardiac cushions. Endocardial cells that have already undergone EMT do not stain for NFATc1, suggesting that NFATc1 is downregulated during EMT, or that the subpopulation of NFATc1-expressing cells does not undergo EMT. Despite convincing evidence that disruption of NFATc1 signaling interrupts endocardial cushion development, few studies have identified upstream regulators or downstream targets of endocardial NFATc1. Three potential signaling partners include VEGF, DSCR1 (Down syndrome critical region 1), and connexin 45 (Figure 3).

**VEGF: Control of NFAT-Dependent Proliferation**

Because of the spatiotemporal overlap between VEGF and NFATc1 expression in the developing cardiac cushions, we hypothesized that VEGF and NFATc1 may interact in valve endothelial cells. In primary cultures of human pulmonary valve endothelial cells, we showed that VEGF, acting through VEGF-R2, increases NFATc1 nuclear localization, and that this activation of NFATc1 markedly increases the proliferation of these cells. The NFAT-mediated proliferative response appears to be specific to valve endothelial cells, because inhibition of NFAT signaling in HUVECs or HDMECs did not significantly decrease the proliferative response to VEGF. Immunohistochemistry suggests that NFATc1 is expressed in a subset of postnatal valve endothelial cells; NFATc1 may therefore play a role in repopulating and reconstituting endothelium of the adult valve. In follow-up studies, we have shown that VEGF-R signaling is required for zebrafish heart valve formation, suggesting that this signaling pathway also operates during valve development (unpublished data, 2004). These results argue that VEGF is an upstream regulator of NFAT signaling in developing and postnatal valve endothelium. The endogenous source of VEGF is unclear, but may be a myocardial paracrine and/or endothelial autocrine pathway.

**Down Syndrome: Inhibition of Calcineurin-NFAT Signaling**

Down syndrome (trisomy 21) is an inherited abnormality of maternal chromosomal meiotic dysjunction. Thirty to sixty percent of patients with Down syndrome have structural anomalies of endocardial cushion-derived structures, and it is estimated that Down syndrome accounts for >60% of atrioventricular septal defects. DSCR1, located in region 21q22.1-q22.2, may play a role in the cardiac anomalies present in Down syndrome. DSCR1 (also known as MCIP1, modulatory calcineurin interacting protein 1) is a member of the calcipressin gene family, which encode a group of endogenous modulators of calcineurin activity. In the murine heart, DSCR1 is expressed throughout the primitive ventricle from E9.5 to E10.5, during the same time period that the cardiac cushions develop. DSCR1 binds to the linker region between the catalytic and regulatory subunits of calcineurin. Because overexpression of DSCR1 inhibits Ca<sup>2+</sup>-dependent translocation of NFAT in cardiac myocytes, it has been suggested that DSCR1 may compete with NFAT for binding to calcineurin. Increased cytosolic Ca<sup>2+</sup> stimulates DSCR1 expression, suggesting that DSCR1 may interact as a negative feedback regulator of NFAT signaling.

In the developing mouse heart, DSCR1 is expressed in endocardial cells overlying the cushion-forming area. In trisomy 16 mice (mouse chromosome 16 contains a syntenic...
Connexins: Diffusion Gradient of \( \text{Ca}^{2+} \) Signaling

Connexins are a heterogeneous group of transmembrane proteins that assemble into hexameric connexons, which associate at the plasma membrane to form gap junctions between cells. Connexin 45 (Cx45) is the first connexin expressed during development and is expressed throughout the developing heart. By E9.5, there is selective upregulation of Cx45 in endocardial cells overlying the AVC and OT, suggesting a role for Cx45 in cardiac cushion development.46

In mice, the Cx45 knockout resulted in cardiac conduction defects, dysregulated contraction, and decreased cardiac cushion formation; the mutation was lethal by E10.47 Interestingly, \textit{connexin 45}\(^{-/-}\) mice had a dramatically decreased percentage of cushion endocardial cells with intranuclear (active) NFATc1 relative to wild-type mice. These results suggest that Cx45 functions upstream of NFATc1 in the developing cardiac cushion. It has been proposed that Cx45 may provide a transcellular route for \( \text{Ca}^{2+} \) to diffuse between endocardial cells, thus creating a gradient of \([\text{Ca}^{2+}]\) across the endocardial cushions.48 In this model, an inductive stimulus (perhaps VEGF signaling) activates \( \text{Ca}^{2+} \) transients in a subset of endothelial cells, and Cx45 gap junctions allow spread of a gradient across the cushion endocardium (Figure 3).

