Asymmetric Involution of the Myocardial Field Drives Heart Tube Formation in Zebrafish

Stefan Rohr, Cécile Otten, Salim Abdelilah-Seyfried

Abstract—Many vertebrate organs are derived from monolayered epithelia that undergo morphogenesis to acquire their shape. Whereas asymmetric left/right gene expression within the zebrafish heart field has been well documented, little is known about the tissue movements and cellular changes underlying early cardiac morphogenesis. Here, we demonstrate that asymmetric involution of the myocardium of the right-posterior heart field generates the ventral floor, whereas the noninvoluting left heart field gives rise to the dorsal roof of the primary heart tube. During heart tube formation, asymmetric left/right gene expression within the myocardium correlates with asymmetric tissue morphogenesis. Disruption of left/right gene expression causes randomized myocardial tissue involution. Time-lapse analysis combined with genetic analyses reveals that motility of the myocardial epithelium is a tissue migration process. Our results demonstrate that asymmetric morphogenetic movements of the 2 bilateral myocardial cell populations generate different dorsoventral regions of the zebrafish heart tube. Failure to generate a heart tube does not affect the acquisition of atrial versus ventricular cardiac cell shapes. Therefore, establishment of basic cardiac cell shapes precedes cardiac function. Together, these results provide the framework for the integration of single cell behaviors during the formation of the vertebrate primary heart tube. 

Key Words: heart tube • cell polarity • protein kinase C iota • left–right asymmetry • southpaw • nagie oko

Heart development in vertebrates involves the fusion of 2 myocardial progenitor fields at the embryonic midline. These heart fields derive from the left and right lateral plate mesoderm (LPM). In zebrafish, the fusion of the 2 heart fields forms the heart cone, a central flat disc that is subsequently transformed into the primary heart tube that generates a 2-chambered heart with an anterior atrium and a posterior ventricle, which initiates circulation at 24 hours postfertilization (hpf). Morphogenetic processes and tissue dynamics required for heart cone-to-tube transition are not well understood. Previous studies have described the asymmetric leftward movement of the primary heart tube (after 24 hpf), followed by the looping of the heart at 36 hpf, processes that depend on the left/right (L/R) signaling pathway and transform the linear heart tube into a looped heart with distinct bean-shaped heart chambers. A key player in the hierarchy of the L/R signaling pathway is the nodal-related gene southpaw (spaw), which also affects the correct expression of downstream genes including pitx2, lefty1, and lefty2. Combinatorial gene expression patterns of L/R signaling genes have been described within the heart cone. However, whether this L/R asymmetric gene expression is underlying asymmetric cell behaviors is currently unknown.

Myocardial precursor cells acquire a polarized epithelial morphology, which is a prerequisite for normal heart development. Mutations in has (heart and soul), which encodes atypical protein kinase C iota (prkci), and nok (nagie oko), which encodes membrane protein palmitoylated 5 (mpp5), result in the loss of apicobasal polarity of cells within the myocardial progenitor fields and a failure to form the heart tube. The deficits in tissue dynamics that result in the failure to form the heart tube have not been assessed.

Several lines of evidence indicate that myocardial cells migrate actively during early heart development. The myocardial progenitor fields are located between the underlying yolk syncytial layer and the overlying pharyngeal endoderm and these neighboring tissues are essential for the directed migration of progenitor cells and for zebrafish heart cone formation. Furthermore, myocardial cell motility depends on cell–substratum interactions and the deposition of fibronectin to the extracellular matrix. Whether myocardial progenitors migrate as individual cells or as coherent population (cohort migration) during cone-to-tube transition remains to be determined.

Here, we characterize morphogenetic movements during heart tube formation in zebrafish. We find that asymmetric involution of the right heart field myocardium initiates the transition of the heart cone into a tube. L/R asymmetric gene expression is maintained during heart tube formation and correlates with asymmetric myocardial tissue behaviors.

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Gene knockdown of spaw randomizes the orientation of myocardial tissue involution. In addition, we show that acquisition of basic myocardial cell shapes precedes and is independent of heart tube formation.

Materials and Methods

Fish Maintenance and Stocks
Zebrafish were maintained at standard conditions.14 Embryos were staged according to somite number or by hours postfertilization at 28.5°C.15 Tg(cmlc2:GFP) was created as described elsewhere.16 The Tg(cmlc2:mRFP) transgenic line was generated by injection of I-SceI cmlc2:mRFP transformation vector9 into 1-cell stage embryos.17 F1 founders were identified based on red fluorescent protein (RFP) expression and used for the establishment of a stable line. The hasmutant fish used in this study carried the has<sup>229</sup> allele.18

DNA Constructs and DNA Injection
The cmlc2:mGFP construct was cloned by introducing a membrane-tagged green fluorescent protein (mGFP) fragment into the I-SceI cmlc2 transformation vector. Clonal analysis of mGFP-positive cells was performed on DNA injection into Tg(cmlc2:mRFP) embryos.

