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Review

Shaping the zebrafish heart: From left–right axis specification to epithelial tissue morphogenesis

Jeroen Bakkers [a,b,*], Manon C. Verhoeven [a], Salim Abdelilah-Seyfried [c,*]

[a] Hubrecht Institute and University Medical Centre Utrecht, 3584 CT, Utrecht, The Netherlands
[b] Interuniversity Cardiology Institute of the Netherlands, 3511 GC, Utrecht, The Netherlands
[c] Max Delbrück Center (MDC) for Molecular Medicine, Berlin, Germany

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ABSTRACT

Although vertebrates appear bilaterally symmetric on the outside, various internal organs, including the heart, are asymmetric with respect to their position and/or their orientation based on the left/right (L/R) axis. The L/R axis is determined during embryo development. Determination of the L/R axis is fundamentally different from the determination of the anterior–posterior or the dorsal–ventral axis. In all vertebrates a ciliated organ has been described that induces a left-sided gene expression program, which includes Nodal expression in the left lateral plate mesoderm. To have a better understanding of organ laterality it is important to understand how L/R patterning induces cellular responses during organogenesis. In this review, we discuss the current understanding of the mechanisms of L/R patterning during zebrafish development and focus on how this affects cardiac morphogenesis. Several recent studies have provided unprecedented insights into the intimate link between L/R signaling and the cellular responses that drive morphogenesis of this organ.

Introduction

In vertebrates internal organs are asymmetric with respect to their position and/or their orientation based on the left/right (L/R) axis. This is a highly conserved feature and the normal asymmetric arrangement (*situs solitus*) occurs in well over 99% of humans. Interestingly, individuals who have all their organs arranged in a mirror-image fashion (*situs inversus*) generally face no harmful physiological consequences and often go through life undiagnosed. In contrast, *situs ambiguous* produces a mixture of normal and abnormal L/R pattern and usually manifests multiple congenital anomalies including asplenia or polysplenia, intestinal malrotation, pulmonary isomerism and various complex heart defects (Ramsdell, 2005). In humans, clinically significant laterality defects occur in at least 1 in 10,000 births (Peeters and Devriendt, 2006). The significant morbidity and mortality associated with laterality disease are almost always attributed to complex congenital heart defects, reflecting the extreme susceptibility of the developing heart to disturbances in L/R patterning.

The process of symmetry breaking is thought to improve organ packaging in a limited space through handed positioning and looping but is also necessary for proper functioning of many organs. The lack of variability of the body situs under normal conditions within and across vertebrate species implies that the determination of L/R identity is strictly controlled. L/R patterning can be subdivided into three distinct phases. The first involves the initial break in L/R symmetry and focuses on actions in and surrounding the nodal area, containing the ciliated laterality organ. Subsequently, the second phase converges on the propagation of the L/R information from the nodal area to the lateral plate mesoderm. Finally, this asymmetric information is interpreted by individual organ primordia, resulting in the asymmetric morphogenesis of several organs. In this review, we discuss the current understanding of the mechanisms of L/R patterning during zebrafish development with a focus on cardiac morphogenesis. Several recent studies have provided unprecedented insights into the intimate link between L/R signaling and the cellular responses that drive morphogenesis of this organ. Although, many of the mechanisms that determine the L/R axis are conserved amongst vertebrate species, some species-specific mechanisms exist, which are also discussed where appropriate.

Initial break in L/R symmetry

Work in different vertebrate model organisms has shown that the establishment of correct L/R asymmetric gene expression requires the presence of a transient ciliated organ at the embryonic midline, which is involved in breaking initial symmetry. In zebrafish, such an organ, termed Kupffer's vesicle (KV), is present between late gastrula and early segmentation stages (Essner et al., 2002).

* Corresponding authors.
E-mail addresses: j.bakkers@niob.knaw.nl (J. Bakkers), salim@mdc-berlin.de (S. Abdelilah-Seyfried).

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Morphogenesis of Kupffer's vesicle

The KV is derived from dorsal non-involuting marginal zone cells known as dorsal forerunner cells (DFCs) (Cooper and D'Amico, 1996; Melby et al., 1996). The DFCs are formed by internalization of surface epithelial cells (the enveloping layer or EVL), a process which requires Nodal signaling (Oteiza et al., 2008). Reducing Nodal signaling, by over-expressing the Nodal antagonist Lefty, reduces the number of DFCs. Conversely, ectopic Nodal signaling was shown to be sufficient for DFC formation since supernumerary DFCs and subsequently ectopic KVs are formed along the margin. In wild-type embryos, upon internalization, DFCs form a single large rosette structure that will generate a single Kupffer's vesicle. Besides Nodal signaling, a number of other factors have been identified that affect KV formation. Calcium, in addition to its suggested role in cilia mobility, regulates KV formation (Kreiling et al., 2008; Schneider et al., 2008). It has been suggested that intracellular calcium is required to inhibit β-catenin signaling in the DFCs. The activation of β-catenin, either by injecting mRNA encoding constitutively active β-catenin or by *axin-1* knock-down, specifically in DFCs, results in KV and laterality defects similar to those observed after reducing the intracellular calcium levels (Schneider et al., 2008). Reducing intracellular calcium levels by thapsagargin treatment results in more dispersed DFCs that fail to form a single KV. A similar affect was observed after morpholino knock-down of the T-box transcription factor, *tbx16*, which is also expressed in the DFCs (Amack et al., 2007). Tbx16 may function parallel to or redundantly with another T-box transcription factor, *no tail* (*ntl*, homologous to mouse *Brachyury*), which is required for formation of the lumen in the KV in a cell-autonomous manner (Amack et al., 2007; Amack and Yost, 2004). Although zebrafish has proven to be an excellent model to study KV morphogenesis and signaling, it will be important to identify the downstream targets for the above mentioned pathways to understand KV morphogenesis at the cellular level.

