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.
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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

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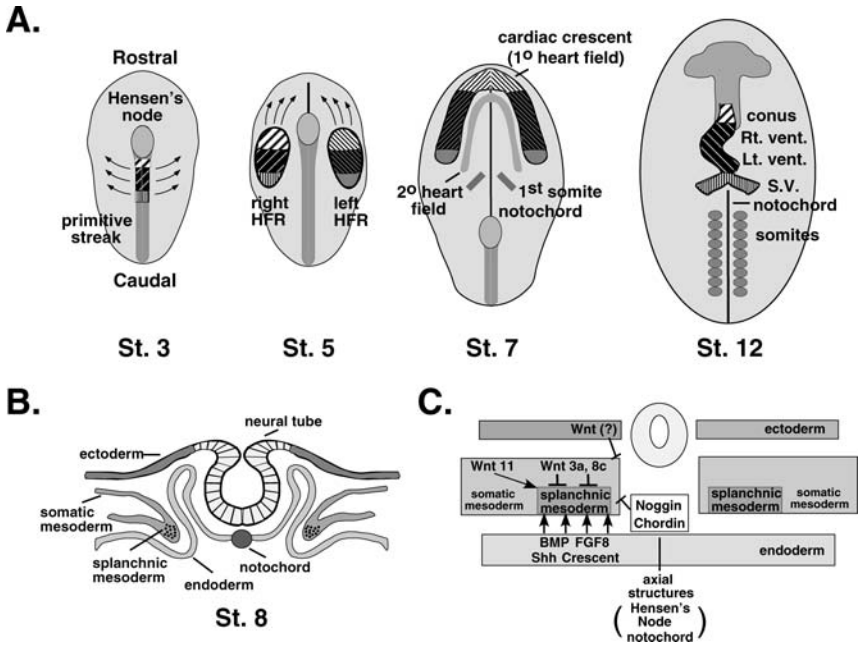

Figure 1. (A) Cardiogenesis during chicken gastrulation. *St.* refers to Hamburger and Hamilton stages. Stage 3: Cardiac progenitor cells caudal to Hensen's node are in the same anteroposterior order as their eventual positions in the tubular heart. Stage 5: Cardiac progenitor cells in the bilateral 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.

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

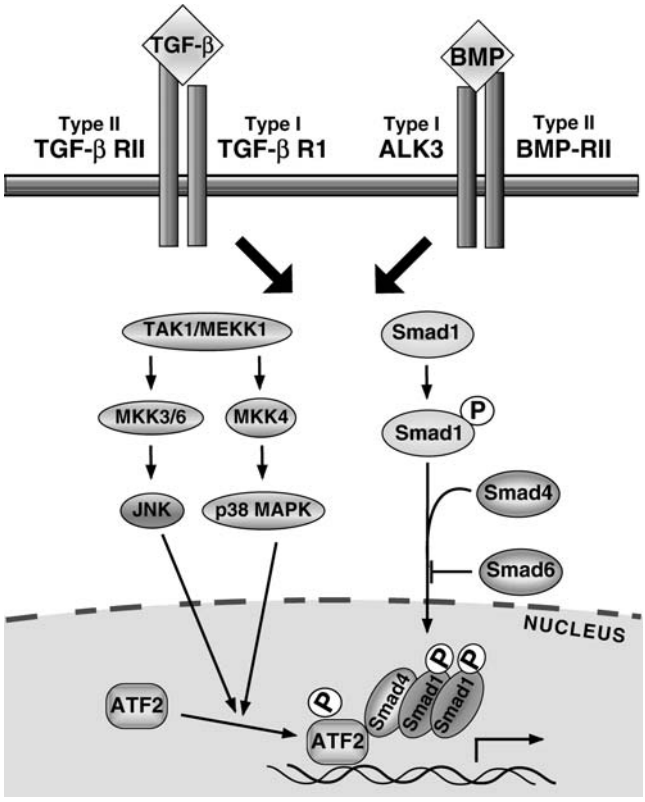

Figure 2. TGF-β and BMP signal transduction pathways involved in cardiogenic induction. The TGF-β or BMP signal can be directed to either the TAKI/MEKK1 or smad pathway and presumably can activate different gene programs through different transcriptional effectors or smad transcription factors. The Smad1/4 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

