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ARTICLE

Left-Right Asymmetry and Cardiac Looping: Implications for Cardiac Development and Congenital Heart Disease

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Proper morphogenesis and positioning of internal organs requires delivery and interpretation of precise signals along the anterior-posterior, dorsal-ventral, and left-right axes. An elegant signaling cascade determines left- versus right-sided identity in visceral organs in a concordant fashion, resulting in a predictable left-right (LR) organ asymmetry in all vertebrates. The complex morphogenesis of the heart and its connections to the vasculature are particularly dependent upon coordinated LR signaling pathways. Disorganization of LR signals can result in myriad congenital heart defects that are a consequence of abnormal looping and remodeling of the primitive heart tube into a multi-chambered organ. A framework for understanding how LR asymmetric signals contribute to normal organogenesis has emerged and begins to explain the basis of many human diseases of LR asymmetry. Here we review the impact of LR signaling pathways on cardiac development and congenital heart disease. Am. J. Med. Genet. (Semin. Med. Genet.) 97:271–279, 2000. © 2001 Wiley-Liss, Inc.

KEY WORDS: left-right asymmetry; cardiac looping; cardiac development; congenital heart disease; heterotaxy

INTRODUCTION

The appearance of a bilateral exterior in vertebrates, including humans, disguises dramatic asymmetries of the interior body plan. The heart and vasculature, along with other organs, such as the lungs, stomach, intestines, and brain, show characteristic left–right (LR) asymmetry. Remarkably, the positioning of organs in the normal body plan (situs solitus) is conserved in all vertebrate species studied to date [Fujinaga, 1997]. Although the embryonic body plan is initially symmetric, the first anatomic indication of a more global establishment of LR asymmetry occurs with the rightward looping of the midline heart tube at human embryonic day 23 (mouse embryonic day 8.0–8.5). As the embryo twists in a counterclockwise direction along the rostrocaudal axis, LR asymmetry of other organ systems and the whole body emerges.

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Such an undeviating body plan suggests that highly conserved genetic pathways may control determination of the LR axis. In recent years, tremendous progress has been achieved in identifying many of the genes responsible for specifying LR patterning.[reviewed in Burdine and Schier, 2000; Capdevila et al., 2000]. Not surprisingly, perturbations in this pathway result in defects of laterality, known as heterotaxy. Translated from its Greek origins to mean "other arrangement," heterotaxy may occur in inherited or sporadic human diseases of LR abnormalities and manifests as aberrant visceral organ position and/or asymmetry [reviewed in Kosaki and Casey, 1998]. Morphogenesis of the heart appears especially sensitive to aberrations in LR positional information [reviewed in Harvey, 1998; Mercola, 1999]. Alterations in establishment of the LR axis are associated with an assortment of cardiac alignment defects, implying that pathways regulating LR asymmetry affect cardiac development (Table I). In this review, we attempt to integrate important findings from the LR field with clinical observations of aberrant cardiac looping, in an effort to understand better the molecular mechanisms involved in human cardiac laterality defects.

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LEFT-RIGHT EMBRYONIC AXIS AFFECTS DIRECTIONALITY OF CARDIAC LOOPING

Elucidation of components in the molecular pathways that establish LR asymmetry has been derived from studies in mouse, chick, frog, and zebrafish (Fig. 1). Many critical steps involve serine/threonine kinase receptors that bind to secreted proteins of the transforming growth factor (TGF)-β family [reviewed in Massagué et al., 2000]. From its midline position, Hensen's node appears to control the establishment of asymmetric LR gene expression throughout the lateral plate mesoderm (LPM) [Levin et al., 1995], which later contributes to most of the visceral organs.

