MINIREVIEW

Signal Transduction in Early Heart Development (II): Ventricular Chamber Specification, Trabeculation, and Heart Valve Formation

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The formation of a four-chambered heart with ventricular chambers aligned in a left-right orientation begins with the rightward looping of the linear heart tube in accordance with the left-right embryonic axis. The functional specification of the ventricular chambers in the looped heart occurs with the formation of a trabeculated myocardium along the outer curvature of the realigned heart tube. Two major signal transduction pathways are involved in this process, the retinoic acid and neuregulin signaling pathways, with the retinoic acid pathway also participating in rightward heart tube looping. With the establishment of the atrial and ventricular chambers, maintenance of a unidirectional flow of blood between the two chambers must be ensured. To achieve this, heart valves develop at the atrioventricular juncture. This process begins with formation of endocardial cushions, the primordia of heart valves, and ends with formation of heart valve leaflets. Underlying this process is a complex network of signal transduction pathways that mediate communication between the endocardial and myocardial cell layers to form the endocardial cushions and nascent heart valve. Some of the signaling molecules involved are vascular endothelial growth factor, *Wnts*, bone morphogenetic proteins, epidermal growth factor, hyaluronic acid, neurofibromin, and calcium. Exp Biol Med 232:866–880, 2007

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Introduction

While many of the overt events of heart formation appear to unfold sequentially—for example, heart tube formation, looping, and septation, followed by chamber formation—the molecular processes underlying these events often have their inception at much earlier time points. Thus, while the focus of this review will be ventricular chamber formation, it is clear that ventricular development begins as early as the specification of cardiac mesoderm, a process already discussed in Part 1 of this review series (1).

Functional Specification of Ventricular Chamber Identity: Formation of Trabeculated Myocardium

Formation of left and right ventricles begins when ventricular progenitors in the bilateral heart-forming regions (HFRs) of the embryo and right ventricular-specific progenitors from the anterior (or secondary) heart field migrate to form the anterior heart tube (Fig. 1; Refs. 2, 3). Specification of heart chamber identity is already evident at this early stage of development with the expression of various cardiogenic genes in distinct domains along the anteroposterior (AP) axis of the heart tube (5–8). This genetic regionalization presages the more apparent morphologic regionalization that comes with the formation of distinct structures, such as the outflow tract (OT), embryonic right and left ventricles, atria, and sinus venosus (Fig. 1). To align these presumptive structures into the appropriate

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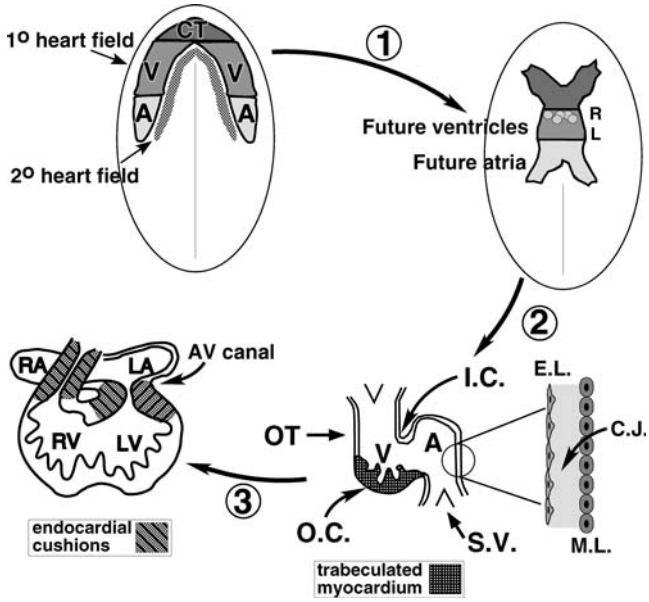

Figure 1. Early heart development and ventricle formation. Ventricular precursors from the primary and secondary heart fields migrate medially and coalesce to form the linear heart tube (1). The anteroposterior organization of these precursors is maintained in the heart tube, with right ventricular precursors (R) anterior to left ventricular precursors (L) and the future atria. Looping leads to the appropriate juxtaposition of these heart regions and the formation of an inner curvature (I.C.) and a trabeculated outer curvature (O.C.), the future ventricular myocardium (2). Further elaboration of this myocardium and septation into right and left atria and ventricles precedes the synchronized pumping of blood between the atria and ventricles and the formation of one-way valves to control blood flow (3). The precursors to these valves are the endocardial cushions that form in the AV canal. A, atria; C.J., cardiac jelly; CT, conotruncus; E.L., endocardial cell layer; LA, left atrium; LV, left ventricle; M.L., myocardial cell layer; RA, right atrium; RV, right ventricle; SV, sinus venosus; V, ventricle. Adapted from Brand (Ref. 2; used with permission of Elsevier) and Iwamoto and Mekada (Ref. 4; used with permission of J-STAGE).

position to form the primitive heart, the heart tube undergoes looping to reorient its anterior portion along the left-right (L/R) axis of the embryo.

Once the heart tube has realigned, it undergoes differential growth along the anteroposterior and dorsoventral axes to form inner and outer curvatures (6). The apical parts of the left and right ventricles expand from defined zones along the outer curvature to form a specialized myocardium called the trabeculated myocardium. Formation of this ventricular myocardium at the correct point along the outer but not the inner curvature of the tube functionally specifies the future ventricular chambers, and its correct positioning is likely to involve complex signaling along both the dorsal-ventral and cranio-caudal axes (6, 9). A candidate source of these signals is the inner endocardial cell layer of the early heart tube. Prior to trabeculation, the heart tube consists of an outer myocardial layer and an inner endocardial cell layer that are separated by an intervening matrix of proteoglycans and glycosamino-glycans called the cardiac jelly. Signaling from the endocardium to the ventricular myocardium initiates the conversion of the myocardium into a thickened ventricular myocardial chamber wall capable of contraction. The first step in this process entails the proliferation, differentiation, and migration of cells out of the myocardial layer and into the lumen of the ventricle to form protrusions or trabeculae at the outer curvature of the looped heart tube (10, 11). The spongy, trabecular myocardium that forms is largely responsible for the maintenance of blood flow at this point in development, since a contractile myocardium has yet to form, a fact underscored by the lethality of mouse gene mutants in which this trabeculated myocardium fails to develop (11).

Two major signal transduction pathways, the retinoic acid (RA) signaling pathway and the neuregulin/ErBb signaling pathway, are involved in formation of the trabeculated myocardium. The involvement of RA in heart development has been studied by removing or adding excess RA to embryos, as well as by eliminating RA synthesis or signaling pathways through knockout of specific RA synthesizing enzymes or receptor genes. These latter studies have shown RA to play an important role in trabeculation of the ventricular myocardium (12–14). Gene knockout studies of neuregulin signaling have demonstrated that this signaling molecule is also critical to formation of a trabeculated myocardium (15–17). In addition to RA and neuregulin-ErbB signaling, other signaling pathways appear to play a role in myocardial trabeculation. One interesting example of this is bone morphogenic protein 10 (BMP-10). Overexpression studies in which BMP-10 signaling was increased or knockout mutations in which BMP-10 signaling was ablated showed hearts that were hypertrabeculated *versus* ones that had profound hypoplasia of the ventricular walls and virtually no ventricular trabeculae, respectively. Further analysis of these mutants showed that BMP-10 plays a critical role in formation of the trabeculated myocardium by regulating both the proliferation of cardiomyocytes and their postnatal hypertrophic growth to provide appropriate numbers of cells to populate trabeculae and ultimately form the compact ventricular wall critical to normal heart function (18, 19). In addition to BMP-10, serotonin signaling to myocardial cells appears necessary for the growth and trabeculation of the ventricles, perhaps through this pathway's ability to maintain ErbB2 receptors at a critical level in myocardial cells (9, 20). As with heart tube looping and chamber specification, these observations underscore the complex network of signal transduction pathways that drive many of the morphogenetic events in heart development (1).

