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Short survey

Cardiac development in zebrafish: coordination of form and function

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Organogenesis is a dynamic process involving multiple phases of pattern formation and morphogenesis. For example, heart formation involves the specification and differentiation of cardiac precursors, the integration of precursors into a tube, and the remodeling of the embryonic tube to create a fully functional organ. Recently, the zebrafish has emerged as a powerful model organism for the analysis of cardiac development. In particular, zebrafish mutations have revealed specific genetic requirements for cardiac fate determination, migration, fusion, tube assembly, looping, and remodeling. These processes ensure proper cardiac function; likewise, cardiac function may influence aspects of cardiac morphogenesis.

Key words: cardiac development / cardiac fusion / heart tube / morphogenesis / zebrafish

Heart formation at a glance

The heart is the first organ to form and function in the vertebrate embryo. In its earliest incarnation, the heart is a simple tube, composed of two concentric layers: an outer muscular layer (the myocardium) and an inner endothelial layer (the endocardium). This two-layered tube is also subdivided into two major chambers, with unidirectional blood flow traveling from the posterior atrium to the anterior ventricle [Figure 1(A) and (C)]. This deceptively uncomplicated architecture belies the complexity of its formation. Cardiac development begins with pattern formation events that specify each essential cell type, including endocardial and myocardial lineages as well as separate ventricular and atrial myocardial lineages. After the initial differentiation of cardiac precursors in lateral positions, a complex series of morphogenetic events are required to assemble the heart tube at the embryonic midline. Finally, to create the ultimate form of the adult heart [Figure 1(B) and (D)], the initial heart tube must be gradually remodeled, continuing its differentiation and morphogenesis while it functions. The spatial and temporal coordination of these processes is essential for the heart to maintain functional integrity throughout development. Therefore, analysis of the genetic regulation of cardiogenesis is a popular pursuit for developmental biologists employing a variety of model organisms, from flies to mice.

Zebrafish as a model organism: a window into the heart

The widely touted benefits of the zebrafish as a model organism provide great motivation for its application to the study of cardiac development.[1,2] The transparency of the zebrafish embryo is a key advantage, allowing high-resolution visualization of the heart during its rapid development.[3] Another useful feature is the robustness of the zebrafish embryo: it can survive without circulating blood until larval stages, facilitating extended study of a dysfunctional heart.[4] These embryonic attributes augment the feasibility of conducting classical genetic screens for zebrafish mutations that affect cardiac development.[5–8] Significant defects in cardiac form or function are easy to observe in live embryos, and more subtle defects can be revealed with molecular markers. Thus, a variety of screening strategies have been successful in identifying mutations that disrupt specific aspects of cardiac patterning, morphogenesis, and function.[5–8] Recent analyses of mutant phenotypes and identification of

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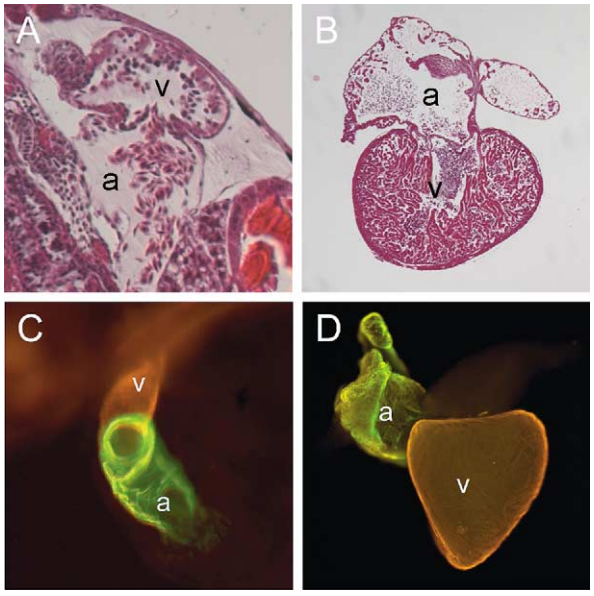

Figure 1. The ventricle and atrium are morphologically distinct chambers within embryonic and adult zebrafish hearts. (A) Longitudinal section through a 5-day-old embryonic heart, anterior to the top. The heart is located within the pericardial sac and features a thick-walled ventricle (v) and a thin-walled atrium (a). The chambers are separated by an atrioventricular valve and both contain blood. (B) Cross-section of an adult heart depicts a heavily trabeculated ventricle (v) and a thin-walled atrium (a). (C) Lateral view of a 48 hpf embryonic heart, anterior to the left. Immunofluorescence with MF20 and S46 antibodies demonstrates distinct expression of chamber-specific myosin heavy chain genes.[5] Red fluorescence (MF20 staining) indicates ventricular (v) myocardium, yellow fluorescence (MF20 and S46 staining) indicates atrial (a) myocardium. (D) Immunofluorescence with MF20 and S46 antibodies indicates morphologically distinct ventricular (v) and atrial (a) chambers of an adult heart.

