Early Signals in Cardiac Development

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Abstract—The heart is the first organ to form during embryogenesis and its circulatory function is critical from early on for the viability of the mammalian embryo. Developmental abnormalities of the heart have also been widely recognized as the underlying cause of many congenital heart malformations. Hence, the developmental mechanisms that orchestrate the formation and morphogenesis of this organ have received much attention among classical and molecular embryologists. Due to the evolutionary conservation of many of these processes, major insights have been gained from the studies of a number of vertebrate and invertebrate models, including mouse, chick, amphibians, zebrafish, and Drosophila. In all of these systems, the heart precursors are generated within bilateral fields in the lateral mesoderm and then converge toward the midline to form a beating linear heart tube. The specification of heart precursors is a result of multiple tissue and cell-cell interactions that involve temporally and spatially integrated programs of inductive signaling events. In the present review, we focus on the molecular and developmental functions of signaling processes during early cardiogenesis that have been defined in both vertebrate and invertebrate models. We discuss the current knowledge on the mechanisms through which signals induce the expression of cardiogenic transcription factors and the relationships between signaling pathways and transcriptional regulators that cooperate to control cardiac induction and the formation of a linear heart tube. (Circ Res. 2002;91:457-469.)

Key Words: cardiac induction • growth factors • cardiogenic transcription factors

Our understanding of vertebrate heart development has been propelled to a large part through studies in a number of model organisms, most notably the chick, amphibians, zebrafish, and mouse. In all of these systems, the heart arises from cells in the anterior lateral plate mesoderm of the early embryo, where they are arranged in bilateral fields on either side of the prechordal plate and rostral notochord. These fields include the precursors of both myocardial and endocardial cells, although there is apparently no common pool of bipotential precursors for these two heart cell lineages.1,2 The cells of the cardiogenic mesoderm are brought to these positions by gastrulation movements that occur in close association with ingressing cells of the rostral endoderm. In mammals and birds, the bilateral fields of the cardiogenic mesoderm merge at their anterior margins to form the so-called “cardiac crescent” (Figures 1A and 2A).3 More recent studies have identified a second type of heart field that is located more medially in the splanchnic mesoderm, directly adjacent to the cardiac crescent. The cells from this bilateral field, termed anterior heart-forming field, are fated to generate anterior heart structures of the outflow tracts (see review4) (Figures 1A and 2B).

Results from experiments with mesodermal tissue explants from quail and chick embryos indicate that the specification...
of cardiomyocytes occurs just before or during the formation of the cardiac crescent, and differentiation markers become expressed shortly thereafter. The earliest steps of assembly of the heart tube are initiated by the convergence and fusion of the bilateral heart primordia along the midline. The cells of the anterior heart-forming field, after having migrated anteriorly, are added to the anterior end of the linear tube during and after this period. The resulting beating tubular heart is composed of an external myocardial and an internal endocardial layer and also possesses a polarity along the anteroposterior axis, in which the prospective tissues of the aortic sac, outflow tract (conotruncus), right ventricle, left ventricle, and atria are present in an anterior to posterior order along the tube (Figure 1A) (see references).

Figure 1. Stages of cardiogenesis. A, Mouse cardiogenesis at E7.5, E8, and E8.5. Major heart forming field is shown in black and the anterior (secondary) heart forming field (AHF) in gray. AS indicates atria, sinus venosa; CT, conotruncus; RV, right ventricle; LV, left ventricle; and A, atria. B, Drosophila cardiogenesis at 6, 12, and 16 hours after fertilization. Heart progenitors (HP) are shown in black and other dorsal mesodermal cell types (DM) in gray. AO indicates aorta; HT, heart.

Figure 2. Early markers for the cardiac crescent (A) and anterior heart forming field (B) in the mouse.
In all vertebrates, the tubular heart undergoes a process known as rightward looping. The morphogenetic steps required to achieve looping are guided by molecular asymmetries that are established in and around the heart by the embryonic left/right axial pathway. Furthermore, in higher vertebrates, septal division of the chambers and formation of the valves, which involves endothelial cells, are essential steps leading to the formation of an integrated 4-chambered heart with separate venous (or inflow) and arterial (or outflow) poles. During the growth process of the cardiac epithelium another distinct cell lineage, the migrating cardiac neural crest cells, populate the heart through the outflow channel and contribute to the formation of the great vessels and outflow septum.

The spatial and temporal orchestration of these processes implies a complex program of genetic control. As we now know, this program is exerted in large part through precisely controlled processes of cell-cell signaling and regulators of gene expression. Many of these processes were initially discovered through embryological studies, which uncovered inductive tissue interactions and candidates for cytokines that transmit these signals. More recently, genetic screens in the zebrafish system have provided an alternative and highly successful route toward the identification of regulatory components in cardiogenesis. Unexpectedly, genetic and molecular studies of mesodermal tissue development in an invertebrate, the fruit fly Drosophila, have also been instrumental in the identification of specific genes and processes in cardiogenesis that appear to be conserved in all higher animals. As we will discuss, these similarities extend to, and are likely a result of, similarities in the molecular and genetic mechanisms that control early heart development in vertebrate and insect embryos.

