DEVELOPMENTAL DYNAMICS 222:552–563 (2001)

REVIEWS

A PEER REVIEWED FORUM

Cardiac Patterning and Morphogenesis in Zebrafish

DEBORAH YELON*

Developmental Genetics Program, Department of Cell Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York

ABSTRACT **Development of the embryonic vertebrate heart requires the precise coordination of pattern formation and cell movement. Taking advantage of the availability of zebrafish mutations that disrupt cardiogenesis, several groups have identified key regulators of specific aspects of cardiac patterning and morphogenesis. Several genes, including *gata5*, *fgf8*, *bmp2b*, *one-eyed pinhead*, and *hand2*, have been shown to be relevant to the patterning events that regulate myocardial differentiation. Studies of mutants with morphogenetic defects have indicated at least six genes that are essential for cardiac fusion and heart tube assembly, including *casanova*, *bonnie and clyde*, *gata5*, *one-eyed pinhead*, *hand2*, *miles apart*, and *heart and soul*. Furthermore, analysis of the *jekyll* gene has indicated its important role during the morphogenesis of the atrioventricular valve. Altogether, these data provide a substantial foundation for future investigations of cardiac patterning, cardiac morphogenesis, and the relationship between these processes.**

Key words: heart; myocardium; ventricle; atrium; cardiac fusion; endoderm; valve; *nkx2.5*; *gata5*; *fgf8*; *hand2*; *miles apart*; *heart and soul*; *jekyll*

INTRODUCTION

Organogenesis is a multifaceted and complex developmental process. Formation of an embryonic organ involves the specification and differentiation of multiple cell lineages. Furthermore, assembly of these different cell types into the final organotypic form requires precise regulation of cell movements and cell–cell interactions. Ultimately, the coordination of patterning and morphogenesis is the key to successful organ formation.

The heart is the first organ to form and function in the vertebrate embryo and has, therefore, been a popular topic for the study of the fundamental principles underlying organogenesis. Even a relatively simple organ like the embryonic heart tube is challenging to assemble. Initially, intricate patterning processes regulate the induction of the diverse cell lineages found in the heart. These cell types include myocardiocytes, muscular cells that provide cardiac contractility; and endocardiocytes, endothelial cells that provide continuity with the vascular endothelium (Fig. 1). Additional lineage diversification creates subpopulations such as ventricular and atrial myocardiocytes that have distinct histologic and physiological characteristics (DeHaan, 1965; Satin et al., 1988; Lyons, 1994; Franco et al., 1998). Following the events of cardiac patterning, multiple phases of morphogenesis are required to organize the diverse cardiac lineages into the form of the heart tube. The myocardium and endocardium form the outer and inner concentric layers of the tube, and the ventricular and atrial cells populate their respective chambers (Fig. 1). Cardiac patterning, cardiac morphogenesis, and the relationships between these processes are presumably tightly regulated to ensure effective embryonic circulation.

Several vertebrate model organisms, including chick, frog, mouse, and zebrafish, are suitable for investigating the genetic regulation of cardiogenesis. Many features of the zebrafish embryo make it especially appealing for cardiac studies (Stainier and Fishman, 1994). First, the externally fertilized and transparent zebrafish embryo permits extensive, high-resolution observation of cardiac cells throughout their development (Fig. 1B). Furthermore, zebrafish do not require a functional cardiovascular system for survival during embryogenesis (Pelster and Burggren, 1996), allowing careful analysis of cardiac defects without the confounding context of a dying embryo. Finally, the zebrafish presents excellent opportunities for large-scale genetic screens, primarily due to its small size, fecundity, and brief generation time (Westerfield, 1995). Thus, it is possible to identify and study mutations affecting many aspects of zebrafish cardiogenesis (Chen et al., 1996; Stainier et al., 1996; Alexander et al., 1998).

*Correspondence to: Deborah Yelon, Developmental Genetics Program, Department of Cell Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016. E-mail: yelon@saturn.med.nyu.edu

Received 6 August 2001; Accepted 6 September 2001

DOI 10.1002/dvdy.1212

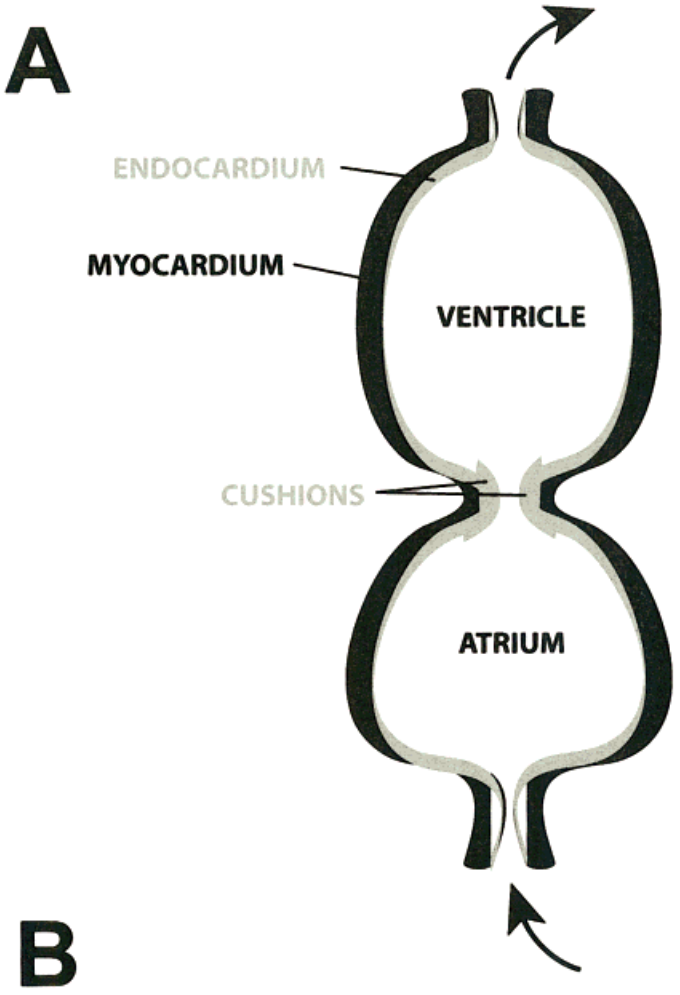

Fig. 1. Morphology of the embryonic vertebrate heart. **A:** Schematic of an embryonic vertebrate heart tube. The heart tube is composed of two concentric layers of tissue: the outer myocardium and the inner endocardium. The heart tube is also divided into two major chambers: the ventricle and the atrium. Cardiac cushions form at the atrioventricular boundary. Arrows indicate direction of blood flow. **B:** Lateral view of a 2-day-old zebrafish embryo, anterior to the right. The heart is located posterior to the head and anterior to the yolk. The ventricle is in focus, and its myocardial and endocardial layers are visible. The atrium (filled with blood) is out of the focal plane.