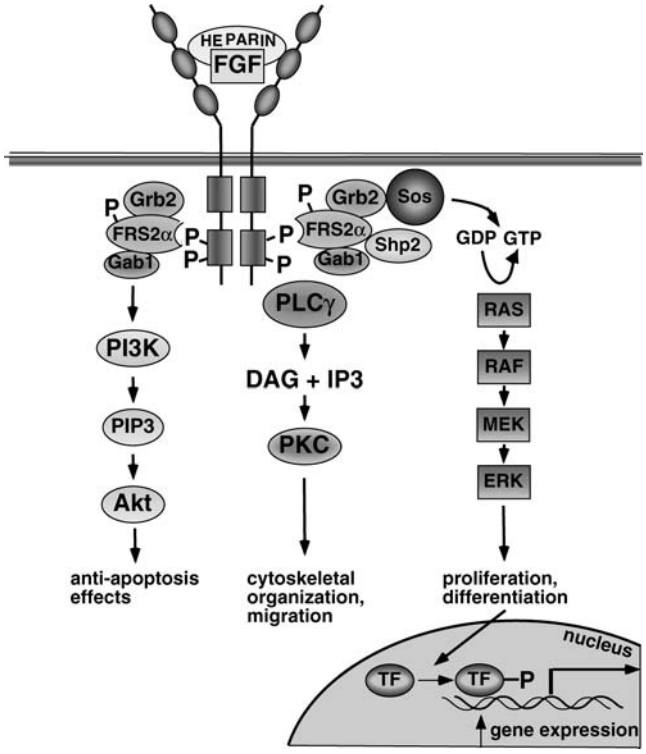

Figure 3. FGF signal transduction pathways. Binding of heparin and FGF to FGF receptors stimulates their phosphorylation and the binding of the FGF receptor docking protein FRS2α. Phosphorylation of FRS2α facilitates recruitment of the Grb2 and Gab1 adaptor proteins, as well as the protein tyrosine phosphatase 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

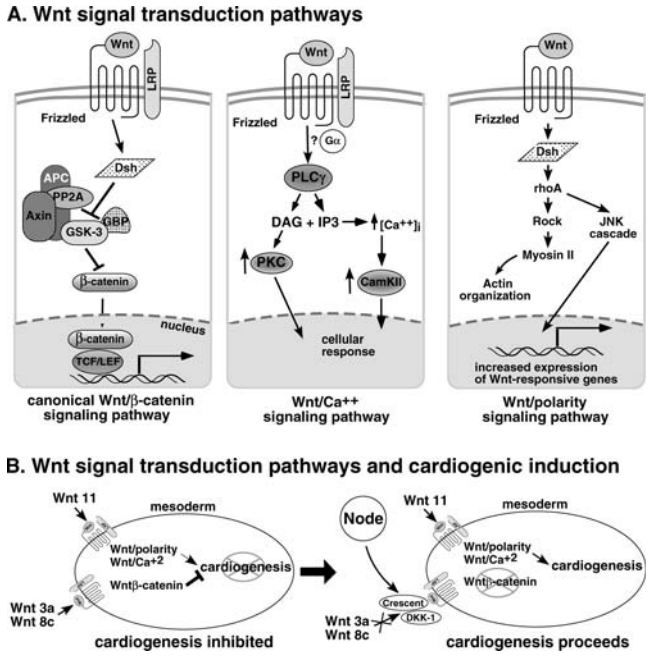

Figure 4. (A) *Wnt* signal transduction pathways. Activation of the canonical Wnt/β-catenin signaling pathway. In the absence of Wnt signal, β-catenin is phosphorylated, leading to its ubiquitination and destruction by the proteasome (not shown). Binding of *Wnt* to its receptor, Frizzled, and its co-receptor, 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