Retinoic Acid

Background. Prior to the elucidation of the RA signaling pathway, the role of retinoids in development was studied by their removal from animals using vitamin A–deficient diets (vitamin A is the biologically active precursor of RA; Ref. 21) or by presenting excess RA to developing embryos. With respect to heart, both vitamin A–deficient

diets and excess RA resulted in a spectrum of morphologic defects, many of which could be attributed to perturbation of RA signaling and its control of genes involved in tissue patterning and cell specification, differentiation, and proliferation. Successful completion of these developmental processes apparently requires tight control of RA ligand concentration, not only in heart but in all developing tissues. This suggests that RA signaling may function in development to translate differential RA levels into differential gene expression. Support for this comes from studies of nervous system development in which differential RA levels imparted positional information to cells along the AP axis of the neural tube by controlling the expression of patterning genes, such as homeobox genes (22, 23).

Role in Cardiogenesis. Blocking RA signaling by removing either RA or its receptors in embryos has been shown to affect heart development. In early studies, removing RA using vitamin A–deficient diets yielded offspring with septal defects and an incompletely formed, spongy myocardium, both indicative of a failure of cardiomyocytes to grow and differentiate into myocardia capable of inducing septation and forming a contractile ventricular chamber wall (24). With the advent of gene knockout technology, RA signaling could be eliminated by knocking out the genes encoding enzymes that synthesize RA or the receptors that transmit the RA signal. In general, removal of RA signaling by gene ablation affected AP patterning of the heart tube and its looping, but experimental results varied in some instances. Ablation of the RA synthesizing gene, retinal dehydrogenase-2 (RALDH-2), did not affect the AP specification of heart compartments but did affect their eventual development, as seen in the reduced size of atria and the sinus venosus (25). On the other hand, providing embryos with excess RA appears to affect AP specification by "posteriorizing" the more anterior regions of the heart tube (26, 27). The later event of heart looping is also affected by altering RA levels. In vitamin A deficiency studies, heart looping was reversed, and the L/R-determining genes *Nodal* and *Pitx2* were downregulated (28). In other studies using a pan-RA antagonist, the direction of heart looping was randomized, and *Nodal*, *Lefty,* and *Pitx2* genes were downregulated (27). In the RALDH-2 knockout study, rightward heart looping did not occur, and *Nodal*, *Lefty,* and *Pitx2* gene expression appeared normal, suggesting that RA signaling can influence heart tube looping independently of its role in L/R axis formation (25). Treatment of embryos with excess RA also led to abnormal looping, *situs inversus* (the complete mirror image reversal of organ asymmetry), and the symmetrical expression of *Lefty* (27, 29). Despite their complexity, these studies support the notion that RA signaling imparts AP positional information to the linear heart tube and participates in both early (i.e., embryonic L/R axis formation) and later stages of heart tube looping. As with the nervous system, these functions appear to be critically dependent on maintaining the appropriate concentration of RA in the developing embryo (23).

A more precise way to delineate the function of RA signaling in heart development would be to prevent transmission of the RA signal altogether by ablating RA receptor genes. Ablation of the RXRalpha receptor gene, the most widely used RA receptor (see below), prevents RA signaling through this pathway in RXRalpha-expressing tissues. In RXRalpha knockout mouse embryos, myocytes in the compact layer of the ventricular wall fail to proliferate, leading to a hypoplastic ventricular chamber and a diminished trabecular myocardium (12–14). In addition to a lack of proliferation, attenuation of the compact layer may also result from the premature differentiation of subepicardial ventricular cardiomyocyte precursors into migratory trabecular myocytes, a process believed to prevent cardiomyocyte precursors from populating the compact zone (12, 25). Aberrant growth control in RA signaling mutants also results in the ventricular septal defects evident in vitamin A–deficient embryos (13, 24) and in the failure of endocardial cushions to expand into septa of the appropriate size for heart valve formation (14, 25). Together, these studies show that RA signaling contributes to heart development on a number of levels by controlling the patterning, differentiation, and proliferation of cardiomyocytes and their precursors (30).

The RA Signaling Pathway. RA signaling is mediated primarily by two major ligands, all-*trans* retinoic acid and 9-*cis*-retinoic acid, that bind either homodimerized or heterodimerized forms of the retinoic acid receptor (RAR) and the RXR receptor (31). These receptors are structurally and functionally similar to nuclear steroid hormone receptors: upon binding ligand, they recognize and bind to RA-responsive elements (RAREs) in the upstream promoters of RA-responsive genes and activate their transcription (32, 33). Each receptor has three isotypes—α, β, and γ—and at least two isoforms within these isotypes (RAR β has four). These isoforms are expressed in different tissues, implying the need for different receptor isoforms to perform different functions (34, 35). The most biologically potent form of RA receptor *in vivo* is a heterodimer formed between RAR and RXR (36), with RXRalpha being the most widely used binding partner for RARs (12). In addition to RAR/RXR heterodimers, RXR homodimer receptors are capable of transmitting the retinoid signal *in vivo*. Both receptor types exhibit ligand specificity: RAR/RXR heterodimers preferentially bind RA and 9-*cis* RA, whereas RXR homodimers bind only 9-*cis* RA (37, 38).

RA signaling plays a major role in the specification and patterning of tissues by activating specific genes, the most prominent being the homeobox genes (39). The ability to activate RA-responsive genes in a precise temporal and spatial manner relies on differential RA receptor expression and the spatial and temporal control of RA synthesis. The biochemical precursor to RA is retinol, which when taken

