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Experimental Biology and Medicine 2007, 232:852-865.

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MINIREVIEW

Signal Transduction in Early Heart Development (I): Cardiogenic Induction and Heart Tube Formation

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Heart development begins with the induction of cardiogenic cells from the embryonic mesoderm, followed by the coalescing of these cells into a linear heart tube. Subsequent looping of the heart tube brings the rudimentary atria and ventricles into alignment for further development into the four-chambered heart. Underlying these morphologic events is a complex program of signaling between cells and tissues that orchestrates their participation in heart development. Among these signals are bone morphogenetic proteins, fibroblast growth factors, *Wnts*, *Hedgehog*, and members of the transforming growth factor-β family of signaling molecules. We review here the various properties of these signaling molecules and their signal transduction pathways in hopes of providing a greater appreciation of the molecular events driving heart development.
Exp Biol Med 232:852–865, 2007

Key words: cardiogenic induction; heart tube looping; left-right embryonic axis; fibroblast growth factor; *Wnt*; *Hedgehog*; *Nodal*

Introduction

Development of the heart is a continuum of highly complex morphogenetic processes that are coordinated both spatially and temporally. Many of these processes involve cell and tissue interactions mediated by signal transduction pathways that allow instructive signals from one cell or tissue to induce changes in the behavior of adjacent cells or tissues. Recent advances in our understanding of the nature of these signals and how they are produced, received, and acted upon has provided insights into the molecular basis of organ morphogenesis, including that of the heart.

Study of the signal transduction pathways that underlie heart and vascular development has been conducted in a number of animal models, ranging from zebrafish to humans. Certain aspects of heart development are shared by different species, making what has been learned from analysis of lower vertebrates applicable to higher vertebrates. To integrate these studies and provide as comprehensive an understanding of the signal transduction pathways underlying heart development as possible, we will focus on the more common features of vertebrate heart development in which signal transduction is critical to morphogenesis, omitting species-specific details for the sake of brevity.

Cardiogenic Induction

As with most organs, the heart develops from one of the germ cell layers established in the early embryo. The early vertebrate embryo is an ovoid disc composed of the endodermal and ectodermal cell layers, between which is situated the mesoderm, the germ cell layer that gives rise to heart muscle (1). Running medially along the long axis of the disc is the primitive streak that forms the embryonic midline. Mesenchymal cells migrate through the streak and move out laterally to form separated but paired left and right heart-forming regions (HFRs; Fig. 1A). These mesodermal cells then migrate cranially and coalesce at the midline to form a crescent of rostrolateral cardiogenic mesodermal

This work was supported by National Institutes of Health grant HL073399 to M.A.Q.S.

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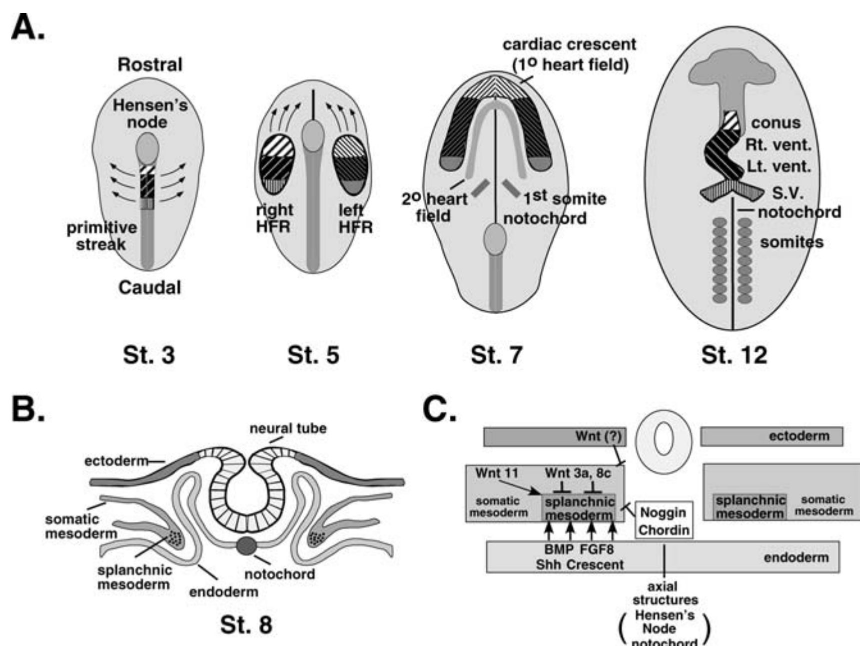

Figure 1. (A) Cardiogenesis during chicken gastrulation. *St.* refers to Hamburger and Hamilton stages. Stage 3: Cardiac progenitor cells caudal to Hensen's node are in the same anteroposterior order as their eventual positions in the tubular heart. Stage 5: Cardiac progenitor cells in the bilateral HFRs in the lateral plate mesoderm. Stage 7: HFR cells migrate to form the cardiac crescent. In addition, the secondary heart field forms. Stage 12: Tubular heart with distinguishable chamber primordia including the conus, primitive right ventricle (Rt. vent.) and left ventricle (Lt. vent.), and *sinus venosus* (SV). Modified from Brand (2). (B) Cross-section of stage 8 chicken embryo depicting the ectodermal and endodermal layers that surround the somatic and splanchnic mesoderm. (C) Signaling pathways between germ cell layers that act to induce cardiogenic mesoderm. Positive acting signals in the endoderm and the mesoderm signal splanchnic mesodermal cells to become cardiogenic. Inhibitory signals from the ectoderm, Hensen's node, the notochord, and from within the mesoderm inhibit cardiogenesis. Modified from Brand (Ref. 2; used with permission of Elsevier).

cells at the cranial border of the disc; this formation often is referred to as the "cardiac crescent" or primary heart field. In mammals this mesoderm is divided into a dorsal somatic mesoderm and a ventral splanchnic mesoderm. In the immediate vicinity of the splanchnic mesoderm lie three neighboring cell layers that emit positive- and negative-acting signals that together result in the induction of cardiogenic cells (Fig. 1B and C). These cell layers are the underlying anterior endoderm, the overlying neurectoderm, and the Node (or Organizer in amphibia; Hensen's Node in chicken), a transitory chordomesodermal structure that migrates rostrocaudally along the primitive streak, depositing cells that eventually form the notochord. Positive-acting signals from these neighboring tissues, most prominently the endoderm, induce mesodermal cells to become cardiogenic. These signals include bone morphogenetic protein 2 (BMP-2; Ref. 3), fibroblast growth factor 8 (FGF-8; Ref. 4), Crescent (5), and mesodermally-derived Wnt11 (6). Negative-acting or inhibitory signals also play a role in early cardiogenesis, perhaps as a means of delimiting cardiogenic induction to a specific population of mesodermal cells (Fig. 1C). Among these signals are *Chordin* (7), *Noggin* (8, 9), Serrate (10), and Wnts 3a and 8 (5). Together, these signals direct mesodermal cells to the cardiogenic cell lineage, presumably by inducing the expression of cardiogenic transcription factor genes, such as Tal 1, Tbxs 2, 3, and 5 (3) Nkx2.5 (11), and cGATA (12), among others.

In addition to the primary heart field, a second heart field, located more medially in the splanchnic mesoderm and directly adjacent to the cardiac crescent (Fig. 1A), contributes cells to the heart. Attempts to delimit the extent of this field and determine what heart structures are derived from it have provided different results (13). The widest area encompasses progenitor cells that contribute to the definitive outflow tract (conus and truncus) as well as the right ventricle, and this area is called the anterior heart-forming field. A more narrowly defined region, the "prepharyngeal" mesoderm (14), contributes both myocardial and smooth muscle cells to the "arterial pole," the myocardial-arterial junction at the base of the aorta and pulmonary trunk (15). These contributions to the ascending limb of the looped heart occur after the ventricular and inflow regions of the primary heart tube are formed (16). Cardiogenic induction of cells in the secondary heart field appears to be controlled by FGF-8 and BMP-2 signaling molecules present in the caudal pharynx and outflow tract (17–19), as well as by *Sonic Hedgehog* (20). These signals direct expression of cardiogenic transcription factors, such as Tbx1 (21–23), Nkx2.5, and GATA4 (17, 24, 25), all of which appear necessary for the determination and differentiation of secondary heart field cardiac progenitors.

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Positive-Acting Signaling Molecules of the Endoderm: BMPs and FGFs

Induction of cardiogenic cells in the primary and secondary heart fields requires positive-acting signals from neighboring germ layers or developing tissues. The two major signaling molecules involved are BMP and FGF.

BMPs. *Background.* BMPs were originally identified as signaling molecules capable of inducing bone and cartilage formation *via* their effects on the differentiation of chondroblasts and osteoblast lineage cells (26). It is now known that BMPs are multifunctional proteins that play a variety of roles in the development and function of various cells and tissues (27). BMPs belong to the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins/inhibins, and Mullerian inhibiting substance (28). BMPs are disulfide-linked dimeric proteins that are structurally similar to other members of the TGF-β superfamily. To date, 15 BMPs have been identified in mammals.