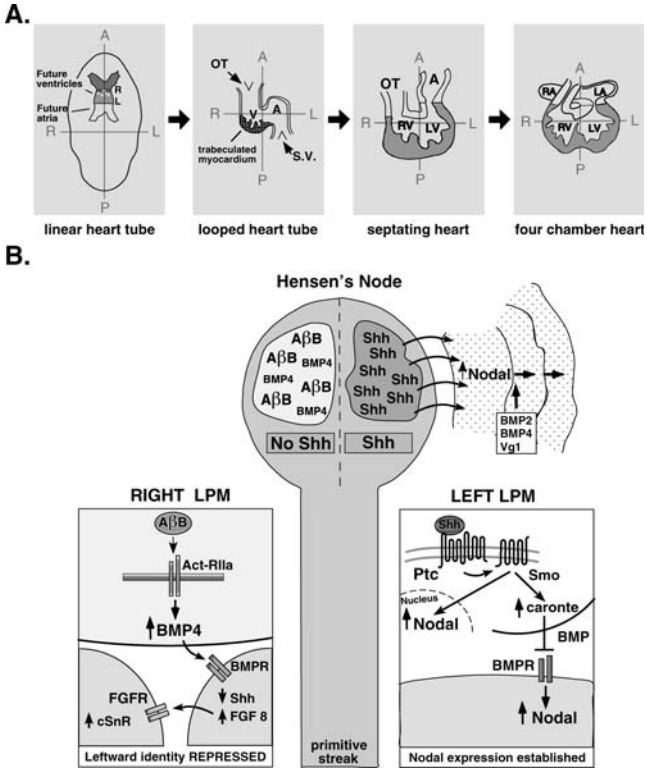

Figure 5. (A) Heart tube looping and the L/R embryonic axis. Chamber precursors aligned in the anteroposterior orientation in the linear heart tube are brought into the appropriate left-right juxtaposition for septation and establishment of the left and right ventricular chambers by looping of the heart 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

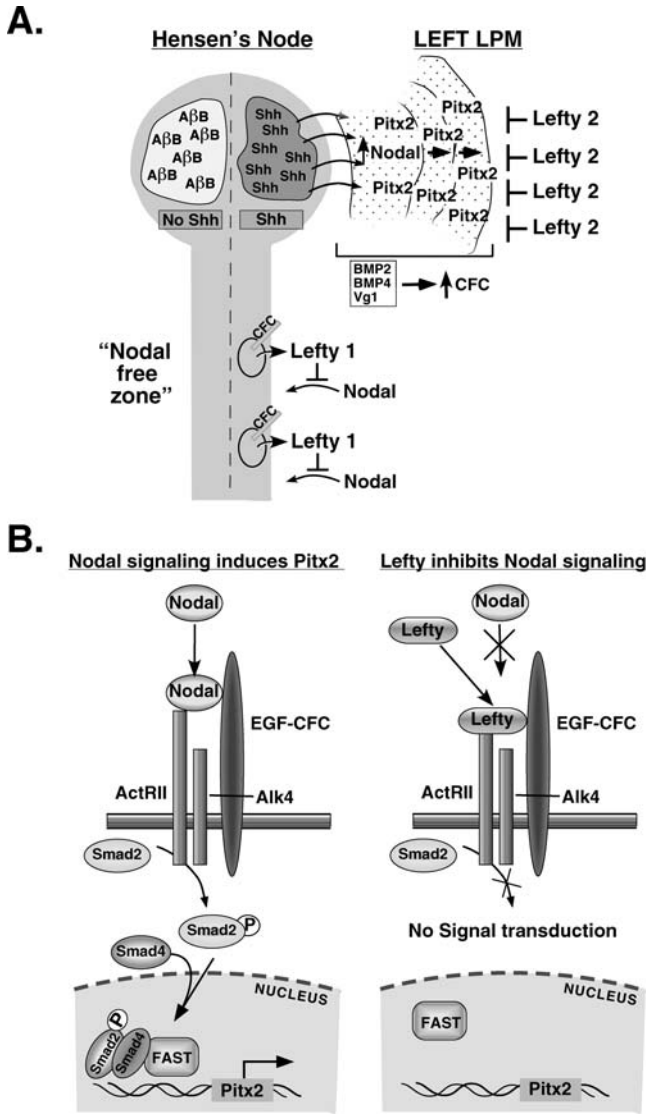

Figure 6. Actions of Nodal and the Lefty proteins in establishing L/R laterality. (A) *Shh*-induced Nodal expression in the left LPM leads to expression of the homedomain protein Pitx2, which establishes a leftward identity in the LPM. BMP-2, BMP-4, and Vg1 act to maintain and expand *Nodal*'s activity 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