mutated genes have provided a number of insights regarding the molecular mechanisms that govern heart formation. Current models for the initial steps of cardiac patterning have been reviewed recently[1,2]; therefore, this review will focus on the assembly of cardiac precursors into a functionally integrated organ. The genetic regulation of vertebrate cardiac development is thought to be highly conserved, especially during the initial stages of heart tube assembly.[9,10] However, since there is currently limited overlap between the specific studies discussed here and those performed in other species, this discussion will emphasize what is known for zebrafish.

Cardiac migration: finding the middle ground

Differentiation of zebrafish myocardial precursors begins around 16 h post-fertilization (hpf), when these cells reside within bilateral regions of anterior lateral plate mesoderm (ALPM).[11] Integration of these lateral populations into a single heart tube requires their migration toward the embryonic midline, which occurs in concert with the medial movement of the entire ALPM [Figure 2(A)–(C)]. Cardiac migration appears to proceed in an organized fashion, with the medially located ventricular precursors advancing ahead of the laterally located atrial precursors.[11]

How is the movement of the myocardial precursors stimulated and regulated, and how is their migration directed toward the midline? Many zebrafish mutations inhibit cardiac migration, causing a condition known as cardia bifida, in which two separate hearts form in lateral positions.[5–7] Studies of several cardia bifida mutations have revealed essential requirements for cardiac migration.[1,2] One key prerequisite for myocardial movement is proper myocardial differentiation: all zebrafish mutations that cause myocardial deficiencies also cause cardia bifida.[1] For example, mutation of the *hands off/hand2* locus, which encodes a bHLH transcription factor expressed in the ALPM, dramatically reduces the number of myocardial precursors.[12] Mutant myocardiocytes do not move to the midline, indicating that some components of myocardial differentiation pathways and/or a critical mass of myocardial precursors are essential for cardiac migration [Figure 3(A) and (B)]. However, normal myocardial differentiation is not sufficient to drive cardiac migration; proper differentiation of the closely juxtaposed endodermal precursors is also necessary. All mutations that disrupt formation of anterior endoderm (e.g. *casanova*, *bonnie and clyde*, *faust*, and *one-eyed pinhead*) also result in cardia bifida [Figure 3(C)].[13–18] Endodermal precursors may provide a substrate for migrating myocardiocytes or supply critical signals that stimulate myocardial motility. Alternatively, intrinsic patterning of the endoderm could provide directional cues for the myocardiocytes. Indeed, it is likely that directional signals, possibly medial attractants, are necessary to recruit myocardiocytes to the midline. Clues to the nature of these signals come from the analysis of cardia bifida mutations that preserve normal myocardial and endodermal differentiation, such as mutation of *miles apart* (*mil*), which encodes a lysosphingolipid