Morpholino Injections
Morpholinos (MOs) (Gene Tools) were injected at a concentration of 100 μmol/L or 150 μmol/L (for spaw MOs). MO sequences were as follows: hasMO, 5′-TGGTAGAGCTTCAACAGACTCTGCA-3′; hasMO2, 5′-TGTCCCGCAGCGTGGGCATTATGGA-3′; spawMO, 5′-GACGCCATATGACTGGTCCATTGCA-3′; spawMO2, 5′-TTGTTAGAGCTTCAACAGACTCTGCA-3′.

In Situ Hybridization and Sectioning
Whole mount in situ hybridization was performed as previously described.19 Digoxigenin-UTP-labeled riboprobes were synthesized according to the instructions of the manufacturer (Boehringer Mannheim). The cmlc2 probe was amplified from cdNA (AF114428) and subcloned into pCS2+. The probe for lefty2 was a gift from J. Essner. Embryos were mounted in Permount (Fisher Scientific) and documented using an Axioscope 2 microscope (Zeiss). Images were processed with Adobe Photoshop software (Adobe Systems). For sectioning of whole mount in situ hybridizations, embryos were dehydrated in 100% methanol for 10 minutes and embedded in JB-4 according to the instructions of the manufacturer (Polysciences Inc). Sections of 10 μm thickness were cut with a microtome (Ultracut E, Leica). Countertaining of nuclei was performed using Neutral Red (Sigma).20 Sections were preserved with Permount and imaged with an Axioscope 2 microscope.

Antibody Staining and Sectioning
Whole mount antibody stainings were performed as previously described.18 Sectioning and subsequent antibody stainings were performed according as described elsewhere.8 The following antibodies were used: rabbit anti-α-tubulin protein kinase (aPKC) (1:100; Santa Cruz Biotechnology), mouse anti–zn5/ALCAM/Dm-GRASP/Neurolin (1:200; ZIRC, Eugene, Ore), S68 (1:20, Developmental Studies Hybridoma Bank), rabbit anti-laminin (1:200) (Jackson ImmunoResearch). F-Actin was stained with rhodamine–phalloidin (1:100, Molecular Probes). Nuclear stainings were performed using 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen; D121490).

Image Acquisition
Samples were analyzed using Leica TCS SP2 or Zeiss LSM510 ConfoCor2 (FCS) confocal microscopes. For reconstruction of myocardial cell morphology, single sections of recorded z-stacks were analyzed using Leica and Zeiss LSM software. For time-lapse analysis, embryos were embedded in 1.5% low-melting-temperature agarose (NuSieve GFT agarose; Cambrex) solved in D-MEM cell culture medium (GIBCO) with 10% FCS (Biochrom) and addition of penicillin/streptomycin/gentamicin. Individual myocardial contrac- tion and beating of the developing heart tube was reduced using 3-aminobenzoic acid ethyl ester (Tricaine) (Sigma). Embryos were imaged with a CoolSnap ES camera (Photometrics) on an Axiosoplan2 microscope. All time-lapse movies were performed using ×10 magnification and a capture rate of 1 frame per 5 minutes. Time-lapse analysis of single-cell migration was performed with a ×20 magnification and a capture rate of 1 frame per minute. Data were collected and analyzed using Metamorph software 6.1 (Visitron Systems). Nuclear stainings were performed by following nuclear GFP expression in Tg(cmlc2:GFP) using ImageJ software.