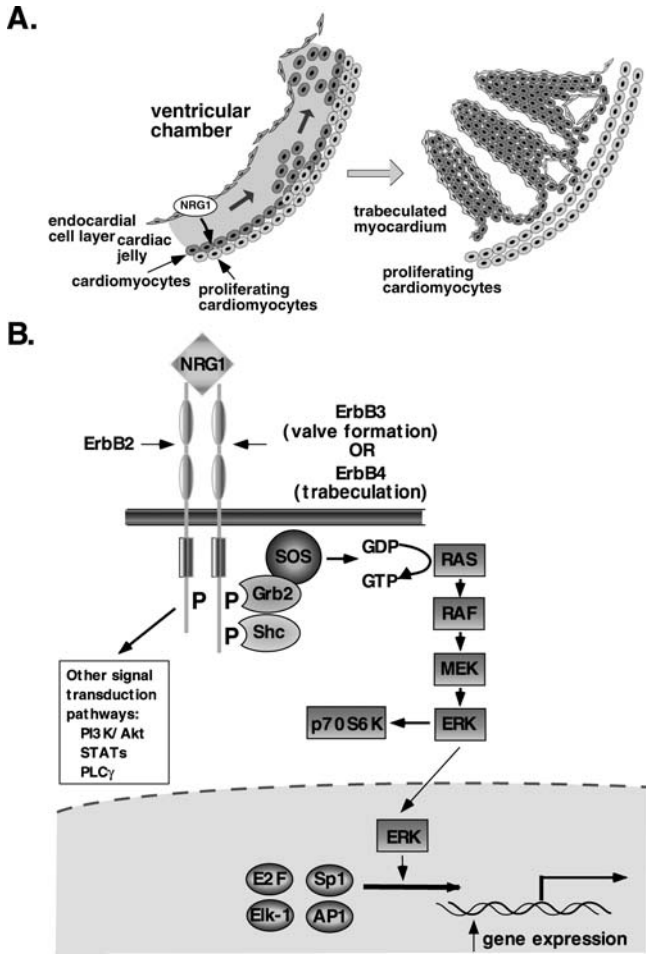

Figure 2. Trabeculation and NRG1 signaling *via* the ErbB signaling pathway. (A) NRG1 from the endocardium signals myocardial cells to proliferate and populate the emerging trabeculae with differentiated cardiomyocytes. (B) Ligand binding induces ErbB2 and ErbB4 receptors to dimerize (for myocardial trabeculation) and activate tyrosine kinase activity directed to specific tyrosine residues (P) within the carboxyl-terminal tail of the receptors. Adaptor proteins (Grb2, Shc) are recruited to activated receptors and direct the ErbB signal down specific signaling pathways, including the Ras-MAPK, PI3K-Akt, PLC-PKC, and the Jak/Stat signaling pathways. Virtually all ErbB receptors signal through the Ras/MEK/ERK signal transduction pathway, leading to upregulation of target genes. MEK, mitogen-activated protein kinase kinase; p70S6K, ribosomal p70-S6 kinase; ERK, mitogen-activated protein kinase. Modified from Marmor et al. (Ref. 47; used with permission of Elsevier).

up by cells is converted to retinal and then RA by the sequential action of retinol and retinal dehydrogenases (40). (9-*cis* RA is formed by 9-*cis* retinol dehydrogenase.) Virtually all RA in the embryo is produced by the action of RALDH-2 (25) and broken down into inert or less active retinoids by the action of a cytochrome P450 enzyme called CYP26 (reviewed in Ref. 41). These two enzymes, together with retinoid binding proteins (42), are largely responsible for maintaining control over RA levels in embryos, a very important task, since many actions of RA *in vivo* require the establishment of appropriate concentrations in responding tissues (43). Once produced in the signaling cell, RA or 9-*cis* RA is sufficiently lipophilic enough to diffuse out and into neighboring cells, where it is taken up by nuclear RA receptors that then go on to activate RA-responsive genes.

Neuregulin 1

Background. Neuregulins, originally identified as signaling molecules for Schwann cell proliferation and acetycholine receptor synthesis, have now been shown to be involved in a number of diverse physiologic processes, including heart development (44). Four neuregulin genes are found in the vertebrate genome, all encoding cell-cell signaling proteins that are ligands for receptor tyrosine kinases of the ErbB family. NRG1, the most studied gene, can express up to 15 different isoforms through alternative splicing of its RNA transcript or the use of multiple upstream promoters. These isoforms can be sorted into different functional categories depending on the presence or absence of various structural domains, the most critical being the epidermal growth factor (EGF)-like domain that activates the ErbB receptor tyrosine kinases (44).

Role in Cardiogenesis. Ablation of NRG1 signaling *via* site-directed removal of functionally important domains, such as the EGF-like domains, or ablation of its associated ErbB receptors has shown the NRG1/ErbB signaling pathway to be critical to the process of trabeculation. In mice lacking a functional NRG1 gene product or the ErbB2 or ErbB4 genes, trabeculae fail to form in the outer curvature of the looped heart that is destined to become the left and right ventricles (15, 17, 45, 46). The similarity between the NRG1 and ErbB2 and four gene knockout phenotypes indicates that NRG1 signaling *via* ErbB receptors is essential for trabeculation of the ventricular myocardium (Fig. 2A). The directionality of this signaling pathway, from endocardium to myocardium, was established by studies showing NRG1 mRNA to be expressed in the endocardium and ErbB2, and four mRNAs to be expressed in the myocardium (15, 45). This expression pattern notwithstanding, the precise mechanism as to how NRG1/ErbB signaling is directed to the outer but not the inner curvature of the heart tube remains an open question. Indirect evidence suggests the involvement of a second, ancillary signal transduction pathway using the cardiac jelly component hyaluronan as a ligand for the CD44 cell adhesion receptor (48, 49). Exactly why NRG1 knockouts fail to form trabeculae is still a matter for speculation (50). Recent studies showing that NRG1 can activate focal adhesion kinase (51), a key requirement for increasing cell motility and migration, point to a possible failure of cardiomyocytes to migrate into and populate trabecular protrusions in NRG1(–/–) embryos. In this case, the NRG1 signal is likely to be sent down the PI3K-PKB/Akt pathway, similar to what is seen in VEGF signaling in migratory endocardial cells (Fig. 3).

The Neuregulin Signal Transduction Pathway. In heart development, NRG1 signals to responsive

cells *via* ErbB receptors consisting of ErbB3 or ErbB4 receptors that heterodimerize with ErbB2, a "ligandless" receptor (Fig. 2B; Refs. 47, 52). ErbB2/ErbB4 heterodimers are involved in trabeculation, whereas ErbB2/ErbB3 heterodimers are important for cardiac valve formation (4, 15). Structurally, ErbB receptors have an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular portion containing a highly conserved tyrosine kinase domain (47). Ligand binding to ErbB receptors leads to phosphorylation of tyrosine residues on the partner receptor, resulting in an increase in its kinase activity. Additional tyrosine phosphorylation enables the recruitment and activation of adaptor proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains (Fig. 2B). These adaptor proteins assemble into multiprotein complexes that direct ligand signaling to various downstream signal transduction pathways. In the case of ErbB signaling, this could be any or all of four different signal transduction pathways: the Ras-mitogen–activated protein kinase (Ras-MAPK) pathway, the phophatidylinositol 3′ kinase–protein kinase B (PI3K-PKB/Akt) pathway, the phospholipase C–protein kinase C (PLC-PKC) pathway, or the Jak/STAT pathway (47). For formation of trabeculae, the signal transduction pathway used by the ErbB2/4 receptor has not yet been formally demonstrated, but since all ErbB ligands and receptors couple to activation of the Ras-MAPK pathway, this pathway is likely to play a role in NRG1 signaling to myocardial cells. The Ras/MAPK adaptor protein complex linking ErbB receptors to the Ras signal transduction pathway consists of Grb2, an adaptor protein, and Sos, a guanine nucleotide exchange factor that is the actual activator of Ras (47, 53–55). This complex brings Sos into close proximity with Ras, where it activates Ras by exchanging a guanosine diphosphate (GDP) nucleotide (Ras bound to GDP is inactive) for guanosine triphosphate (GTP), the nucleotide that binds and activates Ras. Once activated, Ras binds to and activates the Raf kinase, leading to activation of the MEK/ERK kinase cascade and activation of transcription factors, such as Sp1, E2F, Elk-1, and AP1.