Role in Cardiogenesis. BMPs 2 and 4 appear to be the only BMP isoforms capable of inducing the formation of cardiogenic cells in non-precardiac mesoderm *in vitro* (9, 29, 30). Endodermal cells underlying the anteromedial mesoderm produce and secrete BMPs that bind BMP receptors on the surface of precardiogenic mesodermal cells and activate the appropriate signal transduction pathways (Fig. 2). Molecular cloning of BMP-2 has allowed for production of pure BMP-2 and testing of its cardiac-inducing potential *in vivo*. When BMP was ectopically presented to noncardiogenic mesoderm, key cardiogenic transcription factors, such as *Nkx2.5*, GATA4 (9, 33), and Tbxs 2 and 3 (3), were induced. However, limited exposure to BMPs could not fully substitute for the endoderm in upregulating these cardiogenic factors. In this case, addition of FGF restored BMPs' inductive capacity and indicated that *in vivo,* both BMPs and FGFs are necessary for full cardiogenic induction (30). Together, these experiments have led to the conclusion that BMP-2 (and BMP-4) signaling plays an important role in the early steps of cardiogenic induction, inducing mesodermal cells into the cardiogenic lineage and maintaining their cardiogenic potential until later signals complete their differentiation into cardiomyoyctes.

The BMP Signal Transduction Pathway. Activation of the BMP signal tranduction pathway begins when BMP binds two BMP receptors of different subtypes, type I and type II, acting as a bridge to bring these two receptors into juxtaposition and allowing phosphorylation of the type I receptor by the type II receptor kinase (Fig. 2; Ref. 34). Once the type I receptor is phosphorylated, it acts as a kinase to transduce the BMP signal to downstream effectors. At this point, two signal transduction pathways can be activated: the TAK1-MKK3/6-p38/JNK pathway and the Smad pathway (31). TAK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) superfamily (35). Phosphorylation of TAK1 triggers a cascade of phosphorylation reactions that lead to activation of the nuclear transcription factor ATF-2 and upregulation of subordinate genes. Alternatively, the BMP signal can be transmitted *via* the Smad signal transduction pathway (31). Upon binding of the type I receptor by BMPs, smad1 proteins are recruited to the receptor, where they are phosphorylated and released. This BMP ligand–specific smad then associates with Smad4 (which does not bind receptors), and the smad1/4 complex translocates from the cytoplasm into the nucleus, where it binds and activates the ATF-2 transcription factor to transcribe BMP-responsive genes.

FGFs. *Background.* FGFs comprise a large family of polypeptide growth factors, with as many as 22 separate FGFs encoded in the human genome (36). These proteins were first identified as growth-promoting factors when FGF-1 and FGF-2 were shown to stimulate the proliferation of fibroblasts. Since then, FGFs have been found to be involved in a variety of cellular processes, including chemotaxis, cell migration, angiogenesis, differentiation, cell survival, and apoptosis (37). FGF proteins are characterized by their high affinity for heparin, a molecule that facilitates their binding to cell surface FGF receptors, as well as an evolutionarily conserved core domain of 120 amino acids that mediates interaction with FGF receptors. The human FGF family is subdivided into seven subfamilies based on (i) the presence or absence of a signal peptide in the N-terminal region of the protein and (ii) whether the FGF can be secreted to act as a cell-to-cell signaling molecule or is retained in the cell, where it can act intracellularly (36).

Role in Cardiogenesis. Discerning the precise role of FGFs in the induction of cardiogenic precursors has been complicated by their earlier involvement in the induction and patterning of the mesoderm (independent of cardiogenic induction) and by the fact that their role in cardiogenic induction involves synergistic interaction with BMP signaling pathways (30, 38). Despite this, experiments in chicken and mouse embryos have identified certain FGFs—FGFs 1, 2, 4, and 8 in chicken and FGF-8 in mice—that can cooperate with BMP-2 to specify mesodermal cells as cardiogenic (4, 38–40). In mice it appears that FGF-8 action is directed toward cardiac induction in the anterior or secondary heart field (Fig. 1; Refs. 41, 42). The need for both BMP and FGF in cardiac induction was revealed in experiments showing that BMP-2 alone could not promote survival of precardiac or non-precardiac mesoderm cells in culture, whereas FGF-4 could support and maintain cardiogenesis in precardiac mesoderm, although it lacked the ability to induce cardiogenesis in non-precardiac mesoderm (38). More recent studies have shown that maximal induction of cardiogenic cells in non-precardiac mesoderm and expression of cardiac transcription factors, such as *Nkx2.5* and serum response factor (SRF), requires the continual presence of BMP and only a brief earlier exposure to FGF (30). Experiments in chicken have confirmed this by

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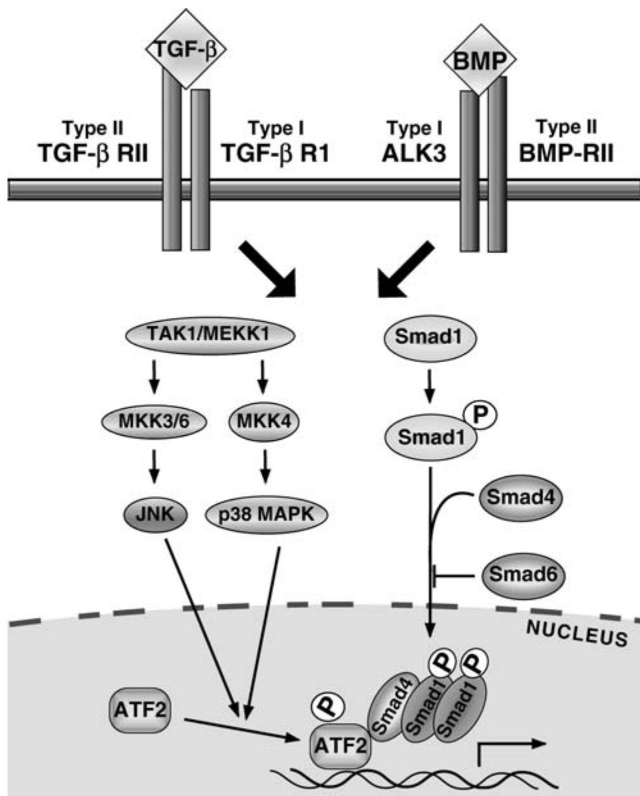

Figure 2. TGF-β and BMP signal transduction pathways involved in cardiogenic induction. The TGF-β or BMP signal can be directed to either the TAKI/MEKK1 or smad pathway and presumably can activate different gene programs through different transcriptional effectors or smad transcription factors. The Smad1/4 heterodimer can bind the ATF-2 transcription factor, activating it to transcribe TGF-β/BMP-responsive genes. Activation of ATF-2 can also be achieved by the alternate TAK1 pathway. Modified from Monzen et al. (Ref. 31; used with permission of Elsevier) and Derynck and Zhang (Ref. 32, used with permission of Nature Publishing Group).

showing that both FGF-8 and BMP-2 are necessary for cardiac induction and the expression of cardiogenic genes, such as *Nkx2.5* and MEF2c (4). Together, these observations suggest that the full cardiogenic potential of mesodermal cells in either the primary or secondary heart fields (19) requires BMPs for inducing mesodermal cells into cardiac progenitors and FGFs for the subsequent proliferation and survival of these differentiated cardiomyocytes.

The FGF Signal Transduction Pathway. There are four FGF receptors (FGFRs) encoded in the mammalian genome, each of which can undergo alternative mRNA splicing to give structural variants that have different FGF ligand specificities (see Table I in Ref. 43; see also Refs. 36, 44). FGFRs are ligand-activated receptor tyrosine kinases. Binding of FGF leads to dimerization of FGFRs and autophosphorylation of tyrosine residues in their intracellular domain, a process that serves as a mechanism for the assembly and recruitment of downstream signaling complexes (Fig. 3). The FGF signal can be transmitted *via* three main pathways: the Ras/MAPK pathway, the phospholipase C-γ/Ca^{+2} pathway, and the phosphatidylinositol 3 (PI3)-kinase/Akt pathway (37, 45). Of these, the major intracellular signaling pathway for FGF is the Ras/MAPK pathway (46, 47). Activation of this pathway occurs when an activated FGFR binds to and phosphorylates tyrosine residues in a membrane-anchored docking protein called FGFR substrate 2alpha (FRS2α). Phosphorylation of FRS2α promotes binding of Grb2, a small adaptor molecule that is complexed with the nucleotide exchange factor Sos. Sos plays a pivotal role in activating the Ras pathway. In cells, Ras is active when it is bound to guanosine triphosphate and inactive when it is bound to guanosine diphosphate. Ras signaling is initiated when the guanosine diphosphate bound to Ras is replaced by guanosine triphosphate, a reaction catalyzed by guanine nucleotide exchange factors, such as Sos. Ras activation initiates a phosphorylation/activation cascade involving Raf, MEK, and the MAP kinases ERK1 and ERK2. The ERKs enter the nucleus, where they complete the transduction of the FGF signal by phosphorylating and activating transcription factors that then transcribe FGF-responsive genes.

Positive and Negative Signaling Molecules of the Mesoderm and Ectoderm: *Wnts* and *Crescent*

The *Wnt* family of signal transducers comprises a third major group of signaling molecules controlling cardiogenic induction of mesoderm. Within this group are both positive-acting *Wnts*, such as *Wnt*11, which promote cardiogenesis, as well as negative-acting *Wnts*, such as *Wnt*3a and *Wnt*8, which inhibit it. For cardiogenesis to take place, these negative-acting *Wnts* must be inhibited. Their inhibition by specific *Wnt* signaling inhibitors, such as *Crescent,* forms part of the inductive process (5).

