Review

Left–right asymmetry and congenital cardiac defects: Getting to the heart of the matter in vertebrate left–right axis determination

Ann F. Ramsdell *

Department of Cell and Developmental Biology and Anatomy, School of Medicine and Program in Women’s Studies, College of Arts and Sciences, University of South Carolina, Columbia, SC 29208, USA

Department of Cell Biology and Anatomy and Cardiovascular Developmental Biology Center, Medical University of South Carolina, Charleston, SC 29425, USA

Received for publication 9 June 2005, revised 21 July 2005, accepted 26 July 2005

Abstract

Cellular and molecular left–right differences that are present in the mesodermal heart fields suggest that the heart is lateralized from its inception. Left–right asymmetry persists as the heart fields coalesce to form the primary heart tube, and overt, morphological asymmetry first becomes evident when the heart tube undergoes looping morphogenesis. Thereafter, chamber formation, differentiation of the inflow and outflow tracts, and position of the heart relative to the midline are additional features of heart development that exhibit left–right differences. Observations made in human clinical studies and in animal models of laterality disease suggest that all of these features of cardiac development are influenced by the embryonic left–right body axis. When errors in left–right axis determination happen, they almost always are associated with complex congenital heart malformations. The purpose of this review is to highlight what is presently known about cardiac development and upstream processes of left–right axis determination, and to consider how perturbation of the left–right body plan might ultimately result in particular types of congenital heart defects.

2005 Elsevier Inc. All rights reserved.

Keywords: Cardiac development; Congenital heart defect; Heterotaxy; Left–right asymmetry; Situs inversus

Introduction

The significant morbidity and mortality of laterality disease almost always are attributed to complex, congenital heart defects (CHDs). This prevalence indicates that the developing heart is extremely susceptible to disturbances in embryonic left–right patterning. In attempt to define the cellular and molecular mechanisms that underlie cardiac asymmetry, most of the focus in the field has been on identifying genes and cell–cell signaling interactions that establish and maintain global left–right asymmetry of the vertebrate body plan. While this line of investigation is certainly critical to unraveling the issue, equally important is to understand how global left–right axial patterning intersects with morphogenetic processes of heart development. In this review, I discuss how heart development necessarily invokes three different types of asymmetric pattern and summarize the upstream molecules and inductive signaling processes that are central to current models of vertebrate left–right axis determination. In addition, I propose how different types of CHDs might arise when left–right axis defects impede normal development of one or more of the three types of asymmetric pattern in the heart.

Overview of left–right axis determination

Left–right development of the heart (and other organs) is critically dependent upon upstream pathways that impose asymmetry onto what is initially a bilaterally symmetric body plan. These early-acting pathways are collectively known as “left–right axis determination” and involve not only the breaking of bilateral symmetry, but also equally important, the directional orientation of asymmetry relative to the anteroposterior and dorsoventral body axes. Deviations in left–right axis determination during embryogenesis result in a
wide spectrum of abnormal laterality phenotypes that are generally classified as either \textit{situs inversus} or \textit{situs ambiguus}. \textit{Situs inversus} is a condition in which the left–right axis is reversed in alignment with the other two body axes, resulting in a mirror image of normal body and organ \textit{situs} (\textit{situs solitus}). Because of the concordant inversion of the body plan, one common misperception is that \textit{situs inversus} is not linked with a higher than normal incidence of CHDs. However, the estimated incidence of CHDs in \textit{situs inversus} patients is significantly higher than that in patients with \textit{situs solitus} (i.e., \textit{\%3} vs. \textit{\%0.08}) (Ferencz et al., 1985; Nugent et al., 1994; Sternick et al., 2004). In addition, the risk for developing laterality disease, and hence complex CHDs, is greatly increased for progeny of individuals with \textit{situs inversus} (Burn, 1991; Gebbia et al., 1997).