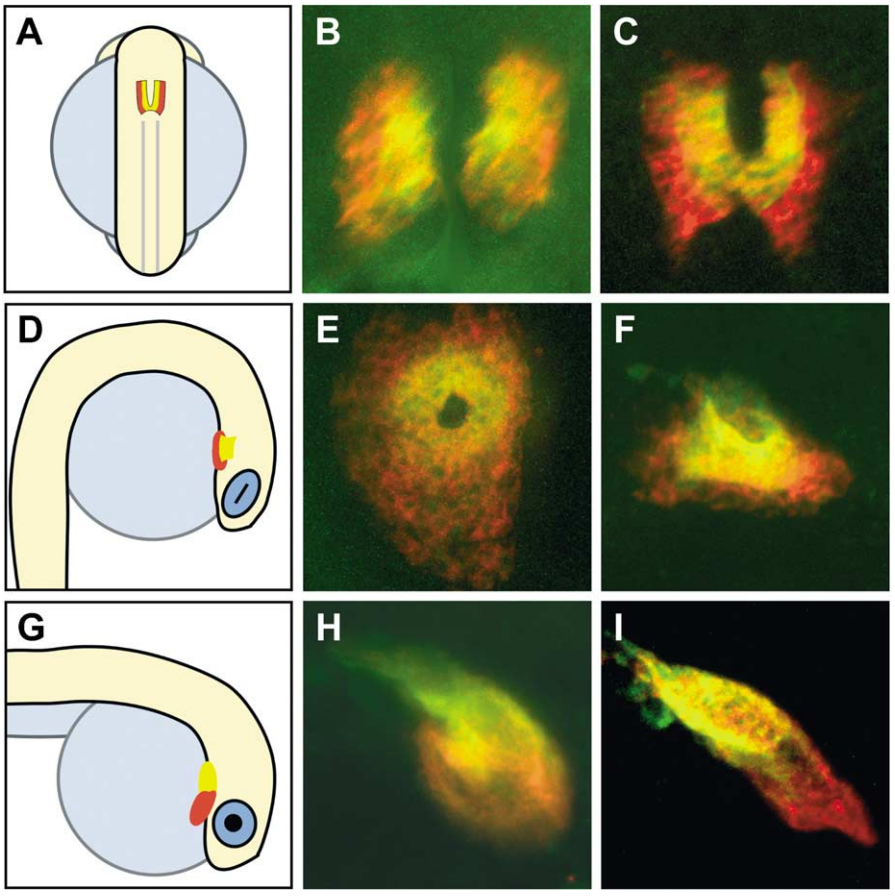

Figure 2. Cardiac morphogenesis involves a complex set of tissue shape changes driven by a series of dynamic cell movements. Schematics in (A), (D), and (G) indicate the location of the myocardiocytes within the embryo. Fluorescent *in situ* hybridizations in (B), (C), (E), (F), (H), and (I) depict *cmlc2* (visualized in red) and *vmhc* (visualized in green) expression patterns in wild-type embryos, revealing the location of ventricular precursors (expressing both *vmhc* and *cmlc2*) and atrial precursors (expressing only *cmlc2*).[11] (A)–(C) Dorsal views with anterior to the top. (A) Schematic depicting the location at which cardiac migration and fusion bring the myocardiocytes together. The notochord is depicted at the midline of the embryonic axis, and the yolk is indicated in blue. (B) At 17.5 hpf, two lateral populations of cardiac precursors are moving toward the midline; ventricular precursors are relatively medial and atrial precursors are relatively lateral. (C) Cardiac fusion begins near the posterior end of both leading edges at 18 hpf. (D) Schematic lateral view, depicting the location of the cardiac cone relative to the embryonic axis, eye, and yolk. (E) Dorsal view with anterior to the top, demonstrating that fusion has completed on the anterior side of a central lumen by 19.5 hpf. (F) Lateral view with anterior to the right. At 19.5 hpf, the cardiac precursors are arranged in a shallow cone, with ventricular precursors at its center and apex. (G) Schematic lateral view, depicting the location of the forming heart tube. (H)–(I) Lateral views with anterior to the right. (H) At 20.5 hpf, the cone tilts and extends toward the posterior of the embryo. (I) Further elongation of the cone results in a tube with distinct ventricular and atrial ends by 24 hpf.

G-protein-coupled receptor [Figure 3(D)].[19] Thus, generation or reception of directional signals may require Mil signaling, fitting with previously suggested roles for Mil-like receptors in endothelial cell migration.[20]

Progress toward understanding the regulation of cardiac migration naturally raises new questions about this process. For instance, the specific cell behaviors involved in initiating and directing cardiac cell movements remain mysterious. Do the myocardial precursors exhibit distinct cell shape changes or adjust their cellular polarity while in motion, and are these cell behaviors dependent upon endodermal signals or upon Mil activity? It is also unclear whether

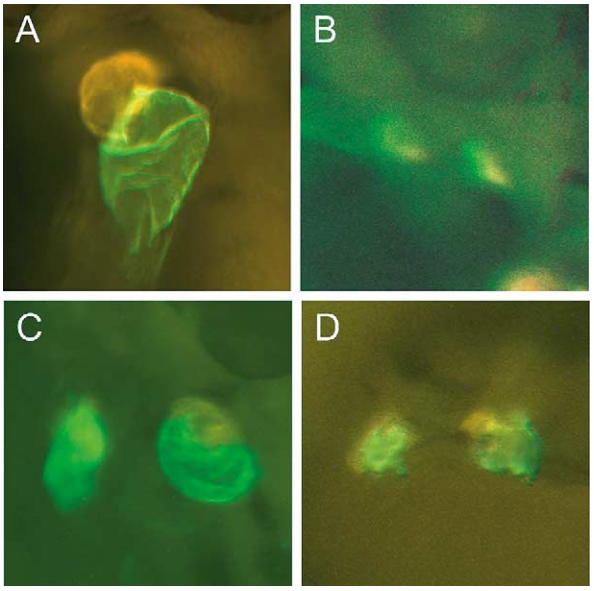

Figure 3. Cardia bifida mutations inhibit cardiac migration and fusion. (A)–(D) Immunofluorescence with MF20 and S46 antibodies demonstrates location of ventricular (red) and atrial (yellow) myocardium in ventral views of embryos at 48 hpf.[5] (A) Wild-type embryos have a single heart tube composed of a ventricle and an atrium; (B) *hands off* mutants have a reduced amount of myocardial tissue, and the existing myocardiocytes remain in lateral positions;[12] (C) *casanova* mutants lack endoderm and exhibit cardia bifida;[14] and (D) *miles apart* mutants have normal endodermal and myocardial differentiation and exhibit cardia bifida.[19]