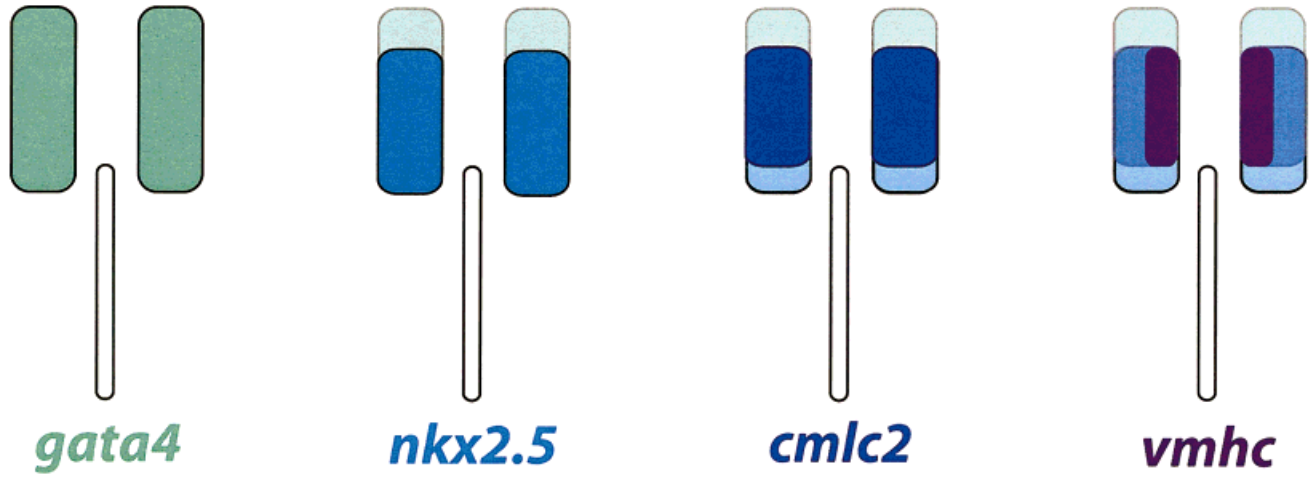

Fig. 2. Gene expression patterns reveal subpopulations within the anterior lateral plate mesoderm (ALPM). Schematics compare overlapping expression patterns at the 15-somite stage, showing dorsal views of the ALPM, with anterior at the top. Notochord is depicted as a white rod. At this stage, *gata4* is expressed in a large portion of the ALPM, including the cells that express *nkx2.5* (Serbedzija et al., 1998). *cmlc2* expression is restricted to the *nkx2.5*-expressing cells that are anterior to the tip of the notochord (Yelon et al., 1999). *vmhc* expression is restricted to a medial subpopulation of the *cmlc2*-expressing cells (Yelon et al., 1999).

Taking advantage of the benefits of the zebrafish as a model organism, several groups have successfully identified key regulators of cardiac development. This review will cover a selection of these recent findings, beginning with a discussion of studies relevant to the pattern formation events that regulate myocardial differentiation. The next section will describe our understanding of the morphogenetic events that organize heart tube assembly and formation of the atrioventricular valve. Finally, discussion will focus on how proper pattern formation sets the stage for proper morphogenesis, as well as speculation regarding promising future directions for the study of cardiac development in zebrafish.

SPECIFICATION AND DIFFERENTIATION OF THE ZEBRAFISH MYOCARDIUM

In all vertebrates, the myocardial progenitors arise bilaterally, within anterior portions of the lateral plate mesoderm (LPM). Fate maps of the zebrafish embryo indicate that the myocardial progenitors are originally located in the ventrolateral marginal zone of the early blastula (512-cell stage; Stainier et al., 1993). In the late blastula (40% epiboly), the myocardial progenitors are found within the three tiers of blastomeres closest to the embryonic margin (Warga and Nüsslein-Volhard, 1999). These progenitors are clustered in two groups, one on either lateral side of the embryo (roughly between 60 and 120 degrees and 240 and 300 degrees, if the dorsal midline of the embryonic margin is defined as 0 degrees). These blastomeres are among the first to involute when gastrulation begins at 50% epiboly (Warga and Kimmel, 1990). During early somitogenesis, the myocardial progenitors, along with the other cell types that compose the LPM, converge toward the embryonic midline and extend toward the anterior of the embryo (Stainier and Fishman, 1992; Stainier et al., 1993).

Although fate maps indicate the location of the myocardial progenitors at early stages, it is not yet known precisely when myocardial specification occurs. However, gene expression patterns reveal that the LPM is already elaborately patterned by the onset of somitogenesis. Anterior-posterior (A-P) patterning is particularly apparent. For example, the mesodermal expression of the transcription factor gene *gata4* is restricted to the anterior lateral plate mesoderm (ALPM) (Serbedzija et al., 1998) (Fig. 2). The expression of another transcription factor gene, *hand2*, overlaps substantially with *gata4* gene expression in the ALPM and is also found in more posterior portions of the LPM (Angelo et al., 2000; Yelon et al., 2000). A subset of ALPM cells express *nkx2.5* (Chen and Fishman, 1996; Lee et al., 1996), a transcription factor gene that is considered a marker of precardiac mesoderm in many vertebrates (Evans, 1999) (Fig. 2). In zebrafish, most, but not all, of the *nkx2.5*-expressing cells terminally differentiate into myocardiocytes (Serbedzija et al., 1998; Yelon et al., 1999). Differentiating myocardiocytes initiate expression of genes that encode cardiac sarcomere proteins, such as *cmlc1* and *cmlc2* (Reiter et al., 1999; Yelon et al., 1999) (Fig. 2). These myocardial precursors are divided into ventricular and atrial subpopulations by the expression pattern of *vmhc*, which encodes a ventricle-specific myosin heavy chain (Yelon et al., 1999). *vmhc* expression initiates in a medial subpopulation of myocardial precursors, revealing medial-lateral patterning within the ALPM (Yelon et al., 1999) (Fig. 2). The medial $vmhc^{+}$ cells are thought to be the

ventricular precursors, and the lateral *vmhc*$^-$ cells are thought to be the atrial precursors.

Altogether, the early expression patterns of cardiac markers in the LPM suggest that several levels of pattern formation are involved in the generation of myocardial tissue, including induction of *nkx2.5* expression, selection of myocardial precursors, and division of myocardial precursors into ventricular and atrial lineages. Analysis of zebrafish mutations that disrupt myocardial differentiation has revealed several genes that are essential for these specific aspects of LPM patterning.

Which Factors Induce *nkx2.5* Expression?

Four genes — *gata5*, *fgf8*, *bmp2b*, and *one-eyed pinhead* — have been shown to play early roles during myocardial specification. The transcription factor gene *gata5* is expressed in a broad region of the ALPM, including the portion that expresses *nkx2.5* (Reiter et al., 1999). Mutation of the *faust/gata5* locus results in a dramatic reduction of *nkx2.5* expression (Reiter et al., 1999). Additionally, few myocardial cells differentiate in *faust* (*fau*) mutants, and differentiation of ventricular precursors is especially inhibited. Overexpression of *gata5* can cause the ectopic expression of *nkx2.5* and several myocardial markers (Reiter et al., 1999). Altogether, these data indicate that Gata5 is necessary and sufficient for the induction of *nkx2.5*-expressing precardiac mesoderm. However, *gata5* is expressed in many cells that do not become myocardium, such as other ALPM populations and the endodermal progenitors. Therefore, it is likely that *gata5* cooperates with other essential regulators during the specification of cardiac precursors.

Like Gata5, the growth factor Fgf8 is also important for the initial induction of *nkx2.5* expression. *fgf8* expression can be detected in *nkx2.5*-expressing precardiac mesoderm and neighboring cells during early somitogenesis, and mutation of the *acerebellar/fgf8* locus causes a reduction of *nkx2.5* expression (Reifers et al., 2000). In addition, *acerebellar* (*ace*) mutants have smaller hearts than their wild-type siblings, with the ventricle being more strongly affected than the atrium. Implanting Fgf8-soaked beads near the ALPM can slightly expand the expression of *nkx2.5* (Reifers et al., 2000). Perhaps regionally restricted Fgf8 signaling collaborates with the more widely distributed Gata5 activity to select the precardiac portion of the ALPM.

Other signaling pathways also contribute to the regulation of myocardial specification. The growth factor Bmp2b is important for the assignment of ventrolateral fates in the zebrafish embryo (Kishimoto et al., 1997; Nguyen et al., 1998). Thus, *swirl/bmp2b* mutants have a severe reduction of the derivatives of ventrolateral blastomeres, including *nkx2.5*-expressing precardiac mesoderm (Kishimoto et al., 1997; Reiter et al., 2001). Accordingly, very little differentiated myocardium forms in *swirl* (*swr*) mutants. *swr* mutants exhibit reduced *gata5* expression during and after gastrulation, and overexpression of *gata5* can rescue *nkx2.5* expression and myocardial differentiation in *swr* mutants (Reiter et al., 2001). Together, these observations suggest that Bmp2b signaling acts through Gata5 to regulate *nkx2.5* induction.

Like Bmp2b, the EGF-CFC protein One-eyed pinhead (Oep) is required for *gata5* expression. Oep is an essential cofactor for Nodal signaling, and *oep* is expressed both maternally and zygotically (Zhang et al., 1998; Gritsman et al., 1999). The defects in mutant embryos lacking only zygotic Oep (*Zoep*) include reduction of *gata5* expression, *nkx2.5* expression, and myocardial differentiation, especially ventricular differentiation (Reiter et al., 2001). *gata5* overexpression can restore *nkx2.5* expression and myocardial differentiation in *Zoep* mutants (Reiter et al., 2001), indicating that Nodal signaling, like Bmp2b signaling, can act through Gata5 to regulate early steps of myocardial development.