Several caveats, however, apply to this model. First, \textit{connexin 45}\(^{-/-}\) mice display a number of abnormalities other than cushion defects, including conduction system blocks and disordered contraction. The dysregulated blood flow across the cushion-forming area could act as an epigenetic factor to alter gene transcription and/or cell fate and cell differentiation in endocardial cells.49 Second, the majority of Cx45 expression is in the myocardium; disruption of Cx45 may therefore have a more profound effect on the elaboration of a myocardial-derived signal necessary for initiating cardiac cushion development.50 Third, \textit{connexin 45}\(^{-/-}\) mice die at E10, approximately three days earlier than mice that succumb to congestive heart failure from valve agenesis.51,52 Therefore, it is possible that cardiac cushion formation in \textit{connexin 45}\(^{-/-}\) mice is delayed, rather than disrupted, and that the proximate cause of death is a disorder of cardiac contraction.

Notch: Specification for Endothelial-Mesenchymal Transdifferentiation

Notch is a transmembrane protein that, on binding to its DSL ligands, is converted from a transmembrane receptor to a transcriptional coactivator.49 Recently, analysis of Notch mutants revealed an essential role for Notch in the control of endocardial cushion EMT (Figure 4).50,51 In addition, disruption of the UDP-glucose dehydrogenase gene, which results in toggling of blood in the heart, causes dysregulation of notch1b expression,52 which is normally confined to the AV boundary.

Based on these observations, mice homozygous for disruptions of notch1 or RBP-Jk were found to have hypoplastic cardiac cushions, suggesting that the endocardium in this region failed to undergo EMT.56 These findings were confirmed using an ex vivo explant assay, which showed a decreased number of cells undergoing EMT. Furthermore, injection of a constitutively active Notch intracellular domain (NICD) into zebrafish increased endocardial mitosis and caused valve hyperplasia. Conversely, antagonism of Notch signaling by addition of DAPT, an inhibitor of \( \gamma \)-secretase (one of the enzymes that cleave Notch from its transmembrane domain) caused marked valve hypoplasia in the developing zebrafish.

The authors found that disruption of Notch signaling, either in Notch1 or RBP-Jk mutants, specifically reduced TGF-\( \beta \) expression in the myocardium. Consistent with this finding, expression of the transcription factor snail (discussed later in relation to TGF-\( \beta \) signaling) was markedly decreased after disruption of Notch signaling. Notch signaling in the endocardium may induce a signal that increases TGF-\( \beta \) expression in the myocardium; TGF-\( \beta \) would then, in turn initiate EMT in endocardial endothelial cells. However, the authors were unable to rescue EMT in ex vivo explant models by adding back TGF-\( \beta \) or TGF-\( \beta \). These results suggest that endocardial Notch signaling activates EMT directly, or that perhaps a combination of Notch and TGF-\( \beta \) signaling function to regulate EMT.

Wnt/\( \beta \)-Catenin: Activation of a Mesenchymal Program

During chick development, wnt signaling from the posterior mesoderm represses cardiogenesis; the gradient of wnt sig-
naling is therefore crucial for patterning of the heart-forming field. New evidence suggests that wnt signaling may also closely regulate valve development.

In the zebrafish, an early missense mutation in the APC gene product resulted in embryonic lethality by 96 hours post-fertilization (hpf). This timing corresponds to cardiac valve formation in zebrafish. Homozygous mutants demonstrated massive expansion of the cardiac cushions throughout the heart, implying that endocardial cells lining most of the heart had undergone EMT. Consistent with this finding, antagonism of wnt/β-catenin signaling in wild-type embryos inhibited cardiac cushion formation. In wild-type embryos, β-catenin was intranuclear in endocardial and myocardial cells overlying the cushion-forming areas. In homozygous APC truncation mutants, β-catenin stained with a nuclear pattern throughout the heart, implying that an expanded population of endocardial cells was capable of proliferating and competent to undergo EMT. These findings demonstrate a new role for wnt/β-catenin signaling in valve development; the authors propose that β-catenin may activate expression of genes crucial for transition from an endocardial to a mesenchymal phenotype.