TABLE I. Mutations Affecting Laterality and Cardiac Looping

Gene	Organism	Laterality and cardiac phenotype	Reference
Node component			
iv/Lrd	Mouse	Heterotaxy, randomized heart looping	Supp et al. [1997, 1999]
KIF3A	Mouse	Heterotaxy, randomized heart looping	Marszalek et al. [1999]; Takeda et al. [1999]
KIF3B	Mouse	Heterotaxy, randomized heart looping	Nonaka et al. [1998]
Signaling molecule			
Shh	Mouse	Randomized heart looping and abnormal heart position	Meyers and Martin [1999]
FGF-8	Mouse	Right pulmonary isomerism, randomized heart looping	Meyers and Martin [1999]
Activin receptor llb	Mouse	"Asplenia syndrome," randomization of heart position, right atrial isomerism, TGA, VSD, ASD	Oh and Li [1997]
ACVR2B	Human	Various situs abnormalities	Kosaki et al. [1999b]
Cryptic	Mouse	Right pulmonary isomerism, randomized cardiac looping, TGA, AVS defects	Gaio et al. [1999]; Yan et al. [1999]
CRYPTIC	Human	Various situs abnormalities	Bamford et al. [2000]
Lefty-1 (LEFTYB)	Mouse	"Polysplenia syndrome," left thoracic isomerism, left atrial isomerism	Meno et al. [1998]
LEFTYA (lefty-2)	Human	Various situs abnormalities	Kosaki et al. [1999a]
Nodal+/− Smad2+/−	Mouse	Right pulmonary isomerism, TGA	Nomura and Li [1998]
Cyclops (Nodal)	Zebrafish	Midline defects, including cyclopia, randomized cardiac looping	Chen et al. [1997]; Schilling et al. [1999]; Bisgrove et al. [2000]
Transcription factor			
Pitx2	Mouse	Right pulmonary isomerism, common AVC	Gage et al. [1999]; Kitamura et al. [1999]; Lin et al. [1999]
ZIC3	Human	X-linked situs abnormalities	Gebbia et al. [1997]
No tail	Zebrafish	Midline defects, randomized heart looping	Danos and Yost [1996]; Chen et al. [1997]; Schilling et al. [1999]; Bisgrove et al. [2000]
Floating head	Zebrafish	Midline defects, randomized heart looping	Danos and Yost [1996]; Chen et al. [1997]; Schilling et al. [1999]; Bisgrove et al. [2000]
Smad5	Mouse	Randomized heart looping, embryos fail to turn	Chang et al. [1999, 2000]
Unknown function			
Momo	Zebrafish	Midline defects, randomized heart looping	Odenthal et al. [1996]; Bisgrove et al. [2000]
Inv/inversin	Mouse	Situs inversus (mirror-image reversal)	Lowe et al. [1996]; Mochizuki et al. [1998]; Morgan et al. [1998]

FGF, fibroblast growth factor; TGA, transposition of the great arteries; VSD, ventricular septal defect; ASD, atrial septal defect; AVS, atrioventricular septum; AVC, atrioventricular canal; Shh, Sonic hedgehog.

At Hensen's node, several genes are expressed asymmetrically (Fig. 1A). On the left in the chick, expression of the morphogen *Sonic hedgehog* (*Shh*) becomes restricted to the nodal region and induces perinodal expression of *Nodal* [Levin et al., 1995; Pagan-Westphal and Tabin, 1998], a member of the TGF-β signaling family. Transfer of this "leftness" positional information from the node to the periphery in the LPM is mediated by a *Cerberus/Dan*-related secreted protein, Caronte (Car) [Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999]. As *Car* expression extends to the left LPM, Car directly antagonizes a subclass of TGF-β family members known as bone morphogenic proteins (BMPs), relieving BMP-mediated suppression of *Nodal* and possibly *lefty-2* in the LPM (Fig. 1B). Similar to other *Cerberus* family members, Car also can inhibit Nodal by direct binding, providing a negative

feedback loop to maintain precisely the levels of Nodal activity. Subsequent left-sided expression of *Nodal* induces expression of a bicoid-type homeodomain protein, Pitx2, in the LPM. Expression of *Pitx2* continues during asymmetric organ development along the left side of the heart tube, gut, and lungs [Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999; Essner et al., 2000; Schweickert et al., 2000]. In this fashion, LR information is transmitted from the node to the visceral organs.