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

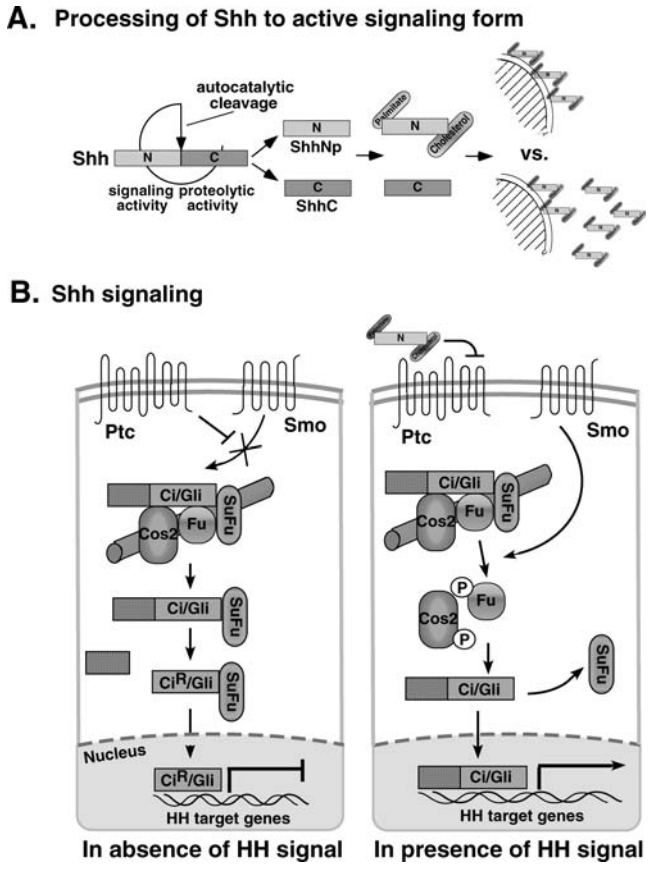

Figure 7. *Hedgehog* (*Hh*) signal transduction pathway. (A) The proteolytic activity of the C-terminal of Shh cleaves it into N- and C-terminal products. Covalent linkage of ShhNp to cholesterol and palmitate increases its hydrophobicity, which improves either binding to the cell surface for short-range 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 derepression 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).

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,

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.