Results

Asymmetric Myocardial Involution Initiates Heart Tube Formation
The transformation of the initially flat myocardial field into the heart tube occurs during a 5-hour period of zebrafish development, ie, between the 20-somite stage (19 hpf) and 30-somite stage (24 hpf) (Figure 1A). At the 20-somite stage, the heart cone shows asymmetric expression of L/R genes.7 To test whether this asymmetric gene expression correlates with asymmetric myocardial cell behaviors and to understand the morphogenetic movements during transition of the heart cone into a tube, we performed a series of immunohistochemical stainings on sections of embryos between the 16-somite stage (17 hpf) and 32 hpf. We used a transgenic line of zebrafish that expresses GFP under the control of the myocardial-specific cmlc2 promoter [Tg(cmlc2:GFP)].16 Cell polarity was assessed using antibodies against the apical marker aPKC9,18 and the basolateral marker zn5/ALCAM.21–23 Before the 20-somite stage, heart cone formation is a symmetrical process (Figures I and II in the online data supplement). We observe the first evidence for an asymmetry at the border of the heart cone opening around the 20-somite stage (19 hpf) (Figure 1B through 1D). In particular, the tissue at the right-posterior border of the heart cone opening begins to turn dorsally (Figure 1B, arrowheads; Figure 1E, yellow marked tissue; supplemental Figure IIB, posterior red section planes), whereas myocardial cells at the opposite, anterior-left side do not change their monolayered morphology (Figure 1B, arrows; supplemental Figure IIB, anterior red section planes). The remaining right myocardial sheet moves along the already-turned area in direct juxtaposition, rather similar to involuting cells at the blastoderm border during gastrulation, which spread over the internal surface of the remaining external cells. We therefore refer to this process as myocardial involution. At the 25-somite stage (21.5 hpf), the site of involution is below the noninvoluting area and progressively motions toward the anterior-left (Figure 1F through 1H). At 27 hpf, right-posterior involution is nearly completed, resulting in an inversion of the right half of the heart cone, which places the basal surface of the involuting tissue toward the lumen of the nascent heart tube (Figure 1J through 1L). This finding is further supported by analysis of transverse sections of the heart tube, where zn5/ALCAM and similarly the basally deposited extracellular matrix marker laminin localize toward the luminal side (supplemental Figure III). At 27 hpf, atrial cells are located anteriorly and can be recognized by their squamous epithelial morphology (Figure 1J, arrows) and atrium-specific immunoreactivity.
Myocardial involution is closing the heart tube ventrally. A, Scheme representing the flat heart cone and the primary heart tube. The rapid remodeling of the myocardium during cone-to-tube transition is an unknown morphogenetic process. B through D, Section along the anterior/posterior axis of the developing heart tube (45° angle away from the embryonic midline) at the 20-somite stage (19 hpf) reveals the initiation of tissue involution at the posterior-right (between arrowheads) of the central heart cone opening, whereas myocardial cells of the anterior-left side (between arrows) do not change their monolayered morphology. Shown are myocardial GFP-positive cells (green), immunohistochemical staining of aPKC (red), and zn5 (blue). C and D, Details of individual color channels (black and white). Central heart cone opening is marked with an asterisk. E, Schematic representation displaying asymmetric myocardial involution of the heart cone. Basal surfaces are represented by blue dotted lines. The tissue-initiating involution is indicated in yellow. The arrow indicates the direction of involution. F through H, Similar section plane of a 25-somite stage (21.5 hpf) heart demonstrates the progression of the myocardial involution process. I, Whereas the dorsal roof of the future heart cone is extending, ventral closure via involution is progressing. J through L, At 27 hpf, ventral closure is still in progress. J, Note cuboidal ventricular (arrowheads) and squamous atrial cells (arrows). K, Asterisks mark the lumen of the posterior heart tube. L, The remaining tissue is involuting (arrow). M, Scheme illustrating the progress of heart tube closure toward anterior at this stage. N, Model representing key stages of heart tube formation attributable to asymmetric involution of the myocardial field. The color code demonstrates the gradual involution starting with yellow tissue at the border of the right-posterior heart cone opening, followed by blue and finally the red area of the posterior myocardium. The section plane shown in the cross sections is indicated by the dashed line. The (supplemental Figure IV). However, at this stage, only the dorsal roof of the atrium is formed, which is derived from cells of the noninvoluting anterior-left half of the heart cone. Ventricular cells are located posteriorly and have a cuboidal epithelial morphology (Figure 1J, arrowheads; supplemental Figure IV). In contrast to the atrium, the ventricular chamber exhibits a dorsal roof and ventral floor. The ventral floor originates from the right half of the heart cone that has involuted, whereas the dorsal roof stems from the left half of the heart cone, which has not involuted (Figure 1E, 1I, and 1M).

To further investigate the final steps of heart tube closure, we analyzed transverse sections at the 27-somite stage (22.5 hpf) and at 27 hpf. This revealed that the myocardial epithelium also involutes on the left-lateral and right-medial sides of the nascent heart tube with respect to the embryonic midline (supplemental Figure VA through VF and VH). Ventral views onto the entire heart field show that lateral and medial involution of the heart tube progresses from posterior to anterior (supplemental Figure VG, VG′, and VI). Our analysis reveals that heart tube formation is driven by an initially asymmetric myocardial involution, which generates the ventral floor of the heart tube (Figure 1N). Progressive lateral and medial involution of the anterior part completes primary heart tube formation.

**lefty2 Expression Marks the Noninvoluting Dorsal Heart Tube**

To analyze whether asymmetric myocardial behavior correlates with L/R asymmetric gene expression, we assayed *lefty2*, which unlike other L/R asymmetry components, is expressed exclusively within the left side of the heart cone at the 21-somite stage (19.5 hpf) (Figure 2B). In comparison, the pan-myocardial marker *cmlc2* is expressed throughout the entire heart cone at the same stage (Figure 2A). At the 27-somite stage (22.5 hpf), *cmlc2* and *lefty2* are expressed along the entire length of the developing heart tube (Figure 2C and 2D). To assess whether *lefty2* expression marks the dorsal heart tube, which is exclusively derived from the left noninvoluting heart cone region, we analyzed transverse sections. This analysis showed that indeed *lefty2* expression is restricted to a dorsal crescent and left-lateral sides of the completed posterior part of the heart tube (Figure 2F and F′), whereas the ventral floor and the right-medial side of the heart tube lack expression of *lefty2*. At the same stage, *cmlc2* expression marks the entire heart tube (Figure 2E and 2E′). Our results correlate molecular differences along the L/R axis of the early myocardial field with asymmetric myocardial morphogenesis (Figure 2G). Moreover, asymmetric gene expression along the L/R axis of the heart cone is maintained along the dorsal/ventral axis of the nascent heart tube.
Disruption of L/R Signaling Randomizes the Orientation of Myocardial Tissue Involution