Wnts

Background. *Wnts* are a family of secreted glycoproteins that have been implicated in developmental processes, such as cell fate determination, establishment of cell polarity, and the differentiation, proliferation, and migration of various cell types (48). Once secreted, Wnt proteins associate with glycosaminoglycans in the extracellular matrix and are bound tightly to the cell surface, where they can act on the producing cell or close neighboring cells (49). Despite this tethering to the *Wnt*-producing cell, *Wnts* also can act as long-range morphogens, eliciting different responses from responding cells at various distances from the *Wnt*-producing cell (50). This could be achieved by the *Wnt* inducing the secretion of signaling molecules from neighboring cells in a sort of cell-to-cell relay mechanism of long-range signaling, or more directly *via* secretion of freely diffusible Wnt proteins into the extracellular space. Recently, a transmembrane protein called *wntless* has been shown to guide Wnt to the plasma membrane for secretion (51–53).

The family of *Wnt* proteins is large, with 19 different

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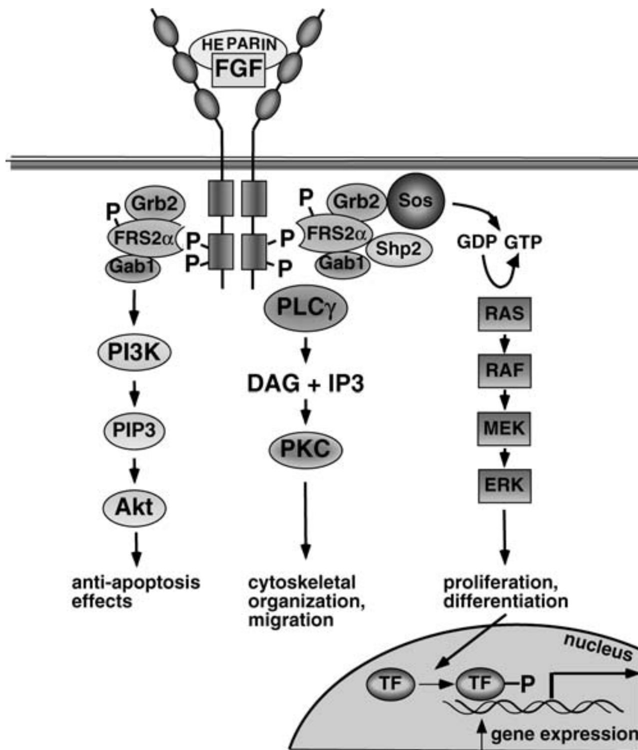

Figure 3. FGF signal transduction pathways. Binding of heparin and FGF to FGF receptors stimulates their phosphorylation and the binding of the FGF receptor docking protein FRS2α. Phosphorylation of FRS2α facilitates recruitment of the Grb2 and Gab1 adaptor proteins, as well as the protein tyrosine phosphatase Shp2, and directs the FGF signal down either the PI-3 kinase–AKT or Ras-MAP kinase pathway. Activation of the PLCγ pathway stimulates PKC. These pathways control a variety of cellular behaviors involved in cardiogenesis and heart formation. Modified from Dailey et al. (Ref. 45; used with permission of Elsevier).

Wnt genes in humans alone (54). Attempts to sort out the function of different *Wnts* have relied on two different biologic assays: the ability to form a secondary axis when injected into early Xenopus embryos and the ability to transform a mammary epithelial cell line called C57mg (55). Those *Wnts* failing to exhibit either of these two properties have been assigned to a class of *Wnts* that can alter cell movements and reduce cell adhesion when introduced into Xenopus embryos. These various activities are likely to result from *Wnt* signaling *via* different signal transduction pathways, and they point to the involvement of *Wnts* in diverse biologic processes, an observation borne out by the varied phenotypes resulting from the ablation of *Wnt* genes in mice (56).

Role in Cardiogenesis. Studies of heart formation in Xenopus and chicken have shown that induction of cardiac mesodermal progenitors requires activation of the Wnt/Ca^{+2} (57) and Wnt/polarity (58) pathways, as well as inhibition of the Wnt/β-catenin pathway (Fig. 4; Ref. 5). Activation of the Wnt/Ca^{+2} pathway leads to activation of protein kinase C (PKC), whereas activation of the Wnt/polarity pathway leads to activation of the Jun amino-terminal kinase (JNK) and upregulation of nuclear gene expression (54). The Wnt/polarity pathway is activated by *Wnt*11, which is expressed prominently in the precardiac mesoderm of Xenopus, mouse, and avian embryos (58). Two other *Wnts*, *Wnt*3a and *Wnt*8c, also are expressed in the cardiogenic mesoderm; however, in contrast to *Wnt*11, they activate the Wnt/β-catenin pathway and inhibit cardiogenesis (5). Realization of the full cardiogenic program thus requires the inhibition of these two *Wnts*, most likely through the secretion of the inhibitory molecules *Dkk-1* and *Crescent* (see below and Fig. 4B). In Xenopus, these *Wnt* inhibitors are expressed in the Spemann organizer (the Xenopus counterpart to the Node in vertebrates), which has cardiac-inducing activity (5). Thus, in terms of *Wnt* signaling, heart formation requires the spatially controlled expression of two opposing activities: inhibition of the cardiac-inhibiting Wnt/β-catenin signaling pathway in anterior lateral mesoderm by *DKK-1* and *Crescent,* and activation of the Wnt/Ca^{+2} and Wnt/polarity pathways in precardiac mesoderm by *Wnt*11.

The Wnt Signal Transduction Pathway. *Wnt* signaling is mediated by cell surface receptors that divide into two distinct families, the *Frizzled* (*Fzd*) gene family and the low-density lipoprotein receptor–related protein (LRP) family (54). As discussed above, the different functions exhibited by *Wnt* proteins when injected into Xenopus embryos indicated that *Wnts* might act through distinct signaling pathways to effect different cell behaviors. Pathway selection is determined in large part by which Frizzled receptor is activated by which *Wnt* ligand (Fig. 4). Signaling through the canonical Wnt/β-catenin pathway is achieved with the activation of a latent group of transcription factors belonging to the LEF/TCF family by a molecule called β-catenin (48, 59, 60). In the absence of Wnt, a multiprotein complex binds to and degrades β-catenin, preventing it from activating LEF/TCF transcription factors. In the presence of Wnt, this degradation process is blocked, resulting in increased levels of free β-catenin that bind and activate LEF/TCF transcription factors and increase target gene expression (Fig. 4A). The Wnt/Ca^{+2} pathway operates independently of β-catenin and is activated by a distinct group of *Wnts* and *Fzd* receptors (e.g., Wnt5a, Wnt11, and Fzd2; Refs. 55, 60). Binding of Wnt5a or Wnt11 to Fzd2 activates a heterotrimeric G protein, which leads to an increase in intracellular Ca^{+2} levels and activation of calcium/calmodulin-regulated kinase II (CamKII) and PKC. The activation of these two signaling pathways can be influenced by secreted modulators of *Wnt* signaling that antagonize or block *Wnt* binding to *Fzd* receptors. Among those inhibitors that appear to play a role in cardiogenesis are *Dickkopf* (*Dkk-1*) and *Crescent* (Fig. 4B; Refs. 5, 61, and reviewed in Ref. 62). *Dkk-1* blocks activation of *Wnt* signaling by interacting with the extracellular domain of LRPs, whereas *Crescent* (not yet found in mammals) is a member of the secreted Frizzled-Related Protein (sFRP) family, the members of which

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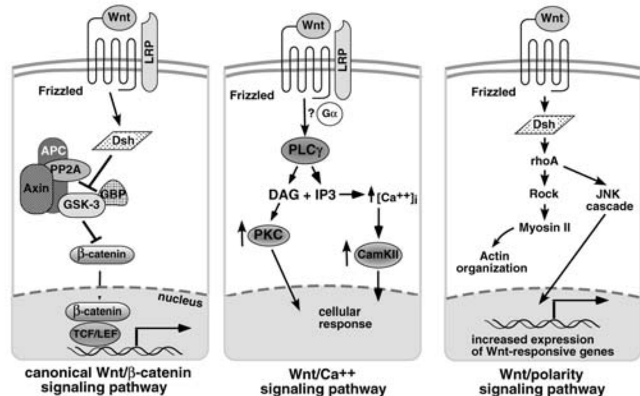
A. Wnt signal transduction pathways



B. Wnt signal transduction pathways and cardiogenic induction

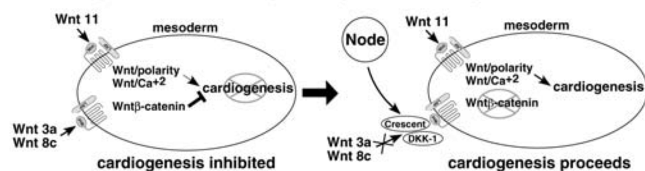


Figure 4. (A) *Wnt* signal transduction pathways. Activation of the canonical Wnt/β-catenin signaling pathway. In the absence of Wnt signal, β-catenin is phosphorylated, leading to its ubiquitination and destruction by the proteasome (not shown). Binding of *Wnt* to its receptor, Frizzled, and its co-receptor, the LRP, prevents the degradation of β-catenin that then transits to the nucleus where it activates target genes. Signaling through the Wnt/Ca^{+2} pathway appears to involve G-protein activation, whereas signaling through the Wnt/polarity pathway, like the canonical pathway, involves Dsh; but in this case, the signal is directed to activation of rhoA and transmitted via rhoA kinase (*Rock*). Modified from Miller (Ref. 54; used with permission of Biomed Central). B. *Wnt* signal transduction pathways and cardiogenic induction. Activation of the Wntβ-catenin pathway prevents complete cardiogenic induction of mesodermal cells. Inhibition of this pathway by *Crescent* and/or *DKK-1* secreted by the node relieves this inhibition and allows for full cardiogenic induction.

appear to bind directly to Wnts and modulate their activity in a context-dependent manner (63).