Fluid flow in Kupffer's vesicle

Cilia within the KV rotate in a counter-clockwise direction, thus generating a fluid flow in the same direction (Essner et al., 2005; Kramer-Zucker et al., 2005). The directional fluid flow in the KV induces asymmetric expression of two conserved L/R signaling genes *pitx2* (a bicoid-related transcription factor) and *southpaw* (*spaw*, a Nodal-related gene) in the lateral plate mesoderm (LPM). By which mechanism intracellular calcium levels are important for ciliary mobility and, consequently, for fluid flow within the KV are not known (Sarmah et al., 2007; Shu et al., 2003; Shu et al., 2007). Knock-down of the calcium exchanger Ncx4a or of the Na,K-ATPase-alpha-2 results in slightly elevated intracellular calcium levels in the KV and reduced cilia mobility and subsequent fluid flow. The G protein-coupled receptor (*gpr161*) is required to control intracellular calcium levels in the KV and perinodal area (Leung et al., 2008). Although it remains to be investigated whether cilia mobility is also affected by the knock-down of *gpr161*, the L/R patterning defects are partially rescued by overexpression of the calcium exchanger *ncx1*. Besides intracellular calcium, soluble inositol phosphates (IPs) are required for cilia mobility (Sarmah et al., 2005). Whether IPs regulate intracellular calcium levels and thereby affect cilia mobility or whether IPs have calcium independent functions remains to be studied. Interestingly, inositol 1,3,4,5,6-pentakisphosphate 2-kinase (Ipk1) that generates inositol hexakisphosphate is located in centrosomes and basal bodies, suggesting a role of Ipk1 in ciliogenesis (Sarmah et al., 2007).

More recently Yost et al. demonstrated a conserved role for Fgf signaling in ciliogenesis not only in the KV but also in other tissues (Neugebauer et al., 2009). Their results show that abrogating Fgf signaling results in downregulating expression of two ciliogenenic transcription factors, *foxj1* and *rfx2*, indicating that Fgf signalling mediates cilia length through an intraflagellar transport pathway. It is currently not clear how these results relate to previous findings that the implantation of Fgf beads in the LPM downregulates expression of *Nodal* in chick embryos and upregulates expression of *Nodal* in mouse embryos (Boettger et al., 1999; Meyers and Martin, 1999).

For the mechanism of how fluid flow (also called nodal flow derived from the node, the structure homologous to the KV in mouse) determines L/R patterning, two models now exist in mice. The first is based on the formation of a chemical gradient, by the asymmetric accumulation of a diffusible morphogen (Nonaka et al., 1998; Okada et al., 2005). In agreement with such a model, extracellular particles that contain growth factors such as Shh and also retinoic acid have been identified in the node of mouse embryos (Tanaka et al., 2005). Since retinoic signaling is symmetric around the node, it remains undetermined what the function is of these growth factor containing particles (Sirbu and Duester, 2006). The second model is based on the physical stimulation of immobile cilia, that lack left–right dynein, situated in the peripheral region of the ventral node. It has been proposed that these immobile cilia are mechanically actuated by nodal flow and that flexing of the immobile cilia on the left side of the node leads to an elevation of intracellular calcium within cells on the left side, which in turn can activate asymmetric patterns of gene expression (McGrath and Brueckner, 2003) (reviewed in Mercola, 2003; Tabin and Vogan, 2003). Similarly, in zebrafish, elevated intracellular calcium levels have been reported on the left side of the KV (Sarmah et al., 2005), even though non-mobile cilia have not been identified in the zebrafish KV yet.

For either model to fit appropriately in the KV, a non-uniform distribution of cilia is necessary to interrupt the cycle of fluid flow. Consistent with this theory, the majority of cilia are located on the anterior-dorsal surface of the KV (Kreiling et al., 2007; Okabe et al., 2008). Such a distribution of cilia in the KV will generate a fast leftward flow in the anterior half and a slow rightward flow in the posterior half (Kramer-Zucker et al., 2005). In addition to cilia distribution, the observed tilting of the cilia in a posterior direction will enhance these differential flow rates within the KV (Cartwright et al., 2004; Kramer-Zucker et al., 2005; Okabe et al., 2008; Supatto et al., 2008). Together, these observations provide solid evidence for differential fluid flow patterns within the KV of zebrafish.

Role of H^+ flux

Although nodal flow has been established as a critical factor in the establishment of L/R asymmetry, evidence suggests that additional symmetry breaking events may exist. H^+/K^+-ATPase activity during blastula stages is necessary for normal L/R asymmetry development, providing early L/R cues based on differences in membrane potential in *Xenopus*, zebrafish and chick (reviewed in Levin, 2005). Loss- and gain of function studies in zebrafish have demonstrated the importance of H^+-V-ATPase-dependent H^+ flux (Adams et al., 2006). The use of H^+-V-ATPase inhibitors at different time points revealed an early requirement, preceding formation of the KV, for correct L/R patterning. Since these inhibitors affect cilia formation in the KV as well, it is not unlikely that H^+-V-ATPase activity is required for proper formation and/or function of the KV.