We next evaluated the possibility that the L/R signaling pathway directly affects the orientation of myocardial tissue involution. To investigate whether myocardial involution is randomized in spaw morphants, we analyzed 25-somite stage (21.5 hpf) Tg(cmlc2:GFP) transgenic embryos that were injected with spaw MOs. Injection of 2 independent spaw MOs yielded the same results. In contrast to wild-type (WT) controls which consistently displayed myocardial involution toward the left anterior (n=145; 100% leftward involution), both MOs caused a randomization of myocardial tissue involution (Figure 2H). Detailed reconstructions of confocal serial images of individual heart cones representing different phenotype classes revealed that the shape and size of invoving myocardial tissue was comparable (Figure 2I through 2K). The majority of spaw morphants displayed myocardial tissue involution along the posterior medial portion of the heart cone, with an orientation toward the anterior midline of the embryo. Therefore, L/R asymmetry signaling controls the asymmetric positioning of myocardial tissue involution within the posterior half of the heart cone, rather than regulating tissue involution per se.

Evidence for Directional Myocardial Cohort Migration During Heart Tube Formation

At the onset of heart tube formation, the myocardial field rapidly moves away from the midline in a left-anterior direction.4 To visualize the mode of motility of myocardial cells during this translocation and during heart cone-to-tube transition, we performed time-lapse analysis over a 6-hour period in transgenic Tg(cmlc2:GFP) embryos.4 We reproducibly found that the majority of WT myocardial cells translocates as a coherent population (Figure 3A through A’ and supplemental Movie 1). Tracking nuclei of migrating myocardial cells showed that cells exhibit a uniform directionality independent of their initial position. (Figure 3D [black lines]). Nuclear trackings of myocardial cells from all areas of the heart field show a uniform movement at a 45° angle away from the embryonic midline (supplemental Figure VI and Movie 2). In time-lapse movies, the posterior involuting area of the heart cone appears slightly brighter (supplemental Figure VIB’). To track single-cell behaviors during migration, we followed individual cells in time-lapse recordings of Tg(cmlc2:mRFP) or Tg(cmlc2:GFP) embryos. In addition, we created myocardial clones of cells expressing mGFP. This analysis showed
that myocardial cells maintain constant neighbor relationships and largely avoid cell mixing during heart tube formation (10 clones analyzed containing 47 cells) (Movie S3 and data not shown). We next asked whether myocardial cells display subcellular cell extensions usually associated with cell motility during cohort migration by creating myocardial cell clones expressing mGFP. Three-dimensional reconstructions of confocal z-stacks of such clones revealed that small cellular extensions are present and restricted to their basal surfaces (Figure 3G and 3G'). Consistency in speed, lack of extensive cell mixing, maintenance of motion-direction, and presence of cellular extensions support the notion that heart tube formation involves directional cohort migration of myocardial cells.

**Loss of Directional Myocardial Cohort Migration in has/prkci and nok/mpp5**

In the absence of Prkci and Nok, heart development arrests at the heart cone stage.9,18,28 To address whether directional cohort migration of myocardial cells is affected in these mutants, we performed time-lapse analysis of has/prkci and nok/mpp5 morphants in the Tg(cmlc2:GFP) background and found that most myocardial cells are immobile and remain at the embryonic midline. In contrast, at the periphery of the heart field, strings of adherent cmlc2:GFP-positive cells frequently lose contact with neighboring cells and initiate nondirectional single-cell migration (Figure 3B through 3B'' and 3C through 3C'' and supplemental Movies 4 and 5). Tracking nuclei of individually migrating cells showed that coordinated directional cohort migration across the midline toward left-anterior does not occur in these mutants (Figure 3E and 3F). High-magnification analysis of single nok/mpp5 mutant cells showed that rapid but randomized protrusion formation occurs during individual myocardial cell migration (Figure 3H and supplemental Movie 6). Directional cohort migration of myocardial cells is replaced by nondirectional migration of single myocardial cells in has/prkci and nok/mpp5 morphants.