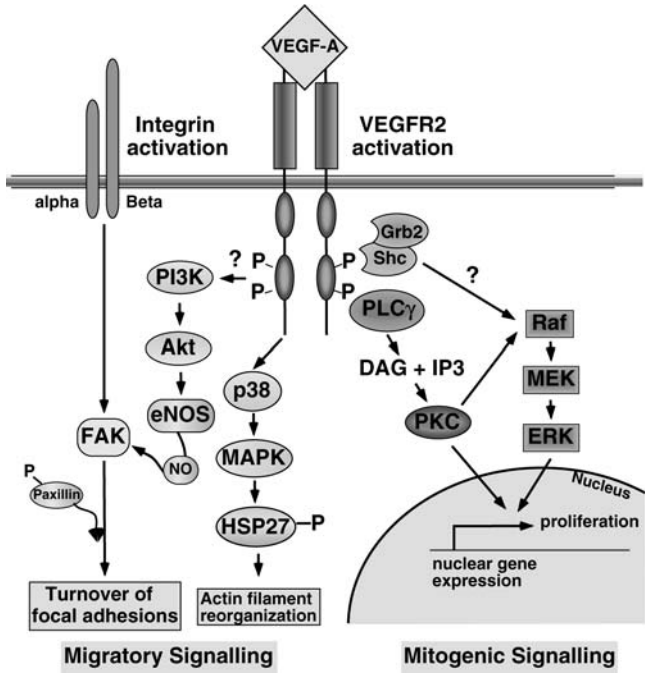

Figure 3. VEGF signaling pathways. Ligand-induced activation of VEGFR2 leads to phosphorylation of tyrosines within the VEGFR2 intracellular domain that act as binding domains for SH2 domain–containing adaptor proteins (Grb2, Shc, PLCγ). The VEGF signal is rendered mitogenic by the Shc/Grb2 and PLCγ adaptor complexes that direct signaling to the Raf/MEK/ERK and PKC pathways. Shc and/or Grb2 adaptor proteins direct the VEGF signal down the Raf/MEK/ERK pathway, possibly *via* Ras activation. PLCγ transmits the VEGF signal *via* activation of PKC, which can either activate ERKs 1/2 *via* Raf-1 and MEK or directly influence nuclear transcription factors to upregulate growth-related gene expression. VEGF migratory signaling is mediated in part *via* PI3K-mediated activation of the anti-apoptotic kinase Akt. Akt activates Ca^{+2}-independent endothelial nitric oxide synthetase (eNOS) through phosphorylation to produce NO, which activates focal adhesion kinase (FAK) by an unknown mechanism. FAK activity may also be induced *via* the integrin receptor $alpha_v/Beta_3$. VEGF signaling *via* the p38/MAPK/HSP27 pathway directs reorganization of the actin cytoskeleton. Adapted from Zachary and Gliki (Ref. 64; used with permission of Elsevier).

Heart Valve Development and Endocardial Cushion Formation

Blood flow through a chambered heart requires synchronized pumping between the different heart chambers (56). To ensure that blood flows in one direction and to prevent backflow in the opposite direction requires the use of one-way valves. The precursors of valves, cardiac cushions, form between the atria and ventricles within the atrioventricular (AV) canal and within the ventricular outflow tract (OT; Fig. 1). We will focus on the signaling pathways underlying formation of the AV cushions, since these have been the more intensely studied.

Early in its development, the single heart tube consists of an outer myocardial layer and an inner endocardial layer that are separated by an extracellular matrix referred to as the cardiac jelly. At this point, the linear heart tube is segmented into outflow and inflow tracts, as well as future atria and ventricles (Fig. 1). The positions where endocardial cushions form along the heart tube are determined in part by a "prepatterning" of endocardial and myocardial cells competent to signal each other to form cushions and also by the correct positioning of these prepatterned regions in the looped heart. Heart tube looping repositions the future atrial and ventricular chambers in such a way as to allow for cushion formation to occur at their juncture, the AV canal. In the AV canal, cushion formation begins when the cardiac jelly expands and swells into cushion primordia that then become "cellularized" *via* the influx of mesenchymal cells (Figs. 1 and 4A; Ref. 57). These cells come from the endocardial cell layer from which they have delaminated

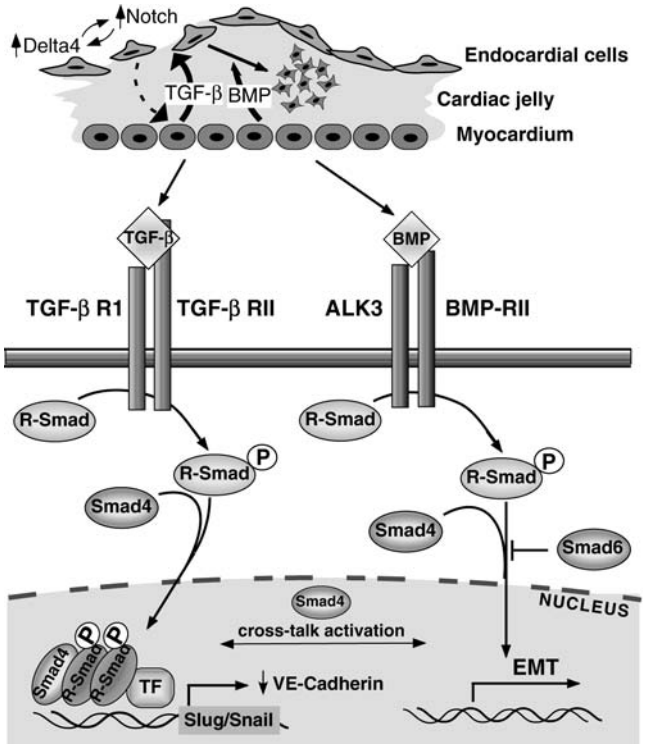

Figure 4. *Notch*, TGF-β, and BMP activity in endocardial cushion formation. High levels of Notch activate neighboring endocardial cells to produce Delta4 by a lateral induction mechanism that leads to delamination from the endocardial layer. Myocardial cells express TGF-β to initiate EMT, and BMP to control mesenchymal cell proliferation. TGF-β signals through TGF-β receptors RI and RII on endocardial cells, whereas BMP signals through heterodimerized ALK3/BMP-RII receptors on mesenchymal cells. Both receptors signal by phosphorylating receptor-activated smad proteins (R-smads), which then heterodimerize with smad4 to yield a transcriptional activator complex. TGF-β signaling activates the *Snail/Slug* transcription factor to repress VE-cadherin expression and endocardial cell adhesion (78, 79). BMP signaling activates genes promoting EMT through activation of ATF-2. The involvement of smad4 in both pathways allows for cross-talk between the TGF-β and BMP signal transduction pathways. Mesenchymal cell number can be limited by turning off BMP-dependent EMT gene expression through the intervention of smad6, a negative transcriptional regulator (80, 81).

and had undergone an epithelial-to-mesenchyme transformation (EMT; Figs. 4 and 6). The selective expansion of these and not other endocardial cells may rely on their being genetically competent to respond to inductive signals from the apposing myocardium, a similarly specialized myocardium able to induce EMT (58–61). Recent evidence has shown that this inductive signaling involves myocardial-derived BMP-2 signaling (61) as well as certain components of the extracellular matrix produced by the endocardial cells in response to myocardial-derived signals (62). Thus, both a myocardial-inducing activity and a patterning of endothelial cells competent to receive inductive signals appear necessary for cushion formation. Among the signals directing endocardium to form the AV endocardial cushions are *Wnts*, *Notch*, TGF-β, vascular endothelial growth factor (VEGF), BMPs, hyaluronic acid (HA), neurofibromin, and EGF.

EMT and cushion formation can be divided into three sequential processes and the signaling molecules involved in each roughly assigned as follows: activation and delamination of cells from the endocardial layer are associated with increased VEGF, *Notch*, and TGF-β signaling; cell migration into the cardiac jelly with increased VEGF and HA signaling occurs; and cell proliferation and its control with an increase in positive regulators such as *Wnt* and BMP, and negative regulators such as EGF and NF1, occurs. Once formed, cushions subsequently develop into heart valve leaflets through a complex signaling process that includes signaling by calcium, VEGF, and nuclear factor of activated T cells (NFAT). The successful integration of these various signaling pathways appears crucial to heart development, since abnormal development of the valves and septa comprise the majority of congenital heart defects (63).