Transfer of information from Kupffer's vesicle to the lateral plate mesoderm

Since most organs that display L/R asymmetry are derivatives of the LPM, it is essential to transfer the L/R information from the KV to the LPM. How this occurs has been studied extensively but is still not fully understood. In zebrafish, the expression of the Nodal-related gene *spaw*/*nodal* in the left LPM is the first sign of asymmetric gene expression and this expression is essential for L/R patterning of all

organs including the gut, heart and brain (also reviewed in Ahmad et al., 2004).

Perinodal area

Preceding the left-sided expression in the LPM, bilateral expression of *spaw/nodal* is initiated around the KV at the 4–6 somite stage and this expression domain is often referred to as the perinodal expression domain (Long et al., 2003). Only at the 10–12 somite stage, *spaw/nodal* expression is initiated in the LPM (Fig. 1). This left-sided *spaw/nodal* expression subsequently expands to the anterior of the embryo, a progression which requires an autoregulatory loop (Long et al., 2003). It remains open whether the Nodal signal is relayed, or whether the ligand itself is transported. Although the asymmetric expression of *spaw/nodal* in the LPM depends on the presence of a functional KV, the initial perinodal *spaw/nodal* expression does not (Gourronc et al., 2007). So far the only known factors that regulate the perinodal *spaw/nodal* expression domains are the Ntl and Tbx16 transcription factors. Both *ntl* and *tbx16* expression overlaps with the perinodal *spaw/nodal* expression and it was suggested that they regulate *spaw/nodal* expression independently from their function during KV morphogenesis (see above) (Gourronc et al., 2007). Like *spaw/nodal*, the expression of the Nodal antagonist *charon* (homologous to mouse *Dante5/Cerl-2*) in the perinodal area is independent of KV function. Possibly, *charon* is a direct target of *ntl* and *tbx16*, which are both expressed in the marginal zone of the gastrula embryo as well as the DFCs, since the *charon* promoter contains a functional T-box binding site (Gourronc et al., 2007). *charon* expression in the perinodal area is not only dependent on the activity of T-box transcription factors. Notch activity is also required for proper *charon* expression, while *spaw/nodal* expression in the same area is not (Gourronc et al., 2007). Although both *spaw/nodal* and *charon* in the perinodal area are required to transfer L/R patterning to the LPM, their expression is symmetric and independent of KV function (Fig. 2). Whereas the perinodal expressions of *spaw/nodal* and *charon/dante5* in zebrafish are symmetric, the perinodal expressions of *Nodal* and *Dante5* in mouse and *Cnr1/Nodal* in chick embryos are transiently asymmetric (Collignon et al., 1996; Levin et al., 1995; Lowe et al., 1996; Marques et al., 2004).

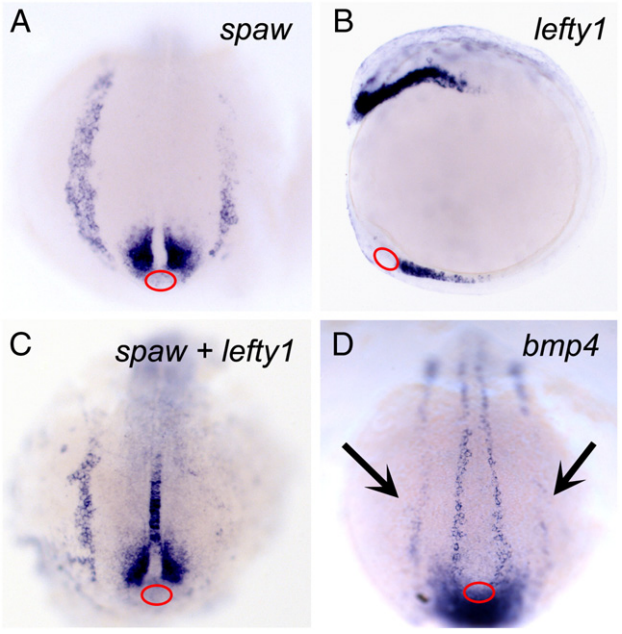

Fig. 1. Expression of *spaw/nodal*, *lefty1* and *bmp4* around the Kupffer's vesicle. Shown are wild-type embryos at 10 som stage stained by *in situ hybridization*. Embryos are shown as dorsal views (A, C, D) or lateral view (B). The red circle indicates the position of the KV. Expression of *spaw/nodal* is observed in 2 separate domains; perinodal area (close to the KV) and in the left LPM. *lefty1* is only expressed in the midline. *bmp4* is expressed in the tailbud mesoderm including the KV (not shown here) and the LPM (indicated by arrows).

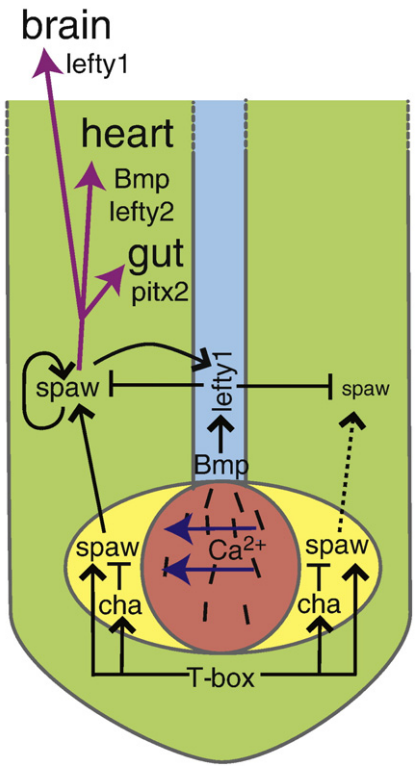

Fig. 2. Schematic overview of L/R patterning from KV to organs. The coloring indicates the different structures that are being described in the main text. Red, Kupffer's vesicle; yelow, perinodal area; green, lateral plate mesoderm; blue, midline.