Endocardial cushion-specific expression of wnt/β-catenin signaling was also recently identified in the developing mouse heart. At E11.5, wnt signaling is restricted to a subset of cells in the AVC and OT, with only a subset of mesenchymal cells staining for wnt/β-catenin signaling. This pattern of expression indicates that the role of the wnt/β-catenin pathway in endocardial cushion development is conserved in mammals.

**Wnt/β-Catenin: Signaling Convergence with CD31 and VEGF?**

In addition to its signaling functions, β-catenin acts as a structural link between actin and VE-cadherin to form the adherens junction, a molecular scaffold that mediates cell-cell adhesion and cell polarity in endothelial cells. Phosphorylated β-catenin also associates with CD31/PECAM-1, a transmembrane protein of the immunoglobulin (Ig) family involved in cell-cell contact. The association of β-catenin with cadherins and PECAM-1 may provide a cellular "sink" to regulate the level of free, cytosolic β-catenin.

The interactions between β-catenin, CD31, and VEGF may be a critical nexus in the control of EMT during endocardial cushion formation if results from generic endothelial cells can be extrapolated to valve endothelial cells. VEGF signaling increases the phosphorylation of β-catenin, leading to increased association of β-catenin with CD31. Sequestration of β-catenin by VEGF signaling could be one mechanism whereby increased levels of VEGF decrease cardiac cushion formation. During endocardial cushion EMT, CD31 is downregulated and α-smooth muscle actin is upregulated as endocardial cells differentiate into a mesenchymal phenotype. It is possible that, as CD31 is downregulated, the cytosolic levels of β-catenin increase and activate proliferation of cells undergoing EMT; β-catenin could thus serve as the link between activation of the mesenchymal program and population of the cardiac jelly with mesenchymal cells. Consistent with this hypothesis, the cushion-forming areas of cd31−/− mice remain competent to undergo EMT in the presence of hyperglycemia, which reduces the level of VEGF.

**BMP/TGF-β: Initiation of Endothelial-Mesenchymal Transdifferentiation**

The TGF-β cytokine superfamily includes bone morphogenetic proteins (BMPs) and TGF-βs. The major TGF-β family members implicated in heart development are BMP-2, BMP-4, TGF-β1, and TGF-β2. All TGF-β family members are homodimeric proteins that interact with transmembrane TGF-β receptors (Figure 5). Ligand binding activates type II receptors to transphosphorylate type I receptors within the ligand-receptor complex. The phosphorylated type I receptor then acts as a serine/threonine kinase to phosphorylate and activate cytosolic Smad proteins, which are the major intracellular mediators of TGF-β signaling. As discussed later, Smad6 competes with Smad1 for binding to Smad4, thereby preventing Smad1-dependent signal transduction. TGF-β and BMP are the most thoroughly studied signaling partners in endocardial cushion formation. Two recent reviews have summarized much of this work.

**BMP is a Myocardial-Derived Signal Required for EMT**

Expression patterns, data from tissue explant studies, and gene disruption studies point toward a critical role of BMPs in the initiation of cardiac cushion EMT. In the developing mouse, BMP-2 and BMP-4 are expressed in the myocardium underlying the developing AVC and OT. The expression of BMP-2 appears to be stronger in the AVC, whereas BMP-4 expression is stronger in the OT. BMP6 is expressed in endocardium, myocardium, and mesenchyme of the developing heart, with some predilection for the AVC. The major BMP receptors, including Alk2, Alk3, and BmprI, are widely expressed in the heart, with no clear spatial restriction.