\textit{Situs ambiguus}, also termed heterotaxy, is a much broader category that refers to any combination of discordant normal and abnormal left–right asymmetries that cannot be strictly classified as \textit{situs solitus} or \textit{situs inversus}. Complex CHDs almost always are present in individuals with \textit{situs ambiguus} with estimates reaching at or greater than 90% (Nugent et al., 1994). Failure to establish asymmetry or errors in relay of axial patterning information during development can cause \textit{situs ambiguus}, such that asymmetry in structure and placement of organs still develops, albeit stochastically, due to the lack of definitive positional information. \textit{Situs ambiguus} also includes isomerism, a condition in which normally lateralized organs instead develop left or right symmetry. Cardiac defects typically occurring with \textit{situs ambiguus} include, but are not limited to, atrial septal defects (ASDs), ventricular septal defects (VSDs), transposition (or corrected transposition) of the great arteries (TGA), double outlet right ventricle (DORV), anomalous venous return, and aortic arch (AA) anomalies (reviewed by Bowers et al., 1996).

Once the body plan is established and positional information has been relayed throughout the embryo, three different endpoints of left–right axial pattern are possible. The first is directionally oriented looping that occurs in organs that essentially begin as a tube (e.g., heart or gut) (Figs. 1A, B). The series of bending and rotational movements in looping morphogenesis are necessary to establish structural asymmetry within organs as well as to establish proper organ placement within the body. A second endpoint of left–right pattern is unilateral regression and/or persistence of structure. One example of this is during spleen development, in which two organ fields are initially present to either side of the midline, but under normal circumstances, only the left-side tissue completes differentiation (Patterson et al., 2000) (Figs. 1C–E). Third, as exemplified by left–right differences in lobulation of the lungs and liver, some organs that first appear symmetric go on to develop structural features that show “handedness” to their asymmetry (Figs. 1F, G). This third endpoint is called lateralization of structure, and it is preceded by cellular and molecular left–right differences that are present even before morphological asymmetry can be observed. As detailed below, because the vertebrate heart must acquire all three endpoints of left–right pattern during its formation, it is especially prone to developing defects if any aspect of left–right axis determination is compromised.

\textbf{Cardiac left–right development}

\textit{Lateralization of the heart fields and the primary heart tube}

Mesoderm cells appear to become specified to a cardiac lineage quite early in development, either just prior to or during their migration at gastrulation (Antin et al., 1994; Yatskevych et al., 1997). As these cells gastrulate, they migrate anteriorly and spread laterally to the left and right of the embryonic midline (i.e., the primitive streak in chick or mouse, and the dorsal midline in fish and frog), where they form two paired fields of cardiac-fated mesoderm called the primary heart fields (Fig. 2A). Lineage analysis of gastrulating cells in the chick embryo has shown that very few cells cross the midline when migrating through the primitive streak (Levy and Khaner, 1998). As discussed below, because left–right positional information is present in the embryo during gastrulation stages, this suggests that left and right mesoderm cells, including those cells destined for the cardiac lineage, may be exposed to
different left–right patterning signals as they become arranged in the newly formed mesodermal germ layer.

The earliest indication of cardiac molecular asymmetry is observed after gastrulation, once cardiac cells are residents of the primary heart fields. In the chick, there are genes and proteins that are expressed either by only one heart field, or genes that are expressed asymmetrically by both heart fields, with expression being higher in one field compared to the other. Three proteins that show relative asymmetry in the primary heart fields are components of the extracellular matrix and include fibrillin-2 (Rongish et al., 1998; Smith et al., 1997), which is predominant on the right, and hLAMP-1 (Smith et al., 1997), and flectin (Tsuda et al., 1996), which are predominant on the left. Pitx2c, a bicoid-related transcription factor, is detected in cells only in the left, but not right, heart field (Campione et al., 2001; St Amand et al., 1998). Flectin and Pitx2c continue to be expressed asymmetrically as cells become incorporated into the primary heart tube.