VEGF

Background. VEGF is required for the activation, proliferation, and eventual modeling of cushion cells into valve leaflets. Initially recognized as a vascular permeability factor (64), VEGF has since been implicated in a wider array of processes, including vasculogenesis and angiogenesis (65). Vertebrates have six VEGF genes, VEGF-A through VEGF-E and placenta growth factor (66). VEGF-A, a gene with eight exons that can be alternatively spliced to give five different VEGF-A isoforms, has been the most studied VEGF gene. The most abundant and biologically active VEGF-A isoform is VEGF165, which is secreted and forms an active signaling molecule upon glycosylation and dimerization. VEGF165 and two other isoforms, VEGF121 and VEGF145, are the biologically active forms of VEGF in endothelial cells (64, 67). VEGF expression can be regulated by a variety of stimuli, including nitric oxide (NO), growth factors such as basic fibroblast growth factor (bFGF), and certain hormones (64).

Role in Valve Formation. Many studies have linked VEGF to endocardial cushion formation *via* its expression pattern in early heart and the effects of overexpressing VEGF in the early cardiovascular system. (Ablation of the VEGF gene is lethal in early embryos.) Early in development, VEGF expression is found in most endocardial cells of the heart tube, whereas at Embryonic Day 9.5 this expression becomes restricted to endocardial cells lining the AV canal and the OT, and also to the myocardial cells underlying cardiac cushions (68). This restricted expression pattern may represent a type of prepatterning of the heart tube with respect to cardiac cushion formation. Expression of VEGF in myocardial cells located at points of cushion formation suggests that the myocardium initiates EMT in endocardial cells by myocardial-to-endocardial VEGF signaling. Once activated, these endocardial cells then produce their own VEGF to induce neighboring cells to undergo EMT (Fig. 6). In this way, paracrine VEGF signaling from the myocardium to the endocardium as well

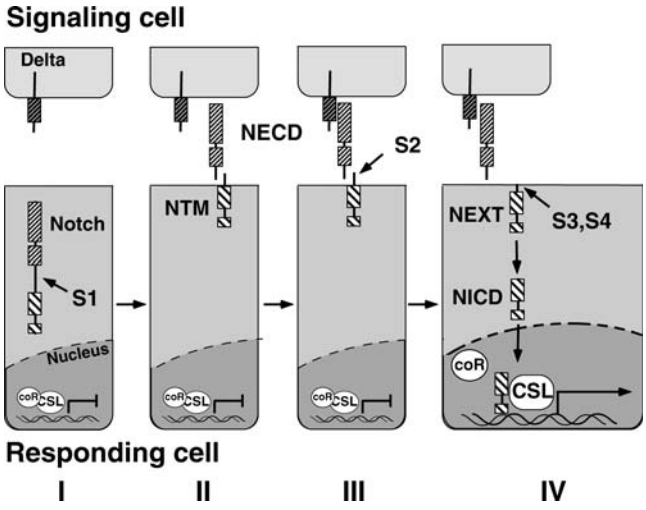

Figure 5. A model for *Delta*-dependent *Notch* signaling to the nuclear transcription factor CSL. I. Preactivation state. Cleavage of *Notch* at S1 forms NTM and NECD. II. Delta at the surface of the signaling cell binds S1-cleaved Notch at the surface of the responding cell. III. Ligand-dependent S2 cleavage of Notch generates an activated membrane-bound form of Notch, NEXT. IV. NEXT is further processed at the S3 and S4 sites to release the NICD that translocates into the nucleus, where it de-represses CSL by displacing the co-repressor coR. Adapted from Schweisguth (Ref. 84; used with permission of Elsevier).

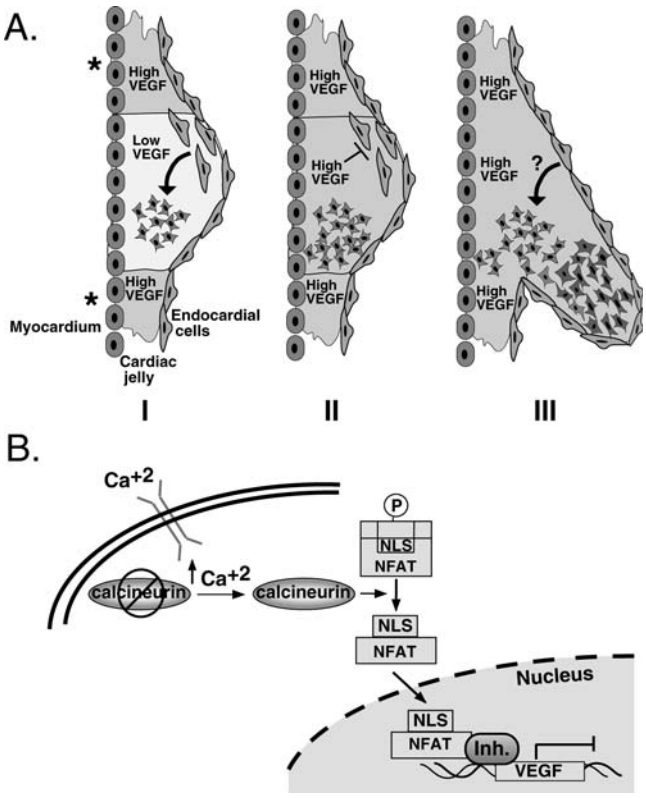

Figure 6. (A) Differential levels of VEGF control EMT in cushion and heart valve formation. Low VEGF expression from myocardial cells permits endocardial EMT (I), whereas high levels terminate EMT (II). High VEGF levels suppress cushion formation outside of cushion-forming regions (*). NFAT nuclear localization and function control VEGF levels, initially in myocardial cells and then in endocardial cells, where they induce expression of a presently undefined signal that initiates valvoseptal cell formation (III). Adapted from Lambrechts and Carmeliet (Ref. 141; used with permission of Elsevier). (B) Ca^{+2} levels can contribute to valve formation by controlling NFAT nuclear localization. Ca^{+2} influx in myocardial cells activates calcineurin to dephosphorylate NFAT and expose a nuclear localization signal that promotes NFAT translocation into the nucleus, where it binds to gene regulatory molecules to either activate or repress target genes. This same pathway can induce VEGF gene expression either through a decrease in Ca^{+2} influx or by switching the NFAT binding partner from a transcriptional inhibitor to a transcriptional activator (141). Inh, transcriptional inhibitor.

as autocrine VEGF signaling between endocardial cells act to initiate and sustain EMT. To provide further evidence for such a role in cushion formation, VEGF was overexpressed in myocardial cells, with the expectation of increasing EMT and the overall size of cardiac cushions. However, the opposite occurred: cardiac cushions failed to form, most likely due to an inability of cells to undergo EMT (69). This suggests that while VEGF remains a positive inducer of EMT, its levels must be tightly regulated for normal cushion formation to occur (70). Together, these studies show that VEGF signaling plays a critical role throughout cardiac cushion formation, from determining where cushions will form to initiating and then maintaining EMT to provide the appropriate number of cushion cells for constructing the semimuscular leaflets of the fully formed heart valve (see below).