Heart Tube Formation and Looping

Linear Heart Tube Formation. Soon after cardiac crescent formation, the flat embryonic disc begins to fold in conjunction with the growth of the cranial neural tube. This folding channels migrating endocardial cells from both sides of the embryo into the developing neck region to form a lumen within the pericardial cavity (64). The endocardial cells then are surrounded by myocardial cells to form a bilaterally symmetric heart tube centrally positioned within the embryo. During this time, cells from the anterior heart-forming field migrate into the cardiac region to populate the cranial pole of the heart tube that will eventually form the outflow tract and the primordium of the right ventricle (65). With this migration, the heart tube becomes progressively more defined into morphologically distinct anterior and posterior regions that eventually give rise to the ventricular and atrial compartments of the developed heart, respectively (see Fig. 1 in Ref. 66). This functional regionalization of the heart tube originates earlier in the HFRs (67) such that an HFR cell with a given anteroposterior position will maintain that same position in the heart tube and contribute to either the atrial or the ventricular chamber (68).

Formation of Left-Right Asymmetry in the Embryo and Its Role in Heart Looping and Chamber Specification

The next step in heart formation is to convert the anterior/posterior (ventricular/atrial) organization of the linear heart tube into a primitive heart with two atrial and two ventricular chambers arranged in a left-right (L/R) orientation. This conversion is critical to normal heart chamber formation and begins with a rightward looping out of the heart tube that realigns the future ventricles into an L/R juxtaposition (Fig. 5A). These events imply the presence of a molecular asymmetry within the heart tube that heart looping passively follows. In fact, such an L/R asymmetry exists, but it appears to be a property of the embryo itself imposing an asymmetry on the earliest cardiogenic regions, the two bilateral heart fields within the lateral plate mesoderm (LPM) and, in the later event, of orienting heart tube looping (67, 69, 70). In this way, the signal transduction events that determine the L/R embryonic axis can be viewed as comprising the first steps in left-to-right heart tube looping and L/R heart chamber determination. For simplicity's sake, the process can be divided into four steps (71): the initial breaking of L/R symmetry in or near the node, which takes place at the late neural fold stage; transfer of L/R biased signals from the node to the LPM; L/R asymmetric expression of signaling molecules, such as the TGF-β–related molecules *Nodal* and *Lefty,* in the LPM on the left side of the embryo; and L/R asymmetric morphogenesis of organs that are induced by these signaling molecules (2, 71–73).

The ultimate goal of breaking the L/R symmetry in or near the node is to establish an asymmetric expression pattern of the signaling molecule *Nodal* in the lateral plate mesoderm immediately adjacent to the node. Depending on the organism under study, this appears to be achieved by either physical or genetic means. The deposition of left-side determinants left of the node could result from a leftward flow of perinodal extra-embryonic fluid propelled by the movement of cell cilia that "sweep" left-side determinants toward the left side of the embryo (71, 74, 75). These same determinants also could migrate *via* intercellular gap junctions that are asymmetrically distributed in node and perinodal cells (71). A third way of initiating asymmetry, based on genetic evidence, entails interactions between local signaling molecules to set up a perinodal asymmetric L/R expression domain that imparts laterality information to the node. Much of this work has been carried out in chicken embryos, and we present it here as a means of introducing the various signaling pathways involved, with the caveat

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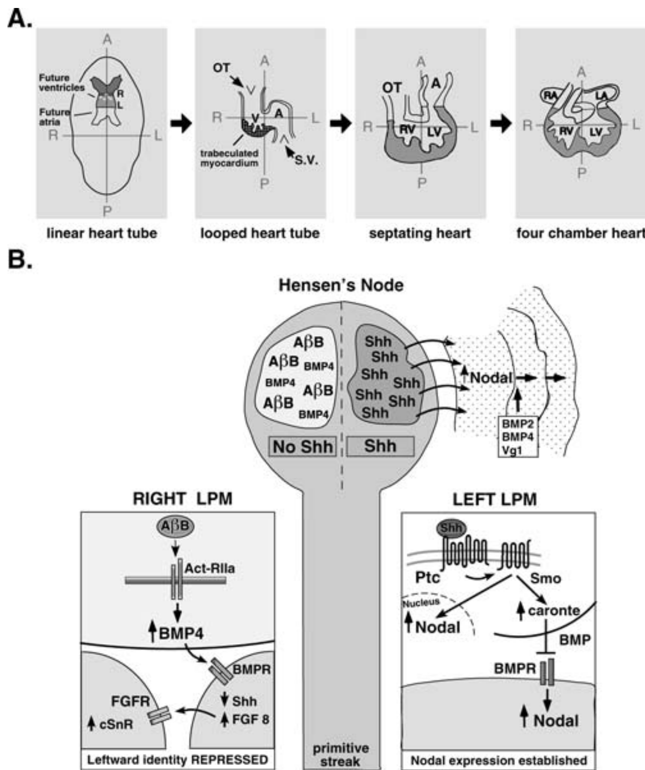

Figure 5. (A) Heart tube looping and the L/R embryonic axis. Chamber precursors aligned in the anteroposterior orientation in the linear heart tube are brought into the appropriate left-right juxtaposition for septation and establishment of the left and right ventricular chambers by looping of the heart tube. (B) Network of interacting signal transduction pathways that institute a left-right asymmetry within the chick embryo. Left/right asymmetry is initiated by asymmetric expression of activinβB (AβB) within Hensen's node. AβB inhibits Shh expression in the right portion of the node, allowing its expression in the left portion, where it diffuses into the adjacent LPM and induces *Nodal* expression either directly or *via caronte*, an antagonist of BMP action. Certain BMPs, such as BMP-2 and BMP-4, and Vg1 maintain Nodal expression in the LPM as its expression domain expands with time. ActivinβB also acts to prevent establishment of a leftward identity in the right LPM by imposing a "Nodal-free zone" by increasing BMP-4 and FGF8 signaling to inhibit *Shh* and activate cSnR.

that certain aspects of this model appear to differ from those in other species (discussed below).

In chicken embryos, the first molecular indications of asymmetry come from the asymmetric expression of the signaling molecule ActivinβB in Hensen's node (76). ActivinβB induces cells on the right side of the node to express BMP-4 (77). BMP-4 antagonizes Shh activity in these cells, thereby restricting *Shh* activity to the left portion of Hensen's node (Fig. 5B). BMP-4 further reinforces this asymmetry by inducing expression of FGF-8 to signal cells on the right side of the embryo to express *cSnR*, a zinc finger protein of the Snail transcription family that is a repressor of leftward identity (2). Once the leftward expression of *Shh* is established, it induces expression of *Nodal*, itself a signaling molecule, in the LPM immediately adjacent to Hensen's node on the left side of the embryo (Fig. 5B). It is unclear whether *Shh* directly induces *Nodal* or does so indirectly by inducing an intermediary molecule called *Caronte*, a BMP antagonist found in chicken (73, 74). As development proceeds, *Nodal* activity assumes an increasingly larger expression domain in the left LPM, which is attributable to a positive feedback loop in which Nodal signaling through its receptor induces expression of more Nodal protein. Maintaining an active *Nodal* receptor appears necessary for this, and recent evidence implicates BMP 2 or 4 and possibly VG1, another member of the TGF-β superfamily (73, 78, 79), in the maintenance of an active *Nodal* receptor complex through expression of the *Nodal* co-receptor *Cryptic*, an EGF-CFC protein (Fig. 6A).

Asymmetric *Nodal* expression is so vital to establishing the L/R laterality that directs normal heart and body morphogenesis that in addition to positive feedback loops, other "autocatalytic-type" mechanisms for maintaining asymmetric *Nodal* expression may be at work in the chicken blastula. On a speculative note, this could explain how *Nodal* expression can be upregulated and maintained not only by the activation of BMP pathways (78, 79), but also by their inactivation by inhibitors such as *Caronte* (80, 81). Unlike *Caronte* and *Shh*, BMPs, which can induce *Nodal* (73, 78, 79), are expressed bilaterally in the chicken blastula (79), a situation that would lead to bilateral *Nodal* expression and organ isomerism. To avoid this, *Nodal* expression must be prevented in the right and established in the left LPM (Fig. 5B, lower left panel). To allow *Shh* to induce expression of *Nodal* in the left LPM, BMPs, which antagonize *Shh* activity (77), must themselves be antagonized. This function is likely to be carried out by *Caronte*, presumably during a transitory yet critical period early in the establishment of L/R laterality. Once established, the leftward *Nodal* expression domain then could be maintained by BMPs (assuming the decay of *Caronte* activity) until "leftwardness" is irrevocably established *via* gene expression programs under the control of *Pitx2* and other transcription factors (Fig. 6). The presumptive transitory nature of both *Shh* and *Caronte* expression as well as differences in avian *versus* mammalian gastrulation (74) could explain why these two important factors in chicken L/R laterality have not yet been found to be similarly expressed in mammals. Alternatively, it is completely possible that ensuring asymmetric *Nodal* expression and L/R laterality in mammals relies on other molecules or mechanisms (79, 82).