1. Gilbert SF. Developmental Biology. Sunderland, MA: Sinauer Associates, Inc., 2006.
2. Brand T. Heart development: molecular insights into cardiac specification and early morphogenesis. Dev Biol 258:1–19, 2003.
3. Yamada M, Revelli JP, Eichele G, Barron M, Schwartz RJ. Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. Dev Biol 228:95–105, 2000.
4. Alsan BH, Schultheiss TM. Regulation of avian cardiogenesis by Fgf8 signaling. Development 129:1935–1943, 2002.
5. Schneider VA, Mercola M. Wnt antagonism initiates cardiogenesis in Xenopus laevis. Genes Dev 15:304–315, 2001.
6. Eisenberg CA, Eisenberg LM. WNT11 promotes cardiac tissue formation of early mesoderm. Dev Dyn 216:45–58, 1999.
7. Matsui H, Ikeda K, Nakatani K, Sakabe M, Yamagishi T, Nakanishi T, Nakajima Y. Induction of initial cardiomyocyte alpha-actin–smooth muscle alpha-actin–in cultured avian pregastrula epiblast: a role for nodal and BMP antagonist. Dev Dyn 233:1419–1429, 2005.
8. Zimmerman LB, De Jesus-Escobar JM, Harland RM. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599–606, 1996.
9. Schultheiss TM, Burch JB, and Lassar AB. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 11:451–462, 1997.
10. Rones MS, McLaughlin KA, Raffin M, Mercola M. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. Development 127:3865–3876, 2000.
11. Moses KA, DeMayo F, Braun RM, Reecy JL, and Schwartz RJ. Embryonic expression of an Nkx2–5/Cre gene using ROSA26 reporter mice. Genesis 31:176–180, 2001.
12. Laverriere AC, MacNeill C, Mueller C, Poelmann RE, Burch JB, Evans T. GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. J Biol Chem 269:23177–23184, 1994.
13. Abu-Issa R, Waldo K, Kirby ML. Heart fields: one, two or more? Dev Biol 272:281–285, 2004.
14. DeVries P. Evolution of precardiac and splanchnic mesoderm in relationship to the infundibulum and truncus. In: Pexieder T, Ed. Mechanisms of Cardiac Morphogenesis and Teratogenesis. New York: Raven Press, pp31–48, 1981.
15. Waldo KL, Hutson MR, Ward CC, Zdanowicz M, Stadt HA, Kumiski D, Abu-Issa R, Kirby ML. Secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. Dev Biol 281:78–90, 2005.
16. de la Cruz MV, Sanchez Gomez C, Arteaga MM, Arguello C. Experimental study of the development of the truncus and the conus in the chick embryo. J Anat 123:661–686, 1977.
17. Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. Conotruncal myocardium arises from a secondary heart field. Development 128: 3179–3188, 2001.
18. Abu-Issa R, Smyth G, Smoak I, Yamamura K, Meyers EN. Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. Development 129:4613–4625, 2002.
19. Yutzey KE and Kirby ML. Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. Dev Dyn 223:307–320, 2002.
20. Garg V, Yamagishi C, Hu T, Kathiriya IS, Yamagishi H, Srivastava D. Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development. Dev Biol 235:62–73, 2001.
21. Jerome LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. Nat Genet 27:286–291, 2001.
22. Merscher S, Funke B, Epstein JA, Heyer J, Puech A, Lu MM, Xavier RJ, Demay MB, Russell RG, Factor S, Tokooya K, Jore BS, Lopez M, Pandita RK, Lia M, Carrion D, Xu H, Schorle H, Kobler JB, Scambler P, Wynshaw-Boris A, Skoultchi AI, Morrow BE, Kucherlapati R. TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. Cell 104:619–629, 2001.
23. Xu H, Morishima M, Wylie JN, Schwartz RJ, Bruneau BG, Lindsay EA, Baldini A. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. Development 131:3217–3227, 2004.
24. Schultheiss TM, Xydas S, Lassar AB. Induction of avian cardiac myogenesis by anterior endoderm. Development 121:4203–4214, 1995.
25. Jiang Y, Tarzami S, Burch JB, Evans T. Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. Dev Genet 22:263–277, 1998.
26. Reddi AH. Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens. Cytokine Growth Factor Rev 8:11–20, 1997.
27. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 22:233–241, 2004.
28. Kawabata M, Imamura T, Miyazono K. Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev 9:49–61, 1998.
29. Ladd AN, Yatskievych TA, Antin PB. Regulation of avian cardiac myogenesis by activin/TGFbeta and bone morphogenetic proteins. Dev Biol 204:407–419, 1998.
30. Barron M, Gao M, Lough J. Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. Dev Dyn 218:383–393, 2000.
31. Monzen K, Nagai R, Komuro I. A role for bone morphogenetic protein signaling in cardiomyocyte differentiation. Trends Cardiovasc Med 12:263–269, 2002.
32. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577–584, 2003.
33. Andree B, Duprez D, Vorbusch B, Arnold HH, Brand T. BMP-2 induces ectopic expression of cardiac lineage markers and interferes with somite formation in chicken embryos. Mech Dev 70:119–131, 1998.
34. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16:251–263, 2005.
35. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of