How Are Myocardial Precursors Selected?

Because a subset of *nkx2.5*-expressing cells do not differentiate into myocardial tissue, it is likely that additional factors, besides those required for *nkx2.5* induction, contribute to the selection of the myocardial precursors. In this regard, it is interesting to note that the bilateral populations of *nkx2.5*-expressing cells are located near the embryonic axis, at the anterior-posterior level of the prechordal plate/notochord boundary (Fig. 2). Most of the *nkx2.5*-expressing cells lie anterior to the rostral tip of the notochord, but the most posterior cells in this population lie beside the notochord (Serbedzija et al., 1998). The *nkx2.5*-expressing cells near the prechordal plate begin to express myocardial differentiation markers around the 13-somite stage (Yelon et al., 1999). However, the cells that are located adjacent to the notochord do not express terminal differentiation markers (Yelon et al., 1999) and do not contribute to the myocardium (Serbedzija et al., 1998). When notochord cells are removed (by means of laser-mediated ablation or mutation of the *no tail* locus), the *nkx2.5*-expressing cells near the notochord are able to become myocardial (Goldstein and Fishman, 1998). Thus, the notochord seems to play a repressive role, restricting *nkx2.5*-expressing cells from initiating myocardial differentiation. The molecular nature of the proposed negative signal from the notochord (and/or proposed positive signal from the prechordal plate) has not been identified.

Several zebrafish genes are required for the formation of the proper number of myocardial precursors. *fau*, *ace*, *swr*, and *Zoep* mutants exhibit reduced numbers of differentiating myocardial precursors; however, these defects may be secondary to the loss of *nkx2.5* expression in these embryos. The effects of *nkx2.5* loss-of-function have not yet been studied in zebrafish, but *nkx2.5* overexpression can induce the ectopic expression of some myocardial markers (Chen and Fishman, 1996). In contrast to the activities of Gata5, Fgf8,

Bmp2b, and Oep, the transcription factor Hand2 is dispensable for *nkx2.5* induction but essential for myocardial differentiation (Yelon et al., 2000). *hand2* is expressed in a broad domain of the ALPM, and mutation of the *hands off*/*hand2* locus results in a dramatic reduction of differentiated myocardium, especially ventricular myocardium, without affecting the initial expression of *nkx2.5* (Fig. 3A). It is not yet clear how the broad expression of *hand2* in the ALPM regulates the selection of myocardial precursors within the *nkx2.5*-expressing population. Further study of the binding partners and transcriptional targets of Hand2 will be required to clarify its role in providing competence for myocardial differentiation downstream of *nkx2.5* induction.

Which Genes Establish the Ventricular and Atrial Lineages?

Fate mapping studies have suggested that the ventricular and atrial lineages are physically separate by the 2,000-cell stage in zebrafish (Stainier et al., 1993). However, it is not understood precisely when or how ventricular and atrial fates are specified. The medially restricted expression of *vmhc* is the earliest known indication of molecular diversification of the myocardial precursors in zebrafish (Yelon et al., 1999). Several genes are known to be required for normal *vmhc* expression. For example, *hand2* is influential during the induction of *vmhc* expression (Yelon et al., 2000). *gata5*, *fgf8*, and *Zoep* also influence the formation of ventricular precursors (Reiter et al., 1999; Reifers et al., 2000; Reiter et al., 2001), but it is not clear whether the ventricular defects in *gata5*, *fgf8*, and *Zoep* mutants are secondary to the loss of *nkx2.5* expression.

It is interesting to note that all mutants known to have *vmhc* expression defects also have problems with myocardial differentiation in general. *han*, *fau*, *ace*, and *Zoep* mutants exhibit reduced numbers of atrial cells, in addition to significant reductions in ventricular tissue. The same is true for *pandora* mutants (Yelon et al., 1999) and other recently identified mutants (Alexander et al., 1998). It is not yet clear what this trend indicates about the molecular mechanisms of cardiac patterning. Perhaps some aspect of general myocardial differentiation is an important prerequisite for ventricular differentiation but is less critical for atrial differentiation. Alternatively, the same molecular pathways may be used during separate processes of myocardial differentiation and ventricular differentiation. Further study of *han*, *fau*, *ace*, and *Zoep* as well as cloning of other genes affecting ventricular development will help to resolve the mechanistic relationship between general myocardial differentiation and ventricle-specific differentiation.

Little is known about the genes that contribute to atrial specification and differentiation in zebrafish. Studies in chick and mouse have implicated retinoic acid (RA) signaling as a positive influence on atrial development (Yutzey et al., 1994; Moss et al., 1998;

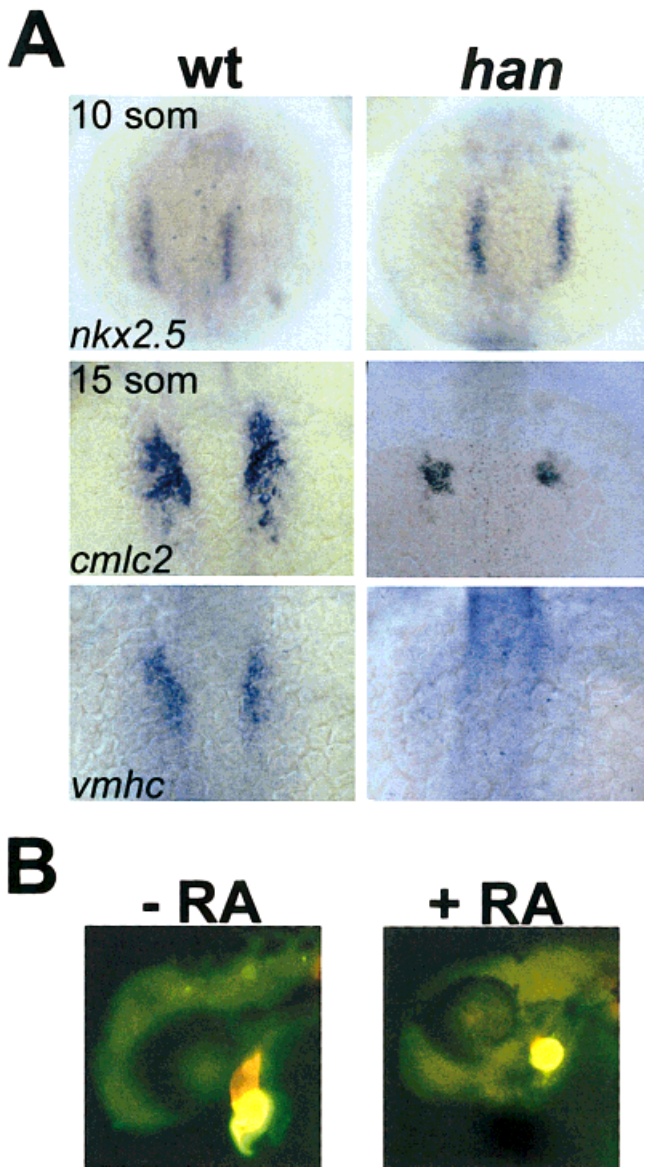

Fig. 3. Examples of zebrafish embryos with cardiac patterning defects. **A:** Hand2 is required for the selection of myocardial precursors and for ventricular differentiation. However, Hand2 is dispensable for *nkx2.5* induction. Images are dorsal views, anterior to the top, comparing expression patterns by in situ hybridization in wild-type and *hands off* (*han*) mutant embryos. At the 10-somite stage, *nkx2.5* expression is identical in wild-type and *han* mutant embryos. At the 15-somite stage, *cmlc2* is expressed in the myocardial precursors in wild-type embryos, but very few cells express *cmlc2* in *han* mutant embryos. Also, although *vmhc* is expressed in the medial myocardial precursors in wild-type embryos, *vmhc* is not expressed in *han* mutant embryos at this stage. Images are adapted from Yelon et al. (2000). **B:** Exogenous retinoic acid (RA) treatments inhibit ventricular development in zebrafish. Late gastrula embryos were treated with RA (as in Stainier and Fishman, 1992) and raised until 30 hours postfertilization (hpf). Immunofluorescent images are lateral views of 30-hpf embryos, anterior to the left, stained with MF20 (TRITC) and S46 (FITC) (Alexander et al., 1998). In these double exposures, red fluorescence indicates ventricular myocardium, whereas yellow fluorescence indicates atrial myocardium. The heart of the untreated embryo has two chambers, a red ventricle and a yellow atrium. The heart of the treated embryo has a yellow atrium and a severely truncated red ventricle.