L/R asymmetry in *Nodal* expression is achieved not only by the maintenance of its expression in the left LPM, but also by its prevention in the right LPM (Fig. 5B). Since *Nodal* activity is diffusible, similar boundaries also must be imposed on the dispersal of its activity. Cells in the LPM and along the midline of the embryo express *Nodal* receptors (CFC-expressing cells), making them responsive to Nodal and its ability to upregulate its own expression. Left unregulated, this would lead to expansion of *Nodal* signaling beyond its normal confines in the left LPM and also into the right side of the embryo, thereby disrupting the

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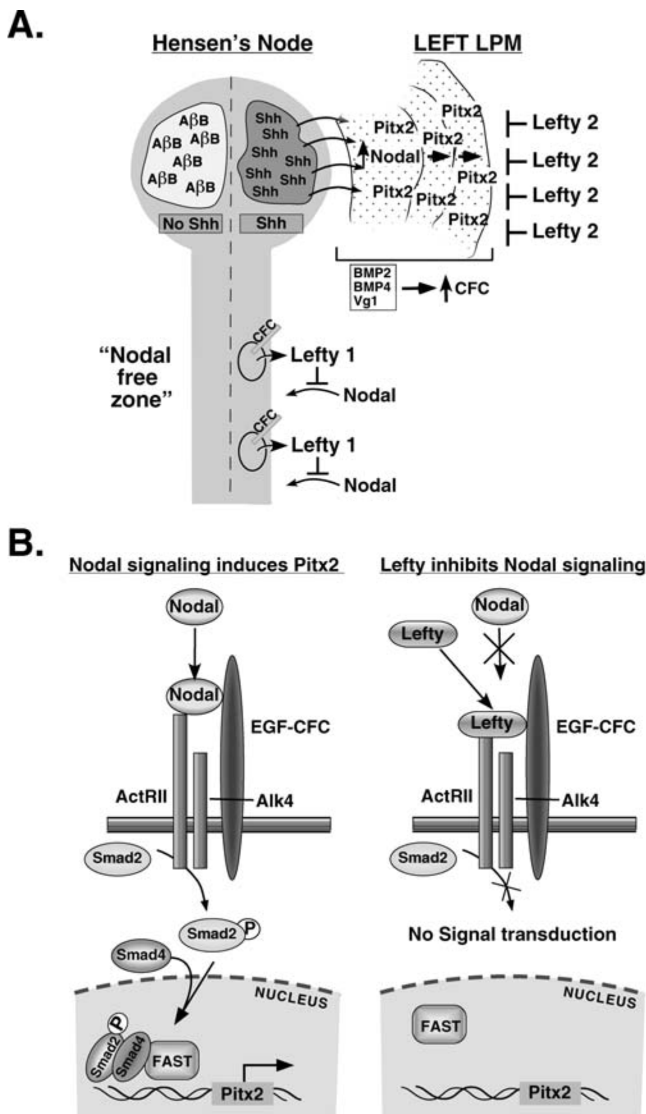

Figure 6. Actions of Nodal and the Lefty proteins in establishing L/R laterality. (A) *Shh*-induced Nodal expression in the left LPM leads to expression of the homedomain protein Pitx2, which establishes a leftward identity in the LPM. BMP-2, BMP-4, and Vg1 act to maintain and expand *Nodal*'s activity by increasing expression of CFC, a *Nodal* co-receptor. Continued expansion of *Nodal* activity is restricted by a *Nodal* antagonist, Lefty2. Nodal also induces expression of another antagonist, Lefty1, in CFC-expressing midline cells to prevent expansion of *Nodal* activity into the right side of the embryo. (B) *Nodal* signaling *via* the ActRII/Alk4 heterodimer receptor and EGF-CFC co-receptor complex activates smad transcription factors to enter the nucleus, bind the *FAST* transcription factor, and upregulate *Pitx2* gene transcription. Lefty proteins act as antagonists of *Nodal* by competing with Nodal for binding to the ActR 11 receptor. Adapted from Hamada et al. (Ref. 71; used with permission of Nature Publishing Group).

asymmetry in *Nodal* expression that is required for establishing L/R laterality. Interestingly, *Nodal* itself prevents this from happening by inducing or maintaining expression of two other genes in these regions, *Lefty*1 and *Lefty*2, two TGF-β–like signaling molecules that are functional antagonists of *Nodal* signaling (Fig. 6B; Ref. 71). Despite their names, these molecules act to inhibit *Nodal* activity. *Lefty*1 is expressed in CFC-expressing midline cells, where it appears to act as a barrier preventing *Nodal* signaling from transgressing into the right side of the embryo (83), whereas *Lefty*2 is expressed in CFC-expressing cells within the left LPM and acts to prevent further spread of the *Nodal* signal and to limit the duration of *Nodal* activity (84). In those regions of the left LPM expressing little or no *Lefty* protein, *Nodal* signaling can proceed unabated to impart "leftwardness" on the left LPM, primarily *via* upregulating expression of *Pitx2*, a homeobox transcription factor responsible for generating left-side morphology of at least some of the visceral organs (Fig. 6B; Refs. 71, 72, 74, 85, 86).

Perhaps the most convincing experiments demonstrating a link between heart looping and L/R laterality involve disruption of this laterality both by changing the normal leftward expression pattern of *Nodal* within the LPM to a bilateral one (69, 87, 88) and by functionally ablating *SnR* (89). In both cases, heart looping became randomized (i.e., the heart tube looped sometimes to the left and sometimes to the right). Interpretation of these results has led to a model for heart tube looping in which a default state of random asymmetry generated by *Shh*, *Nodal*, and *SnR* can be made to establish the correct left-to-right laterality by receiving an initial biasing "push" leftwards, instigated perhaps by an initial asymmetry in activin signaling in the node (Figs. 5 and 6; Ref. 70).

While this model for establishing *Nodal* asymmetry has relied heavily on studies in chicken, differences do exist between how L/R laterality might be achieved in chicken *versus* other species (71, 74, 90). These differences center mainly on the mechanisms proposed for the initial breaking of symmetry in the node and split into two alternative models: a physical one that relies on the leftward accrual of L/R determinants by nodal flow and accounts for asymmetry in the mouse, and a genetic one that relies on asymmetric expression of a "primal" signaling molecule, such as ActivinβB, and accounts for asymmetry in chickens. While these models no doubt reflect differences in how birds and mammals gastrulate (74), the nodal flow model in mammals is, in some ways, more intriguing, because the mechanism for initiating a directional bias is known. This mechanism is the leftward sweeping motion of cilia. Genetic mutations in molecules that generate the vortical motion of nodal cilia give rise to *situs inversus*, the complete mirror image reversal of organ asymmetry (reviewed in Ref. 74), thus implicating cilia in the establishment of L/R laterality. The most recent nodal flow model suggests that the rotation of cilia on the surface of nodal cells causes a leftward flow of extracellular fluid that sweeps vesicular "parcels" containing signaling/inductive molecules to the leftward periphery of the node. These particles trigger mechano-sensory cilia in peripheral node cells to elevate calcium levels and increase calcium-dependent signal transduction to induce genes (e.g., *Lefty* and *Nodal*) that impart "leftness" to the left LPM (75, 91). While ample evidence supports the involvement of

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cilia-generated nodal flow in determination of L/R laterality, the nature of the downstream signal transduction pathways that mediate this remain an area of active research.

L/R Asymmetry in Heart Positioning and Chamber Specification

While much of early heart development and L/R chamber specification depends on the interpretation of the L/R laterality information set up in the embryo, different regions of the heart appear to interpret this information in different ways and at different times. For example, the formation of left and right atria appears to reflect differences in the left and right progenitor pools of the LPM, suggesting early establishment of L/R asymmetry that affects atrial progenitor cell behavior (Fig. 1A; Ref. 92). Ventricles, on the other hand, are initially specified along the A/P axis and later become oriented along the L/R axis by virtue of heart tube looping (Fig. 5A; Ref. 93). Even though A/P patterning may predominate in the linear heart tube, it is apparent that the initial bending of the heart tube occurs in accordance with the L/R asymmetry initially set up in the embryo when the caudal heart tube undergoes a leftward shift prior to looping (70, 94).