the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 270:2008–2011, 1995.
36. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. Trends Genet 20:563–569, 2004.
37. Bottcher RT, Niehrs C. Fibroblast growth factor signaling during early vertebrate development. Endocr Rev 26:63–77, 2005.
38. Lough J, Barron M, Brogley M, Sugi Y, Bolender DL, Zhu X. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. Dev Biol 178: 198–202, 1996.
39. Zhu X, Sasse J, McAllister D, Lough J. Evidence that fibroblast growth factors 1 and 4 participate in regulation of cardiogenesis. Dev Dyn 207:429–438, 1996.
40. Lopez-Sanchez C, Climent V, Schoenwolf GC, Alvarez IS, Garcia-Martinez V. Induction of cardiogenesis by Hensen's node and fibroblast growth factors. Cell Tissue Res 309:237–249, 2002.
41. Ilagan R, Abu-Issa R, Brown D, Yang YP, Jiao K, Schwartz RJ, Klingensmith J, Meyers EN. Fgf8 is required for anterior heart field development. Development 133:2435–2445, 2006.
42. Brown CB, Wenning JM, Lu MM, Epstein DJ, Meyers EN, Epstein JA. Cre-mediated excision of Fgf8 in the Tbx1 expression domain reveals a critical role for Fgf8 in cardiovascular development in the mouse. Dev Biol 267:190–202, 2004.
43. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7:165–197, 2000.
44. Thisse B, Thisse C. Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol 287:390–402, 2005.
45. Dailey L, Ambrosetti D, Mansukhani A, Basilico C. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev 16:233–247, 2005.
46. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 103: 211–225, 2000.
47. Tsang M, Dawid IB. Promotion and attenuation of FGF signaling through the Ras-MAPK pathway. Sci STKE 2004:pe17, 2004.
48. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303:1483–1487, 2004.
49. Reichsman F, Smith L, Cumberledge S. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. J Cell Biol 135:819–827, 1996.
50. Zecca M, Basler K, Struhl G. Direct and long-range action of a wingless morphogen gradient. Cell 87:833–844, 1996.
51. Banziger C, Soldini D, Schutt C, Zipperlen P, Hausmann G, Basler K. Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell 125:509–522, 2006.
52. Bartscherer K, Pelte N, Ingelfinger D, Boutros M. Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell 125: 523–533, 2006.
53. Ching W, Nusse R. A dedicated Wnt secretion factor. Cell 125:432–433, 2006.
54. Miller JR. The Wnts. Genome Biol 3:REVIEWS3001, 2002.
55. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet 16:279–283, 2000.
56. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 11:3286–3305, 1997.
57. Sheldahl LC, Slusarski DC, Pandur P, Miller JR, Kuhl M, Moon RT. Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. J Cell Biol 161:769–777, 2003.
58. Pandur P, Lasche M, Eisenberg LM, Kuhl M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418:636–641, 2002.
59. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810, 2004.
60. Miller JR, Hocking AM, Brown JD, Moon RT. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. Oncogene 18:7860–7872, 1999.