In the present review, we summarize our current knowledge of the major signaling mechanisms controlling early heart development, with a particular focus on the specification of myocardial cells and heart patterning. We highlight particular signaling processes using the most informative findings in specific model organisms as examples and discuss the general insights that can be drawn from different systems. Hence, we will restrict our focus to the stages between mid gastrulation and the formation of the linear heart tube because it appears that parallels between vertebrate and insect systems can be best drawn during this period.
Early Inductive Processes in Heart Specification and Determination

Transplantation experiments in chick and mouse embryos demonstrate that the cardiogenic regions contain inductive activities that promote cardiac differentiation.1,14 When cells from regions that are normally not cardiogenic, such as the posterior primitive streak in the chick embryo, are transplanted into the cardiogenic region, they are induced to form heart instead of blood cells.14 Moreover, coculture experiments suggest that the heart-inducing activity originates from the anterior endoderm and not from the cardiogenic mesoderm itself.15,16 Removal of early endoderm in newt and frog embryos blocks heart formation showing that, at least in amphibians, endoderm is not only sufficient but also required for cardiac induction.17,18 However, genetic ablation of the endoderm in zebrafish and surgical ablation in the chick does not block early cardiogenesis.19,20 Although this result implies that differentiated endoderm is not necessary in all vertebrates, signaling from unspecified endodermal precursors may still be required for heart induction. From these and other observations, it can be concluded that cells of the anterior endoderm and/or their precursors have a major role in inducing cardiogenesis. Additional cardiogenic signals are derived from the organizer,21 which act either indirectly by patterning the early adjacent endoderm or directly by inducing cardiogenesis in combination with anterior endodermal signals. Ectodermal influences on cardiac induction have also been described and are thought to serve mainly in counteracting negative influences from the neural plate.22 Of note, observations in amphibians and the chick show that not the entire precardiac mesoderm (the “heart field,” being the mesoderm that differentiates into cardiac muscle on explantation into tissue culture) gives rise to heart in vivo.23,24 In particular, cardiogenesis is restricted to lateral areas of the precardiac mesoderm on either side of the prechordal plate. Medial areas fail to form heart tissue in vivo, although medial and lateral endoderm do not differ intrinsically in their heart-inducing activity.15,25 It appears that this difference in cardiac determination of lateral versus medial areas of the heart field is largely due to negative signals from the anterior neural plate.17,26,27 In addition, ablation studies in the zebrafish demonstrated that negative signals from the notochord play a role in limiting heart formation to areas anterior to the notochord.28 Altogether, it has become apparent that positive and negative signals act successively and perhaps combinatorially to induce early cardiogenesis within a defined area of the anterior lateral plate mesoderm of vertebrate embryos.

Whereas the small size of the Drosophila embryo has prevented embryological studies on heart induction, experiments of this type have been successfully performed in a larger dipteran, the lacewing fly.29 These cell ablation studies showed that induction across germ layers is also required for the specification of the fly dorsal vessel. Unlike in vertebrates, where the heart-inducing activity is predominantly of endodermal origin, in the fly embryo the essential inductive activity resides in the dorsal ectoderm on either side of the germ band.29

Cardiogenic Transcription Factors Responding to Inductive Signals in Myocardial Development

Although classical studies have mainly used morphological criteria and myocardial differentiation genes as markers of cardiac induction, more recent studies have shown that the earliest responses involve the induction of regulatory genes that encode specific transcription factors. Genes encoding factors of the NK homeodomain, GATA, T-box, and other families were found to exert the functions of inductive signals during specification, patterning, and differentiation of the heart. In addition, it appears that most, if not all, developmental signaling pathways are required to act in combination with tissue-specific transcriptional cofactors to elicit inductive responses. Studies of the expression and function of early cardiogenic transcription factors have therefore significantly advanced our understanding of inductive signaling processes during cardiogenesis and the major representatives will be described in the following sections.

NK Homeodomain Proteins

The prototype of cardiac NK homeodomain proteins is the product of the Drosophila tinman gene. tinman was the first regulatory gene in any species known to be expressed in the precardiac mesoderm and to function in specifying cardiac precursors. Hence, the discovery of tinman and homologous NK-homeobox genes in vertebrates has provided a major impulse in the field. The activity of Drosophila tinman is absolutely required for the formation of the dorsal vessel and for the specification of all of its progenitor cells.30,31 The expression of tinman has 3 distinct phases, with the first occurring before gastrulation in the entire mesoderm, except for the prospective hemocytes. The second phase occurs after mesoderm migration, when tinman expression becomes restricted to the dorsal (ie, lateral) mesoderm.30–32 which includes not only the cardiogenic mesoderm but also mesodermal cells fated to form visceral and dorsal body wall muscles (Figure 4A). The function of tinman is required, presumably during this stage, for the specification of all 3 of these tissues. In the third phase, tinman expression becomes further restricted to precursor cells of the dorsal vessel, both cardioblasts and pericardial cells. This expression pattern is maintained throughout development, but includes only a subset of dorsal vessel cells in each segment (Figure 3C).13,33,34 During this third expression phase, tinman is thought to function in the diversification and differentiation of dorsal vessel cells.