Xavier-Neto et al., 1999; Niederreither et al., 2001). In zebrafish, exogenous RA treatments appear to inhibit ventricular development, suggesting that endogenous RA signaling influences the balance between atrial and ventricular specification (Stainier and Fishman, 1992) (Fig. 3B). Further study will be required to determine exactly how RA signaling affects the promotion of atrial development and/or the repression of ventricular development. It will be particularly important to establish whether RA signaling is required within the myocardium or within other tissues that influence myocardial development.

Conservation of Patterning Mechanisms

These investigations have identified a panel of key players during cardiac patterning in zebrafish. Although not all of these factors have been studied in the same manner in other organisms, it is likely that their functions are conserved across taxa. For example, many experiments suggest that the roles of the Bmp, Nodal, and RA signaling pathways during heart development are similar in different species (e.g., Schultheiss et al., 1997; Xu et al., 1998, 1999; Rosenthal and Xavier-Neto, 2000). Rather than discuss all of the experiments that demonstrate similarities in cardiac patterning mechanisms, this section will simply describe data relevant to the conserved roles of Gata transcription factors during cardiogenesis. In *Drosophila*, the Gata factor gene *pannier* is required for formation of cardiac cells, and misexpression of *pannier* can produce ectopic cardiac cells (Gajewski et al., 1999); thus, *pannier* appears to function like *gata5*. In vertebrates, including zebrafish, there are three Gata transcription factor genes (*gata4*, *gata5*, and *gata6*) expressed in the ALPM (Charron and Nemer, 1999). Overexpression studies in frog and chick have demonstrated that Gata4, Gata5, and Gata6 can induce expression of myocardial genes (Jiang and Evans, 1996; Jiang et al., 1998). Furthermore, promoter analysis in transgenic mice and frogs has demonstrated that Gata factor consensus sites are required for the regulation of *nkx2.5* expression (Searcy et al., 1998; Lien et al., 1999; Reecy et al., 1999; Sparrow et al., 2000). Even so, mice lacking any one of the *gata4*, *gata5*, or *gata6* genes do not exhibit significant defects in cardiac patterning (Kuo et al., 1997; Molkentin et al., 1997, 2000; Morrisey et al., 1998; Koutsourakis et al., 1999), suggesting some functional redundancy between Gata factors. Overall, these data strongly suggest conservation of Gata factor function during cardiac patterning in species ranging from insects to mammals.

Although the roles of many cardiogenic factors are likely to be conserved, precise mechanisms could also vary between species. For example, although *hand2* regulates early aspects of myocardial differentiation in zebrafish, *hand2* mutant mice do not exhibit cardiac defects until after heart tube formation (Srivastava et al., 1997). One possible explanation for this discrepancy is that the related mouse gene *hand1* may be able to compensate for a loss of *hand2* function at early stages. Further investigation of the early roles of mouse *hand* genes will resolve whether the requirements for Hand activity are similar across taxa.

ASSEMBLY AND MORPHOGENESIS OF THE ZEBRAFISH HEART TUBE

The transformation of bilateral populations of patterned LPM into a midline heart tube involves a complex series of cell movements. By the 16-somite stage, zebrafish myocardiocytes are positioned in bilateral sheets with the ventricular precursors more medial than the atrial precursors (Yelon et al., 1999). With the ventricular cells in the lead, the myocardiocytes move toward the midline where they meet each other, initiating the process called "cardiac fusion" (Fig. 4A). Specifically, fusion begins as the two sheets of myocardial precursors bring together the posterior portions of their medial edges, followed by an anterior closure that creates a central lumen. Thus, the myocardial precursors form a shallow cone, with the apex of the cone raised dorsally around the lumen. Next, the myocardial cone is transformed into a linear tube. This process begins when the apex of the cone tilts posteriorly and to the right, shifting the cone's axis from a dorsal-ventral plane to an anterior-posterior plane. The tilted cone then extends, elongating its anterior-posterior axis until the base of the cone coalesces into a tube. At this point, the heart tube is angled such that its ventricular end is to the right of its atrial end.

Cardiac morphogenesis continues to be highly dynamic after the primitive heart tube has formed. For example, gradual movements transform the linear heart tube into a looped organ, with the ventricle positioned to the right of the atrium (Fig. 4B). Additionally, by 48 hour postfertilization (hpf), the separation of the ventricle and the atrium is further defined by the formation of cardiac cushions at the atrioventricular boundary (Hu et al., 2000). Cardiac cushion formation is thought to begin with the induction of specialized subtypes of myocardial and endocardial cells at the junction between the ventricle and the atrium (Eisenberg and Markwald, 1995). Communication between the myocardial and endocardial layers then results in an epithelial-to-mesenchymal transition of the endocardial cells, thereby creating cushions. With time, the atrioventricular cushions differentiate into the flaps of the atrioventricular valve.

The diverse aspects of cardiac morphogenesis involve many levels of genetic regulation. Recent studies of zebrafish mutations that cause morphogenetic defects have revealed several genes that are essential for specific aspects of heart formation, including cardiac fusion and cardiac cushion formation.

What Drives Cardiac Fusion?

A simple model of the regulation of cardiac fusion predicts at least three requirements for the movement of myocardiocytes toward the midline. First, the myo-