Role for BMP signaling

In addition to Nodal and calcium signaling, Bmp signaling plays an important role in transducing the left determining signal from the KV to the LPM (Chocron et al., 2007). The *bmp4* ligand is strongly expressed in the LPM and the KV (Figs. 1 and 2). Blocking Bmp signaling at the end of gastrulation, which avoids major defects in dorsal–ventral patterning, results in a bilateral *spaw/nodal* expression in the LPM while ectopic Bmp activation at that stage results in the absence of *spaw/nodal* expression in the LPM. Two type II Bmp receptors are involved in transducing the Bmp signal to the LPM. Interestingly, the signaling properties of these two type II BMP receptors exhibited a striking difference in their ligand binding and signal transducing capacities (Monteiro et al., 2008). Knock-down of *bmp4* in the KV or the knock-down of one of the BMP type II receptors results in a reduced expression of *lefty1* in the midline (Chocron et al., 2007; Monteiro et al., 2008). Since Lefty1 is a Nodal antagonist, this suggests a model in which Bmp signaling regulates *spaw/nodal* expression in the LPM indirectly by activating *lefty1* expression in the midline (Fig. 2). *Lefty1* expression in the midline also requires *spaw/nodal* expression in the LPM (Long et al., 2003; Wang and Yost, 2008). It was proposed that in mouse embryos Nodal and Lefty maintain the L/R asymmetry by a self-enhancement and lateral-inhibition mechanism (Nakamura et al., 2006). The SELI model is able to convert a small difference between two separated regions into a robust difference through local activation and long-range inhibition (Meinhardt and Gierer, 2000). According to the model, the nodal flow generates only a small difference in Nodal levels around the node and the interplay of Nodal and Lefty converts this small asymmetry into an amplified and

exclusively left-sided Nodal expression spanning the left LPM. With this model it is easier to comprehend how dramatic differences in gene expression between the left and right side of the embryo can be initiated by, for instance, leftward nodal flow. By construction of a mathematical model it is possible to both simulate and predict certain asymmetry determining events in mouse embryos (Nakamura et al., 2006). Since *lefty* expression in the zebrafish is not overlapping with *spaw*/*nodal* expression and the regulation of *lefty* expression does not depend on Nodal activity only but also on Bmp activity, it remains unanswered whether a similar SELI model is active or not during zebrafish L/R patterning.

Organ laterality

A crucial feature in establishing asymmetric organogenesis is the interpretation of L/R specific information that is initiated around the node and subsequently propagated through the LPM. This information needs to be received by different organ primordia and is often translated into asymmetric gene expression patterns in or surrounding these primordia. Several signaling factors, including BMPs and Nodals, are important players in mediating L/R asymmetry during organ morphogenesis. While these factors have been mainly implicated in cell fate specification processes, newly emerging evidence suggests that a Nodal-Bmp signaling cascade regulates cell behaviors, including cell migration and tissue involution. Presently, the challenge is to elucidate the exact roles of each signaling pathway in modulating such cellular responses. This issue is further complicated by the fact that different regions within an organ primordium may respond differentially to L/R signals. As a consequence, cells will undergo asymmetric morphogenesis, which is tightly regulated and very specific for each organ.

Under normal conditions, there is a high degree of concordance between the laterality of various organs. However, in both humans and model organisms, diverse laterality defects have been described, which exhibit different degrees of discordance in brain, heart and gut asymmetry. This suggests that the L/R orientation of each of these organs is regulated separately (Bisgrove et al., 2000). In zebrafish, misexpression of *shh* on the right side of the embryo, for instance, which is an early regulator of the L/R signaling cascade, reverses asymmetric gene expression patterns in precursors of both heart and viscera. Under these conditions, the reversals of heart and gut are uncoordinated, suggesting that each organ interprets the signal (or the absence of a signal) in its own way (Schilling et al., 1999). Discordance has also been demonstrated in several other zebrafish mutants with notochord defects (*floating head*, *bozozok*, and *no tail*) (Chin et al., 2000). The differential patterning of the heart and viscera observed in these mutants suggests that normal development of midline structures is important for the coordination between heart and intestinal looping (Chin et al., 2000). As a final example, embryos with a mutant form of the Bmp receptor *laf*/*alk8* or embryos in which Bmp signaling has been inhibited during late somite stages exhibit discordance by a loss of heart laterality but normal positioning of internal organs (Chen et al., 2001; Chocron et al., 2007). These data demonstrate that there are different control mechanisms by which organs regulate laterality.

Heart morphogenesis

In zebrafish, as in many other vertebrates, the heart is the first organ to develop asymmetrically. Many of the molecules and mechanisms involved in the specification of myocardial cells and in the rapid morphogenesis of cardiac tissues during primary heart tube formation are evolutionarily conserved. Recent work in zebrafish has begun to elucidate the link between L/R signaling and tissue morphogenetic events involved in cardiogenesis at the cellular level. These studies have revealed a close correlation between asymmetric L/R signaling and cellular behaviors including directional cell migration and epithelial tissue involution.