Unlike Drosophila, vertebrates contain several members of this subgroup of homeobox genes, which are named Nkx2-3 through Nkx2-10 (see review35; not all members are present in every species and Nkx2-1, 2-2, and 2-4 belong to a different, noncardiac subgroup). In mouse, chick, frog, and zebrafish, the main representative, Nkx2-5, is expressed in the lateral plate mesoderm within the heart field. In the chick and frog, the onset and pattern of early Nkx2-5 expression roughly coincide with the timing and area of cardiac specification, as defined by the previously discussed explant studies, thus suggesting that these genes respond to and perhaps exert early inductive signals.15,36 As tinman in Drosophila, vertebrate...
Nkx2-5 genes continue to be expressed throughout development in the heart.\textsuperscript{37–39} Mouse embryos lacking Nkx2-5 gene activity show early heart defects. Although loss-of-function of Nkx2-5 does not block heart tube formation as in Drosophila tinman mutants, cardiogenesis arrests before looping morphogenesis.\textsuperscript{40} Human NKX2-5 is haploinsufficient because dominant putative loss-of-function mutations have been identified, which cause congenital cardiac disease by disrupting cardiac morphogenesis and, in particular, septation.\textsuperscript{41} The less severe heart phenotypes of murine Nkx2-5 mutant embryos as compared with tinman mutant fly embryos raise the question of whether there is functional redundancy among members of this Nkx2 subgroup. At least in the mouse, the other known Nkx2 members are expressed only in subareas or outside of the Nkx2-5 expression domain.\textsuperscript{42–44} Accordingly, the heart defects in mouse Nkx2-5/Nkx2-6 double mutant embryos are only slightly more severe than those observed in Nkx2-5 single mutants.\textsuperscript{45} These data appear to suggest there is only limited redundancy among Nkx2 genes in early cardiogenic tissue. A detailed reexamination of the Nkx2-5 expression profile in the chick has revealed that its expression in this and perhaps other species does not occur in the entire cardiogenic mesoderm, but rather is largely restricted to the anterior, presumptive ventricular portions.\textsuperscript{11} Although it is premature to generalize, most of the available data argue against a strict requirement of cardiac Nkx2 genes in the general specification of myocardial fates in vertebrates.

The complete block of cardiogenesis on overexpression of dominant-negative forms of Nkx2-3 and Nkx2-5 in Xenopus has been used in support of the argument of a redundant activity of these genes in the specification of cardiac fates.\textsuperscript{46,47} However, there is increasing evidence of the importance of protein-protein interactions between Nkx2 proteins and other cardiogenic transcription factors (see next two sections), and thus it is possible that the loss of cardiogenesis in these experiments is due to the interference with the cardiogenic activity of other regulators. For the normal situation, this interpretation would imply that different types of transcription factors have overlapping and partially redundant functions in cardiac specification.

### GATA Factors and Cardiogenesis

Members of the cardiac GATA subfamily of factors bind to the WGATAR motif in the promoter regions of many cardiac- and gut-specific genes (see review\textsuperscript{48}). Gata4 and 2 related genes, Gata5 and 6, are expressed in the precardiac mesoderm and developing heart of different vertebrate species almost simultaneously and spatially overlapping with Nkx2-5, although their expression also includes the associated endodermal layer.\textsuperscript{49–54} Functional knockout experiments with Gata4 in mouse, Gata5 in zebrafish, and simultaneous knockdown experiments with all 3 family members in the chick show early heart defects, including the lack of fusion of the bilateral heart primordia (cardia bifida), decrease of Nkx2-5 expression, and a reduction in the number of cardiomyocytes expressing myocardial differentiation genes.\textsuperscript{55–58} With genetically chimeric embryos in the mouse, it was shown that Gata4 function is required in the endoderm and not within the presumptive heart cells for cardiac fusion and myocardial differentiation.\textsuperscript{55,59} However, in zebrafish, there is strong genetic evidence for a mesoderm-autonomous contri-
bution of *Gata* genes in Nkx2-5 regulation and cardiomyocyte differentiation. The identification of functionally important GATA-binding sites or *Gata*-responsive enhancers upstream of mouse and frog Nkx2-5 as well as many myocardial differentiation genes provides additional support for a role of these genes in early myocardial differentiation and perhaps specification. Cross-regulation between *Gata* and Nkx genes, the importance of protein-protein interactions between GATA and Nkx factors, and functional significance of combinatorial GATA and Nkx binding sites in cardiac gene regulation have also been demonstrated.