61. Marvin MJ, Di Rocco G, Gardiner A, Bush SM, Lassar AB. Inhibition of Wnt activity induces heart formation from posterior mesoderm. Genes Dev 15:316–327, 2001.
62. Eisenberg LM, Eisenberg CA. Wnt signal transduction and the formation of the myocardium. Dev Biol 293:305–315, 2006.
63. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116:2627–2634, 2003.
64. Moorman A, Webb S, Brown NA, Lamers W, Anderson RH. Development of the heart: (1) formation of the cardiac chambers and arterial trunks. Heart 89:806–814, 2003.
65. Kelly RG, Buckingham ME. The anterior heart-forming field: voyage to the arterial pole of the heart. Trends Genet 18:210–216, 2002.
66. Srivastava D, Olson EN. A genetic blueprint for cardiac development. Nature 407:221–226, 2000.
67. Fishman MC, Chien KR. Fashioning the vertebrate heart: earliest embryonic decisions. Development 124:2099–2117, 1997.
68. Fishman MC, Olson EN. Parsing the heart: genetic modules for organ assembly. Cell 91: 153–156, 1997.
69. Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C. A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell 82:803–814, 1995.
70. Harvey RP. Cardiac looping–an uneasy deal with laterality. Semin Cell Dev Biol 9:101–108, 1998.
71. Hamada H, Meno C, Watanabe D, Saijoh Y. Establishment of vertebrate left-right asymmetry. Nat Rev Genet 3:103–113, 2002.
72. Harvey RP. Links in the left/right axial pathway. Cell 94:273–276, 1998.
73. Raya A, Belmonte JC. Sequential transfer of left-right information during vertebrate embryo development. Curr Opin Genet Dev 14:575–581, 2004.
74. Capdevila J, Vogan KJ, Tabin CJ, Izpisua Belmonte JC. Mechanisms of left-right determination in vertebrates. Cell 101:9–21, 2000.
75. Hirokawa N, Tanaka Y, Okada Y, Takeda S. Nodal flow and the generation of left-right asymmetry. Cell 125:33–45, 2006.
76. Levin M, Pagan S, Roberts DJ, Cooke J, Kuehn MR, Tabin CJ. Left/right patterning signals and the independent regulation of different aspects of situs in the chick embryo. Dev Biol 189:57–67, 1997.
77. Monsoro-Burq A, Le Douarin NM. BMP4 plays a key role in left-right patterning in chick embryos by maintaining Sonic Hedgehog asymmetry. Mol Cell 7:789–799, 2001.
78. Fujiwara T, Dehart DB, Sulik KK, Hogan BL. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. Development 129:4685–4696, 2002.
79. Schlange T, Arnold HH, Brand T. BMP2 is a positive regulator of Nodal signaling during left-right axis formation in the chicken embryo. Development 129:3421–3429, 2002.
80. Rodriguez Esteban C, Capdevila J, Economides AN, Pascual J, Ortiz A, Izpisua Belmonte JC. The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. Nature 401:243–251, 1999.
81. Yokouchi Y, Vogan KJ, Pearse RV, 2nd, Tabin CJ. Antagonistic signaling by Caronte, a novel Cerberus-related gene, establishes left-right asymmetric gene expression. Cell 98:573–583, 1999.
82. Hirokawa N, Tanaka Y, Okada Y, Takeda S. Nodal flow and the generation of left-right asymmetry. Cell 125:33–45, 2006.
83. Meno C, Shimono A, Saijoh Y, Yashiro K, Mochida K, Ohishi S, Noji S, Kondoh H, Hamada H. lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. Cell 94:287–297, 1998.