Chick cushion explant models have verified that the expression pattern of BMPs during development has a functional consequence. In the chick model, knock down of BMP-2 mRNA transcripts resulted in profound reduction of mesenchymal cell invasion into a collagen lattice. In a recent study using mouse AV explants, BMP-2 was shown to be a functional substitute for myocardium in terms of mediating EMT. Furthermore, the BMP-2-treated AV endothelial explants were induced to express TGF-β2, suggesting that in mice, BMP-2 may initiate EMT by activating an endocardial TGF-β autocrine loop.

Genetic manipulations in mice have also confirmed an important role for BMP signaling in the control of EMT. Gene disruption of BMP-6 or BMP-7 does not result in a cardiac phenotype, but bmp6−/−;bmp7−/− double mutants have a delay in OT formation and hypoplastic cardiac cushions. This study was the first genetic evidence in mice that BMP signaling is required for cardiac cushion formation; further studies will be necessary to determine whether the phenotype is caused by a dysregulation of myocardial proliferation/differentiation, or a defect in endocardial competence to undergo EMT.

Genetic disruption of BMP receptors also causes dysregulated cardiac cushion formation. Creation of a cardiac myocyte-specific disruption of ALK3, which encodes a type Ia BMP
receptor, bypassed the early lethality of this gene disruption. Homozygous mutants initially underwent normal EMT in the cardiac cushions, but by E10.5, the cushions were hypoplastic and failed to fuse properly. Expression of TGF-β2 was also decreased in ALK3−/− mutants; the cardiac cushions of mutant mice may therefore be hypoplastic secondary to decreased TGF-β2–mediated endocardial cushion EMT. A hypomorphic allele of BMP-RII (a type II BMP receptor) that has reduced signaling capability resulted in failure to form OT valves, but did not affect AVC cushion formation. These results suggest either that this receptor is required specifically in the formation of OT valves, or that the OT cushion is more sensitive to decreases in BMP-RII activity than the AVC.

**Increased BMP Signaling and Hypertrophic Valves**

BMP ligand and/or receptor disruption results in specific valve phenotypes that decrease valve formation. The converse experiment, to increase BMP signaling, was elegantly performed by disrupting Madh6, which encodes Smad6, a downstream target of BMP signaling. During development, Smad6 is expressed in endothelial cells, with increased immunostaining detected specifically in the AVC and OT. During adulthood, Smad6 expression persists throughout the endothelium, including the cardiac valvular endocardium. Madh6−/− mice display markedly thickened valves, and also have hypertension and decreased endothelial cell-mediated vasodilation. In vitro evidence suggests that BMP-2 potently induces the expression of Smad6. Therefore, inhibitory Smad6 signaling may play a physiologic feedback role in development by limiting the number of endocardial cells that undergo EMT. Notably, the madh6−/− mouse is one of the few genetic valve manipulations that is not uniformly embryonic lethal. Although speculative, the continued expression of Smad6 in postnatal valvular endocardium suggests a potential continued role for BMP signaling in control of postnatal valve maintenance.

**Transcriptional Targets of TGF-β: Snail/Slug and E-Cadherin**

The downstream effects of TGF-β and BMP signaling in the developing cardiac cushions are still not well defined but may include transcription factors of the Snail/Slug family. (Slug is the homologue in chick and Drosophila. The mouse has two genes, sna and slug, with close homology.) This family consists of a group of zinc-finger transcription factors that act as transcriptional repressors. A known function of Slug in the chick provides a paradigm for elucidating its role in valve development: inactivation of Slug with antisense oligonucleotides impairs epithelial-to-mesenchymal transdifferentiation.

The expression patterns of Slug/Slit in the developing heart suggest that these transcription factors could also regulate EMT in cardiac cushion formation. In chick hearts, Slug immunoreactivity occurs within the mesenchyme of developing cushions and in a subset of endocardial cells overlying the cushions, where it is thought to be a target of TGF-β, during EMT. Similarly in the embryonic mouse, Slit is expressed in the mesenchyme of many regions that undergo epithelial-to-mesenchymal transdifferentiation, including lung, kidney, and teeth, and in the endocardium and mesenchyme of developing heart valves. Analogous to E-cadherin in epithelia, VE-cadherin expression appears to be reciprocal to Snail, as revealed in studies of Notch signaling mutants. Notch1 mutants retain strong VE-cadherin, fail to express Snail, and fail to undergo EMT. A potential model would be that TGF-β induced by Notch signaling in the developing cushion activates Snail, which decreases the expression of cell adhesion molecules and thereby downregulates endocardial cell-cell adhesion and allows endocardial cells to initiate invasion into the cardiac jelly.