VEGF Signal Transduction Pathway. VEGF ligands signal through two receptor tyrosine kinases, VEGFR1 (or Flt1) and VEGFR2 (or KDR; Ref. 71). Most biologically relevant VEGF signaling in endocardial cells is mediated *via* VEGFR2. As with all receptor tyrosine kinases, VEGF signaling begins when it binds to and stimulates VEGFR2 receptor dimerization and trans(auto)-phosphorylation of distinct tyrosine residues in the cytoplasmic domain of the receptors (Fig. 3). Different SH2 domain-containing adaptor proteins bind to these phosphotyrosine residues and direct the VEGF signal down different intracellular signaling pathways, allowing VEGF to participate in many diverse biologic processes (64, 71). Two of these processes, cell proliferation and migration, are important aspects of endothelial cell behavior in angiogenesis that also appear to be involved in endocardial cushion formation. For proliferation, activated VEGFR2 recruits adaptor proteins, such as Shc, Grb2, and phospholipase C-γ (PLC-γ) to send the VEGF signal down two pathways, one being the Raf-1/MEK/ERK pathway which, in this case, might not be dependent on Ras activation, and the other being the phospholipase C-γ pathway, also Ras independent (64). Both converge in the nucleus, where they activate transcription factors to promote expression of mitogenic or angiogenic genes as the case might be, including transcription factor genes, such as NFAT (72, 73).

VEGF also appears to control the migration of mesenchymal cells into the cardiac jelly. Cells migrate by the repeated breaking and reforming of adhesive bonds to

the extracellular matrix and by stretching and retracting filopodia *via* constant actin filament reorganization. VEGF regulates these processes by signaling to multiple downstream pathways that include focal adhesion kinase (FAK), p38 kinase, and PI 3-kinase (PI3K; Fig. 3; Ref. 64). Human umbilical vein endothelial cells, or HUVECs, often are used to study the effects of VEGF on endothelial cell behavior. In these cells, VEGF induces tyrosine phosphorylation of FAK and the focal adhesion-associated protein paxillin to promote recruitment of FAK to new focal adhesions, a necessary step in generating cell motility (74). VEGF can also activate p38 MAP kinase in HUVECs, leading to actin reorganization and cell migration (75). A third pathway promotes production of NO, a molecule that influences endothelial cell migration by regulating focal adhesion integrity and FAK phosphorylation (76). Since all these processes are likely to contribute to endocardial cell migration in cushion formation, their associated signal transduction pathways are also likely to be active in migratory endocardial/mesenchymal cells.

Notch

Background. The *Notch* signaling system plays a key role in determining the fate of cells and their patterning in complex tissues. In heart valve development, an additional feature of *Notch* signaling appears to be at work; namely, *Notch*'s ability to downregulate cell adhesion (77). This signaling activity is believed to activate endocardial cells to undergo EMT by decreasing their cell-to-cell adhesion and allowing for their delamination from the endocardial layer and transformation into migratory mesenchymal cells (Fig. 4). Mediating this activity is the *Delta*/*Notch* (ligand/receptor) signal transduction pathway. The four vertebrate *Notch* receptors are very similar to the *Notch* receptors originally identified and studied in Drosophila, which have a single membrane-spanning domain connecting an extracellular, ligand-binding domain to a cytoplasmic domain required for signal transduction (82). The Notch extracellular domain contains a tandem array of 29 to 36 repeated elements homologous to a sequence in the EGF protein. Their spatial arrangement appears critical for binding the ligands Jagged and Delta. The cytoplasmic domain is the active signaling component of the receptor, and much of *Notch* signaling is directed to the release of this domain and its transit to the nucleus, where it activates specific transcription factors (Fig. 5; Refs. 82, 83).

The *Notch* ligands *Jagged* and *Delta* are structurally similar to the *Notch* receptor in that they also have multiple EGF repeats in their extracellular domains. Like Notch, but unlike most other ligands, Jagged and Delta are membrane bound. The cytoplasmic domain of these ligands facilitates their dimerization into an active ligand recognized by *Notch* receptors on neighboring cells. Signaling between membrane-bound ligand and receptor limits the extent of *Notch*-activated cells, a characteristic of *Notch* signaling that appears essential for normal development (85).

Role in Formation of Cardiac Valves, AV Canal, and Aortic Development. As mentioned, a common feature of *Notch*-activated cells is their loss of adhesion (77), and this property has been put to use in promoting EMT in endocardial cells of the OT and the AV canal. Both *Notch* and its ligand *Delta4* are expressed in the Embryonic Day 8.5 mouse endocardium, with *Notch* expression visible in endocardial cushion mesenchyme when EMT begins (78). *Notch* appears to work *via* TGF-β2 and BMP signaling to effect EMT (Fig. 4). This is supported by the observation that TGF-β2, a BMP family member that is normally expressed in the OT and the AV canal myocardium (86), is severely reduced in *Notch* signaling mutants (78). Reduced TGF-β2 signaling, in turn, results in the absence of *Slug*, a member of the *Snail* gene family that is a TGF-β2–responsive transcriptional repressor normally expressed in the OT and the AV canal endocardium during the onset of EMT (79). In epithelial tumor cells, *Snail* induces EMT by repressing expression of the gene for E-cadherin, a cell adhesion molecule (87, 88). In endocardial cushions, *Snail* induces EMT by repressing the gene for vascular endothelial cadherin (VE-cadherin), resulting in decreased adhesion between endothelial cells and their delamination from the endocardial cell layer (78). In mouse *Notch* mutants, little or no *Snail* gene product is expressed, resulting in the failure of endocardial cells to delaminate and "cellularize" the cardiac cushion (78). These observations were confirmed by experiments designed to inactivate *Slug*/*Snail* in endocardial cushion explant cultures by antisense RNA technology. As with *Notch* mutants, reduced expression of the *Slug* gene impaired EMT, suggesting that the transcriptional repressor activity of *Slug* normally promotes EMT *in vivo* (89). In keeping with this, *Slug* has been shown to be expressed in cushion mesenchyme and subsets of endocardial cells overlying the cushions (90). These observations have led to a model in which high levels of Notch induce high levels of the *Notch* ligand Delta4 in endocardial cells by a type of lateral induction mechanism (Fig. 4). This leads to premigratory activation of endocardial cells and the production of an endocardial cell–derived signal that induces the nearby myocardium to produce TGF-β2. TGF-β2 then signals back to activated endocardial cells to express the *Slug*/*Snail* transcription factor, which results in the reduction of VE-cadherin expression and overall cell adhesiveness. Endocardial cells then delaminate and migrate into the cardiac jelly to form cellularized endocardial cushions under the control of BMP and VEGF (reviewed in Refs. 70, 91, 92).