On the right side, an inferred activin-like activity inhibits *Shh* expression [Levin et al., 1995], while *fibroblast growth factor* (FGF)-8 is up-regulated near the node [Boettger et al., 1999], where it inhibits *Car* expression [Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999]. Consequently, unrestrained BMP-mediated pathways repress right-sided *Nodal* expression and up-regulate the snail-related zinc finger transcription factor (SnR) [Isaac et al., 1997] in the right LPM. Together, these signals establish the LR axis and convey the positional information necessary for body situs and asymmetric organogenesis.

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Studies in the mouse have shown that ciliary projections on the node beat in a vortical movement to create a "nodal flow" that may displace molecules functioning as left determinants to the left of the node [Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999]. It is believed that determination of the LR axis in mice is initiated by ciliary beating that establishes asymmetric gene expression near the node, while a midline barrier, possibly lefty-1, maintains the accumulation of morphogens on the left side (Fig. 1A). The inversus viscerum (*iv/iv*) mouse strain displays situs solitus (normal position), situs inversus (mirror-image reversal), or situs *ambiguous*, a condition in which the arrangement of LR organization is uncoupled in visceral organs, similar to heterotaxy syndrome. Randomization of heart, lung, and gut asymmetry reflects a lack of coordination by the LR signaling pathways and often is associated with abnormal organogenesis. Expression of molecular markers for the LR pathway, such as *Nodal* [Supp et al., 1997] and *Pitx2* [Piedra et al., 1998; Ryan et al., 1998; Campione et al., 1999] in the *iv/iv* mouse is normal, reversed, bilateral, or absent, suggesting a malfunction in the biasing mechanism that distinguishes left from right.

The *iv* gene product encodes for *left-right dynein* (Lrd), which is an axonemal-type dynein that acts as a force-generating component in cilia that is expressed at many sites, including nodal cells [Supp et al., 1997, 1999]. Mice lacking *Lrd* or genes that encode other components of the ciliary motor, such as KIF3A or KIF3B, have immotile nodal cilia and thus fail to generate initial LR asymmetry around the node [Nonaka et al., 1998; Marszalek et al., 1999; Okada et al., 1999; Supp et al., 1999; Takeda et al., 1999]. With such evidence, a molecular model can begin to explain the clinical finding that situs abnormalities correlate with ciliary dysfunction in immotile cilia syndrome, also known as Kartagener syndrome [Afzelius, 1976].

Intriguing evolutionary questions have been raised by differences in chick and mouse with regard to the establishment of LR asymmetry. It is not yet known whether there are motile cilia in the chick node. However, the initial nodal asymmetry of FGF-8 and Shh is reversed in mice compared with chick, as are some downstream events, such as asymmetric expression of *Nkx3.2* [Meyers and Martin, 1999; Rodriguez-Esteban et al., 1999; Schneider et al., 1999]. Despite the disparity in early events, LR asymmetry of downstream events involving *Nodal* and *Pitx2* appear to be conserved in all species studied to date [Yost, 1999]. The discordance of early LR asymmetry among species has yet to be explained, but it may reflect the presence of undiscovered pathways regulating transmission of LR information from the node to the LPM.

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In the murine model of situs inversus (*inv*), nearly all of the *inv* mutants show complete reversal of the LR axis as it relates to organ asymmetry and position. Molecular analyses show *Nodal* and *Pitx2* expression along the right rather than the left LPM, suggesting an inversion in LR signaling [Lowe et al., 1996; Yoshioka et al., 1998; Campione et al., 1999]. In the clinical setting, patients that have situs inversus have a well-coordinated reversal of LR visceral asymmetry and thus have a low incidence of defects in organogenesis, which is similar to the findings in the *inv* mouse model (Fig. 2D). Although the *inv* gene product, inversin, is known, its function remains a mystery [Mochizuki et al., 1998; Morgan et al., 1998].

Like the phenotype of the *iv/iv* mouse, the phenotype of the majority of patients with LR defects consists of situs ambiguous (visceral heterotaxy) and possible defects in almost all aspects of cardiogenesis. Often, one side predominates; patients show signs of either bilateral right-sidedness, a condition known as "asplenia syndrome," or bilateral left-sidedness, a condition known as "polysplenia syndrome." Commonly, one side of a visceral organ is duplicated, and this is referred to as an isomerism. Several examples exist in which components of the LR signaling cascade are disrupted in mice, resulting in heterotaxy.