The asymmetric gene expression in the primary heart tube is consistent with much earlier studies that demonstrated cellular differences between the left and right heart fields. For example, cardiomyocyte differentiation (Patten and Kramer, 1933) and striated myofibril formation (Lindner, 1960) happen slightly earlier in the right heart field compared to the left. The right cardiogenic fold appears slightly more advanced than the left (Stalsberg and DeHaan, 1969), and when cardia bifida is
elicited by preventing fusion of the two heart fields, two hearts form that show left–right differences (Van Praagh and DeHaan, 1967). The anterior portion of the heart tube is larger in the right heart structure and the posterior portion of the heart tube is larger in the left heart structure. Examination of the relative contributions of the primary heart fields to the chick heart indicates that the anterior and posterior regions of the heart differ in their composition of cells that were derived from each field. Specifically, a larger population of cells derived from the right heart field contributes to the posterior (inflow) region (Stalsberg, 1969). The cells that contribute to the posterior region of the heart tube are the same subset of cells that previously expressed nodal, lefty-2, and cSnr prior to their incorporation into the primary heart tube. As discussed below, nodal, lefty-2, and cSnr are left–right asymmetry genes that are expressed specifically in the left or right (but not both) lateral plate mesoderm, with their anterior expression domains reaching into the posterior primary heart field. Although these genes are no longer expressed once cardiac cells become incorporated into the heart tube, this observation suggests that this subset of posterior cardiac cells possesses a “history” of molecular asymmetries that distinguishes it from other cells in the heart. These observations suggest not only that the nascent heart tube exhibits cellular and molecular laterality, but also that laterality of the heart tube varies along the length of its anterior–posterior axis.

Besides the primary heart fields, another source of mesoderm cells, termed the “secondary”, or “anterior”, heart field (hereafter called the secondary/anterior heart field, SAHF) contributes to the heart tube (Franco et al., 2001; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001,2005a,b; Zaffran et al., 2004). The precise location of the SANF has been debated, with some investigators defining this field as a small area adjacent to the ventral pharynx (Waldo et al., 2001), and others defining it as a broader area to include mesoderm surrounding the aortic sac (AS) and extending into all of the pharyngeal arches (Franco et al., 2001; Mjaatvedt et al., 2001). Once cells from the left and right primary heart fields coalesce to form the ventricular and inflow regions of the heart tube, mesoderm cells from the SANF are added to the heart tube to complete its formation. In chick (and perhaps in frog), the SANF cells contribute to the conotruncus, or outflow tract (OFT) of the heart (Martinsen et al., 2004; Mjaatvedt et al., 2001; Waldo et al., 2001), in addition to the AS (Waldo et al., 2005a,b). Moreover, there is evidence that the SANF contributes to both the OFT and the right ventricle in the mouse heart (Kelly et al., 2001; Zaffran et al., 2004). The successful recruitment of cells from the SANF to the heart requires the presence of a second cell type, the cardiac neural crest, which migrates through the pharyngeal arches in order to eventually populate the OFT, and to a lesser extent, the inflow region of the heart (reviewed by Hutson and Kirby, 2003). The importance of cardiac neural crest with respect to the SANF is that the neural crest is thought to regulate “availability” of inductive factors that mediate addition of SANF cells to the OFT myocardium of the heart (Farrell et al., 1999, 2001; Waldo et al., 2005a,b; Yelbuz et al., 2002, 2003). Whether this regulation is direct or indirect is not known. Nevertheless, neural crest ablation experiments result in hearts that have shortened OFTs, in addition to several other types of defects that are discussed below (Farrell et al., 2001; Martinsen et al., 2004; Yelbuz et al., 2002, 2003). Similar to the primary heart fields, the SANF exhibits asymmetric Pitx2c expression that is detected in the left, but not right, AS mesoderm and left pharyngeal arch mesenchyme (Liu et al., 2002), suggesting that molecular and cellular laterality is present also in the SANF. In addition, Pitx2c plus two Pitx2 isoforms (Pitx2a and Pitx2b) are expressed—albeit symmetrically—in cardiac neural crest cells as they migrate into the heart (Hamblet et al., 2002; Kioussi et al., 2002).