It is clear from genetic studies that the L/R laterality information residing in the early embryo can influence heart development at two levels: the situation of the heart within the embryo (i.e., its ultimate placement within the left of the adult chest cavity), and the formation of L/R chambers within the heart. Failure to establish the L/R axial polarity upon which organ primordia take their spatial cues can often result in the inverted deposition of organs, referred to as *situs inversus* or heterotaxia. For example, when genes controlling *Nodal* expression are ablated, affected embryos exhibit defects in heart looping and heart structure (e.g., right isomerism of atria; Refs. 83, 95, 96) as well as random positioning of the heart within the chest (97). And in the well-characterized mouse laterality mutant *situs inversus viscerum* (*Iv*), a mutation in the early steps of the laterality pathway results in a dramatic scrambling of *Nodal* expression (98). These results show that the *Nodal* signaling pathway is clearly involved in positioning of the heart. *Nodal* and *Lefty* may also contribute to establishing L/R chamber identity within the heart independently of their role in directing right-to-left heart tube looping. For example, the domain of *Nodal* and *Lefty* expression in the left LPM includes the left caudal precursors of the heart tube, an expression domain consistent with these signaling molecules' participation in setting up caudal heart asymmetries (i.e., determining left and right ventricular identities). One indication that these leftward determinants play a role in L/R ventricular patterning comes from analysis of *Pitx2* gene expression. In *Lefty* mutant mice, *Pitx2* is expressed bilaterally in the LPM leading to left atrial isomerism and formation of a double-outlet right ventricle (83). Thus, in addition to atrial L/R patterning, LPM leftward determinants, such as *Nodal*, *Lefty* and *Pitx2,* also can play a direct role in ventricular patterning. Together, these results show that establishment of an asymmetric biologic difference or polarity along the L/R embryonic axis is critical to the formation of heart chambers and also to the position of the heart within the body (74). Signaling molecules critical to this process, such as BMPs, activin, and FGFs, have already been discussed. The signaling pathways for the two other critical determinants of L/R laterality, *Shh* and *Nodal*, are detailed below.

Hedgehogs

Background. There are three *Hedgehog* (*Hh*) genes in vertebrates: Sonic Hedgehog (*Shh*), Indian Hedgehog (*Ihh*), and Desert Hedgehog (*Dhh*). Of these, *Shh* has the most widespread biologic activity and is the most studied. Hedgehog proteins are secreted proteins that function in short-range signaling to neighboring cells (i.e., on the order of around a few dozen or so cell diameters; Ref. 99). This property results from a type of posttranslational modification that appears unique among most signaling molecules (Fig. 7A). Once synthesized, Hh undergoes an internal autoproteolytic cleavage into separate N-terminal (ShhNp) and C-terminal (ShhC) peptides, followed by the covalent addition of lipid molecules to the N-terminal peptide (101, 102). The N-terminal peptide is the functional signaling molecule, whereas the C-terminal peptide acts as an intramolecular cholesterol transferase; as such, it has no real signaling activity (103). The proteolytic processing and lipid modification of ShhNp facilitates both short-range and long-range signaling by Hh, the former *via* tight association of Hh with the producing cell to create a steep, high-to-low Hh concentration gradient, and the latter by increasing the "diffusibility" of Hh *via* its multimerization (99, 100). Studies in Drosophila have shown how cells can interpret differential Hh levels to produce either the activator or repressor form of the *Hh*-responsive *Cubitus interruptus* (*Ci*) transcription factor: in the absence of Hh, Ci is cleaved to give a transcriptional repressor peptide termed CiR, which inhibits *Hh*-responsive gene expression. With high concentrations of Hh this cleavage is prevented, and cells produce the activator form of Ci that promotes gene expression (Fig. 7B). With low concentrations of Hh, cells do not accumulate either form of Ci, and gene activation occurs by other non-*Hh* pathways (99). Vertebrates express two homologs of *Ci*, *Gli*2 and *Gli*3, each with transcriptional activation and repression domains as well as sequences required for proteolysis (104). This suggests that vertebrate cells may respond to Hh gradients in much the same way as Drosophila cells do. The concentration-dependent activation of genes, many of which are transcription factors important in specifying cell type, is a critical feature of the ability of *Hh* to pattern complex tissues (105).

Role in Cardiogenesis. As discussed, *Hh* plays a

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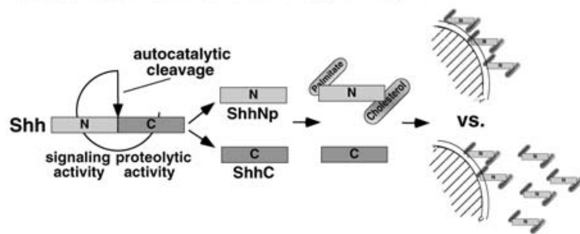

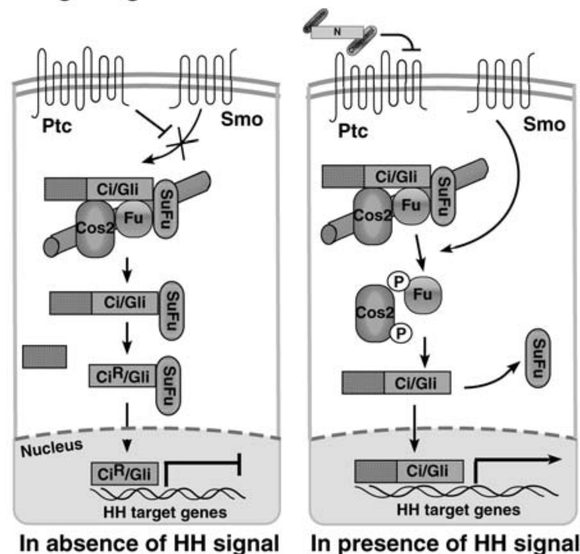

Figure 7. *Hedgehog* (*Hh*) signal transduction pathway. (A) The proteolytic activity of the C-terminal of Shh cleaves it into N- and C-terminal products. Covalent linkage of ShhNp to cholesterol and palmitate increases its hydrophobicity, which improves either binding to the cell surface for short-range signaling or diffusion to act as a long-range signal (100). (B) In the absence of *Shh*, Ptc inhibits Smo, allowing the complex of Fused (Fu), Costal2 (Cos2), and Suppressor of Fused (SuFu) to sequester the transcription factor Gli in a complex bound to microtubules. Sequestered Gli is cleaved to release a fragment that suppresses *Hh* target genes. Binding of Shh to Ptc nullifies its inhibition of Smo. Active *Smo* signals through unknown mechanisms to the Fu-Cos2-SuFu complex, where phosphorylation of Fu and Cos2 disrupts the complex releasing full-length Gli, which then transits to the nucleus to activate transcription of *Hh* target genes. Adapted from Bijlsma et al. (Ref. 101; used with permission of Wiley).

major role in establishing the L/R embryonic axis, and in this way it is critical to heart tube looping. However, *Hh* signaling also appears to be involved in the earlier event of specifying mesodermal cells to become cardiogenic. Studies of mice mutant for *Hh* signal transduction show a delay in *Nkx2.5* expression and delayed heart development (106), suggesting the involvement of *Hh* in cardiac induction. Further support for this comes from analysis of *Ptc*, the *Hh* receptor. *Hh* signaling invariably involves upregulation of *Ptc*, such that increased *Ptc* expression is a hallmark of *Hh* signaling. In normal mice, increased *Ptc* expression is seen in the yolk sac mesoderm and node periphery, suggesting active *Hh* signaling during this time in development. In addition to these *in vivo* studies, *in vitro* studies have shown that when *Shh* is introduced into P19 cells, a line of embryonic stem cells, these cells will undergo cardiogenesis upon the formation of three-dimensional cell aggregates (107). Taken together, these studies suggest that in addition to L/R heart tube looping, *Hh* signaling plays an important role in specifying mesodermal cells to enter the cardiac muscle cell lineage.

The *Hh* Signal Transduction Pathway. *Hh* signaling can be viewed as the transition between two states: one a basal state of transcriptional repression that occurs in the absence of Hh, and the other a state of transcriptional de-repression and gene activation that is initiated by Hh binding to its receptor, *Patched* (*Ptc*). The transition between these two transcriptional states is mediated by a molecular "gatekeeper" of sorts called *Smoothened* (*Smo*), another transmembrane protein that controls the accessibility of the *Gli* family of transcription factors to *Hh*-responsive gene promoters (Fig. 7C). In the basal, repressed transcription state (i.e., absence of Hh ligand), the unoccupied *Hh* receptor, Ptc, inhibits the activity of Smo, thereby allowing formation of a multiprotein complex that prevents access of *Gli* transcription factors to *Hh*-controlled genes. This complex consists of at least three proteins: the kinase Fused protein (Fu), the kinesin motor protein Costal2 (Cos2), and the Suppressor of Fused (SuFu), an antagonist of *Hh* signaling. These proteins tightly bind Gli proteins and sequester them into a complex with cytosolic microtubules that facilitates cleavage of Gli into two fragments, one of which contains the zinc-finger DNA-binding domain minus any transcriptional activation domains. This fragment translocates to the nucleus and binds to sites within the promoter of *Hh*-controlled genes, effectively preventing functional full-length Gli proteins from binding and activating transcription. This state of transcriptional repression is relieved with the binding of *Hh* ligand to *Ptc*. The Hh-Ptc ligand-receptor complex is brought into the cell by endocytosis and degraded by the lysosomal pathway. Destruction of Ptc relieves the inhibition of Smo, which then activates the Hh pathway by promoting release of the Fu-Cos2-SuFu-Gli complex from microtubules and freeing Gli proteins from proteolytic cleavage. Free, full-length Gli translocates to the nucleus, where it binds promoter elements in Hh-responsive genes, activating their transcription and completing the Hh signaling pathway (101).