Targeted disruption of *Pitx2* in the mouse results in right pulmonary iso-

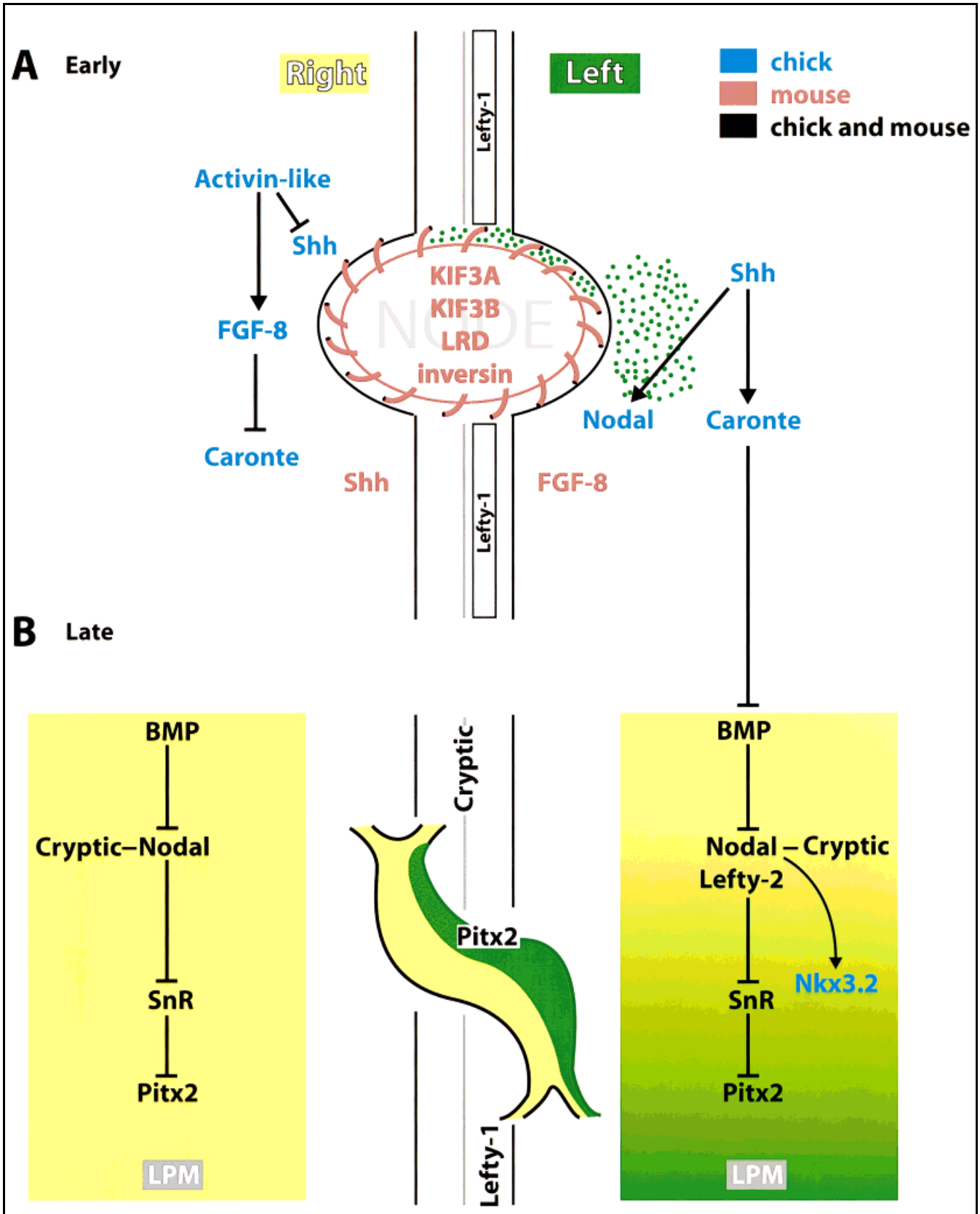

Figure 1. Cascades regulating left-right (LR) asymmetry. Early asymmetric gene expression around the node (**A**) results in activation or repression of Sonic hedgehog (Shh)– or fibroblast growth factor (FGF)-8–dependent pathways on the right or left (ventral view). Early roles of Shh and FGF8 are reversed in mouse and chick, as indicated in color-coded fashion. Leftward flow of morphogens (green dots) by nodal cilia establishes the asymmetric gradient around the node in mice. Expression of Lefty-1 near the midline may serve as a barrier to maintain left-sided asymmetry of morphogens. At later stages of organogenesis, LR asymmetric information at the node is transferred to the lateral plate mesoderm (LPM) by Caronte. Caronte relieves bone morphogenic protein (BMP) inhibition on the left, initiating a cascade of events that culminates in expression of Pitx2 in the left LPM and on the left side of the heart tube (**B**). Consequently, a "leftness" (green) signal appears to be propagated actively to overcome a default "rightness" (yellow) program [adapted from Capdevila et al., 2000].