**ErbB: Integration of Extracellular Matrix Signals**

An increasing number of studies are substantiating a role for the erbB family of receptors in the control of cushion remodeling into the thin, fibrous valve (Figure 6). ErbB proteins mediate cell proliferation, migration, differentiation, adhesion, and apoptosis in numerous cell types. ErbB family receptors are receptor tyrosine kinases (RTK) referred to as ErbB1/EGFR/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. The four erbB proteins bind ligands that include epidermal growth factor (EGF), members of the heregulin/neuregulin (HRG) family, heparin-binding epidermal growth factor (HB-EGF), TGFα, amphiregulins, betacellulin, and epiregulin. Each of these ligands has different receptor affinities; for example, EGF binds with high affinity to erbB1, whereas HB-EGF binds to erbB1 and erbB4.

**ErbB Regulates Cardiac Cushion Proliferation**

Gene disruption and the use of hypomorphic alleles have provided basic insight into ErbB signaling in cardiac cushion development. A hypomorphic egrf, termed waved-2, contains a point mutation in the tyrosine kinase region of egrf; the resulting protein product possesses only 10% to 20% of the intrinsic kinase activity relative to erbB1/EGFR. The resulting protein product possesses only 10% to 20% of the intrinsic kinase activity relative to erbB1/EGFR. It was therefore reasoned that generation of egrf+/−/waved-2, Ptpn11−/− mice might sufficiently
These observations suggest that EMT initiates normally in HB-EGF+/− and TACE−/− mice, but that mesenchymal proliferation proceeds unchecked. Consistent with this hypothesis, developing valves in HB-EGF+/− and TACE−/− mice have increased staining for bromodeoxyuridine (BrdU, a marker of cell proliferation), but no change in the relative number of cells undergoing apoptosis. It seems likely that HB-EGF, after activation by TACE, signals through EGFRT to limit mesenchymal cell proliferation, because erbB4 knockout mice display only moderately decreased endocardial cushion size, but have no defects in fully developed valves.83

**ErbB Signaling and the Cardiac Jelly**

Numerous studies from the past decade have emphasized the crucial importance of the cardiac jelly in providing a signal that initiates endocardial differentiation (Figure 6). The initial focus was on soluble factors present in the jelly; for example, an EDTA-soluble fraction of cardiac jelly can initiate EMT when applied to competent cardiac cushion endocardial cells.84 Increasingly, it is becoming apparent that the superstructure of ECM regulates growth factor activity. An area of recent focus in this regard has been the role of hyaluronic acid (HA) in mediating erbB signaling.85

HA is a glycosaminoglycan composed of alternating glucuronic acid and N-acetylglucosamine (NAG) residues. In the extracellular matrix, HA exists as a hydrated gel that expands the extracellular space and regulates ligand availability. HA also interacts directly with numerous ECM proteins, including the proteoglycan versican, a major constituent of the cardiac jelly.86

Mammals possess three HAS genes, termed has1, has2, and has3.87 Has2 appears to be the major enzyme responsible for HA synthesis during development. Has2−/− mice die by E9.5 and display pericardial edema, disordered vessel growth, and complete absence of cardiac jelly.88 In the absence of cardiac jelly, the endocardial cushions are unable to form. In addition to its structural role, HA can modulate cell signaling events.89 Endocardial cells overlying the cushion-forming region in has2−/− mice display reduced EMT and migration, an effect that can be rescued by adding back HA or by transfection with constitutively active Ras.88 Conversely, transfection with a dominant-negative Ras blocked the ability of HA to promote EMT. Further studies revealed that heregulin (a ligand for ErbB3) rescued the has2−/− phenotype in ex vivo cushion explant models.90 Furthermore, has2−/− mice possess decreased endocardial cushion ErbB2/ErbB3 phosphorylation relative to wild-type embryos. Addition of HA to has2−/− tissue explants restored ErbB3 phosphorylation. ErbB3−/− mice die by E13.5, and have hypoplastic cardiac cushions, with decreased mesenchyme.91 Notably, erbB3 is expressed by endocardial cushion cells and mesenchymal cells undergoing EMT. In contrast, EGF, erbB2, and erbB4 expression is limited largely to cardiomyocytes during the critical cushion-forming window (E9.5 to E10.5).