Nodal

Background. The *Nodal* gene was discovered in mice using retrovirus-based gene mutation methods to generate developmental mutants (108, 109). One such mutant exhibited an inability to induce mesoderm and successfully undergo gastrulation. Further analysis showed the mutated gene, called *Nodal*, to be a novel member of the TGF-β family of signaling molecules. *Nodal* genes were subsequently isolated from other vertebrates and shown to be involved in mesoderm and endoderm formation as well as in the establishment of the embryonic L/R axis (110).

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Unlike many other signaling molecules, *Nodal* genes do not have homologous counterparts in nonvertebrates. Within vertebrates, however, the function of *Nodal* as a mesoderm inducer has been evolutionarily conserved. These observations suggest that the evolution of *Nodal* is very much tied in with the developmental processes of mesoderm and endoderm induction as well as L/R axis determination, since these processes do not take place in nonvertebrates (110).

Role in Cardiogenesis. As discussed, much of early heart development is devoted to interpreting the L/R laterality information setup by a complex network of interacting signal transduction events within the node and LPM. Critical to this is the formation of an L/R asymmetry in *Nodal* gene expression and the extent and duration of *Nodal* signaling in the left LPM, a process controlled by the *Nodal* antagonists, *Lefty*1 and *Lefty*2 (Fig. 6; Ref. 84). The cardiac phenotypes of mice genetically engineered to disrupt *Nodal* signaling and its regulation vividly demonstrate the dependence of heart development on the embryonic L/R axis. For example, mice lacking the ActRIIB (97) or *Cryptic* gene (95, 96) display defects that include right pulmonary isomerism with *Cryptic* mutants additionally showing randomization of cardiac looping, abdominal situs, and vascular heterotaxia. These mutant phenotypes are consistent with a failure to implement a *Nodal*-dependent program of gene expression within the left LPM. Further testing of this model was done with genetically engineered mice bearing *Lefty*2 genes that could not be asymmetrically expressed within the left LPM (84). These mice had heart malformations, such as atrial left isomerism (i.e., two left atrias), a positional isomerism wherein the pulmonary trunk is side by side with the aorta (normally the pulmonary trunk is situated ventral left relative to the aorta), transposition of the great arteries, a single (left) ventricle, and a common atrioventricular canal. In these mutants, *Nodal* signaling was found to be aberrantly prolonged, allowing for diffusion of Nodal into the right LPM and causing the left-sided isomerism apparent in *Lefty*2 mutant hearts.

Three properties of *Lefty*2 might explain its function and role in delimiting *Nodal* signaling and thus prepatterning the mesoderm for eventual heart development. The first is that the *Lefty*2 gene is regulated by enhancer elements similar to those in the *Nodal* promoter, making *Nodal*-*Lefty*2 co-expression likely (111, 112). This would allow for a type of autoregulatory inhibition of *Nodal* signaling when levels of *Lefty*2 increase to a point at which they can prevent binding of *Nodal* to its receptor (113). Second, *Lefty*2 antagonizes *Nodal* signaling in one of two ways: either by binding the EGF-CFC co-receptor *Cryptic* and preventing Nodal from forming an active type1-type II receptor complex (Fig. 6B; Ref. 113) or by directly binding to Nodal and preventing its interaction with its receptor (114). A third property of Lefty2 relevant to its regulation of Nodal is its ability, despite being induced by *Nodal*, to diffuse more readily than Nodal into neighboring cells. This could set up a perimeter of cells "preantagonistic" to *Nodal* activity, nullifying its activity and confining it to the left LPM (115). Together, these properties suggest that *Lefty*2 can delimit *Nodal* signaling temporally and spatially by "outracing" Nodal to cells with unoccupied EGF-CFC receptors, binding to them or to Nodal itself, and precluding *Nodal*-dependent assembly of active receptors.

The *Nodal* Signal Transduction Pathway. *Nodal* is a member of the TGF-β family of signaling molecules. This family is roughly divided into two classes that depend upon through which type I/type II receptor complex a ligand signals (116). *Nodal* is a member of the class of TGF-β–like signaling molecules that use the ALK4 receptor as its type I receptor and the ActR-IIB receptor as its type II receptor. Included in this class of ligands is activin, the ligand whose initial asymmetric expression in the node appears to set in motion the signaling cascades that determine L/R asymmetry. For the most part, all TGF-β and TGF-β–like molecules transduce their signals to the nucleus *via* smad proteins, cytoplasmic effector molecules that translocate to the nucleus where they regulate gene expression. The Nodal/activin ligand class transduces its signal *via* smads 2 and 3 *versus* smads 1, 5, and 8, which are used predominantly by BMP and BMP-like signaling molecules (116). Another defining feature that distinguishes *Nodal*/activin signaling from BMPs is their higher affinity for type II receptors as opposed to BMPs' affinity for type I receptors (116). *Nodal* signaling begins when Nodal ligands dimerize and bind to the type II ActR-IIb receptor (Fig. 6B; Refs. 110, 116, 117). This facilitates recruitment and complexing with the type I ALK4 receptors that are already bound to the co-receptor Cryptic. This co-receptor is a member of the EGF-CFC family of extracellular, GPI-linked proteins that are essential for *Nodal* signaling. Once this trimeric receptor complex is formed, the type II receptor kinase phosphorylates the type I receptor activating its kinase activity. Activated type I receptor then directly phosphorylates Smads 2 and 3, which then translocate into the nucleus. Once in the nucleus, these smads complex with cell type–specific DNA-binding transcriptional activators, such as *FAST1* (or FoxH1 from the *Forkhead* family of activators), and homeobox transcription factors, such as *Mixer,* to turn on *Nodal*-dependent genes.

Perspectives

Although the signal transduction pathways used by cells to communicate have been well elucidated, understanding how they interact to drive mesodermal cells into the cardiogenic lineage and these cells into a rudimentary heart has proven to be a more difficult question to answer. The delineation of two different heart fields with cells that contribute to different parts of the heart has shown cardiac progenitors to be more defined and diverse than previously believed. It also has raised questions as to whether these different heart fields are functionally distinct or reflect an early patterning or parsing of a single field into cell cohorts that map to different functional units of the heart (13, 118,

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119). Mapping these cell cohorts using more functionally relevant markers, such as cell-specific transcription factors, might reveal a greater variety of cardiac progenitors within the early heart field(s), indicating the need for a more complex network of signaling pathways than that already provided by the FGF and BMP pathways. Counter to this is the situation of heart tube looping and L/R laterality, where despite the involvement of numerous signal transduction pathways, a clear, unified model has yet to emerge. This is likely to reflect the inherent complexity in the processes of looping and laterality as well as species differences. While it might be unwise to "meld" the results from different species into a (misleading) model for the sake of having a model, certain aspects demand more investigation. In this regard, it might be advantageous to determine whether the nodal flow model can be extended to other species, such as birds, and if some aspects of avian L/R laterality can provide insights into how nodal flow in mammals might set up the initiating nodal asymmetry. Clearly, these and other questions will continue to provide interesting insights into early heart development for the foreseeable future.

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Michael Wagner and M. A. Q. Siddiqui

Experimental Biology and Medicine 2007, 232:866-880.

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MINIREVIEW

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

MICHAEL WAGNER[1] AND M. A. Q. SIDDIQUI[1]

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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

Key words: ventricular specification; trabeculation; endocardial cushions; retinoic acid; neuregulins; vascular endothelial growth factor; *Notch*

This work was supported by National Institutes of Health grant HL073399 to M.A.Q.S.

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1535-3702/07/2327-0866$15.00
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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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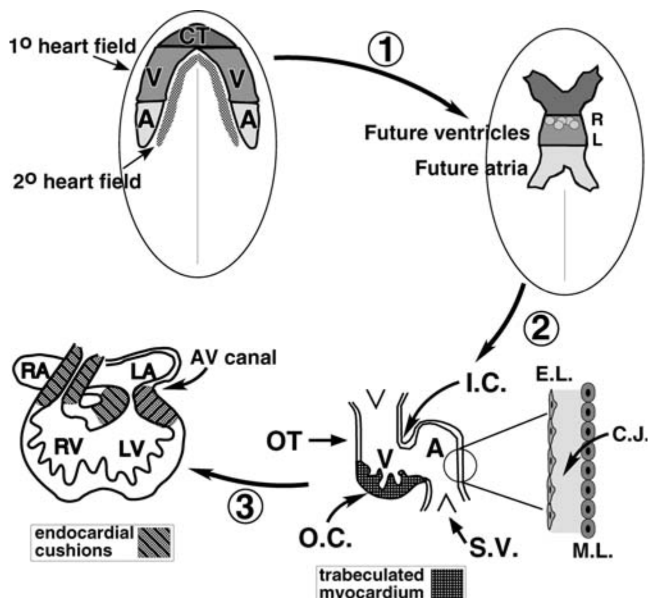

Figure 1. Early heart development and ventricle formation. Ventricular precursors from the primary and secondary heart fields migrate medially and coalesce to form the linear heart tube (1). The anteroposterior organization of these precursors is maintained in the heart tube, with right ventricular precursors (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

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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