In addition to endocardial cushion formation, the *Notch* signaling pathway plays an important role in a number of different processes, from establishment of L/R asymmetry in embryos to angiogenesis. Embryos with either a gain or loss-of-function mutation in *Notch* signaling exhibited disrupted L/R asymmetry and decreased or disrupted *Nodal*

gene expression, suggesting a direct regulation of *Nodal*'s asymmetric expression pattern by *Notch* signaling (93, 94). Mutations in *Notch* signaling effectors cause cardiovascular anomalies often associated with the clinical disorder called Alagille syndrome (AGS; Refs. 95–98). These developmental anomalies include Tetralogy of Fallot as well as stenosis of the pulmonary valve and the branch pulmonary artery in addition to other left- and right-sided anomalies and septal defects (97). Various lines of evidence have linked these cardiovascular syndromes to aberrant developmental processes arising from mutations in the *Notch* ligand *Jagged*, the *Notch* target genes *Hey1* and *Hey2*, and *Notch* itself. An early indication of *Notch's* involvement in angiogenesis came from analysis of a mutation in zebrafish called *gridlock* (*grl*), the zebrafish ortholog of the mammalian *Hey2* gene, a member of the *Hes* family of transcriptional repressors that are targets of *Notch* signaling (99). This mutation results in abnormal assembly of the aorta from the embryonic vasculature and has been attributed to a defect in the specification of angioblast precursors to the arterial cell fate (99–102). These observations received further support when similar anomalies (i.e., missing or poorly formed dorsal aortae) were seen in mice lacking *Notch1* and *Notch4* or the *Hey1* and *Hey2* genes (103, 104). Mutations in the *Jagged* gene (*JAG1*) give rise to anomalies in outflow tract development similarly to that seen in human AGS patients, and studies have linked *JAG1* to AGS (95) and, more recently, to its specific cardiovascular complications (e.g., pulmonary stenosis and Tetralogy of Fallot; Refs. 96, 97). Finally, returning to early heart development, recent studies of the *Notch* signaling pathway have shown it to be involved in demarcating the non–chamber-forming regions of the heart tube to the AV canal and inner curvature myocardium (105). This patterning function involves the repression of BMP-2 expression by the *Hey* transcriptional repressors. Perturbation of *Notch* signaling could alter this patterning function to give atrial and ventricular septal defects; roughly 10% of AGS patients exhibit such defects (97). Together, these studies show that the well-documented role of *Notch* signaling in determining cell fates and patterning of tissues is active in early heart and vascular development.

***Notch* Signaling Pathway.** The *Notch* signaling pathway is unlike any of the signaling pathways discussed so far in that it relies not on kinase cascades to transmit the *Jagged*/*Delta* signal, but on sequential proteolytic cleavages that release the Notch cytoplasmic domain to translocate to the nucleus and activate genes. Notch proteins are synthesized as single polypeptides that are proteolytically cleaved at a site within the molecule (called S1) to form a heterodimerized receptor of sorts on the cell surface (Fig. 5; Refs. 84, 106). One proteolytic product consists of an ectodomain called Notch Extra-Cellular Domain (NECD) and a membrane-tethered intracellular domain called Notch Trans-Membrane (NTM). Notch signaling from neighboring cells begins when the ligands Delta or Serrate bind the NECD, causing the NTM to be cleaved by extracellular proteases at a site called S2. S2 cleavage releases the ectodomain of Notch and generates an activated membrane-bound form of Notch called Notch Extracellular Truncation (NEXT). NEXT is further cleaved at two sites, S3 and S4, to release a peptide called Notch Intra-Cellular Domain (NICD) into the interior of the cell. NICD translocates into the nucleus and assembles into a ternary complex with a DNA-binding protein called CSL. In the absence of Notch signaling and nuclear NICD, CSL recruits transcriptional repressors to *Notch* target genes to repress their expression. With active *Notch* signaling, these repressors (e.g., coR) are displaced by NICD, and CSL is converted to a transcriptional activator.

The *Wnt*, BMP, EGF, HA, and NF1 Signaling Pathways and Their Role in Cardiac Cushion Formation

More often than not, signals serving a function in one tissue or developmental context can be found to act as signals in other tissues or developmental contexts. The *Wnt*, BMP, and ErbB signaling pathways, which are involved in cardiogenic induction and trabeculation of ventricular myocardium, also play roles in cardiac cushion formation. Since their backgrounds and signaling pathways have already been discussed (1), only their roles in cardiac cushion formation will be detailed here.

Wnts. Studies in zebrafish, an increasingly useful model for study of heart development, have shown that when *Wnt* signaling is rendered constitutive *via* stabilization of β-catenin, *Wnt*-responsive transcription factors are activated, and cardiac cushions undergo massive expansion *via* hyperproliferation of endocardial and/or mesenchymal cells (107). Conversely, antagonizing the *Wnt*/β-catenin pathway inhibits cardiac cushion formation. In early mouse heart tube, components of the *Wnt*/β-catenin pathway have been localized to a subset of cells in the endocardial cell layer and the mesenchyme of OT and AV canal cardiac jelly, further supporting a role for *Wnt* signaling in endocardial cushion formation (108). Based on these analyses, it appears that the *Wnt*/β-catenin pathway is the major *Wnt* signal transducer involved in EMT and that controlling the level of *Wnt* activity and the expression of Wnt-controlled genes may be one means of regulating endocardial cell proliferation and cushion size, critical requisites for normal heart valve formation.

BMPs. In the developing mouse heart tube, BMP-2 and BMP-4 expression is restricted to the mesenchyme and myocardium underlying the developing AV canal and OT cardiac cushions, indicating a role in EMT (109–112). The functional analysis of BMPs and TGF-β in EMT has greatly benefited from the use of an *in vitro* collagen gel system that allows endocardial epithelial cells to transform into mesenchymal cells that can invade and migrate through the collagen lattice (113). Using this system, addition of

purified BMP-2 to the media was found to functionally substitute for the myocardium in promoting EMT (114). The role of BMP signaling in EMT was further confirmed by genetic ablation of the BMP receptor, Alk3 (115). In these mutants, cardiac cushions were hypoplastic and failed to fuse properly. In addition, TGF-β expression was decreased, suggesting a potential regulatory control of TGF-β by BMP. Other studies have shown a synergistic relation between BMP and TGF-β activities in the formation of endocardial cushions (116, 117). "Cross-talk" between the BMP and TGF-β pathways could occur *via* smad4, a receptor-activated transcription factor responsive to both signaling molecules (Fig. 4). BMP signals, specifically BMP-2, also appear to provide an inhibitory feedback control regulating the number of cells undergoing EMT, and thus cardiac cushion size (which is important, given that the valves that eventually develop must form a tight and precisely "engineered fit"). This inhibition is carried out by Smad6, a negative transcriptional regulator and a downstream target of BMP signaling (80). In accordance with these findings, ablation of the Smad6 gene results in overproliferation of mesenchymal cells, resulting in hyperplastic endocardial cushions and thickened heart valves (81).

EGF. EGF appears to play a role in controlling the proliferation of mesenchymal cells within the cardiac jelly of endocardial cushions (70). EGF ligands that signal through the ErbB1 and ErbB4 receptors are strongly expressed in the endocardium overlying the cushion-forming areas of the heart tube (118). Mouse mutants defective for EGF signaling exhibit enlarged, hyperplastic valves of the AV canal and OT and die shortly after birth, presumably due to poor valve function and inadequate cardiac pumping (118, 119). Together, these observations suggest that EGF signaling from the endocardium is required late in EMT to limit mesenchymal cell proliferation to ensure formation of functional valve leaflets of the appropriate size.

HA. Components of the cardiac jelly extracellular matrix can either directly signal or modulate other cell-derived signals in the EMT process (57, 70). One of these, HA, is a glycosaminoglycan that not only has a structural role in the extracellular matrix, but also a signaling function *via* ErbB receptors (see Fig. 2 for the ErbB signal transduction pathway; Ref. 120). There are three genes in the mammalian genome devoted to HA synthesis: *has 1, 2,* and *3*. When *has2* is ablated in mice, the heart tube endocardium cannot produce HA and, as a result, endocardial cushion explants onto collagen gels show diminished endocardial cell migration and EMT (49). This phenotype can be rescued by activating the ErbB signaling pathway either by addition of exogenous HA to the endocardial cushion explants (or a different ligand, such as heregulin) or by introduction of constitutively active Ras into endocardial cells. Together, these studies suggest that upon invading the cardiac jelly, further migration of activated endocardial/mesenchymal cells is dependent upon receipt of signals from the extracellular matrix (e.g., HA) that activate the Ras signal transduction pathway (120).