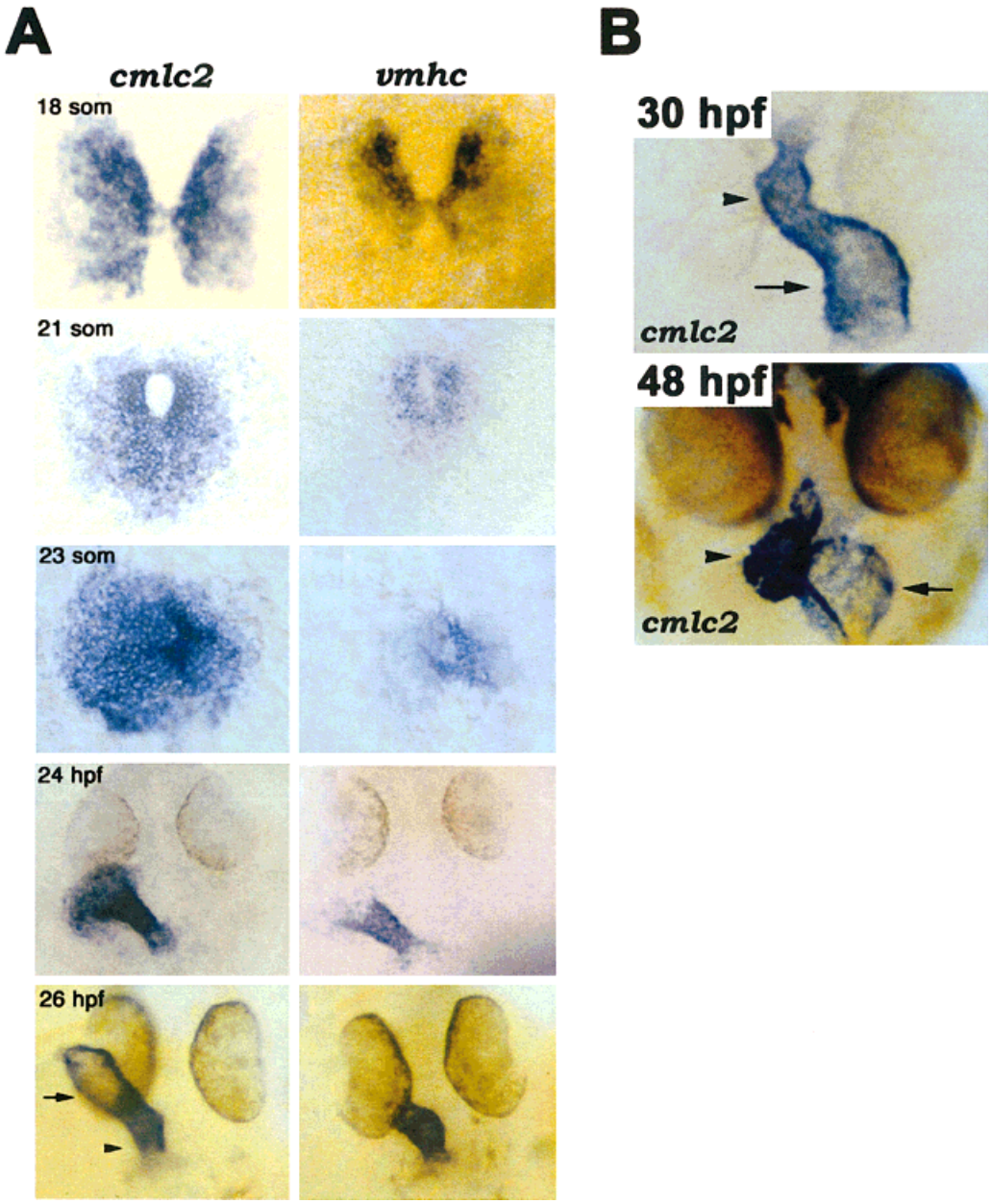

Fig. 4. Cardiac morphogenesis involves a series of dynamic cell movements. **A:** Comparison of *cmlc2* and *vmhc* expression patterns by in situ hybridization illustrates the complex reorganization of myocardiocytes during cardiac fusion and heart tube assembly (Yelon et al., 1999). Images are dorsal views of wild-type embryos, anterior at the top. The embryo's left side is to the left of the figure. At the 18-somite stage, cardiac fusion begins as the ventricular precursors initiate contact. At the 21-somite stage, a shallow cardiac cone has formed, with the ventricular precursors raised slightly dorsally at its apex. At the 23-somite stage, the apex of the cone has tilted posteriorly and to the right. By 24 hours postfertilization (hpf), the cone has begun to extend into a tube. By 26 hpf, tube formation is complete. The ventricular portion of the linear tube is indicated with an arrowhead, and the atrial portion of the linear tube is indicated with an arrow. Images are adapted from Yelon et al. (1999). **B:** Cardiac looping gradually reorients the ventricle and the atrium. Images are head-on views, comparing *cmlc2* expression in a looping heart (30 hpf) and a looped heart (48 hpf). The embryo's left side is to the right of the figure. *cmlc2* is expressed in both the ventricle and the atrium (Yelon et al., 1999). The ventricle is indicated with arrowheads, and the atrium is indicated with arrows. By 30 hpf, the linear tube has become curved. By 48 hpf, the ventricle is to the right of the atrium, and constriction is evident at the atrioventricular boundary. Images are adapted from Yelon et al. (1999).

cardiocytes would require a substrate, either cells or extracellular matrix, to crawl across. Second, active migration would require proper differentiation of the myocardiocytes. Finally, to direct migration, some type of signal would attract the cells to the midline or repel them from lateral locations. Multiple zebrafish muta-

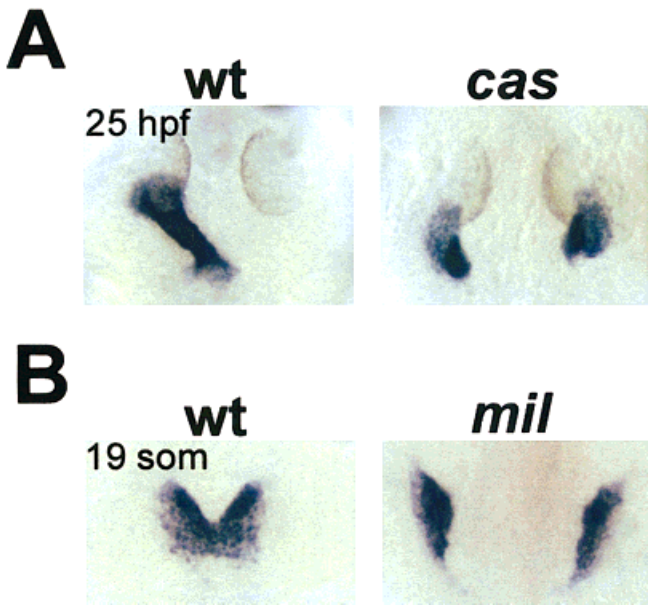

Fig. 5. Examples of zebrafish mutants with defects in cardiac fusion. **A:** *casanova* (*cas*) mutants exhibit cardia bifida. Images are dorsal views, anterior to the top, displaying *cmlc2* expression in wild-type and *cas* mutant siblings. By 25 hpf, heart tube assembly is nearly complete in the wild-type embryo. However, in the *cas* mutant embryo, the myocardiocytes have not migrated toward the midline. Instead, these cells have formed bilateral cardiac cones (Yelon et al., 1999). **B:** *mil* mutants exhibit cardia bifida. Images are dorsal views, anterior to the top, displaying *cmlc2* expression in wild-type and *mil* mutant siblings. At the 19-somite stage, cardiac fusion is under way in the wild-type embryo, but the myocardiocytes in the *mil* mutant embryo have not moved to the midline. As shown above for *cas*, these cells will remain in bilateral positions (Kupperman et al., 2000).

tions interfere with the movement of myocardiocytes toward the midline. In these cases, cardiac fusion does not occur, and mutant embryos exhibit two lateral myocardial clusters instead of a single midline heart. This condition is called "cardia bifida." Characterization of cardia bifida mutations and cloning of the affected genes has revealed genes involved with each of the three proposed requirements for myocardial migration.

As the myocardiocytes move toward the midline, they are in close contact with a layer of endodermal cells that are also moving medially to form the anterior portion of the gut tube. This proximity and coordinated timing of movement suggests that the migrations of these two populations could be interdependent. In support of this hypothesis, all mutations known to inhibit the formation of endodermal progenitors — *casanova*, *bonnie and clyde*, *fau*, and *Zoep* — also cause cardia bifida (Alexander et al., 1999; Kikuchi et al., 2000; Reiter et al., 1999, 2001) (Fig. 5A). Each of these four genes has been cloned (*casanova=sox* transcription factor (Dickmeis et al., 2001; Kikuchi et al., 2001], *bonnie and clyde=mix* transcription factor [Kikuchi et al., 2000]), and in each case, the cloned genes are thought to function autonomously during the formation of endodermal progenitors. In the case of *Zoep*, formation of a midline heart tube in mutant embryos can be restored if the endodermal population is rescued, suggesting that the loss of endoderm (as opposed to the myocardial defects) is responsible for the cardiac fusion defects in this particular case (Peyrieras et al., 1998).

Although anterior endoderm is necessary for normal cardiac fusion, it is not solely sufficient to drive the process. Cardia bifida is also observed in mutants that form a gut tube normally, indicating that myocardial migration is not an entirely passive process and also that endodermal migration does not depend on myocardial migration. For example, *hands off* mutants exhibit cardia bifida in the presence of a normal gut, suggesting that *hand2* plays a LPM-autonomous role in the regulation of cardiac fusion (Yelon et al., 2000). In addition to *han*, all other characterized mutants with defects in myocardial and ventricular differentiation also exhibit a delay or complete failure of cardiac fusion (Reiter et al., 1999; Yelon et al., 1999, 2001; and D.Y., unpublished data). These observations suggest that some aspect of normal myocardial differentiation and patterning creates competence for myocardial migration.

In addition to requirements in the endoderm and in the myocardium, cardiac fusion is likely to require a signal directing cardiac migration toward the midline. The precise nature of this signal is not yet known. However, generation or reception of this signal seems to involve the function of *miles apart* (*mil*), which encodes a lysosphingolipid G-protein–coupled receptor (Kupperman et al., 2000). Mutation of *mil* causes cardia bifida without disrupting gut formation or myocardial differentiation (Fig. 5B). Because *mil* is broadly expressed, it is not yet clear which tissue(s) require Mil activity to facilitate cardiac fusion. Mosaic analysis indicates that *mil* mutant cells can contribute to a midline heart tube in a wild-type embryo, and wild-type cells will not move to the midline on their own in the context of a *mil* mutant embryo. These data are compatible with the hypothesis that Mil activity is not required in the myocardium. However, it is also possible that *mil* could play a myocardium-autonomous role. In this alternate interpretation, activation of Mil signaling would result in directed migration only if a critical mass of myocardiocytes received the signal. Thus, *mil* mutant cells could be carried along with their wild-type neighbors, and individual wild-type cells would not be able to manifest directed migration. Further analysis will be required to determine where, when, and how activation of the Mil receptor influences myocardial migration. It will be especially interesting to study whether myocardial migration is an orchestrated group activity or the cumulative effect of many cells migrating individually.