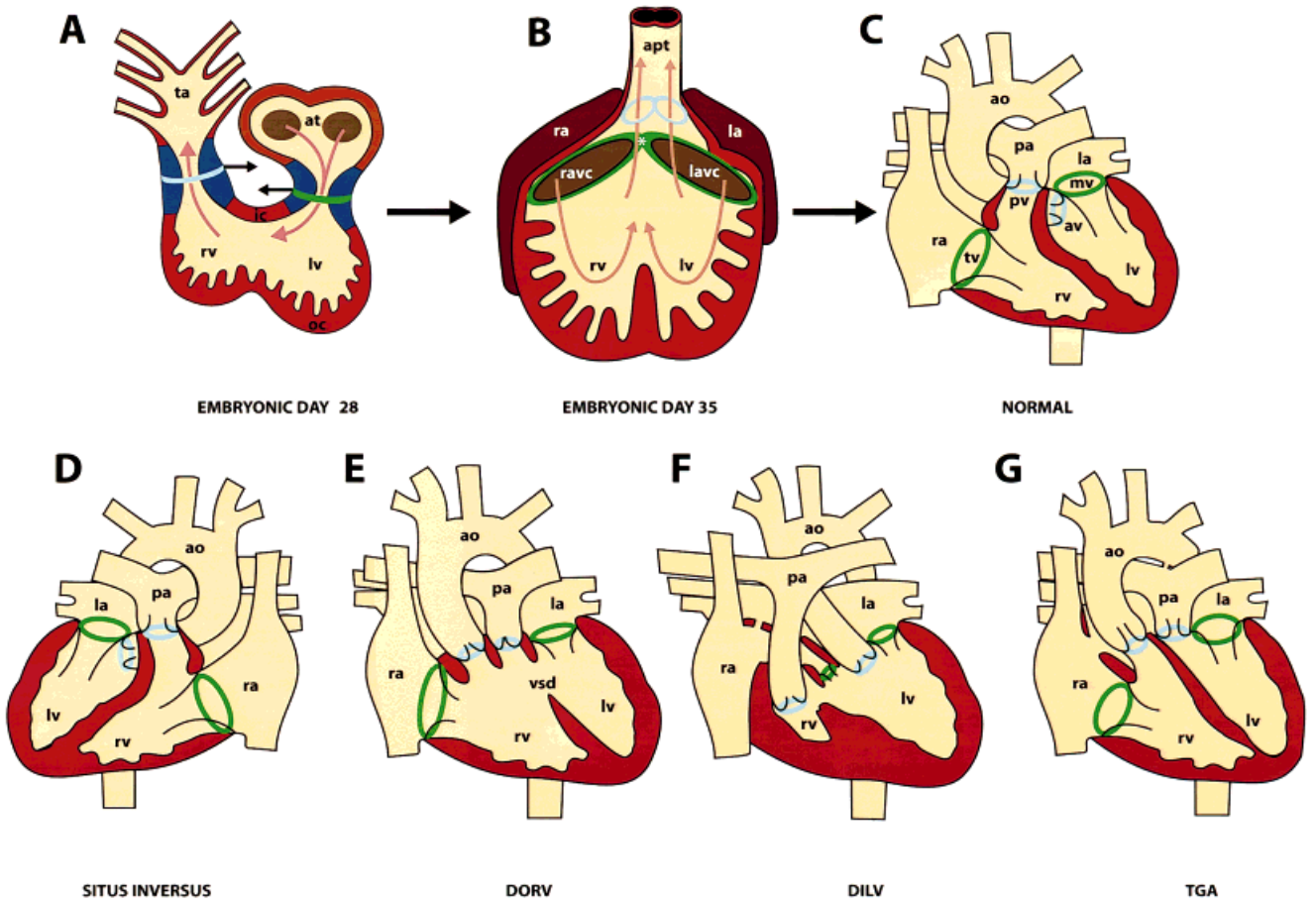

Figure 2. Normal and abnormal cardiac morphogenesis associated with left-right signaling. **A:** As the linear heart tube loops rightward with inner curvature (ic) remodeling and outer curvature (oc) proliferation, the endocardial cushions (dark blue) of the inflow (green) and outflow (light blue) tracts become adjacent to each other. Subsequently, the atrioventricular septum shifts to the right, while the aortopulmonary trunk shifts to the left. ta, truncus arteriosus; at, atrium; rv, right ventricle; lv, left ventricle. **B:** The inflow tract is divided into the right (ravc) and left (lavc) atrioventricular canal by the atrioventricular septum (asterisk). The outflow tract, known as the truncus arteriosus, becomes the aortopulmonary trunk (apt) upon septation. ra, right atrium; la, left atrium. **C:** Ultimately, the left atrium and right atrium are aligned with the left ventricle and right ventricle, respectively. The left ventricle and right ventricle become aligned with the aorta (ao) and the pulmonary artery (pa), respectively, after 180° rotation of the great vessels. mv, mitral valve; pv, pulmonary valve; tv, tricuspid valve. **D:** If the determinants of the left-right axis are reversed coordinately, then a condition known as situs inversus results. **E:** If the aortopulmonary trunk fails to shift to the left, a condition known as double-outlet right ventricle (DORV) results, in which the right ventricle is aligned with both the aorta and the pulmonary artery. vsd, ventricular septal