**Acquisition of Chamber-Specific Myocardial Cell Shapes Is Independent of Heart Tube Formation**

Myocardial cell shape changes have been shown to contribute to the elongation of the heart tube. Within the early heart field, myocardial cells differentiate into atrial and ventricular cell types.29 The acquisition of chamber-specific myocardial cell shapes depends, in part, on physical forces like blood flow or coordinated contractility and occurs during the primary heart tube stage and at later stages of heart development.24,29 To monitor cell shapes of atrial and ventricular myocardial cells during myocardial involution, we established a stable transgenic line of zebrafish expressing membrane-tagged RFP (mRFP) under the control of the zebrafish myocardial-specific promoter cmlc2 [Tg(cmlc2:mRFP)]. Analysis of myocardial cells in these transgenic embryos revealed that atrial and ventricular myocardial cells acquire chamber-specific cell shapes already at the heart cone stage.
before the onset of involution. Previously, Yelon et al demonstrated, based on chamber-specific gene expression, that atrial cells surround ventricular cells at the heart cone stage. To further correlate chamber identity and cell shape, we used the atrium-specific S46 antibody. At the 20-somite stage (19 hpf), cuboidal ventricular cells with high levels of mRFP expression around the central heart cone opening (asterisk) are surrounded by squamous atrial cells (arrowheads) before the onset of heart tube formation. Higher magnification of A, C, Scheme illustrating ventricular cells (red) and atrial cells (green); dorsal view above, cross section along the white dotted line below. D and E, At the 22-somite stage (20 hpf), the myocardial field exhibits an asymmetric shape. D, Squamous atrial cells in the left-anterior area of the cone are expanded (arrowheads). E, Higher magnification shows that atrial cells weakly marked by the atrium-specific S46 antibody (green) are located within the periphery of the heart cone and display elongated shapes (arrowheads). F, Scheme illustrating the initiation of involution of ventricular cells. G through H, Ventral view at the 27-somite stage (22.5 hpf) heart primordium shows a closed ventricle which forms a tube-like structure (between arrowheads), whereas the atrium is comprised of the outstretched anterior atrial roof (between arrows). H, mRFP expression reveals shapes of the atrial roof cells and (H) GFP highlights nuclei and part of the cytoskeleton within a Tg(cmlc2:mRFP)/Tg(cmlc2:GFP) double-transgenic heart. I, Scheme indicating the trailing edge of the posterior involuting tissue and lateral and medial portions flanking the open atrium. J through L, At 32 hpf, heart tube formation is completed. K, Optical section through the heart tube demonstrates the difference between the large opening of the atrial inflow region (IR) and the narrow muscular outflow region (OR) of the ventricle. L, The border between ventricle and atrium is marked by cuboidal vs squamous cell shapes (arrow). Atrial cells are marked by S46 antibody (green) in a Tg(cmlc2:mRFP) transgenic heart (red). M and N, Myocardial cell shapes in a has/prkci mutant heart at 32 hpf revealed by immunohistochemical staining with zn5 antibody. M', Higher magnification shows that although heart tube formation does not occur, centrally located cuboidal ventricular cells are surrounded by squamous-elongated atrial cells (arrowheads). N, Atrial myocardial cells labeled with the S46 antibody (green) are located in the periphery surrounding central ventricular myocardial cells in a has/prkci mutant Tg(cmlc2:mRFP) heart. O, Scheme representing the 32-hpf heart in a has/prkci mutant that shows similarity to the WT heart cone stage. A indicates anterior; P, posterior; L, left; R, right; At, atrium; Ve, ventricle.

The roof of the open atrial chamber is expanded (Figure 4G, arrows), whereas the ventricular chamber is narrow and closed (Figure 4G, arrowheads). Therefore, before and simultaneously with myocardial involution, atrial myocardial cells undergo cell shape changes.

To further analyze whether chamber-specific cell shape changes occur independently of heart tube formation, we visualized myocardial cell shapes in has/prkci mutants that fail to form a heart tube. We found that myocardial cells acquired chamber-specific cell shapes in the has/prkci mutant heart, with centrally located cuboidal ventricular cells surrounded by squamous elongated S46-labeled atrial cells (Figure 4M through 4O). This finding suggests that chamber-
specific myocardial cell shape changes occur independently of heart tube formation. Moreover, cell shape acquisition is partially independent of physical forces.

Discussion

In this study, we describe the transition of the flat heart field into the primary linear heart tube in zebrafish, a process previously not well understood in any vertebrate organism. We show that the asymmetric involution of the myocardial epithelium from the right side of the heart field initiates a complex tissue inversion that creates the ventral floor and medial side of the primary heart tube. Myocardial cells that are derived from the left side of the heart field contribute exclusively to the dorsal roof and lateral side of the heart tube. Disruption of L/R signaling randomizes the orientation of myocardial involution. In addition, we show that directional cohort migration of myocardial cells and extensive cell shape changes contribute to the extension of the primary heart tube toward left-anterior.

We have shown that asymmetric myocardial cell behaviors along the L/R axis drive heart tube formation in zebrafish. Classic labeling experiments in which the right or left side in the early chick embryo was marked resulted in a labeled dorsal or ventral part of the central primitive heart tube. This observation points toward a conserved role in the formation of dorsal and ventral parts of the primitive heart tube derived from left and right parts of the myocardium in the zebrafish heart cone and the chick cardiac crescent. However, in zebrafish, the left myocardial progenitor field gives rise to the dorsal side of the primitive heart tube, whereas fate map studies during chick heart development demonstrated that the left side of the cardiac crescent gives rise to the ventral part of the heart tube. Therefore, not only the anterior-atrial, posterior-ventricular position of the zebrafish primary heart tube is reversed compared with the anterior/posterior axis of the primary heart tube in higher vertebrates but also the contribution of left and right myocardial progenitor fields to dorsal and ventral aspects of the heart tube.