Further studies will be necessary to determine how HA signaling interacts with other erbB ligands, such as neuregulin and HB-EGF. In zebrafish, the jekyll mutant results in failure of cardiac cushion formation.92 Synteny cloning revealed that the jekyll mutant contains a point mutation in the
NF1/Ras: Feedback Control of EMT

Neurofibromatosis is an autosomal dominant disorder characterized by café au-lait spots, neurofibromas, and an increased risk of neural crest cell–derived malignancy. Approximately 2% of patients with neurofibromatosis have cardiovascular malformations; the most striking predilection is a 6-fold increased risk of pulmonic stenosis. Neurofibromatosis is caused by mutations in the gene NF1, which encodes the protein product neurofibromin (Figure 7), a Ras-specific GTPase activating protein (GAP). Neurofibromatosis is caused by mutations in the gene NF1, which encodes the protein product neurofibromin (Figure 7), a Ras-specific GTPase activating protein (GAP). Neurofibromin therefore inactivates Ras, by cycling Ras from its active, GDP-bound conformation to an inactive, GDP-bound conformation. Consequently, patients with neurofibromatosis have dysregulated activation of Ras.

Disruption of nf1 leads to embryonic lethality by E14.5. Embryos display markedly enlarged cardiac cushions, double outlet right ventricle, hyperplasia of sympathetic ganglia, and delay in renal and hepatic growth. The cardiac defects have some morphological similarities to the effects of experimental neural crest cell ablation. Based on this observation, and the realization that other manifestations of neurofibromatosis are caused by abnormalities in neural crest differentiation, it was assumed until recently that the cardiovascular defects in nf1−/− mice were caused by defects in neural crest cell development or migration.

Two lines of evidence now show that endothelial cell loss of NF1 is sufficient to recapitulate the cardiovascular defects observed in nf1−/− mice. Tissue explants of E10.5 cushion tissue from nf1−/− mice displayed increased EMT; adenovirus transfection with a dominant-negative Ras inhibited EMT, whereas transfection of wild-type cushion explants with a constitutively active Ras increased EMT. These results suggest that the increased cardiac cushions of nf1−/− mice result from increased endocardial cell EMT, rather than a defect in neural crest cell migration.

Tissue-specific gene disruption has provided the second line of evidence for the crucial role of endocardium in NF1 signaling. Using a Cre-loxP system with tissue-specific promoters, it was shown that disruption of NF1 expression in endothelial cells, but not in myocardial cells or neural crest cells, accounts for all of the cardiovascular defects observed in nf1−/− mice. In contrast, neural crest cell disruption of NF1 expression caused hyperplasia of sympathetic ganglia, but no cardiovascular defects. It appears, therefore, that the cardiovascular defects in nf1−/− mice result from endothelial cell, not neural crest cell, disruption of NF1 expression.

Figure 7. Model for NF1 in feedback control of EMT. Neurofibromin is a Ras-specific GTPase activating protein (GAP) that cycles Ras from an active, GTP-bound state to an inactive, GDP-bound, state. Ras signaling is activated by receptor tyrosine kinases (RTKs) and usually proceeds through activation of downstream targets to increase mesenchymal proliferation. Evidence also suggests that the downstream signaling targets of Ras interact with NFATc1 to alter gene transcription. Neurofibromin may therefore decrease endothelial and/or mesenchymal cell proliferation by modulating Ras signaling.

E10.5), suggesting that Ras is upstream of NFATc1, and that NF1 modulates the timing of NFATc1 intranuclear localization. One possibility is that premature intranuclear localization of NFATc1 in nf1−/− mice causes earlier initiation of endocardial cushion EMT, a longer duration of EMT, and therefore hyperplastic cardiac cushions.