defect. **F:** Likewise, if the AVS fails to shift to the right, both atria communicate with the left ventricle in a condition known as double-inlet left ventricle (DILV). **G:** Transposition of the great arteries (TGA) results if the aortopulmonary trunk fails to twist, resulting in communication of the right ventricle with the aorta and the left ventricle with the pulmonary artery.

merism [Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999]. Although the hearts of most *Pitx2* mutant embryos loop in the correct direction, the morphologic features of the heart appear abnormal, perhaps as a result of an anomalous process of looping. It remains possible that a low percentage of *Pitx2* mutants may display reversal of cardiac looping. Mice mutant for *activin receptor IIb* have a predominance of right-sidedness, similar to asplenia syndrome, and heart defects, which include randomization of position, transposition of the great arteries (TGA), ventricular septal defect, and atrial septal defect [Oh and Li, 1997]. Similarly, mice deficient for *cryptic*, the extracellular cofactor of Nodal, show signs of right pulmonary isomerism, randomization of cardiac looping, TGA, and atrioventricular septal defects [Gaio et al., 1999; Yan et al., 1999]. Finally, targeted disruption of *lefty-1* in mice leads to left rather than right thoracic isomerism, as in polysplenia syndrome [Meno et al., 1998]. This evidence suggests that in the absence of coordinated LR decisions, malalignments of ventriculo-arterial or atrioventricular connections may occur.