Myocardial progenitor cell migration to the midline

Initially, the heart is derived from bilateral populations of mesodermal cells that undergo symmetrical and coherent medial movements towards the midline where they fuse to form a cardiac cone (Holtzman et al., 2007; Stainier et al., 1993). During midline migration, medial myocardial progenitor cells adopt a polarized epithelial organization with basolateral distribution of β-catenin, apical localization of atypical protein kinase C (aPKC) and junctional localization of zonula occludens 1 (ZO-1) (Rohr et al., 2006; Trinh and Stainier, 2004). In zebrafish, myocardial progenitor epithelial polarization is achieved by the concerted activities of the Heart and soul (Has)/aPKCi and Nagie oko (Nok)/Membrane protein palmitoylated 5 (Mpp5) cell polarity proteins. These evolutionarily conserved proteins are components of the larger Partition defective (Par) and Crumbs multiprotein complexes, respectively, that control the establishment of cellular asymmetry in various polarization processes. Moreover, the maintenance of adherens junctions between myocardial progenitor cells depends on deposits of the extracellular matrix (ECM) protein, Fibronectin, which laterally surrounds myocardial progenitor cells (Trinh and Stainier, 2004; Trinh et al., 2005). In *natter* mutants that lack Fibronectin, myocardial progenitor cells lose coherence and fail to migrate to the midline, causing cardia bifida. These findings suggested that cell-ECM interactions are necessary for epithelial organization of the myocardium and that organization of the myocardium as an epithelial layer is essential for directional tissue migration. However, more recently this interpretation has been challenged by the observation that loss of the cell polarity proteins Has/aPKCi or Nok/Mpp5, which completely disrupts the epithelial coherence of myocardial progenitor cells, prevents neither their midline migration nor the formation of a heart cone (Rohr et al., 2006). Therefore, the failure of *natter*/*fibronectin* mutant myocardial cells to migrate correctly suggests that Fibronectin functions as a substrate which paves the path during myocardial migration rather than that organizing epithelial polarity of the myocardium is essential for migration (Sakaguchi et al., 2006; Trinh and Stainier, 2004). Similar to the *natter*/*fibronectin* phenotype, lack of the sphingosine-1-phosphate (S1P) receptor Miles Apart or of spinster-like putative S1P transmembrane transporter Two of Hearts/Spns2 causes identical cardia bifida phenotypes (Kawahara et al., 2009; Kupperman et al., 2000; Osborne et al., 2008). It is likely, that the spinster-like transporter is involved in trafficking or extracellular release of S1P and that availability of this bioactive lipid for receptor–ligand interactions affects myocardial migration. One mode by which S1P receptor signaling may control myocardial migration is by modulating cell–Fibronectin interactions which was demonstrated within a primary tissue culture system of embryonic zebrafish cells (Matsui et al., 2007).

Endocardial/myocardial interactions during heart tube formation

Currently much less is known about the organization and role of endocardial progenitor cells that migrate in close association with the myocardial progenitor cell layer prior to heart tube assembly. The endothelial progenitor cell marker *flk-1*/*kdr-l* is expressed in endocardial cells and the analysis of a transgenic fish line using the *flk-1*/*kdr-l* promoter fused to GFP (*Tg*(*vegfr4:GFP*)) demonstrated that endocardial cells arrive at the midline already at the 16-somite stage (Bussmann et al., 2007; Liao et al., 1997; Trinh and Stainier, 2004) at which time myocardial progenitor cells are still in bilateral positions from the midline (Holtzman et al., 2007). In *cloche* mutant embryos, which lack all endothelial cells, including the endocardium, myocardial progenitor cells migrate towards the midline, albeit with some delay, and with altered modes of cell movements (Holtzman et al.,

2007). Therefore, myocardial–endocardial interactions play only a minor role during myocardial migration. Moreover, in the absence of endocardial cells, formation of a heart cone is only mildly affected (Holtzman et al., 2007; Stainier et al., 1995).

The first bilateral symmetry breaking event during heart development in zebrafish is the leftward displacement of the heart cone or cardiac 'jogging' (Chen et al., 1997). This process is under the control of the Nodal co-receptor One-eyed pinhead (Yan et al., 1999) and the left-sided Spaw/Nodal signaling cascade (Long et al., 2003). The heart cone has its base on the yolk and its central cells will give rise to the future ventricle (arterial pole), whereas the peripheral cells will form the future atrium (venous pole) (reviewed in Stainier, 2001). Although myocardial and endocardial cells form distinct populations, both cell types are displaced towards the left side simultaneously (Bussmann et al., 2007). Since cardiac jogging is unaffected and the subsequent elongation of the heart tube towards the left is only slightly delayed in *cloche* mutant embryos, endocardial cells are dispensable for this displacement of myocardial cells (Stainier et al., 1995). The direction of cardiac jogging is regulated by L/R signaling and is a good predictor of subsequent cardiac looping in wt embryos. Under normal conditions, leftward displacement of the heart tube will be accompanied by rightward (D) looping whereas a reversed rightward displacement of the tube will be accompanied by reversed leftward (L) looping (Chen et al., 1997). Some mutants however, have a high frequency of the "no-jog" phenotype combined with either a D-loop or a L-loop (Chen et al., 1997; Chin et al., 2000; Long et al., 2003; Yan et al., 1999). This demonstrates that cardiac placement to the left can be separated from cardiac looping and that there are likely separate mechanisms that regulate these processes. The mutants displaying this discordance of cardiac placement and cardiac looping are either defective in midline formation (e.g. *no tail*/*ntl* or *floatinghead*/*flh*) or have reduced Nodal signaling (e.g. *cyclops*/*cyc*, *MZoep* or *spaw*/*nodal* knock-down). This would suggest that either bilateral Nodal signaling (in case of the midline defects) or no Nodal signaling (in case of *spaw*/*nodal* knock-down) prevents cardiac displacement to the left, but does not prevent cardiac looping.