NFAT proteins act in concert with other transcriptional coactivators to modulate gene expression. In most cell types, NFAT-dependent gene transcription is dependent on concomitant signaling through Ras/MAPK pathways. Because neurofibromin negatively regulates Ras activity, it may secondarily decrease the activation of NFAT-dependent gene transcription (Figure 7). In nf1−/− mice, the increased Ras signaling may result in increased activation of NFAT, as suggested by the nuclear-localized NFATc1 seen in these mice. This, in turn, may lead to activation of NFATc1-dependent genes and coactivators. Hence, the physiological role of neurofibromin in cardiac cushion development may be to limit the extent of NFAT signaling and thereby attenuate expression of NFAT gene targets and availability of transcriptional coactivators.

A Signaling Network Model for Heart Valve Formation and Remodeling

The seven signaling modules discussed in this review (VEGF, NFATc1, Notch, Wnt/β-catenin, BMP/TGF-β, erbB, and NF1) have been studied largely in isolation and to varying degrees. An increasing number of studies have suggested links between signaling pathways. Rather than viewing each signaling molecule as a separate entity, the signaling modules can now be integrated into physiologic steps that occur during the process of cardiac cushion development. Therefore, we propose a network model for the control of cardiac cushion EMT during development, with an emphasis on potential points of interaction between signaling pathways (Figure 8).
In this model, the development of a cardiac valve occurs in four overlapping steps. First, the endocardial cells overlying the AVC and OT must be specified to undergo EMT. The exact events initiating this process are controversial; it has been suggested that endocardial cells competent to undergo EMT derive from a unique area of the anterior heart-forming field, and are therefore patterned to undergo EMT even before heart tube formation. Alternatively, the field properties of Notch signaling could create a subset of endothelial cells competent to undergo EMT. Consistent with this hypothesis, ex vivo explant studies have shown that only 10% to 20% of endothelial cells in the cushion-forming area undergo EMT. At the same time, Wnt/β-catenin signaling may control production of the growth factors (eg, BMPs) and structural elements (eg, versican and hyaluronic acid) required for initiation of EMT.

After specification to undergo EMT, cells fated to transdifferentiate must delaminate and activate a mesenchymal program. TGF-β signaling through snail/slug results in decreased VE-cadherin expression, allowing endocardial cells to separate. Concomitant with the delamination process, endocardial cells must repopulate to replace the cells that will undergo EMT, thereby maintaining a continuous endothelium. VEGF signaling through NFATc1 increases endothelial cell proliferation, perhaps contributing to repopulation. VEGF signaling may also maintain an endothelial cell fate, and thereby prevent excess endothelial cell transdifferentiation into mesenchymal cells.

Cells that delaminate from their endothelial cell-cell junctions begin the process of transdifferentiation and migration into the cardiac jelly. TGF-β synergizes with BMP to promote EMT. Hyaluronic acid signaling through ErbB2/3 heterodimers may also mediate the migratory process into the cardiac jelly. Conversely, other ErbB signals, including EGFR, may limit the extent of EMT.

The process of creating a cardiac cushion must be self-limited to ensure that the localized cushion swellings can remodel into appropriately thin fibrous sheets. Little is known about the processes that remodel cardiac cushions, but evidence suggests that neurofibromin acts through inhibition of Ras signaling to limit the extent of EMT. Because Ras signaling comediates NFAT transcriptional activity, neurofibromin may also regulate the extent of endocardial cell repopulation. Smad6 appears to attenuate TGF-β signaling in the developing valves; the precise step at which it intervenes to limit TGF-β signaling is not known but it clearly contributes to proper development. The pathways controlling the final events of remodeling are not known but may involve apoptotic signals.

Genetic studies using mouse models, combined with ex vivo studies on avian endocardial cushions, have identified players in valvulogenesis. More recently, in vitro cell culture of cardiac valve–derived endothelial cells has provided insights on specific cellular responses to VEGF and TGF-β. We hope this schematic will foster ideas for future investigations into the precise cellular and biochemical mechanisms that orchestrate these events. The goal then will be to determine the extent to which these mechanisms can be applied to the treatment of congenital valve defects and/or heart valve disease in adult life.

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