Beyond recruitment of myocardial precursors to the midline, the regulation of cardiac cone formation and conversion of the cone into a tube is poorly understood.

Studies of the zebrafish mutation *heart and soul* (*has*) provide the first insight into the relevant signaling pathways. In *has* mutants, myocardiocytes migrate toward the midline relatively normally, although fusion appears aberrant (Yelon et al., 1999). Furthermore, the cardiac cone does not properly convert into a tube. Rather than tilt to the right, the ventricular apex remains stationary in *has* mutants. As a result, the atrium cannot form properly and the *has* mutant heart exhibits atrial tissue outside of the ventricle (Fishman and Chien, 1997; Yelon et al., 1999). Recently, the *has* locus has been shown to encode an atypical PKC molecule, similar to PKCλ (Horne-Badovinac et al., 2001; Peterson et al., 2001). In *Drosophila*, PKCλ forms a complex with two other proteins (Par-3/Bazooka and Par-6) (Muller and Wieschaus, 1996; Kuchinke et al., 1998; Wodarz et al., 2000; Petronczki and Knoblich, 2001). This complex is found at the apicolateral membrane of epithelial cells, and integrity of the complex is required for the maintenance of epithelial polarity. In *has* mutants, defects in epithelial polarity have been described in the swim bladder, the retina, and the neural tube but the relationship of epithelial polarity to heart tube assembly is not yet clear (Horne-Badovinac et al., 2001). Perhaps the migrating and fusing myocardiocytes behave as a polarized epithelium. Alternatively, the integrity of a different epithelial tissue (possibly the migrating endodermal progenitors) may be essential for myocardial movements within the cardiac cone. Future experiments examining the autonomy of the myocardial requirement for PKCλ may provide a connection between the role of *has* in the cardiac cone and the regulation of myocardial migration at earlier stages.

Which Signals Initiate Cardiac Cushion Formation?

Although most studies of zebrafish cardiac morphogenesis have focused on cardiac fusion, investigations of the *jekyll* (*jek*) mutation have provided an entry point to the molecular mechanisms of valve morphogenesis (Walsh and Stainier, 2001). *jek* mutants fail to form cardiac cushions at the atrioventricular boundary (Stainier et al., 1996). Recently, the *jek* locus has been shown to encode UDP-glucose dehydrogenase, an enzyme required for the synthesis of several proteoglycans (Walsh and Stainier, 2001). In *Drosophila*, the enzyme Sugarless (also a UDP-glucose dehydrogenase) is involved in the synthesis of heparan sulfate proteoglycans that are thought to be required for transduction of Fgf and Wg signals (Hacker et al., 1997; Lin et al., 1999). The signaling pathways inhibited by a loss of Jek function have not been identified. It is interesting to note that *jek* mutants do not exhibit normal restriction of gene expression in myocardial and endocardial cells at the atrioventricular boundary (Walsh and Stainier, 2001). This aspect of the *jek* mutant phenotype suggests that the loss of Jek activity affects proper definition of the atrioventricular region, without which cushion morphogenesis cannot proceed. It will be exciting to determine which signaling pathways, mediated by Jek-modified proteoglycans, establish cell identity at the atrioventricular boundary and whether these pathways operate in the myocardium, the endocardium, or both tissues.

Conservation of Morphogenesis Mechanisms

In contrast to the topic of cardiac patterning, cardiac morphogenesis has not been explored as thoroughly in other model organisms, making it difficult to discuss the conservation of roles of specific players. However, it is possible to comment on the conserved role of endoderm during cardiac fusion. As in zebrafish, mouse mutants with severe endodermal deficiencies exhibit cardiac fusion defects. For example, mice lacking *gata4* have dramatic reductions of endodermal tissue as well as cardiac fusion defects (Kuo et al., 1997; Molkentin et al., 1997). Furthermore, cardiac fusion can be rescued in *gata4* mutant mice by reconstituting the endoderm (Narita et al., 1997). Future studies will reveal whether additional requirements for cardiac fusion, such as Mil activity or the early functions of *hand* genes, also apply in other vertebrates.

PATTERNING AND MORPHOGENESIS ARE INTERTWINED

Having reviewed the processes of cardiac patterning and cardiac morphogenesis in zebrafish, it becomes apparent that these aspects of cardiogenesis are intimately related. Specifically, proper cardiac patterning is a prerequisite for proper cardiac morphogenesis. When aberrant patterning results in poor myocardial differentiation and reduction of ventricular myocardium, cardiac fusion does not proceed normally. When pattern formation in the myocardium or endocardium fails to properly establish the atrioventricular boundary, cushion morphogenesis does not occur. To understand the connection between patterning and morphogenesis thoroughly, it will be important to identify the specific genes, regulated by cardiac differentiation programs, that mediate heart formation. For instance, how does myocardial differentiation create competence to receive and respond to signals directing migration? Which components of the ventricle-specific differentiation program prepare the ventricular precursors to behave as the leading edge of a migrating population? How does specification of juxtaposed ventricular and atrial lineages lead to induction of an epithelial-mesenchymal transition at the atrioventricular boundary? These critical molecular links may emerge as the genes downstream of *gata5*, *fgf8*, *hand2*, and other regulators of cardiac patterning are identified. Given the rapid pace of zebrafish cardiac research, it should soon be possible to describe genetic pathways connecting early steps of pattern formation with later morphogenetic consequences.

PERSPECTIVES AND FUTURE DIRECTIONS

Characterization of zebrafish mutants and cloning of the affected genes has shed light on many aspects of the regulation of cardiac patterning and morphogenesis. Ongoing genetic screens focused on cardiac phenotypes will continue to provide new resources for the discovery of relevant zebrafish genes (Alexander et al., 1998). In addition, the advent of morpholino technology makes it possible to conduct reverse genetics in zebrafish. Morpholinos are highly stable antisense oligonucleotides that can be designed to reduce translation of a desired gene (Summerton and Weller, 1997). Thus, injection of morpholinos provides a rapid method to test the developmental role of a gene in zebrafish (Nasevicius and Ekker, 2000). This strategy will be very helpful in investigating potential regulatory genes that are expressed in the zebrafish heart. For example, morpholinos could allow analysis of the functions of *tbx5* and *tbx20*, T-box transcription factor genes expressed in zebrafish myocardiocytes (Begemann and Ingham, 2000; Griffin et al., 2000; Ahn et al., 2000). Studies in frogs and mice have suggested that *tbx5* may regulate early steps of myocardial differentiation, especially atrial differentiation (Horb and Thomsen, 1999; Liberatore et al., 2000; Hatcher et al., 2001). Similarly, morpholinos directed against *irx4*, an Iroquois transcription factor gene expressed in the zebrafish ventricle (D. Yelon and D.Y.R. Stainier, unpublished data), could reveal whether the role of *irx4* in ventricular differentiation, as proposed by mouse and chick studies (Bao et al., 1999; Chirstoffels et al., 2000; Bruneau et al., 2001), is conserved in zebrafish. Furthermore, morpholino technology could be broadly applied to investigate the functions of the many zebrafish ESTs generated from embryonic and adult heart cDNA libraries (Chen et al., 1998, unpublished observations; Ton et al., 2000).

As an additional approach for identification of key regulators, advances in combinatorial chemistry have created large libraries of small molecules that can be screened for their ability to cause desired embryonic phenotypes (Stockwell, 2000). A small-scale chemical screen has already identified small molecules that disrupt cardiac fusion (Peterson et al., 2000). Treatment with one of these molecules, called concentramide, can phenocopy the *heart and soul* mutant phenotype (Peterson et al., 2001). It will be exciting to determine which endogenous regulators are affected by concentramide and other potent small molecules.

To delve deeper into the genetic regulation of cardiogenesis, it will be critical for future studies to use other new technologies available for the zebrafish. In particular, to understand morphogenesis fully, it will be important to follow cell movements in live embryos. Recently, many groups have reported successful zebrafish transgenesis by using a variety of promoters to drive expression of GFP (e.g., Motoike et al., 2000; Park et al., 2000; Koster and Fraser, 2001). Transgenic lines expressing GFP in cardiac precursors would allow real-time imaging of cardiac morphogenesis in wild-type and mutant embryos, bringing a new level of resolution to our understanding of the wild-type process and the precise roles of key players.