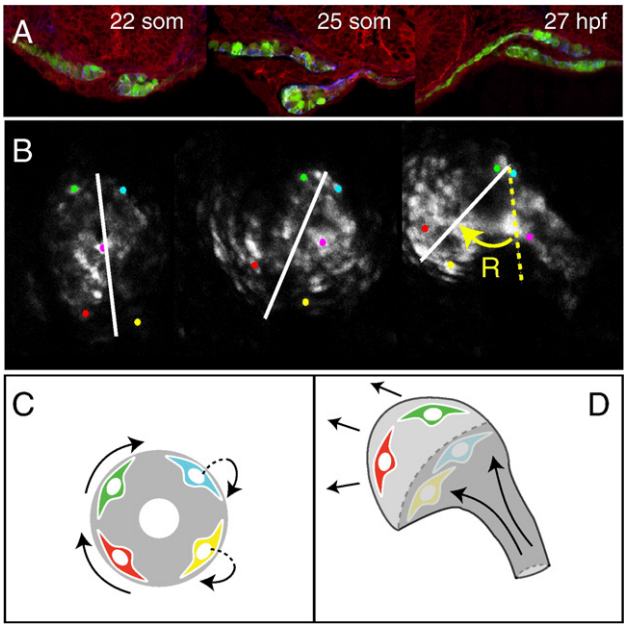

Fig. 3. Migration, rotation and involution of the cardiac tissue during cardiac tube formation. (A) Cross sections through the cardiac tissue during indicated different stages of heart tube formation. Note that the cardiac tissue derived from the right side of the heart field involutes and forms the ventral part of the cardiac tube. Sections where stained for GFP (green) expressed in the cardiomyocytes from the *Tg(cmlc2:eGFP)* transgene, PKC (red) and Zn5 (blue). (B) Cell tracking of individual cardiomyocytes during heart tube formation. The pictures shown are from timelapse confocal images of *Tg(cmlc2:eGFP)* embryos shown as dorsal views with anterior to the top and left to the left. The colored dots indicate individual cardiomyocytes (4 cells positioned in the lateral part of the cardiac disk (future atrium) and 1 cell (pink) positioned in the medial part of the cardiac disk (future ventricle). The white line represents an imaginary line dividing the cardiac disk in a left and a right half. The yellow arrow indicates the clockwise rotation (R) of the cardiac disk during heart tube formation. (C, D) Cartoon in which the migration, rotation and involution of the cardiac tissue during heart tube formation is indicated. The color-coding of the 4 cells is identical with the coloring in panel B. For clarity the pink (ventricular) cell was left out from the drawings.

Asymmetric myocardial tissue involution generates the heart tube

Recent detailed cell biological analyses have revealed that dynamic cellular rearrangements occur during these initial stages of cardiac tube formation. Within the heart cone, the myocardial epithelium shows cell shape differences between medial and lateral cells which indicates that cardiomyocytes undergo a process of epithelial maturation. Cardiomyocytes within more lateral positions of the heart cone have cuboidal epithelial shapes (they are as wide as they are tall) whereas those in more medial positions acquire highly elongated columnar shapes (they are taller than they are wide). Consistent with these cell shape changes, localization of the apical marker aPKC is restricted to apicolateral membranes within lateral myocardial cells whereas the aPKC localization domain is more prominent and extended within medial myocardial cells (Trinh and Stainier, 2004). At this stage, the epithelial organization of myocardial cells is essential for cardiac morphogenesis since loss of the cell polarity proteins Has/aPKCi (Horne-Badovinac et al., 2003; Peterson et al., 2001; Rohr et al., 2006) or Nok/Mpp5 (Rohr et al., 2006) causes an arrest of cardiac morphogenesis at the heart cone stage. This finding suggested that an essential part of L/R asymmetric cardiac development is an epithelial folding process. Indeed, at the onset of cardiac jogging one of the first morphological signs of asymmetry that arises within the zebrafish myocardial field is the occurrence of an involution fold of myocardial cells that are located within the right half of the cardiac cone (Fig. 3A) (Rohr et al., 2008). As this involution commences, myocardial cells derived from the right half of the heart cone move ventrally and towards the anterior/left. By this process of myocardial epithelial tissue involution, cardiomyocytes derived from the right heart field will form the future ventral part of the cardiac tube, while cardiomyocytes derived from the left heart field will contribute to the dorsal roof of the tube. As a consequence, the original L/R asymmetry of the cardiac cone is transferred into the dorsal–ventral asymmetry of the cardiac tube and endocardial cells that where located ventrally of the myocardial cone will be included within the lumen of the cardiac tube (Baker et al., 2008; Bussmann et al., 2007; Rohr et al., 2008; Smith et al., 2008). Disruption of Nodal signaling within the heart field, either by knock-down of *spaw*/*nodal* within the left LPM, or in *switch hitter* mutants that display randomization of *lefty2* expression, showed that the orientation of myocardial tissue involution was randomized, whereas the process of tissue involution itself appeared unaffected (Baker et al., 2008; Rohr et al., 2008). These findings suggest that Nodal signaling does not control the process of myocardial epithelial tissue involution per se.