In zebrafish, the morphogenesis of the left and right LPM is under the control of the L/R asymmetry cascade and requires intact epithelial integrity. During gut development, cells from the right LPM move ventrally, whereas cells from the left LPM migrate dorsally toward the embryonic midline. As a consequence of these asymmetric mesodermal migration behaviors, L/R looping of the gut tube is established. Hence, both cardiac and noncardiac lateral plate mesodermal tissue movements apparently represent variations of a similar morphogenetic program in which the initial asymmetric expression of L/R signaling components instructs asymmetric cellular behaviors that drive organ morphogenesis. In the case of gut development, morphogenesis is regulated extrinsically via the LPM, whereas heart morphogenesis of the myocardial tissue is regulated intrinsically.

Nodal-related genes have been shown to control the L/R asymmetry of the embryonic body. The restricted expression of several L/R asymmetry components is evolutionarily conserved. We have shown that asymmetric L/R gene expression within the myocardium largely corresponds with asymmetric morphogenetic tissue behaviors. Expression of lefty2 within the left side of the heart cone is maintained at later stages in dorsal myocardial cells, most of which fail to involute, with the exception of some lateral cells that eventually involute during closure of the atrium. However, lefty2 may not be responsible directly for the control of involution movements. Knockdown of spaw, which is required for correct lefty2 expression, results in an elongated heart tube, albeit with randomized chirality. In casanova and other cardia bifida mutants, 2 heart tubes or rudiments of tubes form, which suggested that L/R asymmetry signals and the fusion of left and right myocardial progenitor fields at the embryonic midline are not required for heart tube formation. However, the nodal homolog cyclops is expressed in subpopulations of both cardia bifida heart fields of casanova embryos, which is in agreement with our model that heart tube formation requires asymmetric cell behaviors downstream of the L/R signaling cascade. In addition, the directionality of heart tubes in cardia bifida mutants is not uniform toward the left side but linear, random, or mirror-symmetrical. Here, we have shown that L/R signaling via spaw restricts the cellular mechanisms for myocardial involution. This symmetry-breaking event directs heart tube formation toward the left embryonic side (Figure 2G). Surprisingly, loss of spaw does not randomize the orientation of myocardial involution along the anterior/posterior axis of the heart cone. This is indicative of a separate, yet unknown, control mechanism involved in positioning the involution fold in the posterior part of the heart cone. Interestingly, the posterior-right sector of the heart cone, where asymmetric involution initiates, is molecularly unique because of the absence of nodal or nodal downstream target gene expression including ndr2, lft2, and pitx2.

hasl/prkci and nok/mpp5 mutant cardiomyocytes display nondirectional migration patterns, which indicates that these cells fail to sense or fail to react to putative guidance cues within their environment. Previous work by our group demonstrated that transgenic expression of WT Prkci or WT nok exclusively within the myocardium is sufficient to rescue heart development in otherwise hasl/prkci or nok/mpp5 mutant embryos. This finding argues against the possibility that myocardial guidance cues are missing within the mutants and points toward a role of these cell polarity regulators in directional cell migration.

Several lines of evidence indicate that myocardial migration contributes to heart tube formation. First, life imaging revealed that the majority of myocardial cells migrate uniformly as a cohort toward anterior-left. Second, myocardial cells display protrusions at their basal side that are indicative of migratory cells. Third, hasl/prkci and nok/mpp5 mutants display active but nondirectional migration of single detached cardiomyocytes. These findings are in agreement with previous observations that progenitor myocardial cells migrate as epithelial sheets before heart cone formation. Additional mechanisms for cellular and tissue reshaping are required to form the heart tube. Distinct origins of dorsal versus ventral myocardial populations within the primary heart tube could be responsible for further subdivisions of the developing heart chambers, including the outer versus inner curva-
ture. It has recently been shown that members of the planar cell polarity pathway are involved in outflow tract development during mouse heart development. Similar mechanisms are likely to act during zebrafish heart development. The identification of cellular mechanisms, molecular components, and the experimental accessibility make the zebrafish heart an ideal system to analyze the integration of the L/R asymmetry system with the apicobasal and planar cell polarity pathway systems during organogenesis.

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Disclosures

None.