the myocardial precursors move as a coherent population or as independent cells that are simultaneously responding to environmental cues. In this regard, it will be interesting to examine whether the ventricular precursors at the leading edge of each migrating population exhibit cell behaviors that are distinct from their lateral neighbors.

Cardiac fusion: a friendly merger

Once the myocardial precursors reach the midline (around 18 hpf), they begin to interact and combine, in a process called cardiac fusion.[11] Initial contact between the two populations of ventricular precursors is established at a relatively posterior point [Figure 2(C)]. Next, the myocardiocytes posterior to the initial junction join each other, creating a V-shape. Finally, the most anterior portions of both populations connect, creating an anterior border to a central lumen [Figure 2(D)–(F)]. Viewed dorsally, this configuration looks like a ring of ventricular cells surrounded by a ring of atrial cells [Figure 2(E)]. Viewed laterally, it is apparent that the myocardiocytes have formed a shallow cone, with its ventricular apex raised dorsally above its atrial base [Figure 2(F)].[11]

The orderly sequence of events that produces the cardiac cone suggests that the locations and timing of cell–cell interactions during cardiac fusion are tightly regulated. However, little is known about the mechanisms that establish the initial site of fusion, the sequence of posterior and anterior fusion, and a lumen of the correct dimensions. Perhaps endodermal patterning distinguishes specific embryonic locations as preferential sites for fusion. Alternatively, the endocardial precursors, thought to be clustered at the embryonic midline at this stage,[21] may set the boundaries of the lumen. Zebrafish mutations that cause cardiac fusion defects, such as *heart and soul* (*has*),[22,23] are likely to illuminate the genetic regulation of this process. *has* mutants exhibit an abnormal pattern of fusion, in which the anterior edges fuse first, with the posterior side fusing subsequently.[23] The *has* locus encodes an atypical protein kinase C (PKCλ);[22,23] in *Drosophila*, PKCλ function is critical for the maintenance of epithelial polarity.[24] In zebrafish, *has* is essential for epithelial polarity in the retina and the neural tube,[22] but it is not yet clear how *has* function influences cardiac fusion. One possibility is that maintenance of epithelial polarity within myocardial precursors is essential for the cell behaviors that orchestrate fusion. Alternatively, proper polarity of another interacting tissue may be required to establish the cues that order posterior and anterior fusion events.

Heart tube assembly: from a cone to a tube, with a twist

Following cardiac fusion, the cardiac cone extends, gradually converting into a linear heart tube by 24 hpf. Cone extension is initiated by the tilting of its apex toward the right side of the embryo.[11] The cone then lengthens, stretching in a posterior direction [Figure 2(G) and (H)]. Ultimately, all of the myocardiocytes coalesce into a tube with discrete ventricular and atrial ends [Figure 2(I)]. As the myocardial tube forms, the endocardial cells form its inner lining, presumably spreading out from their initial position inside the lumen of the cone.[21] Between 24 and 48 hpf, the linear heart tube gradually bends at the boundary

between the ventricle and the atrium to create an S-shaped loop.[25,26] As a result of cardiac looping, the ventricle comes to lie to the right of the atrium.

The regulation of heart tube assembly and looping remains poorly understood, but, once again, zebrafish mutations promise to provide valuable insights. For instance, in *has* mutants, the cardiac cone does not tilt and fails to elongate into a tube, suggesting that PKCλ-regulated epithelial polarity may also be essential for these aspects of morphogenesis.[11] Additionally, a number of zebrafish mutants exhibit defects in cardiac looping.[8,25–28] In some cases, mutant hearts are looped in the reverse orientation, such that the ventricle is positioned to the left of the atrium; these mutants are likely to have defects in the initial assignment of the embryonic left–right (L–R) axis. [8,25–28] In other cases, mutant hearts fail to loop and retain a linear form; in these scenarios, defects could reside in the molecular mechanisms that allow the heart to interpret L–R cues or to enact looping morphogenesis.[8,25–28] It is interesting to note that many of the mutations that cause cardiac looping defects also cause defects in L–R morphogenesis of endodermal organs, suggesting common mechanisms for the generation of visceral asymmetry.[8,27,28]