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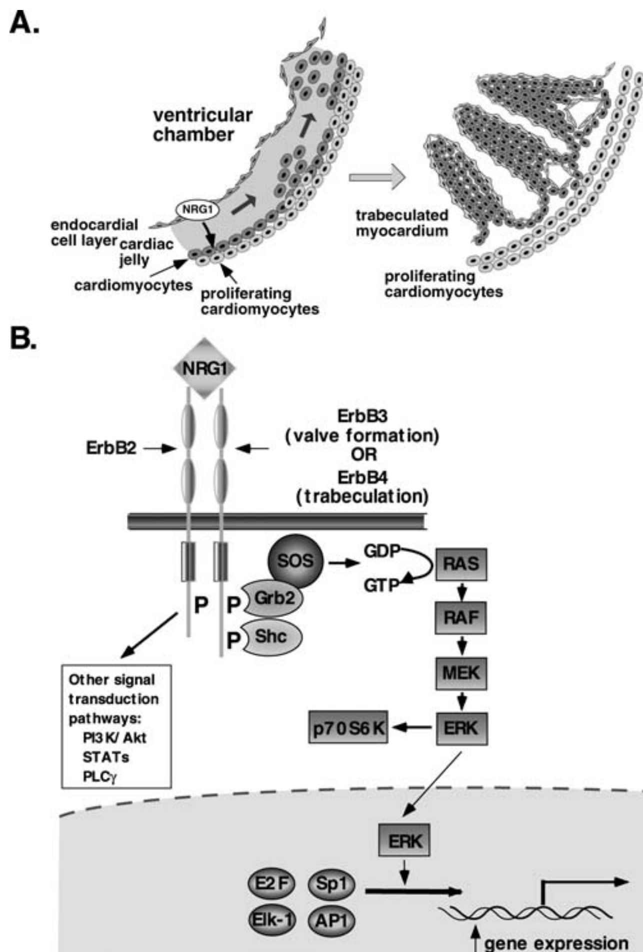

Figure 2. Trabeculation and NRG1 signaling *via* the ErbB signaling pathway. (A) NRG1 from the endocardium signals myocardial cells to proliferate and populate the emerging trabeculae with differentiated cardiomyocytes. (B) Ligand binding induces ErbB2 and ErbB4 receptors to dimerize (for myocardial 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

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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.

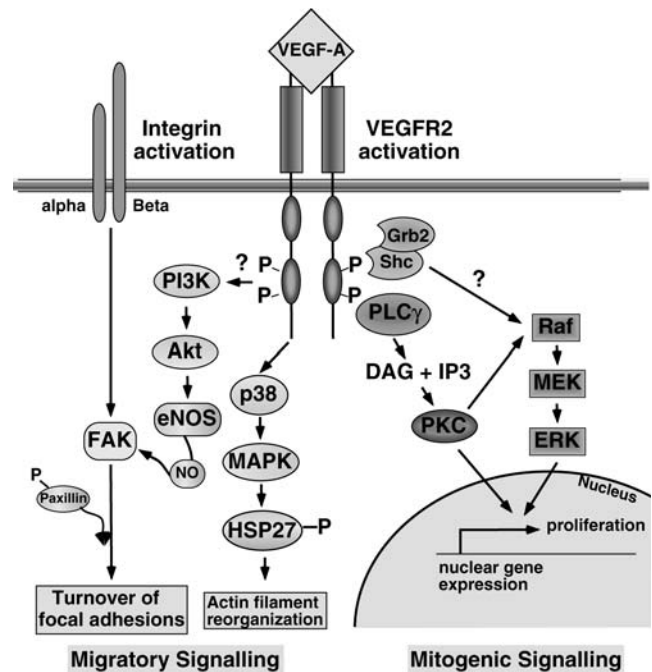

Figure 3. VEGF signaling pathways. Ligand-induced activation of VEGFR2 leads to phosphorylation of tyrosines within the VEGFR2 intracellular domain that act as binding domains for SH2 domain–containing adaptor proteins (Grb2, Shc, PLCγ). The VEGF signal is rendered mitogenic by the Shc/Grb2 and PLCγ 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

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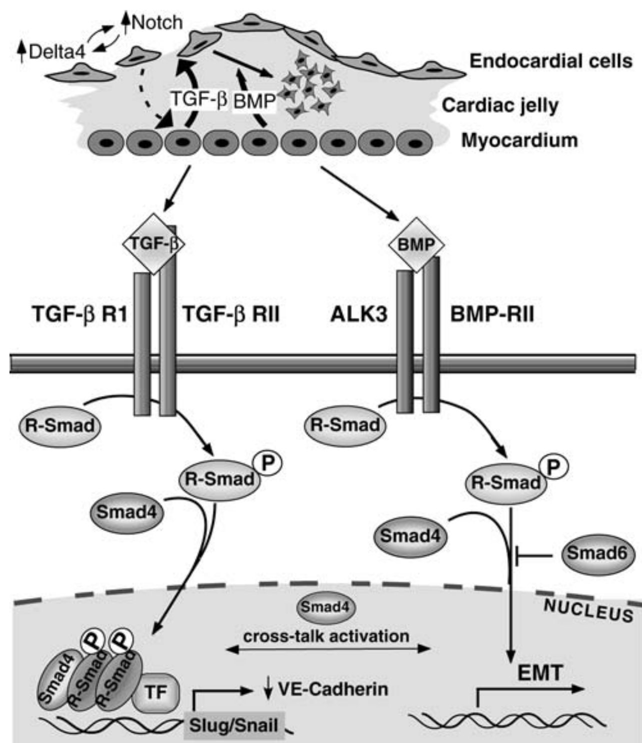

Figure 4. *Notch*, TGF-β, and BMP activity in endocardial cushion formation. High levels of Notch activate neighboring endocardial cells to produce Delta4 by a lateral induction mechanism that leads to delamination from the endocardial layer. Myocardial cells express TGF-β to initiate EMT, and BMP to 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

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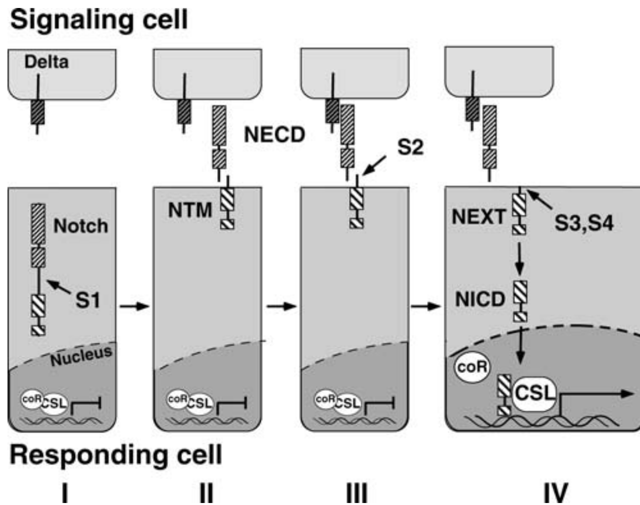

Figure 5. A model for *Delta*-dependent *Notch* signaling to the nuclear transcription factor CSL. I. Preactivation state. Cleavage of *Notch* at S1 forms NTM and NECD. II. Delta at the surface of the signaling cell binds S1-cleaved Notch at the surface of the responding cell. III. Ligand-dependent S2 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).

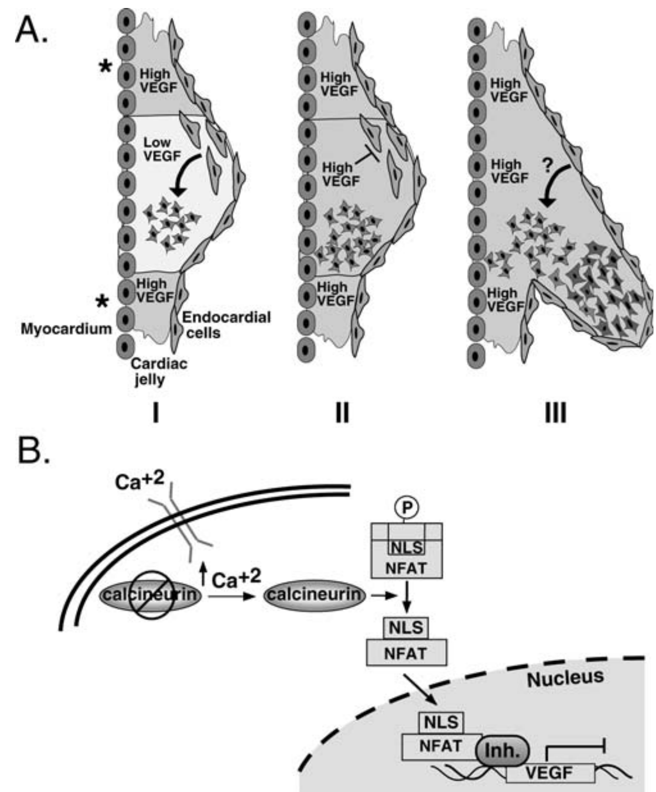

Figure 6. (A) Differential levels of VEGF control EMT in cushion and heart valve formation. Low VEGF expression from myocardial cells permits endocardial EMT (I), whereas high levels terminate EMT (II). High VEGF levels suppress cushion formation outside of cushion-forming regions (*). NFAT nuclear localization 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

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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*

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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

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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

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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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