References

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SUPPLEMENTAL DATA

Fig. S1
Apico-basal polarity of myocardial precursors is maintained prior to and during heart tube formation

(A-E) Transverse section of a 16-somite stage (17 hpf) embryo. Immunohistochemical-staining with aPKC (red) and zn5 (blue), myocardial cells expressing GFP [Tg(cmlc2:GFP)]. (A) Two bilateral groups of myocardial precursors originating from the left and the right side of the embryo are symmetrically located on both sides of the embryonic midline prior to heart cone formation. (B) High magnification shows the localization of the myocardial precursors below the neural tube (NT) and the endoderm (asterisks). The myocardial progenitors display apico-basal polarization. (A, B, D) aPKC marks cell-cell contacts, the apical domain of myocardial cells and the apical surface of cells of the neural tube (arrowheads). (A, B, E) zn5 expression is restricted to the basal and lateral sides of cardiomyocytes and to tissues surrounding the neural tube. (C-E) Separations of individual confocal channels in black and white. (F-K) Sagittal section of a 21-somite stage (19.5 hpf) embryo. (G) The heart cone opening is located at the level of the developing brain ventricle. (J) zn5 staining marks the myocardium on both sides of the heart cone opening (K) Nuclear counter-staining with DAPI (gray) and myocardial-specific GFP (green) reveals the presence of cells in the myocardial-free center of the heart cone, presumably endocardial cells (blue arrow). Note that nuclei are retracted towards dorsal in myocardial cells initiating involution at the posterior heart cone prior to cone-to-tube transition (red arrow). (H-J) Separations of individual confocal channels in black and white. (I) Apical localization of aPKC is indicated (arrowhead). (L-N) Sagittal section of a 23-somite stage (20.5 hpf) embryo. (L) Lateral view onto the side of the heart field, zn5 is strongly expressed in cardiomyocytes and the overlying otic placode. (M) False-colored image of zn5 localization (green) at the basal side
of a row of cells. (N) Myocardial-specific GFP expression identifies these cells as cardiomyocytes. Red lines in schemes represent the level and orientation of section planes in accompanying images. A, anterior; P, posterior; L, left; R, right; D, dorsal; V, ventral; NT, neural tube.

Fig. S2
The first morphological myocardial tissue asymmetry arises at the heart cone stage. (A,B) Shown are ventral views of reconstructions of confocal serial images of (A) a 20-somite stage Tg(cmlc2:GFP) transgenic heart cone and (B) a 22-somite stage Tg(cmlc2:GFP)/Tg(cmlc2:mRFP) double transgenic heart cone. Note that early cmlc2:GFP expression-levels are not uniform within myocardial cells which results in stronger and weaker signals in corresponding cross sections. Red lines indicate cross section planes along the L/R axis of the heart cone perpendicular to the embryonic midline, green lines cross section planes along the embryonic A/P axis and the respective sections are shown within red or green insets. (A) Whereas the 20-somite stage heart cone is symmetric along the L/R axis, (B) serial cross section planes along the L/R axis of the 22-somite stage heart cone reveal myocardial tissue involution within the right and posterior quadrant (section planes 3 and 4). A, anterior; P, posterior; L, left; R, right; D, dorsal; V, ventral.

Fig. S3
Myocardial cells are facing the heart tube lumen with their basal surfaces
(A-D) Cross-sections of the heart tube at 30 hpf reveals the localization of the baso-lateral marker zn5 at the luminal side of myocardial cells. (A) Nuclear staining with DAPI (blue), immunohistochemical-staining with zn5 (red) and myocardial-specific GFP expression [Tg(cmlc2:GFP)] (green). (B) A ring of GFP expressing myocardial cells is formed after completion of the primary heart tube. (C) Basal zn5 localization facing the tube lumen. (D)
DAPI staining reveals the presence of nucleated erythrocytes and endocardial cells inside the heart tube lumen. (E) Diagrammatic representation of the radially-symmetric organization of the primary heart tube. The myocardial cells (green) are facing the tube lumen with their basal surfaces (blue-dotted line), whereas the apical side (black line) is facing towards the lumen of the pericardial cavity in which the heart tube resides. (F-I) Cross-sections of a Tg(cmlc2:mRFP) heart tube (red) at 32 hpf with immunohistochemical-staining of Laminin (green) and nuclear staining with DAPI (blue). (G) Magnification reveals the basally deposited ECM marker Laminin at the luminal side of the heart tube (arrowhead). (H) DAPI staining reveals that endocardial cells are not tightly connected at sites of Laminin staining (indicating the deposition of Laminin by myocardial cells [arrowhead]). (I) Overlap of myocardial-specific mRFP expression (gray) and Laminin staining (green) at the luminal side (arrowhead). Red lines in schemes represent the level and orientation of section planes in accompanying images. L, left; R, right; D, dorsal; V, ventral.