Cardiac remodeling: relationship of form to function, and *vice versa*

The heart tube begins to function as soon as it forms, driving circulation with regular, peristaltic contractions by 24 hpf.[29] By 36 hpf, the ventricle and the atrium exhibit distinct sequential contractions.[29] While the heart is working, cardiac differentiation and morphogenesis continue, building on the foundation of the initial embryonic tube to produce the final form of the adult heart. In addition to cardiac looping, aspects of cardiac remodeling include valve formation, thickening of the ventricular wall, and formation of ventricular trabeculae (finger-like projections of myocardium) [Figure 1(A) and (B)].[30,31] Recent work has begun to investigate the molecular mechanisms responsible for these modifications that enhance cardiac function.

Atrioventricular valve formation begins with the formation of endocardial cushions at the atrioventricular boundary by 48 hpf; these cushions are later replaced by valve flaps that prevent retrograde blood flow.[30,31] Endocardial cushions are created by an epithelial–mesenchymal transition that is stimulated by signaling between specialized endocardial and myocardial cells at the atrioventricular boundary.[32] Zebrafish mutations that exhibit defects in endocardial cushion formation can reveal key players in this process. For example, the *jekyll* (*jek*) mutation inhibits endocardial cushion formation and disrupts expression of molecular markers that normally demarcate the atrioventricular boundary.[33] *jek* encodes UDP-glucose dehydrogenase, an enzyme required for the synthesis of heparan sulfate proteoglycans (HSPGs).[33] HSPGs play many roles during cell signaling, including contributions to the transduction of Fgf and Wnt signals.[34] Thus, HSPG-mediated signaling plays a critical role in establishing the atrioventricular boundary.

It is interesting to note that many aspects of cardiac morphogenesis are regulated independently of cardiac function. For example, mutation of the *silent heart* (*sih*) gene, which encodes the sarcomere component Cardiac troponin T, completely prevents cardiac contractility; even so, cardiac migration, cardiac fusion, heart tube assembly, and cardiac looping occur normally in *sih* mutants.[35] However, the influence of cardiac function on aspects of cardiac remodeling, particularly valve formation and ventricular maturation, has not been fully explored. Evidence hinting at a complex relationship of function and form is emerging as the genes responsible for functional mutations are being identified. For example, the zebrafish *pickwick* (*pik*) locus encodes the sarcomere scaffold protein Titin, an essential component for effective cardiac contraction.[36] In *pik* mutants, myocardiocytes are abnormally thin, resulting in dilation of the cardiac chambers; this phenotype indicates the importance of Titin for maintenance of myocardiocyte shape as well as function. Another example relevant to both function and form involves a component of the cardiac electrical conduction apparatus. The *island beat* (*isl*) locus encodes a L-type calcium channel subunit that is critical for normal cardiac contraction.[37] Interestingly, *isl* mutants are also deficient in the ventricular growth that is normally observed between 48 and 72 hpf. These two aspects of the *isl* mutant phenotype may reflect separate roles of cardiac calcium signaling, one role in controlling contraction and one role in controlling ventricular proliferation. The independence of these functions is suggested by the phenotype of another mutation (*tell tale heart*) that causes a similar contractility defect and yet does not exhibit defects in ventricular growth.[37] Future studies of other mutants with functional defects will clarify whether the relationship of form to function is reciprocated by an impact of normal function on the development of normal morphology.

And the beat goes on . . .

The wide variety of cardiac phenotypes caused by zebrafish mutations has provided fertile ground for investigation of multiple aspects of cardiac development. With many interesting mutations still unexplored, future work will contribute depth and breadth to our understanding of heart formation. Future studies are also likely to feature evolving techniques that complement classical forward genetic approaches, including morpholino-mediated reverse genetics[38] and chemical genetics *via* pathway inhibition with small molecules.[23, 39] Furthermore, analyses of cardiac morphogenesis are certain to be enriched by new strategies for analyzing myocardiocyte behaviors in live embryos. The identification of promoters suitable for driving cardiac expression of GFP, combined with techniques for efficient transgenesis and 4-D analysis, will facilitate real-time examination of cardiac cell movements, shape changes, proliferation, and cell–cell interactions. These studies will provide important connections between essential genes and the cellular processes that they regulate. Altogether, analysis of cardiac development in zebrafish will ultimately provide not only a high-resolution model for the regulation of vertebrate heart formation but also a paradigm for the more general process of coordinating organ form and function.

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