Neurofibromin. Neurofibromin (NF1) is a Ras-specific GTPase activation protein that inactivates Ras by cycling it from an active GTP-bound conformation to an inactive, GDP-bound conformation (121). The NF1 gene was identified originally in patients with von Recklinghausen neurofibromatosis, an autosomal dominant disorder that manifests in a variety of pathologies (121–123). When the NF1 gene is ablated in mice (124) or specifically ablated in endothelial cells (125), embryos exhibit enlarged cardiac cushions, among other cardiac abnormalities. Given NF1's function, this phenotype is most likely due to abnormally high levels of Ras activity in endothelial cells. Support for this notion comes from the results of two complementary experiments: one in which constitutively active Ras introduced into normal endocardial cushion cells promoted EMT, and a second in which a dominant negative Ras introduced into NF1(–/–) endocardial cushion cells (with high Ras activity) was able to reverse endocardial cell hyperproliferation (126). These experimental results show that NF1 acts to control EMT by its ability to downregulate the Ras signal transduction pathway. Various studies have implicated the transcription factor NFAT as the most likely target of the Ras/Raf/MEK/ERK pathway in endocardial cells, suggesting that in NF1(–/–) cells, elevated Ras leads to increased NFAT transcriptional activity (125, 127). Together, these observations raise the possibility that the physiologic role of NF1 in normal cardiac cushion development may be to limit the extent of NFAT activity and, in so doing, modulate endocardial cell transformation and proliferation in order to form functional valve leaflets of the appropriate size.

In addition to NF1, mutations in other components of the *Ras* signal transduction pathway can lead to congenital heart defects, particularly defective valvulogenesis. Mutations in GTP exchange factors, such as Sos, or protein tyrosine phosphatases, such as Shp-2, or mutations in Ras itself that retard its cycling back to the inactive GDP-bound form all lead to an overactive *Ras* signal transduction pathway that appears to be the primary cause of the disorder called Noonan syndrome (128, 129). Noonan syndrome is a genetic disorder with multiple manifestations, including cardiac defects that give rise to pulmonary valvular stenosis, hypertrophic cardiomyopathy, and atrial septal defects (130, 131). Mutations in Shp-2, Sos, and Ras have been isolated from patients with Noonan syndrome, and *in vitro* studies of these mutated proteins have shown them to deregulate the *Ras* pathway, resulting in increased *Ras* activity. Mutations in Sos proteins from Noonan patients prevent autoinhibition of Sos, leading to increased GTP exchange, elevated Ras activity, and increased downstream ERK kinase signaling (132, 133). Mutations in Ras proteins from Noonan patients impair the intrinsic GTPase activity of Ras and prevent the binding of GTPase activating proteins; together, these

mutations lead to higher levels of Ras-GTP and elevated Ras activity (134). Mutations in Shp-2 proteins (PTPN 11 gene mutations) are seen in 50% of Noonan cases (135), and studies have linked it to aberrant endocardial cushion formation and calcium-dependent activation of the NFAT transcription factor (see below). Shp-2 proteins from Noonan patients exhibit gain-of-function mutations, leading to increased phosphatase activity and an increase in the growth and proliferation of cushion mesenchymal cells *in vitro* through the activation of the MEK-1 kinases ERK1/2 (136). *In vivo*, genetic engineering of mice to express the PTPN11 mutated form of Shp-2 leads to enlarged valve primordia in both the AV canal and the OT (137). Additional studies also have shown Noonan-type gain-of-function Shp-2 mutants to increase the oscillatory frequency of intracellular Ca^{+2} transients in a way that perturbs calcineurin function and eventually leads to decreased NFAT activation, a process that could affect endocardial cushion formation *via* its effect on VEGF gene expression (see below and Ref. 138). These studies, along with those of the NF1 mutation, show that signal transduction through the *Ras* pathway plays a prominent role in the development of the heart and its valves and that deregulation of this pathway is a major cause of congenital heart disease (128, 129).

The Ca^{+2}/Calcineurin/NFAT/VEGF Axis in Heart Valve Formation

The tight control of NFAT signaling by NF1 suggests that NFAT itself regulates important downstream events crucial to proper heart valve formation. In accordance with this, recent studies have shown that NFAT plays a central role in both the establishment of the cushion and its transformation into the thin, fibrous semimuscular leaflets that comprise the mature valve (139). NFAT achieves this in a rather unique way—as a calcium-sensitive transcription factor that regulates a second signal transduction pathway, the VEGF pathway. Calcium controls numerous cellular processes, and the Ca^{+2} ion signal can be transduced by various signaling pathways, one of which is the activation of the NFAT transcription factor. The most direct pathway to activation of NFAT is *via* calcineurin: Ca^{+2} influx activates calcineurin, a Ca^{+2}/calmodulin-dependent phosphatase, which dephosphorylates NFAT to expose a nuclear localization signal and promote NFAT translocation into the nucleus, where it activates or represses target genes (Fig. 6B; Ref. 140). In the first stage of endocardial cushion formation, activated NFAT in myocardial cells represses VEGF gene expression, resulting in lower levels of VEGF in the cardiac jelly of the cushion (Fig. 6A; Ref. 139). Low VEGF levels permit the transformation and migration of mesenchymal cells into the cardiac jelly, whereas high levels prevent this transformation (69). In the second phase of valve formation, mesenchymal cell proliferation is arrested, and existing cells are induced to differentiate into valve leaflet precursors. These events are triggered when VEGF increases to levels that inhibit further cell proliferation, presumably from de-repression of the VEGF gene in myocardial cells *via* a decrease in Ca^{+2} activation of the NFAT repressor or a switch in NFAT-binding partner from a transcriptional repressor to an activator. The final step, differentiation of mesenchymal cells into valvuloseptal fibroblastlike cells and their maturation into valve leaflet precursors (57), also involves NFAT activity. Control of this process appears to shift from the myocardium to the endocardium and is paralleled by a similar shift of NFAT expression and activation to the endocardium. Genetic ablation of the endocardial-specific NFAT isoform NFATc1 eliminates Ca^{+2}/calcineurin/NFAT signaling and hinders valve elongation (142). Valve formation can be restored in these knockout embryos by expression of an NFATc1 transgene targeted to endocardial cells, thereby confirming the critical role of endocardium-derived NFATc1 in valve leaflet maturation. The downstream NFATc-dependent signaling molecules that mediate this process are presently not known.

Perspectives

As indicated by many of the mutations in the signaling pathways discussed above, a high level of precision is required in the way these pathways control the growth and assemblage of cells into a functional heart. Such precision appears not to be achieved by the strict regulation of each pathway in and of itself, but rather through the use of a network of interacting and cross-regulating pathways that by their sheer complexity provide for a system of "checks and balances" that fine-tunes the behavior of cells. A particularly instructive example of this is the formation of heart valves. In their review (70), Armstrong and Bischoff present a comprehensive view of valve development, breaking it down into four sequential stages and the eight signal transduction pathways involved. Most importantly, they show how signal transducers between the eight pathways can cross-regulate each other to modulate the growth and size of the endocardial cushions in order to achieve the precision fit of heart valve leaflets. The heart is likely to use similar means of controlling its growth and development, suggesting the need to elucidate the network of signaling interactions involved. While many of the individual signal transduction pathways in heart development have been described, the more daunting task ahead may be left to systems biologists to place these pathways into a higher-order interactive map or "interactome" (143) that comprehensively describes heart development at the molecular level.

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