**Fig. S4**

Atrial versus ventricular cell shapes within the elongating heart tube

(A-D) Section along the anterior-posterior axis of the developing heart tube (45° angle away from the embryonic midline) at 27 hpf reveals distinct cell shapes of squamous atrial (marked with S46, blue) versus cuboidal ventricular myocardial cells. Myocardial GFP-positive cells (green), rhodamine phalloidin staining of F-actin (red), and atrium-specific S46 (blue). (B-D) Details of individual color channels (black and white). (B and D) Arrows indicate the border between atrial and ventricular myocardial cells (arrows). Red line in inset diagram represents the level and orientation of section plane in accompanying images. A, anterior; P, posterior; D, dorsal; V, ventral; At, atrial cells; Ve, ventricular cells.
**Fig. S5**

**Medial and lateral involution proceeds from posterior towards anterior during heart tube formation**

*(A-D)* Consecutive transverse optical sections from anterior towards posterior of a 27-somite stage (22.5 hpf) heart reveal a tissue fold on the medial side of the atrial heart tube which expands from anterior towards posterior (arrows in magnifications A’– D’). At this stage, the future atrial chamber is not closed ventrally and consists only of a dorsal roof. *(E,F)* Similar lateral closure processes occur on the right and left sides of the 27 hpf anterior heart tube. *(E)* Plastic section of a 27-somite stage (22.5 hpf) embryo after whole mount in situ hybridization using the myocardial marker *cmlc2* (blue) reveals a closure process on the left-lateral side of the heart tube (black arrow, inset shows magnified area). Nuclear counterstaining with neutral red. *(F)* Immunohistochemical staining of a transversal section of the 27 hpf heart shows the right-medial fold is closing the heart tube ventrally. *(G)* Ventral view of the heart primordium at the 27-somite stage (22.5 hpf) reveals V-shaped folds on its medial and lateral side and their posterior fusion (arrows). *(G’)* Magnification of the heart primordium (shown in G) with false-colored zn5 staining (green) and nuclear DAPI (blue) shows the fusion of lateral, medial and posterior involution folds. *(H)* Diagrammatic 3D representation of the heart tube at the 27-somite stage (22.5 hpf). Yellow line indicates section plane through medial and lateral folds. Orange line indicates section plane through the ventral floor during posterior involution. *(I)* Ventral view of reconstructions of confocal serial images of a 25-somite stage *Tg(cmlc:GFP)* transgenic heart tube. White lines indicate serial cross section planes along the L/R axis of the heart tube, green line a sagittal section plane along the A/P axis and the respective sections are shown within white (dorsal to the left) or green insets (dorsal to the top). *(A-F)* Red lines in inset diagrams represent the level and orientation of section planes in accompanying images. A, anterior; P, posterior; L, left; R, right; D, dorsal; V, ventral.
Fig. S6

Myocardial cells migrate uniformly along a 45 degree angle

(A-B) Stills from a high-magnification time-lapse recording (Movie S2) of the center of the heart cone beginning at the 20-somite stage (19 hpf) for two hours (t=0). Myocardial cells are GFP-positive [Tg(cmlc2:GFP)]. (B) Only cells which will form the ventricular outflow region remain at the midline (arrowheads). (B’) Dotted line indicates the area of the involuted posterior myocardium which appears slightly brighter. (C) Summary of nuclear trackings illustrates an uniform myocardial migration direction along a 45° angle away from the embryonic midline (yellow dotted line). A, Anterior; P, posterior; L, left; R, right.

Legends for Supplementary Movies

Movie S1
Time-lapse movie of a transgenic Tg(cmlc2:GFP) embryo following the myocardial-specific GFP expression over a six hour period beginning at the 22-somite stage (20 hpf). The great majority of WT myocardial cells migrates as a coherent population. Only a few single cells leave the heart field rapidly within the first 2 hours of recording.

Movie S2
High magnification time-lapse movie of a transgenic Tg(cmlc2:GFP) embryo following the myocardial-specific GFP expression over a two hour period beginning at the 20-somite stage (19 hpf). Nearly all myocardial cells leave the embryonic midline within this period at a 45 degree angle towards left-anterior. Only cells which will form the ventricular outflow region remain at the midline.
Movie S3
Transient expression of single myocardial cells expressing membrane-tagged GFP (cmlc2:mGFP). Myocardial cells maintain constant neighbor relationships and largely avoid cell mixing during heart tube formation. The movie covers 55 min beginning at the 25 somite stage (21.5 hpf). Inverse representation of black and white signal.

Movie S4
Time-lapse movie of a has/prkci morphant embryo within the Tg(cmlc2:GFP) background following the myocardial-specific GFP expression over a six hour period beginning at the 22-somite stage (20 hpf). Most cmlc2:GFP-positive cells are immobile and remain at the embryonic midline. At the periphery of the heart field, strings of adherent cmlc2:GFP-positive cells lose contact to neighboring cells and migrate randomly.

Movie S5
Time-lapse movie of a nok/mpp5 morphant embryo within the Tg(cmlc2:GFP) background following the myocardial-specific GFP expression over a six hour period beginning at the 22-somite stage (20 hpf). Most cmlc2:GFP-positive cells are immobile and remain at the embryonic midline. At the periphery of the heart field, strings of adherent cmlc2:GFP-positive cells lose frequently contact to neighboring cells followed by random single cell migration.

Movie S6
High magnification time-lapse movie of a nok/mpp5 morphant embryo within the Tg(cmlc2:GFP) background following the myocardial-specific GFP expression of randomly migrating cells over a period of one hour. Large protrusions are forming at sites of migration direction.