Clear evidence for a requirement for *Gata* genes in cardiomyocyte specification has been obtained in the *Drosophila* system. Embryos mutant for the *Gata* gene *pannier* lack all cardiomyocytes, but have supernumerary pericardial cells. *pannier* is coexpressed with *tinman* during the second *tinman* expression phase, although within a narrower dorsal domain, and the two genes appear to act synergistically in cardiomyocyte specification. Tinman and Pannier can heterodimerize like their corresponding vertebrate factors. The two genes are also part of a cross-regulatory loop, since the early expression of *pannier* is directly controlled by *tinman* while the later cardioblast expression of *tinman* (perhaps indirectly) requires *pannier*. These data suggest that many aspects of *Gata* and Nkx genes in cardiogenesis have been widely conserved during evolution.

**T-Box Factors and the Homeobox Gene Irx4 in Cardiac Patterning**

The involvement of the T-box genes in heart development is accentuated by the heart defects of mice and humans carrying mutations in *TBX1* and *TBX5*, which have been associated with the human DiGeorge and Holt-Oram syndromes, respectively. Like Nkx2-5 and the cardiac *Gata* genes, *Tbx5* genes are expressed in the bilateral cardiac primordia of mouse, *Xenopus*, chick, and fish embryos, although their expression becomes restricted to posterior areas of the prospective atria and sinus venosa, as well as the left ventricle. In agreement with the expression patterns, dominant-negative approaches in frog embryos and homozgyous null mutations in the mouse cause strong disruptions of heart development, which particularly affect sinoatrial structures and are accompanied by reductions in Nkx2-5 and *Gata4* expression. By contrast, forced *Tbx5* expression in the anterior region of the cardiac crescent results in aberrant ventricular morphogenesis and reduced *mlc2v* expression. Altogether, these data suggest a role of *Tbx5* in the early diversification of atrial versus ventricular cell identities along the anteroposterior extent of the heart primordia. The demonstration of physical interactions between Tbx5 and Nkx2-5 and their coexpression within parts of the heart field suggests functional cooperation of these two cardiac factors during this process.

The particular roles of other T-box factors, including *Tbx1* and zebrafish *hrT*, during early cardiogenesis have been less well defined. *hrT* is expressed within the heart field before *Tbx5* in a pattern that is very similar to *Gata5*. In *Drosophila*, potential homologs of *Tbx5* and *hrT* are also expressed in the developing dorsal vessel, but their functions are not yet known due to a lack of genetic data.

*Irx4*, a member of the *iroquois* subgroup of homeobox genes, is specifically expressed in the prospective ventricular subarea of the early linear heart tube and expression persists in the ventricular chambers. The expression of *Irx4* is downstream of *Nkx2-5*. Functional knockout of *Irx4* in the mouse results in expansion of atrial and suppression of ventricular differentiation markers, whereas forced *Irx4* expression in the chick heart has the opposite effect. These data provide strong evidence that *Irx4* promotes ventricular and suppresses atrial identities within the heart tube.

**Signaling Pathways During the Induction of Cardiogenic Mesoderm**

**BMP/Dpp Signaling**

Evidence for a direct involvement of BMP signaling in early cardiogenesis was initially obtained in the *Drosophila* system through studies of Dpp, a member of the BMP family of TGF-β proteins. During gastrulation and mesoderm migration, Dpp is expressed in a broad band of cells within the dorsal ectoderm, which in another fly species was shown to possess heart-inducing activity (see above). Significantly, *Drosophila* mutant embryos that lack the activity of Dpp form neither a dorsal vessel nor any of its progenitor cells, indicating that Dpp is one of the essential signals conferring the heart-inducing activity of the dorsal ectoderm. A crucial molecular target of Dpp in this pathway is the *tinman* gene, whose expression in the dorsal mesoderm is induced by Dpp (Figure 4A). Embryos that are mutant for *dpp* or other components of the Dpp pathway lack dorsal mesodermal expression of *tinman*. Ectopic activation of the Dpp pathway ultimately leads to ectopic formation of dorsal vessel cells in the ventral-most areas of the mesoderm. However, in spite of their ectopic expression of *tinman*, cells in lateral areas of the mesoderm cannot be transformed into cardiac tissue by ectopic Dpp, presumably because of a requirement for additional signals that are restricted to the dorsal- and ventral-most areas of the germ band.

The molecular mechanisms of *tinman* induction by Dpp have largely been clarified. In the mesoderm, Dpp signals are mediated through the activation of the effectors Mad (*Smad1*) and Medea (*Smad4*) (Figure 3A), which can bind to several specific Smad-binding sites within a Dpp-responsive enhancer element that is located downstream of the *tinman* gene. Smad binding to these sites is however not sufficient because the synergistic activities of Smad and Tinman itself, which bind to adjacent sites, are required to induce *tinman* expression. The source of the autocatalytic Tinman activity is derived from the transient early activation of the *tinman* gene by Twist in the entire mesoderm. In essence, Twist-activated Tinman provides the mesoderm with the competence to respond to Dpp by inducing *tinman* in a dorsally restricted domain during its second phase of expression and, ultimately, to induce cardiac progenitors.