Future studies using emerging technologies are likely to extend our understanding of myocardial patterning and morphogenesis. Furthermore, future experiments are likely to address other areas of interplay between pattern formation and movement in the zebrafish heart. In particular, one would expect advances in the study of left-right (L-R) axis formation to reveal genetic regulators of cardiac looping morphogenesis. In zebrafish and other vertebrates, initial establishment of the embryonic L-R axis leads to asymmetric gene expression patterns, such as the left-sided expression of the transcription factor gene *pitx2* in the LPM (for a recent review of L-R patterning, see Burdine and Schier, 2000). At later stages, L-R asymmetric cues somehow control the rightward tilting of the ventricular apex of the cardiac cone and the looping morphogenesis that positions the ventricle to the right of the atrium. Although significant attention has been paid to zebrafish mutations that randomize L-R asymmetry (e.g. Chen et al., 1997; Yan et al., 1999; Bisgrove et al., 2000; Chin et al., 2000), little is known regarding the molecular mechanisms that translate L-R patterning into directed cardiac morphogenesis. Future studies of mutations that interfere with cardiac looping without disrupting initial L-R patterning will be particularly informative in this regard.

Together, the provocative nature of recent findings and the exciting potential of new technologies should rapidly lead to new levels of comprehension of zebrafish cardiogenesis. Based on the conservation of developmental mechanisms, it is likely that these future discoveries will be relevant to cardiac development in other organisms, although it will be particularly interesting to learn how the details of regulation differ between species. As conserved paradigms emerge, it will be rewarding to determine whether the principles that govern cardiogenesis apply to the control and coordination of patterning and morphogenesis of other embryonic organs as well.

ACKNOWLEDGMENTS

I thank Alex Schier and the members of the Yelon laboratory for helpful discussions and Didier Stainier, Sally Horne-Badovinac, and Emily Walsh for sharing data before publication. I regret that space constraints have made it necessary to limit the number of studies discussed in this review. Research in the Yelon laboratory is supported by a Burroughs Wellcome Fund Career Award and by the New York City Council Speaker's Fund for Biomedical Research.

REFERENCES

Ahn DG, Ruvinsky I, Oates AC, Silver LM, Ho RK. 2000. *tbx20*, a new vertebrate T-box gene expressed in the cranial motor neurons and

developing cardiovascular structures in zebrafish. Mech Dev 95: 253–258.
Alexander J, Stainier DY, Yelon D. 1998. Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. Dev Genet 22:288–299.
Alexander J, Rothenberg M, Henry GL, Stainier DY. 1999. *casanova* plays an early and essential role in endoderm formation in zebrafish. Dev Biol 215:343–357.
Angelo S, Lohr J, Lee KH, Ticho BS, Breitbart RE, Hill S, Yost HJ, Srivastava D. 2000. Conservation of sequence and expression of xenopus and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development. Mech Dev 95:231–237.
Bao ZZ, Bruneau BG, Seidman JG, Seidman CE, Cepko CL. 1999. Regulation of chamber-specific gene expression in the developing heart by Irx4. Science 283:1161–1164.
Begemann G, Ingham PW. 2000. Developmental regulation of Tbx5 in zebrafish embryogenesis. Mech Dev 90:299–304.
Bisgrove BW, Essner JJ, Yost HJ. 2000. Multiple pathways in the midline regulate concordant brain, heart and gut left-right asymmetry. Development 127:3567–3579.
Bruneau BG, Bao ZZ, Fatkin D, Xavier-Neto J, Georgakopoulos D, Maguire CT, Berul CI, Kass DA, Kuroski-de Bold ML, de Bold AM, Conner DA, Rosenthal N, Cepko CL, Seidman CE, Seidman JG. 2001. Cardiomyopathy in Irx4-deficient mice is preceded by abnormal ventricular gene expression. Mol Cell Biol 21:1730–1736.
Burdine RD, Schier AF. 2000. Conserved and divergent mechanisms in left-right axis formation. Genes Dev 14:763–776.
Charron F, Nemer M. 1999. GATA transcription factors and cardiac development. Semin Cell Dev Biol 10:85–91.
Chen J-N, Fishman MC. 1996. Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation. Development 122:3809–3816.
Chen J-N, Haffter P, Odenthal J, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang Y-J, Kane DA, Kelsh RN, Mullins MC, Nüsslein-Volhard C. 1996. Mutations affecting the cardiovascular system and other internal organs in zebrafish. Development 123:293–302.
Chen J-N, van Eeden FJM, Warren KS, Chin AS, Nusslein-Volhard C, Haffter P, Fishman MC. 1997. Left-right pattern of cardiac *BMP4* may drive asymmetry of the heart in zebrafish. Development 124: 4373–4382.
Chin AJ, Tsang M, Weinberg ES. 2000. Heart and gut chiralities are controlled independently from initial heart position in the developing zebrafish. Dev Biol 227:403–421.
Christoffels VM, Keijser AG, Houweling AC, Clout DE, Moorman AF. 2000. Patterning the embryonic heart: identification of five mouse Iroquois homeobox genes in the developing heart. Dev Biol 224:263–274.
DeHaan RL. 1965. Morphogenesis of the vertebrate heart. In: DeHaan RL, Ursprung H, editors. Organogenesis. New York: Holt, Rinehart and Winston. p 377–419.
Dickmeis T, Mourrain P, Saint-Etienne L, Fischer N, Aanstad P, Clark M, Strahle U, Rosa F. 2001. A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. Genes Dev 15:1487–1492.
Eisenberg LM, Markwald RR. 1995. Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res 77:1–6.
Evans SM. 1999. Vertebrate *tinman* homologues and cardiac differentiation. Semin Cell Dev Biol 10:73–83.
Fishman MC, Chien KR. 1997. Fashioning the vertebrate heart: earliest embryonic decisions. Development 124:2099–2117.
Franco D, Lamers WH, Moorman AF. 1998. Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model. Cardiovasc Res 38:25–53.
Gajewski K, Fossett N, Molkentin JD, Schulz RA. 1999. The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. Development 126:5679–88.
Goldstein AM, Fishman MC. 1998. Notochord regulates cardiac lineage in zebrafish embryos. Dev Biol 201:247–252.
Griffin KJ, Stoller J, Gibson M, Chen S, Yelon D, Stainier DY, Kimelman D. 2000. A conserved role for *H15*-related T-box transcription factors in zebrafish and *Drosophila* heart formation. Dev Biol 218: 235–247.
Gritsman K, Zhang J, Cheng S, Heckscher E, Talbot WS, Schier AF. 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell 97:121–132.
Hacker U, Lin X, Perrimon N. 1997. The Drosophila *sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. Development 124:3565–3573.
Hatcher CJ, Kim MS, Mah CS, Goldstein MM, Wong B, Mikawa T, Basson CT. 2001. TBX5 transcription factor regulates cell proliferation during cardiogenesis. Dev Biol 230:177–188.
Horb ME, Thomsen GH. 1999. Tbx5 is essential for heart development. Development 126:1739–1751.
Horne-Badovinac S, Lin D, Waldron S, Schwarz M, Mbamalu G, Pawson T, Jan Y-N, Stainier DYR, Abdelilah-Seyfried S. 2001. Positional cloning of *heart and soul* reveals multiple roles for PKCλ in zebrafish organogenesis. Curr Biol 11:1492–1502.
Hu N, Sedmera D, Yost HJ, Clark EB. 2000. Structure and function of the developing zebrafish heart. Anat Rec 260:148–157.
Jiang Y, Evans T. 1996. The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. Dev Biol 174:258–270.
Jiang Y, Tarzami S, Burch JB, Evans T. 1998. Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. Dev Genet 22:263–277.
Kikuchi Y, Trinh LA, Reiter JF, Alexander J, Yelon D, Stainier DY. 2000. The zebrafish *bonnie and clyde* gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors. Genes Dev 14:1279–1289.
Kikuchi Y, Agathon A, Alexander J, Thisse C, Waldron S, Yelon D, Thisse B, Stainier DY. 2001. *casanova* encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev 15:1493–1505.
Kishimoto Y, Lee KH, Zon L, Hammerschmidt M, Schulte-Merker S. 1997. The molecular nature of zebrafish *swirl*: BMP2 function is essential during early dorsoventral patterning. Development 124: 4457–4466.
Koster RW, Fraser SE. 2001. Tracing transgene expression in living zebrafish embryos. Dev Biol 233:329–346.
Koutsourakis M, Langeveld A, Patient R, Beddington R, Grosveld F. 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126:723–732.
Kuchinke U, Grawe F, Knust E. 1998. Control of spindle orientation in *Drosophila* by the Par-3-related PDZ- domain protein Bazooka. Curr Biol 8:1357–1365.
Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM. 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev 11:1048–1060.
Kupperman E, An S, Osborne N, Waldron S, Stainier DY. 2000. A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. Nature 406:192–195.
Lee KH, Xu Q, Breitbart RE. 1996. A new tinman-related gene, *nkx2.7*, anticipates the expression of *nkx2.5* and *nkx2.3* in zebrafish heart and pharyngeal endoderm. Dev Biol 180:722–731.
Lien CL, Wu C, Mercer B, Webb R, Richardson JA, Olson EN. 1999. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. Development 126:75–84.
Liberatore CM, Searchy-Schrick RD, Yutzey KE. 2000. Ventricular expression of *tbx5* inhibits normal heart chamber development. Dev Biol 223:169–180.
Lin X, Buff EM, Perrimon N, Michelson AM. 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. Development 126:3715–3723.
Lyons GE. 1994. In situ analysis of the cardiac muscle gene program during embryogenesis. Trends Cardiovasc Med. 3:184–190.
Molkentin JD, Lin Q, Duncan SA, Olson EN. 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 11:1061–1072.
Molkentin JD, Tymitz KM, Richardson JA, Olson EN. 2000. Abnormalities of the genitourinary tract in female mice lacking GATA5. Mol Cell Biol 20:5256–5260.