Experimental manipulations made in gastrula and neurula stage embryos can alter left–right development of the heart, including asymmetric gene expression in the heart fields and directionality of looping morphogenesis. In the African clawed frog, Xenopus laevis, perturbation of the ectodermal extracellular matrix in the blastocoel roof causes reversed heart looping (Yost, 1992). Treating embryos at early neurula stages with heparan sulfate proteoglycan synthesis inhibitors prevents heart tube looping (Yost, 1990), and extirpations of midline tissues causes inverted or bilateral expression patterns of normally asymmetric genes in the lateral plate mesoderm, in addition to reversed heart tube looping if performed prior to closure of the neural tube (Danos and Yost, 1996; Lohr et al., 1997). Likewise, treatments that cause left–right axis perturbations in chick embryos cause both abnormal heart looping and inverted expression of the genes and proteins that are normally asymmetric in the heart fields. When explants of chick (Stalsberg, 1970) or Xenopus (Danos and Yost, 1996; Yost, 1990) precardiac mesoderm and its associated ectoderm are cultured in isolation, these tissues form a tube-shaped structure that somewhat resembles the primary heart tube including looping. Depending on the stage of the embryo from which the tissue is harvested, the direction of looping is either normal or stochastic. In Xenopus, the ability of the explant structures to consistently loop in a normal direction increases as the age of the donor embryo reaches late neurulation stages (Danos and Yost, 1996; Yost, 1990). Collectively, the observations made in Xenopus and chick demonstrate not only that cellular and molecular laterality is present in the heart from its inception, but also that some aspects of cardiac left–right asymmetry are regulated by processes that precede the appearance of the heart fields and the primary heart tube. This means that as cardiac mesoderm becomes established and as it contributes to the heart tube, these cells are already specified, at least to some extent, for left–right identities.

**Looping morphogenesis is necessary for septation and chamber and vessel concordance**

Even before the heart tube has completed its formation, it starts to undergo looping morphogenesis. Because cardiac looping is a highly conserved process that occurs quite early in vertebrate development, the directionality of the heart loop is commonly used as a “readout” of body situs. However, despite
it being one of the most widely recognized aspects of cardiac asymmetry, it is often less well appreciated that looping is a complex process involving both bending and rotational movements (reviewed by Manner, 2000). As the heart tube continues to elongate, it first develops a dextral loop ("d" loop) that results from the coordinated activities of ventral bending and rightward rotation. Dextral looping occurs concomitantly with increased growth at the outer vs. inner curvature, a process that accompanies chamber formation and that ultimately causes the heart tube to take on a "C"-shaped appearance (Christoffels et al., 2000; Rumyantsev, 1977; Thompson et al., 1990). Under the influence of the left–right body axis, the dextral loop normally orients to the right side of the embryonic midline, aligning the primordial cardiac chambers to face the outer curvature (Christoffels et al., 2000), and it is this aspect of looping that most investigators equate with "rightward" looping. Thereafter, the dextrally looped heart transitions to an "S" shape, a process that shifts the ventricular bend caudally toward the atria. The final phase of looping morphogenesis is characterized by the "wedging" of the distal OFT toward the right atrium. At this point of development, the heart has lost its tubular character, and the anterior (arterial) and posterior (venous) poles of the heart are brought together in close proximity. Current models of the "biomechanics" of looping suggest that forces that are present on both the left and right sides of the heart tube drive looping morphogenesis. Importantly, the left- and right-side forces are thought not to be equivalent and are thought to differ at the cranial vs. caudal aspects of the heart, such that the cranial portion of the tube undergoes a rightward rotation and the caudal portion of the tube undergoes a leftward rotation (Manner, 2004; Voronov et al., 2004). The opposing polarity of rotations at the two ends of the tube implies that asymmetry genes that control looping may be expressed in opposite left–right patterns at the distal ends of the heart tube (Manner, 2004; Voronov et al., 2004).

It should be emphasized that both the process of looping per se and the directionality of the loop are important for normal heart development. Because most cardiac structures arise from cells derived from more than one area of the heart tube, the significant outcome of looping is to rearrange regions of the heart tube so they are appropriately positioned for proper formation and alignment of chambers, valves, and septa (Fig. 2B). Thus, although left–right differences in the ventricles are established by anteroposterior, rather than left–right patterning processes (Franco et al., 2001; Thomas et al., 1998), the directionality of looping determines whether the morphological left ventricle underlies the left atrium, and the morphological right ventricle beneath the right atrium. When the topological sittus of the ventricles is correct, the heart is said to have atrioventricular (A-V) concordance.