Loss- and gain-of-function experiments show that although *tinman* is required, it is not sufficient to promote cardiogenesis. One explanation for this observation is that *tinman*...
may be functional only in conjunction with Dpp signaling in the early mesoderm. Indeed, there is increasing evidence that, similar to tinman itself, there are additional genes that are induced by the synergistic effects of Dpp+Tinman within the cardiogenic mesoderm and control heart development. A more recently studied example is the homeobox gene even-skipped, which is induced during early cardiogenesis in a subset of pericardial cell progenitors and is required for their normal differentiation (Figure 3).93,94 The induction of even-skipped involves a specific enhancer element that also contains a combination of essential Smad and Tinman binding sites.95,96 The Gata gene punnier may provide yet another example of an important cardiac regulatory gene that is induced by synergistic Dpp+Tinman activities.71

Given the overt similarities of the expression and function of tinman and vertebrate Nkx2-5 genes, it has been examined whether the similarities extend to the regulation of these genes, and of cardiogenesis, by Dpp-related signals. In chick and Xenopus, solid evidence for a direct role of BMPs in cardiac induction has been obtained. In the chick, the expression of at least three dpp-like genes, Bmp2, 4, and 7, includes the anterior lateral region of the embryo, which overlaps with the precardiac region that expresses Nkx2-5 and Gata4.97,98 At this stage, Bmp2 and, to a lesser degree Bmp7, are expressed in the adjacent endoderm, which is known to possess the main inductive activity (see above), whereas Bmp4 is expressed within the mesoderm itself and, together with Bmp7, also in the ectoderm. Ectopic application of BMP-2 or BMP-4–releasing implants in vivo cause ectopic induction of Nkx2-5 and Gata4, although not of terminal differentiation markers, and the exposure of tissue explants to soluble BMP-2 or -4 induces both the early cardiac regulators and also terminal differentiation of cardiac tissue.97–99 Of note, these effects are only obtained with BMP-releasing implants into anterior mesoderm, such as anterior/medial areas that normally develop into head mesenchyme, whereas posterior mesoderm is not competent to elicit any cardiogenic responses in these assays. Because posterior mesoderm is able to generate heart in response to anterior endoderm, these observations strongly suggest that the heart-inducing activity consists of BMPs in combination with a second endodermal signal.

The heart-inducing activity of BMP signaling has been further confirmed by studies using inhibitors of BMP signaling, including the BMP inhibitor noggin, truncated versions of type I (tALK3) or type II (tBMPRII) BMP receptors, and inhibitory SMAD6.97,100–102 These studies show that BMP signal transduction is indeed required within the cardiogenic mesoderm, and not only within the anterior endoderm, to promote cardiac differentiation (Figure 4B). A second important conclusion from these data are that BMP signaling is required for the maintenance of Nkx2-5 and Gata gene expression at stages during and after the fusion of the bilateral heart primordia, but apparently not for the initial activation of these genes in earlier stage embryos. This finding is reminiscent of the biphasic regulation of Drosophila tinman (see previous page). As previously demonstrated for the Dpp-responsive enhancer of Drosophila tinman, mouse Nkx2-5 is also controlled by enhancer sequences that contain functionally important SMAD binding sites.103,104

In addition to Nkx2-5, BMP signals in vertebrates are also likely to provide direct inputs in controlling several different cardiac regulatory genes, which may include Gata genes,97,102,105 T-box genes,106 and the bHLH-factor encoding Hand genes.107 Moreover, BMP signals may need the presence of specific cardiac transcription factors such as Nkx2-5 to be able to induce myocardial differentiation in a synergistic fashion,108 as was shown molecularly in the Drosophila system.

Wnt/Wingless Signaling

Neither in Drosophila nor in vertebrates are the spatial domains of BMP signaling sufficient to define cardiogenic mesoderm. In both the insect and vertebrate systems, it has been shown that Wnt-signaling has a major role in further restricting the domains in which BMPs can elicit cardiogenic response. However, as discussed below, the particular mechanisms through which this outcome is achieved differ between the systems.

The activity of the major Wnt signaling protein in Drosophila, Wingless, is essential for all aspects of cardiogenesis.109 The cardiogenic activity of Wingless is required during and probably shortly after the period when Dpp is expressed in the dorsal ectoderm adjacent to the cardiogenic mesoderm.109 During this period, Wingless is expressed in periodic transverse stripes in the ectoderm, which intersect dorsally with the longitudinal Dpp domains. The progenitors of the dorsal vessel are induced precisely within the areas of the mesoderm where the Wingless domains intersect with the dorsal Dpp domain. By contrast, the areas that are exposed to Dpp but not Wingless form visceral muscle progenitors. Combined with existing genetic data, a simple model has been proposed that the combination of Dpp and Wingless signals is required to elicit a cardiogenic response in the adjacent mesoderm while Dpp alone induces visceral mesoderm.13

Additional studies have addressed the genetic and molecular mechanisms of how combined Dpp and Wingless signaling promotes cardiogenesis. The Wingless signals are transduced in the mesoderm via the canonical Wingless/Wnt pathway110 and participate in the induction of target genes that are required for heart specification. One of the two direct mesodermal targets of the Wingless signaling cascade that are known to date is the fork head domain encoding gene sloppy paired (slp).111 Hence, sloppy paired becomes expressed in a pattern of transverse stripes directly beneath the ectodermal Wingless stripes (Figure 3B). As for wingless itself, loss of slp activity results in a complete absence of cardiogenic precursors, showing that slp is an essential mediator of Wingless signaling in this developmental pathway. It is presently not firmly established whether slp serves to activate specific cardiac downstream targets in a direct manner or whether it acts indirectly by inhibiting the inappropriate activation of visceral mesoderm genes that would block cardiogenesis.