Studies in zebrafish and *Xenopus* have augmented our understanding of the common themes involved in vertebrate LR asymmetry. For example, in *Xenopus*, as early as the one-cell stage

[reviewed in Yost, 1995], a "left-right coordinator" is presumed to influence the Spemann organizer at the midline via the activity of another TGF-β family member, Vg1 [Hyatt and Yost, 1998; Ramsdell and Yost, 1999]. In turn, the Spemann organizer relays LR positional information to the rest of the developing embryo. On the left, expression of Nodal may be induced by Vg1, while an ALK2-dependent BMP pathway antagonizes Vg1 on the right [Ramsdell and Yost, 1999]. In zebrafish, though less is known, studies have established the key role of genes expressed in midline structures. For example, *cyclops* (*cyc*), an ortholog of Nodal, *no tail* (*ntl*), *floating head* (*flh*), and *momo* (*mom*) are necessary for establishing a midline domain that differentiates into midline structures, such as the anterior notochord and prechordal plate [Chen et al., 1997; Bisgrove et al., 2000]. It is thought that these tissues act as physical or molecular barriers that restrict left-sided signals from crossing the midline. Mutations in *cyc*, *ntl*, *flh*, and *mom* result in aberrations in LR asymmetry that include abnormal directionality of heart looping. In the heart field, BMP4 is expressed asymmetrically, but misexpression of BMP4 appears to affect only the direction of looping of the heart [Chen et al., 1997; Schilling et al., 1999]. This evidence suggests that BMP4 has a specific role in cardiac looping in zebrafish.

In humans, relatively few genes have been associated directly with laterality defects, although positional cloning and characterization of candidate genes in some families have provided insight into human laterality defects. For example, conventional genetic linkage analysis showed that ZIC3, a zinc finger transcription factor, is a causative factor in X-linked heterotaxy syndrome in several affected families [Gebbia et al., 1997]. Conversely, mutations in the human orthologs of *activin receptor IIb*, *ACVR2B* [Kosaki et al., 1999b], *lefty-2* (*LEFTYA*) [Kosaki et al., 1999a], and *CRYPTIC* [Bamford et al., 2000] were found in persons with LR asymmetry by a candidate gene approach, based on laterality defects found in mouse models.

LR AXIS AFFECTS THE PROCESS OF CARDIAC LOOPING

Soon after gastrulation, cardiogenic precursors from mesodermal cells in the anterior lateral mesoderm form a crescent that is specified to produce distinct segments of the linear heart tube, patterned along the anterior-posterior axis to make up the conotruncus (outflow tract), right (pulmonary) and left (systemic) ventricles, and atria. The left and right ventricular precursors initially are mixed along the anterior-posterior axis at the cardiac crescent stage and consequently assume their final LR position as a result of rightward looping of the heart tube. In contrast, the right atrium appears to develop from right-sided atrial progenitors, while the left atrium develops from those on the left [reviewed in Srivastava and Olson, 2000]. Mouse models mutant for *activin receptor IIb* [Oh and Li, 1997] or *lefty-1* [Meno et al., 1998] show right or left atrial isomerism, respectively, suggesting responsiveness by the left and right atria to LR signals.

The process of cardiac looping intricately establishes the relative positions of cardiac chambers and their vascular connections (Fig. 2A–C). The inflow and outflow tract cushions, swellings of extracellular matrix that remodel into valve tissue, become positioned adjacent to one another by folding of the heart tube (Fig. 2A). Subsequently, the left and right atria must align with the appropriate ventricular chambers, and each ventricle must connect with the aorta or pulmonary artery. This process is mediated by morphogenesis of the atrioventricular septum (AVS), which divides the inflow tract, known as the common atrioventricular canal (AVC), into a right and left AVC (Fig. 2B). As the AVS shifts to the right to lie above the ventricular septum, the AVCs follow and consequently become aligned over their respective ventricles. In conjunction, the common outflow tract, known as the truncus arteriosus, septates to become the aortopulmonary trunk. As it shifts to the left to situate itself over the AVS, the vascular trunk twists 180° to position the aorta properly over the left ventricle and the pulmonary artery correctly over the right ventricle (Fig. 2C). In this manner, the cardiovascular system is converted from a circuit in series to a parallel circulation, in preparation for terrestrial life away from the womb.