A recurring process: Asymmetric involution of the LPM during gut looping

These recent analyses of asymmetric myocardial tissue behavior find a correlate in the morphogenetic events that underlie the L/R asymmetric formation of the digestive system, which is also subjected to L/R positional cues. This includes the bending of the intestine for proper space management in the abdominal cavity and the asymmetrical positioning of liver and pancreas with respect to the midline. In zebrafish, the first leftward bend of the developing intestine is referred to as gut looping, and takes place between 26 and 30 h post fertilization (hpf). During this process, cells from the

neighboring right lateral plate mesoderm migrate ventrally, whereas cells from the left lateral plate mesoderm move dorsally toward the embryonic midline thereby pushing the gut leftwards with respect to the midline. Therefore, the process of gut looping is an indirect consequence of asymmetric LPM movements which are under the control of left-sided Nodal signaling (Horne-Badovinac et al., 2003). Asymmetric migration of the LPM and the secondary gut looping is inhibited in *has/aPKCi* mutants that lack an epithelial organization within the LPM. The process of gut looping provides a good example of a complex organ morphogenetic process which is regulated in a tissue non-autonomous fashion whereby a neighboring tissue which responds to L/R signaling (the LPM) is exerting the morphogenetic force.

In comparison, midgut looping in the chicken embryo does not depend on asymmetric tissue migration but rather on left–right asymmetries of cellular morphologies within the dorsal mesentery, a tissue that connects the gut with the body wall. Left-sided Nodal signaling instructs epithelial cells within the left dorsal mesentery to adopt high columnar shapes and mesenchymal cells to condense whereas right sided epithelial cells are cuboidal (Davis et al., 2008). The asymmetric packing of mesenchymal cells is controlled by the left-sided expression of the adhesion molecule N-cadherin (Kurpios et al., 2008).

Rotation of the heart tube by differential myocardial cell migration

In addition to involution of myocardial cells, cardiac jogging involves differential migration behaviors within the myocardial field. Analyses of the cellular displacements of myocardial cells originating from selected areas within the cardiac cone (anterior left and right versus posterior left and right) revealed that myocardial cells located in the posterior half of the cardiac cone initially move slowly but gain speed when moving into the left-anterior direction and slow down again after reaching their left-sided destination. This is in contrast to myocardial cells in anterior positions that move with a constant and slower speed (de Campos-Baptista et al., 2008; Smith et al., 2008). As a consequence, the cardiac cone rotates in a clockwise direction when observed from a dorsal view (Fig. 3B). Not the entire cardiac tissue rotates equally, since reduced rotation is observed in the central part of the elongating heart tube, which is connected to the main vessels and which will form the future outflow region. The different rates of heart tube rotation result in some torsion of the cardiac tube that may have consequences for the looping events occurring later (Baker et al., 2008; de Campos-Baptista et al., 2008; Smith et al., 2008). The rotation of the peripheral region of the cardiac cone is constant and about equal to the angle of cardiac jogging along the L/R axis (Smith et al., 2008). Moreover, in embryos with mirror-imaged rightward cardiac jogging heart cone rotation is reversed as well (Baker et al., 2008). For their migration into the left-anterior direction, myocardial cells require *hyaluronan synthase 2* (*has2*). Has2 is expressed within the myocardial cells located at the leading edge of the cardiac cone but not within the surrounding LPM (Smith et al., 2008). Has2 produces the extracellular glycosaminoglycan hyaluronic acid, which can stimulate Rac1 activation, local lamellipodia formation and cell migration in cultured cells (Bourguignon et al., 2000; Oliferenko et al., 2000). During gastrulation Has2 is required for the dorsal migration of mesodermal cells during gastrulation by activating the small GTPase Rac1 and thereby inducing lamellipodia formation (Bakkers et al., 2004). Similar lamellipodial extensions are present on myocardial cells during cardiac jogging (Rohr et al., 2008). Although it is generally believed that hyaluronic acid is extruded into the extracellular space directly after its Has2-catalyzed synthesis at the cytoplasmic site of the plasma membrane, its effect on lamellipodia formation is cell-autonomous, suggesting that hyaluronic acids acts as an autocrine signal (Bakkers et al., 2004). Such an autocrine effect would allow a differential cellular regulation of migration behavior within tissues such as the LPM. Besides a requirement for Has2 during rotation and displacement of the heart tube towards the left side, Has2 is also required for normal cardiac looping in zebrafish (Smith et al., 2008) as well as in mouse embryos (Camenisch et al., 2000).