The second known example of a direct Wingless target in the mesoderm is even-skipped. It has been shown that the
pericardial enhancer element of even-skipped (see previous page) contains several binding sites for the Wingless effector dTCF, which are required in addition to the Smad and Tinman binding sites for its normal activity in pericardial progenitors.95,96 These findings identify even-skipped as a paradigm for a cardiac target on which the activities of Tinman as well as the Dpp and Wingless signaling cascades converge. Interestingly, whereas partial inactivation of the dTCF binding sites within this even-skipped enhancer leads to a reduction of its activity,95 the inactivation of all sites results in ectopic activity within the dorsal mesoderm, which occurs even in wingless mutant embryos.96 These and other observations (M. Frasch, unpublished data, 2001) indicate that Wingless signaling is mainly required to relieve the repressible activity of the HMG protein dTCF to allow induction of even-skipped and perhaps other cardiac genes by Dpp and other signals (Figure 5B).

In contrast to Drosophila, where Wingless is essential for cardiac induction, studies in vertebrates have uncovered an important role of Wnt signaling in blocking cardiogenesis. Specifically, overexpression of Wnt3A and Wnt8 in injected frog embryos or exposure of mesoderm from the heart field of chick embryos to Wnt3A and Wnt-1 blocks expression of Nkx2-5, Tbx5, and cardiac differentiation.112,113 In the chick, this treatment results in a transformation from cardiogenic mesoderm into blood. Conversely, injection of an inhibitory component of the canonical Wnt signaling cascade, GSK3β′, causes ectopic cardiac differentiation in cells of the ventral marginal zone from frog embryos that are normally destined to form blood cells.113

Strong candidates for endogenous Wnt antagonists that function to derepress cardiac induction have been identified in both chick and frog embryos. These are the secreted factors Crescent and Dkk-1, which can inhibit specific Wnt ligands, including Wnt3A and Wnt-8. In posterior lateral plate mesoderm and ventral marginal zone explants from chick and frog embryos, respectively, Crescent and Dkk-1 are potent inducers of the expression of Nkx2-5, Tbx5, and of cardiac differentiation.112,113 Moreover, both of these Wnt antagonists are expressed in the organizer and organizer-derived anterior endoderm that is known to possess heart-inducing activity, whereas Wnt-3A and -8 are present predominantly in the posterior lateral plate and paraxial mesoderm during this stage. In addition, Wnt-3A and Wnt-1 are expressed in the anterior neural tube and serve as inhibitors of cardiogenesis in the adjacent anterior paraxial mesoderm.114

Taken together, these findings are able to explain many of the embryological data on cardiac induction and provide a model for the signaling processes that restrict cardiogenesis to the anterior lateral mesoderm. In this model, Wnt antagonists act as signals from the organizer/anterior endoderm to initiate cardiogenesis in the adjacent mesoderm by establishing a zone of reduced Wnt-3a/Wnt-8 activity and expression. Elevated levels of Wnt-1 and Wnt-3a from the anterior neural tube restrict this zone to lateral portions of the anterior mesoderm (Figure 4B). Reduced Wnt signaling then allows, either by default or through the activity of yet undefined signals from the endoderm, expression of Nkx2-5 and other cardiac regulatory genes in the anterior lateral mesoderm and provides it with the competence to respond to BMP signals from the endoderm and lateral mesoderm. In turn, BMP signaling acts to maintain Nkx2-5 expression and may continue to act synergistically with Nkx2-5 and other transcriptional regulators to promote cardiac differentiation (Figure 5A).

In light of the numerous similarities between Drosophila and vertebrate cardiogenesis, it is puzzling that Wnt signaling appears to have opposite effects on cardiac induction, namely a positive one in Drosophila and a negative one in vertebrates. It is conceivable that there are other Wnt family members in vertebrates that act positively in cardiac induc-
tion and cannot be inhibited by Dkk-1 and Crescent (see Note Added in Proof). A role of negatively-acting Wnt family members in Drosophila can also not be excluded, although genes encoding soluble Wnt antagonists are not detectable in the genome. We speculate that, with the advent of soluble Wnt antagonists in ancestors of the vertebrate lineage, negative regulation of cardiogenesis by Wnts and its release by antagonists may have become the dominant mode of vertebrate cardiac induction.