84. Meno C, Takeuchi J, Sakuma R, Koshiba-Takeuchi K, Ohishi S, Saijoh Y, Miyazaki J, ten Dijke P, Ogura T, Hamada H. Diffusion of nodal signaling activity in the absence of the feedback inhibitor Lefty2. Dev Cell 1:127–138, 2001.
85. Logan M, Pagan-Westphal SM, Smith DM, Paganessi L, Tabin CJ. The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. Cell 94:307–317, 1998.
86. Shiratori H, Sakuma R, Watanabe M, Hashiguchi H, Mochida K, Sakai Y, Nishino J, Saijoh Y, Whitman M, Hamada H. Two-step regulation of left-right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. Mol Cell 7:137–149, 2001.
87. Ramsdell AF, Yost HJ. Cardiac looping and the vertebrate left-right axis: antagonism of left-sided Vg1 activity by a right-sided ALK2-dependent BMP pathway. Development 126:5195–5205, 1999.
88. Breckenridge RA, Mohun TJ, Amaya E. A role for BMP signalling in heart looping morphogenesis in Xenopus. Dev Biol 232:191–203, 2001.
89. Isaac A, Sargent MG, Cooke J. Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. Science 275:1301–1304, 1997.
90. Mercola M. Embryological basis for cardiac left-right asymmetry. Semin Cell Dev Biol 10:109–116, 1999.
91. Norris D. Breaking the left-right axis: do nodal parcels pass a signal to the left? Bioessays 27:991–994, 2005
92. Campione M, Ros MA, Icardo JM, Piedra E, Christoffels VM, Schweickert A, Blum M, Franco D, Moorman AF. Pitx2 expression defines a left cardiac lineage of cells: evidence for atrial and ventricular molecular isomerism in the iv/iv mice. Dev Biol 231: 252–264, 2001.
93. Harvey RP. Patterning the vertebrate heart. Nat Rev Genet 3:544–556, 2002.
94. Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet 16:154–160, 1997.
95. Gaio U, Schweickert A, Fischer A, Garratt AN, Muller T, Ozcelik C, Lankes W, Strehle M, Britsch S, Blum M, Birchmeier C. A role of the cryptic gene in the correct establishment of the left-right axis. Curr Biol 9:1339–1342, 1999.
96. Yan YT, Gritsman K, Ding J, Burdine RD, Corrales JD, Price SM, Talbot WS, Schier AF, Shen MM. Conserved requirement for EGF-CFC genes in vertebrate left-right axis formation. Genes Dev 13: 2527–2537, 1999.
97. Oh SP, Li E. The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymmetry in the mouse. Genes Dev 11:1812–1826, 1997.
98. Lowe LA, Supp DM, Sampath K, Yokoyama T, Wright CV, Potter SS, Overbeek P, Kuehn MR. Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. Nature 381:158–161, 1996.
99. Hooper JE, Scott MP. Communicating with Hedgehogs. Nat Rev Mol Cell Biol 6:306–317, 2005.
100. Nybakken K, Perrimon N. Hedgehog signal transduction: recent findings. Curr Opin Genet Dev 12:503–511, 2002.
101. Bijlsma MF, Spek CA, Peppelenbosch MP. Hedgehog: an unusual signal transducer. Bioessays 26:387–394, 2004.
102. Hammerschmidt M, Brook A, McMahon AP. The world according to hedgehog. Trends Genet 13:14–21, 1997.
103. Porter JA, Young KE, Beachy PA. Cholesterol modification of hedgehog signaling proteins in animal development. Science 274: 255–259, 1996.
104. Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126:3915–3924, 1999.
105. Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1:20–29, 2000.
106. Zhang XM, Ramalho-Santos M, McMahon AP. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105:781–792, 2001.
107. Gianakopoulos PJ, Skerjanc IS. Hedgehog signaling induces cardiomyogenesis in P19 cells. J Biol Chem 280:21022–21028, 2005.
108. Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature 361:543–547, 1993.
109. Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120:1919–1928, 1994.
110. Schier AF. Nodal signaling in vertebrate development. Annu Rev Cell Dev Biol 19:589–621, 2003.
111. Adachi H, Saijoh Y, Mochida K, Ohishi S, Hashiguchi H, Hirao A, Hamada H. Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. Genes Dev 13:1589–1600, 1999.
112. Saijoh Y, Adachi H, Sakuma R, Yeo CY, Yashiro K, Watanabe M, Hashiguchi H, Mochida K, Ohishi S, Kawabata M, Miyazono K, Whitman M, Hamada H. Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. Mol Cell 5:35–47, 2000.
113. Branford WW, Yost HJ. Nodal signaling: Cryptic/Lefty mechanism of antagonism decoded. Curr Biol 14:R341–R343, 2004.
114. Chen C, Shen MM. Two modes by which Lefty proteins inhibit nodal signaling. Curr Biol 14:618–624, 2004.
115. Sakuma R, Ohnishi Yi Y, Meno C, Fujii H, Juan H, Takeuchi J, Ogura T, Li E, Miyazono K, Hamada H. Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion. Genes Cells 7:401–412, 2002.
116. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685–700, 2003.
117. Feng XH, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol 21:659–693, 2005.
118. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5:877–889, 2003.
119. Sun Y, Liang X, Najafi N, Cass M, Lin L, Cai C, Chen J, Evans S. Islet 1 is expressed in distinct cardiovascular lineages, including pacemaker and coronary vascular cells. Dev Biol 304:286–296, 2007.