BMP and Nodal signals control myocardial cell migration

While *has2* acts as a permissive factor required for myocardial cell migration, Bmp signaling plays an instructive role by directing myocardial cell migration. Prior to the migration of myocardial progenitor cells towards the left side, *bmp4* is expressed asymmetrically around the cardiac cone with higher expression in the left LPM compared to the right LPM (Fig. 4). This asymmetric expression is only temporary (around 20–22 somite stage) and depends on Nodal signaling (Chen et al., 1997; Chocron et al., 2007; Schilling et al., 1999). This asymmetric expression of *bmp4* within the left LPM is also translated into a left-sided bias of Bmp signaling activity (Smith et al., 2008), which is required for left-sided cardiac jogging between the 18 and 25 somite stages (Chocron et al., 2007). Indeed, blocking all Bmp signaling at the 18 somite stage only affects heart morphogenesis but not gut looping. When Bmp signaling is blocked, myocardial cells located in the posterior half of the cardiac cone move with a constant and slow speed. Furthermore, ectopic activation of Bmp signaling in the entire heart region results in random migration paths of myocardial cells, suggesting that Bmps are acting as a directional cue in cardiac jogging. Consistent with this hypothesis, implanting Bmp-impregnated beads in various locations in the LPM redirects myocardial migration and is capable of recruiting the cardiac tube towards the Bmp bead. Together, these observations suggest that Bmp signaling in the left LPM plays an instructive role in the asymmetric myocardial tissue responses during cardiac jogging and directs the migration of cardiac progenitor cells (Smith et al., 2008). It remains to

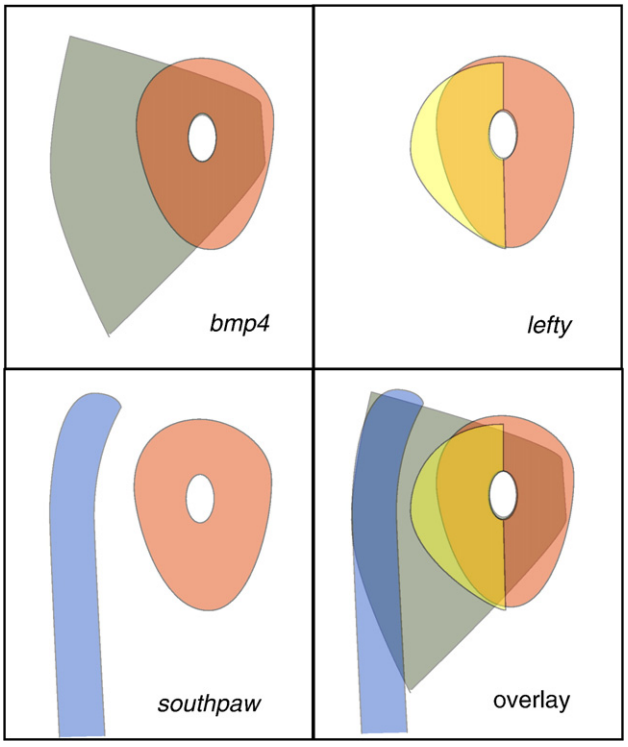

Fig. 4. Schematic overview of asymmetric gene expression around the cardiac field. At the 22–25 somite stage *bmp4*, *lefty1,2* and *southpaw* are expressed in the left LPM in and around the heart field (indicated in red). Cartoons are based on expression patterns reported in Chocron et al. (2007) and de Campos-Baptista et al. (2008).

be studied whether Bmps act directly as a chemo-attractant or have a more indirect role, as observed during gastrulation when Bmps affect the adhesion properties of the migrating cells (von der Hardt et al., 2007).

Similar roles for Nodal signaling in regulating cell migration during cardiac jogging have been suggested. Cardiomyocytes in mutants lacking One-eyed pinhead move more slowly and with less directionality (de Campos-Baptista et al., 2008). Interestingly, whilst this Nodal co-receptor is expressed bilaterally in all LPM cells and within the developing heart, *spaw* and *lefty2*, two other components of the Nodal signaling cascade, exhibit non-overlapping expression patterns within the left heart field and LPM (Fig. 4) (Bisgrove et al., 1999; Bisgrove et al., 2000; de Campos-Baptista et al., 2008; Zhang et al., 1998). How Nodal signaling affects cardiac rotation and differential cell migration remains open. Since the asymmetric *bmp4* expression in the LPM and heart field depends on Nodal signaling (Chen et al., 1997; Chocron et al., 2007), it is conceivable that the effects of asymmetric Nodal signaling are mediated via asymmetric Bmp signaling. Since Bmp signaling is also required to repress *spaw* expression in the right LPM, possibly by inducing *lefty1* expression in the midline, BMP signaling can be placed upstream of Nodal signaling as well (Fig. 2) (Chocron et al., 2007).

Currently it is not clear how left-sided Nodal or Bmp signaling affects cellular responses, including tissue involution and rapid directional migration, within the right heart cone. One possibility is that local signaling events within the left heart cone initiate more complex morphogenetic events that affect the right heart cone via local inductions or via propagation of mechanical forces. What is crucial is that L/R patterning influences cell behavior by providing directional cues in the LPM. Although several factors have been identified in the last year that control heart morphogenesis as a response to L/R patterning, it is likely that many have remained undiscovered. In particular we know very little about the later steps during heart morphogenesis that result in the looping of the linear heart tube. Rotation of the linear heart tube resulting in the change of initial L/R polarity into dorsal–ventral polarity is recognized in other vertebrates, including human (Campione et al., 2001; De la Cruz, 1998). The strong conservation of this mechanism amongst different vertebrate species might be explained by a requirement of rotation for (directional) cardiac looping. Some researchers have proposed by biomechanical modeling that rotation at the poles of the heart tube is even sufficient to drive cardiac looping (Männer, 2004; Voronov et al., 2004). Forward and reverse genetic approaches will hopefully contribute to fill the gaps in our understanding of this complex process in the future.

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