Fibroblast Growth Factor Signaling

There is substantial evidence from both Drosophila and vertebrates that fibroblast growth factor (FGF) signaling is making a direct contribution to the specification of heart progenitors. However, progress toward the understanding of the specific role of FGF signals in this pathway and its relationship with other pathway components has lagged due to the indirect effects of these signals on heart development as a result of their involvement in early mesoderm induction and cell migration during gastrulation (see reviews115,116).

In Drosophila, one of the two FGF receptors from this species, named Heartless (Htl), is expressed specifically in the mesoderm, starting from early gastrulation until differentiation (Figure 4). The observed absence of the dorsal vessel in heartless mutant embryos is, at least in part, an indirect consequence of an early requirement for FGF signaling in mesoderm migration (see review116). Hence, the majority of mesodermal cells fail to reach the dorsal ectoderm in htl mutants, which in turn prevents cardiac induction via Dpp and other signals. In addition, a more direct role of FGF signaling in Drosophila cardiomyocytes and pericardial cell specification has been revealed in experiments in which the FGF signaling pathway is inhibited only at a time after mesoderm migration is completed.117,118 Transmission of the FGF signal within the mesoderm involves the Ras pathway versus Serrate/Jagged ligands, although this specific aspect is not yet known.

In the chick system, it has been shown by in vitro cultures that FGF-2 and -4 can induce cardiogenesis in non-precardiac mesoderm, although induction of Nkx2-5, Gata4, and cardiac differentiation occurs efficiently only if BMP-2 or -4 is also provided.121,122 FGF is only required transiently, whereas BMPs are required continuously for cardiogenic induction in this system. Although FGFs are expressed in the early chick embryo at the correct time and place to be able to fulfill an analogous function in cardiac specification in vivo, the available data cannot distinguish between direct and possible indirect effects of FGF signals, as for example in the regulation of cell migration.

Apart from Drosophila, the clearest data on the in vivo function of FGF signaling in cardiac induction has been obtained in the zebrafish system. Zebrafish FGF8 is expressed in the cardiogenic fields of the lateral plate, as well as in specific areas of the neural tube. fgf8 (acerebellar) mutant embryos display strong heart defects with a particular loss of ventricular structures.123 At earlier stages, strong reductions of Nkx2-5 and Gata4 are observed from the onset of their expression. Importantly, incubation of embryos during early somitogenesis (ie, after the induction and migration of the mesoderm) with a specific inhibitor of the FGF-receptor Fgfr1, SU5402, results in a phenocopy of the acerebellar heart phenotype, including the block of Nkx2-5 expression.123 Together, these data provide strong evidence that FGF signals not only act during early mesoderm development but are also required more directly for the induction of cardiogenic transcription factors during subsequent stages (Figure 4B). It is possible that different members of the FGF family have partially redundant activities during this process, which may account for the normal expression of Gata6 and the formation of residual heart (particularly atrial) tissue in acerebellar mutants.

As shown in the mouse, the cells of the anterior heart-forming field already express FGF-10 at the cardiac crescent stage (Figure 2B).4 Therefore, it is tempting to speculate that apart from a possible role in the anterior migration of these cells, FGF-10 may also be involved, perhaps in combination with BMPs, in the induction of Nkx2-5 and other regulators that drive arterial pole development.

Notch Signaling

Signaling via Notch receptors plays an important role in early cardiogenesis of both Drosophila and vertebrates. Notch, which is involved in a wide range of developmental contexts, is activated by its transmembrane ligands Delta (Drosophila and vertebrates) as well as Serrate (Drosophila) and its vertebrate homologs Jagged. The glycosyl transferase Fringe differentially modulates the responsiveness of Notch to Delta versus Serrate/Jagged ligands, although this specific aspect has not been examined in early heart development. In addition, the transmission of the signal to target genes involves the nuclear factor Su(H)/RBP-J (see review124).

During Drosophila cardiogenesis, Notch functions during two distinct developmental events, namely the process of lateral inhibition and the control of lineage decisions during asymmetric cell divisions of heart progenitors. Lateral inhibition involves reciprocal interactions between neighboring cells through Notch and its ligands, which occur within groups of cells that have equivalent potentials to develop into specific heart progenitors. As a result of these mutual inhibitions, the Notch pathway becomes inactivated within one cell of an equivalence group, which allows it to become a heart progenitor (provided it receives the proper combination of other signals and cues, such as Dpp, Wingless, Timman, etc). By contrast, continued activation of the Notch pathway in the surrounding cells prevents them from being specified as heart progenitors despite the presence of cardiogenic regulators and signals. It is thought that Notch activity blocks the functions of cardiogenic signals in these cells although the molecular mechanisms of this interference are not known. Based on several genetic observations in Drosophila, it appears that this type of reciprocal signaling controls how both the cardioblast and pericardial cell progenitors are singled out from larger cell clusters. In particular, mutant
embryos in which Notch or Delta is inactive produce strongly increased numbers of cardioblasts\textsuperscript{125,126} and pericardial progenitors.\textsuperscript{127} Although these embryos form disrupted dorsal vessels with highly increased numbers of cardioblasts, their pericardial progenitors fail to generate any mature pericardial cells due to a subsequent requirement for Notch in this lineage (see next paragraph).