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Molecular modifications in cellular proliferation, transformation, migration, and death are thought to be involved in the process of looping, but the relative contributions of these cellular mechanisms remain unknown. The inner curvature of the looping heart tube appears to be remodeled, while the outer curvature actively proliferates. A phenomenon known as *myocardialization* may explain why trabeculations in the ventricles are found on the outer curvature while the inner curvature remains smooth [reviewed in Mjaatvedt et al., 1999]. Along the inner curvature, cardiomyocytes evacuate and migrate to the cushions, where they invade and muscularize without proliferating, resulting in relocation of myocardium from the inner curvature to the cushions. Mice with trisomy 16 (syntenic to parts of human chromosome 21 and 22) have defects in myocardialization, such that the myocardium of the inner curvature cannot be removed or remodeled in the absence of cushion development, resulting in defects in the process of looping [Webb et al., 1996].

Genes differentially expressed along the outer or inner curvatures of the looping heart tube have been discovered and may provide a molecular basis for their morphologic differences. Genes encoding atrial natriuretic factor (ANF) and SERCA2a, the sarcoplasmic reticulum calcium pump necessary for cardiac excitation and contraction, are expressed in the outer curvature of the ventricles

and atria but are absent in the inner curvature [Christoffels et al., 2000]. Additionally, two members of the basic helix-loop-helix family of transcription factors, *dHAND* and *eHAND*, are expressed in a complementary and ventricle-specific fashion, predominantly along the outer curvature of the heart [Biben and Harvey, 1997; Srivastava et al., 1997; Thomas et al., 1998]. This evidence suggests that intrinsic properties of the two surfaces may be central to cardiac looping.

Aberrations in the process of cardiac looping may result in many congenital heart diseases involving alignment defects. After the heart tube has initiated cardiac looping, any arrest or delay in the positioning of the AVS or the conotruncal region may result in malalignment of the inflow and outflow tracts with the ventricles (Fig. 2D–G). For example, if the conotruncus is unable to migrate to the left, the right ventricle communicates with the aorta and pulmonary artery, resulting in a condition known as double-outlet right ventricle (DORV) (Fig. 2E). Likewise, the AVS may fail to move to the right, resulting in a condition known as double-inlet left ventricle (DILV), in which the left ventricle communicates with both the left and right AVC (Fig. 2F). Genetic evidence for this has been discovered from embryos containing a targeted disruption for *Fog-2/Zfpm-2* that have a single AVC, which appears to communicate with only the left ventricle [Svensson et al., 2000; Tevosian et al., 2000]. However, the molecular basis for the shifts necessary for proper alignment remains unknown.

If the conotruncus moves appropriately to lie over the AVS but fails to twist, a condition known as TGA may result (Fig. 2G). In TGA, the pulmonary artery communicates with the left ventricle while the aorta communicates with the right ventricle, resulting in two separate circuits in which blood in the systemic circulation fails to be oxygenated. Molecular evidence for TGA has been gathered from embryos that are *trans*-heterozygous for mutations of *nodal* and *Smad2*, a downstream intracellular signaling component of the Nodal/TGF-β pathway [reviewed by Schier and Shen, 2000], which display laterality defects and an outflow alignment defect that is limited to TGA [Nomura and Li, 1998].

Together, these clinical observations and molecular evidence hint at the interaction of pathways that regulate the process and the directionality of cardiac looping. Indeed, some matrix proteins in the chick, such as flectin [Tsuda et al., 1998], are expressed asymmetrically along the linear heart tube and may play a role in the mechanical forces guiding cardiac looping. The discovery that Pitx2 is expressed asymmetrically in the LPM and on the left side of developing organs provides the first transcriptional inroad into understanding how LR signals might affect organogenesis. In the heart, targets of Pitx2 on the left side of the heart tube may interact with cascades governing cardiac development to promote appropriate direction and remodeling of the looping heart tube.

FUTURE DIRECTIONS

We have witnessed a remarkably rapid pace of discovery over the past five years in the field of LR asymmetry. An intricate LR biasing mechanism appears to be initiated early during embryogenesis, well before any evidence of organogenesis. However, many questions remain. Why are the early events of LR asymmetry less conserved across species than later events? What intermediate steps remain undiscovered between early node signals and subsequent LPM gene expression? What are the mechanisms through which LR asymmetric gene expression exerts its influence on individual organs? Ultimately, how does disruption of LR signals result in defects of organogenesis? Undoubtedly, these and other similar questions will be explored in the coming years and may provide a deeper understanding of human diseases associated with anomalies of laterality.

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