Morrisey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS, Parmacek MS. 1998. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev 12: 3579–3590.

Moss JB, Xavier-Neto J, Shapiro MD, Nayeem SM, McCaffery P, Drager UC, Rosenthal N. 1998. Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. Dev Biol 199:55–71.

Motoike T, Loughna S, Perens E, Roman BL, Liao W, Chau TC, Richardson CD, Kawate T, Kuno J, Weinstein BM, Stainier DY, Sato TN. 2000. Universal GFP reporter for the study of vascular development. Genesis 28:75–81.

Muller HA, Wieschaus E. 1996. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. J Cell Biol 134:149–163.

Narita N, Bielinska M, Wilson DB. 1997. Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. Dev Biol 189:270–274.

Nasevicius A, Ekker SC. 2000. Effective targeted gene "knockdown" in zebrafish. Nat Genet 26:216–220.

Nguyen VH, Schmid B, Trout J, Connors SA, Ekker M, Mullins MC. 1998. Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. Dev Biol 199:93–110.

Niederreither K, Vermot J, Messaddeq N, Schuhbaur B, Chambon P, Dolle P. 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 128:1019–1031.

Park HC, Kim CH, Bae YK, Yeo SY, Kim SH, Hong SK, Shin J, Yoo KW, Hibi M, Hirano T, Miki N, Chitnis AB, Huh TL. 2000. Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. Dev Biol 227:279–293.

Pelster B, Burggren WW. 1996. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebrafish (*Danio rerio*). Circ Res 79:358–362.

Peterson RT, Link BA, Dowling JE, Schreiber SL. 2000. Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc Natl Acad Sci U S A 97:12965–12969.

Peterson RT, Mably JD, Chen J-N, Fishman MC. 2001. Convergence of distinct heart patterning pathways revealed by the small molecule concentramide and the mutation *heart and soul*. Curr Biol 11:1481–1491.

Petronczki M, Knoblich JA. 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat Cell Biol 3:43–49.

Peyrieras N, Strahle U, Rosa F. 1998. Conversion of zebrafish blastomeres to an endodermal fate by TGF-beta-related signaling. Curr Biol 8:783–786.

Reecy JM, Li X, Yamada M, DeMayo FJ, Newman CS, Harvey RP, Schwartz RJ. 1999. Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. Development 126:839–849.

Reifers F, Walsh EC, Léger S, Stainier DYR, Brand M. 2000. Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (*fgf8/acerebellar*). Development 127:225–235.

Reiter JF, Alexander J, Rodaway A, Yelon D, Patient R, Holder N, Stainier DYR. 1999. Gata5 is required for the development of the heart and endoderm in zebrafish. Genes Dev 13:2983–2995.

Reiter JF, Verkade H, Stainier DY. 2001. Bmp2b and Oep promote early myocardial differentiation through their regulation of *gata5*. Dev Biol 234:330–338.

Rosenthal N, Xavier-Neto J. 2000. From the bottom of the heart: anteroposterior decisions in cardiac muscle differentiation. Curr Opin Cell Biol 12:742–746.

Satin J, Fujii S, DeHaan RL. 1988. Development of cardiac beat rate in early chick embryos is regulated by regional cues. Dev Biol 129:103–113.

Schultheiss TM, Burch JB, Lassar AB. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 11:451–462.

Searcy RD, Vincent EB, Liberatore CM, Yutzey KE. 1998. A GATA-dependent *nkx-2.5* regulatory element activates early cardiac gene expression in transgenic mice. Development 125:4461–4470.

Serbedzija GN, Chen JN, Fishman MC. 1998. Regulation in the heart field of zebrafish. Development 125:1095–1101.

Sparrow DB, Cai C, Kotecha S, Latinkic B, Cooper B, Towers N, Evans SM, Mohun TJ. 2000. Regulation of the *tinman* homologues in *Xenopus* embryos. Dev Biol 227:65–79.

Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. 1997. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet 16:154–160.

Stainier DYR, Fishman MC. 1992. Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. Dev Biol 153:91–101.

Stainier DYR, Fishman MC. 1994. The zebrafish as a model system to study cardiovascular development. Trends Cardiovasc Med 4:207–212.

Stainier DYR, Lee RK, Fishman MC. 1993. Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. Development 119:31–40.

Stainier DYR, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W, Fishman MC. 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. Development 123:285–292.

Stockwell BR. 2000. Chemical genetics: ligand-based discovery of gene function. Nat Rev Genet 1:116–125.

Summerton J, Weller D. 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 7:187–195.

Ton C, Hwang DM, Dempsey AA, Tang HC, Yoon J, Lim M, Mably JD, Fishman MC, Liew CC. 2000. Identification, characterization, and mapping of expressed sequence tags from an embryonic zebrafish heart cDNA library. Genome Res 10:1915–1927.

Walsh EC, Stainier DYR. 2001. UDP-glucose dehydrogenase required for cardiac valve formation in zebrafish. Science 293:1670–1673.

Warga RM, Kimmel CB. 1990. Cell movements during epiboly and gastrulation in zebrafish. Development 108:569–580.

Warga RM, Nüsslein-Volhard C. 1999. Origin and development of the zebrafish endoderm. Development 126:827–838.

Westerfield M. 1995. The zebrafish book, 3rd ed. Eugene, OR: University of Oregon Press.

Wodarz A, Ramrath A, Grimm A, Knust E. 2000. Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. J Cell Biol 150:1361–1374.

Xavier-Neto J, Neville CM, Shapiro MD, Houghton L, Wang GF, Nikovits WJ, Stockdale FE, Rosenthal N. 1999. A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart. Development 126:2677–2687.

Xu C, Liguori G, Adamson ED, Persico MG. 1998. Specific arrest of cardiogenesis in cultured embryonic stem cells lacking Cripto-1. Dev Biol 196:237–247.

Xu C, Liguori G, Persico MG, Adamson ED. 1999. Abrogation of the Cripto gene in mouse leads to failure of postgastrulation morphogenesis and lack of differentiation of cardiomyocytes. Development 126:483–494.

Yan YT, Gritsman K, Ding J, Burdine RD, Corrales JD, Price SM, Talbot WS, Schier AF, Shen MM. 1999. Conserved requirement for EGF-CFC genes in vertebrate left-right axis formation. Genes Dev 13:2527–2537.

Yelon D, Horne SA, Stainier DY. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214:23–37.

Yelon D, Ticho B, Halpern ME, Ruvinsky I, Ho RK, Silver LM, Stainier DYR. 2000. The bHLH transcription factor Hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 127:2573–2582.

Yutzey KE, Rhee JT, Bader D. 1994. Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. Development 120: 871–883.

Zhang J, Talbot WS, Schier AF. 1998. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. Cell 92:241–251.