The specification of cardiac progenitor cells in \textit{Drosophila} is followed by a cell division which in many, if not all, cases is asymmetric and generates two daughter cells with different identities. For example, one particular progenitor divides to generate one pericardial founder and one somatic muscle founder cell, whereas another lineage generates one cardioblast and another type of pericardial cell from a common progenitor.\textsuperscript{33,94,127} Similar to analogous processes during neuronal development, differential activity of Notch is responsible for the acquisition of asymmetric cardiac cell fates in the two daughter cells. Differential Notch signaling in the two daughters is achieved by the differential segregation of an intracellular inhibitor of the Notch pathway during mitosis, which is encoded by the \textit{numb} gene. In \textit{numb} mutant embryos, there are equal levels of Notch signaling activity in the two daughter cells and therefore two identical cell identities are generated. Through this mechanism, Notch signaling and asymmetrically segregating Notch pathway inhibitors play key roles in the diversification of cardiac cell fates.

The most informative studies on the role of Notch signaling in vertebrate cardiogenesis have been performed in the \textit{Xenopus} system.\textsuperscript{128} The available data indicate that the function of Notch in vertebrate cardiogenesis may be analogous to the Notch-dependent lateral inhibition processes in \textit{Drosophila}. \textit{Xenopus Serrate1} and \textit{Notch1} are initially expressed in a pattern overlapping with one another and with \textit{Nkx2-5}, while during subsequent stages (before and during myocardial differentiation) expression refines such that \textit{Serrate1} becomes restricted largely to dorsolateral (presumptive mesocardial and pericardial roof) areas of the heart field. Conditional activation of the Notch pathway using activated versions of Notch or \textit{Su(H)} results in upregulation of \textit{Serrate1} and inhibition of myocardial differentiation, which reflects the situation that is normally observed in the dorsolateral areas of the heart field. Conversely, conditional inactivation of the pathway with dominant-negative components causes ectopic expression of myocardial markers and an expansion of the heart.\textsuperscript{128} Based on these observations and the observed lack of an effect on \textit{Nkx2-5} and \textit{Gata4} expression in these experiments, it has been proposed that endogenous Notch signaling influences the selection between myocardial and mesocardial/pericardial roof cell fates within the \textit{Nkx2-5}–expressing area of the heart field.

The direct target genes of Notch in heart development are not known. At least in certain contexts, the \textit{Hrt} (=\textit{Hesr1/Hey}) genes of the \textit{Hairy/Enhancer of Split} family were proposed as candidates,\textsuperscript{129} although there is presently no evidence that the differential expression of these genes along the anteroposterior axis of the heart tube is controlled via Notch signaling. Nevertheless, the observations in \textit{Xenopus}, the heart defects of hypomorphic \textit{Notch2} mouse mutant embryos,\textsuperscript{130} and the heart abnormalities associated with \textit{Jagged1} haploinsufficiencies in human Alagille syndrome patients (see review\textsuperscript{131}) indicate that Notch signaling is likely to be required during multiple processes in early vertebrate cardiogenesis. In this context, it is interesting to note that the Notch-dependent areas in the frog heart field probably correspond to the anterior heart-forming fields of the mouse and chick.\textsuperscript{4} Indeed, the heart defects in the Alagille syndrome, which focus on the anterior outflow tracts, could indicate that in amniotes, Notch signaling has an analogous role in the medial-lateral subdivision of the heart field as in frogs that, in this case, may involve the delayed specification of myocardium of the outflow region.

\textbf{Conclusion and Perspectives}

Through the powerful combination of embryological, molecular, and genetic approaches in a number of vertebrate and invertebrate model systems, we have gained significant insight into the molecular programs controlling early events of cardiogenesis. The properties that have been ascribed to particular signaling molecules and some of their targets provide molecular explanations for many of the classical embryological findings on cardiac induction.

It has become apparent that, although a complete disruption of any of the major pathways in early heart development results in embryonic lethality, more subtle disruptions by haploinsufficiencies and hypomorphic or dominant-negative mutations can lead to malfunctioning of the heart at later stages during the lifespan of an organism.\textsuperscript{51,132,133} From a clinical perspective, we can therefore anticipate that the growing knowledge on early pathways in cardiogenesis and the essential genes will be increasingly useful for the understanding and diagnosis of heart disease. Moreover, there is mounting evidence of cardiomyocyte regeneration in the mammalian heart, be it through the contribution of resident stem cells, circulating stem cells, or bone marrow cells that can be mobilized to the heart (see review\textsuperscript{134}). It is therefore conceivable that the knowledge gained on the roles of early signals in normal cardiogenesis will provide valuable guidelines in designing future stem cell therapies that would require expansion, reprogramming, and proper differentiation of cardiogenic stem cell populations.

\textbf{Note Added in Proof}

A recently published study (Pandur et al\textsuperscript{135}) has indeed demonstrated that Wnt-11 is positively required for heart induction through a noncanonical Wnt signaling pathway.

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