In addition to A-V relations, looping also affects septation of the heart. As looping occurs, cushion tissues (progenitor valvuloseptal tissues) that have formed in the atrioventricular canal (AVC) and the OFT regions of the heart are brought together at the inner curvature of the looped heart tube. This repositioning allows two important processes to occur (Fig. 2C). First, it facilitates septation by bringing AV and OFT cushion tissues together for formation of the atrioventricular septum (AVS) and the OFT septum. If either end of the heart tube is delayed or somehow impaired in its looping, then this would cause cushion tissues to be out of alignment at the inner curvature of the heart, increasing the chance for septal defects to occur (Figs. 3B, G). Second, remodeling of the myocardium at the inner curvature is necessary in order for the OFT and the AVC to merge to form the future mitral-aortic continuity. One potential mechanism by which this remodeling occurs is through a process termed myocardialization, in which the OFT cushion tissues at the level of the inner curvature become invaded by overlying myocardial cells (van den Hoff et al., 1999). Abnormal myocardialization in hearts of trisomy 16 and other mouse mutant models is correlated with incomplete looping and failure to properly remodel the inner curvature, suggesting that myocardialization plays an important role in facilitating the final

---

**Fig. 3.** Congenital heart defects that are frequently associated with laterality disease. A schematic depiction of a normal heart is shown in panel A. Red and blue indicate oxygenated and deoxygenated blood, respectively, and purple shading in panels B–G indicates mixing of oxygenated and deoxygenated blood. A ventricular septal defect (VSD) is shown in panel B. An atrial septal defect is shown in panel C. Double-inlet left ventricle (DILV) is shown in panel D. Double-outlet right ventricle (DORV) is shown in panel E. Transposition of the great arteries (TGA) is shown in panel F. Persistent truncus arteriosus (PTA) is shown in panel G. All hearts are drawn in ventral view.
aspects of looping morphogenesis (Waller et al., 2000; Bartram et al., 2001; Boot et al., 2004). One probable outcome of myocardialization, therefore, is that it allows completion of wedging, which in turn, is necessary to establish properly aligned inflow and outflow tracts. In addition, this process also creates the muscular outflow septum below the level of the valves.

Differentiation of the inflow tract (IFT) and OFT occurs during wedging. In the IFT, the common AVC must become divided into left and right components by an AVS that shifts rightward during the final phase of looping to become positioned directly atop the ventricular septum. This results in alignment of the AVC with the ventricles. If the IFT does not fully undergo this rightward shift, a condition known as double-inlet left ventricle (DILV) persists (Fig. 3C). DILV results in persistence of blood flowing into the left ventricle from both the left and right atriums.

Meanwhile, at the OFT region of the heart, the conotruncus shifts leftward and simultaneously twists 180° to become positioned atop the AVS. This repositioning of the conotruncus serves two purposes. First, it brings proximal conal cushion tissues into alignment with AV and ventricular cushions so that septation can finish. Second, the rotational movement of the conotruncus is necessary to position the future base of the aorta and the pulmonary artery (which form from the distal conotruncus) with the appropriate ventricles. Failure to correctly align the conotruncus during looping morphogenesis can result in double-inlet right ventricle (DORV), a condition in which the right ventricle communicates with both the aorta and the pulmonary artery and the left ventricle has no outlet (Fig. 3D). This is different from the type of defect that would occur if the rotational aspect of conotruncal wedging was disturbed. Defects specifically in its rotational component would misalign the base of the aorta and the pulmonary artery with the left and right ventricles, resulting in a condition called transposition of the great arteries (TGA) (Fig. 3E).

In instances where normal conotruncal wedging and rotation happen, an outflow defect known as persistent truncus arteriosus (PTA) still can occur (Fig. 3F). The base of the aorta and the pulmonary artery form from the distal portion of the conotruncus plus the AS. Both the AS and the conotruncus must become divided in order to separate systemic and pulmonary blood flow through this region. When this septation process does not occur normally, PTA is the result. It has been appreciated for many years that aorticopulmonary septation requires the population of this region by the cardiac neural crest that pass through the pharyngeal arches to form the APS, which divides the AS (Nishibatake et al., 1987). Specifically, neural crest arising from between the fourth and sixth pharyngeal arches extends into the AS and ultimately merges with the fused OFT cushion tissues. Perturbation of this process in vivo, by neural crest cell ablation, results in PTA (Nishibatake et al., 1987).

Lateralization and differentiation of the inflow tract and AV canal

Unlike the ventricles, which form in “series”, the atria derive from a common progenitor, the common atrium, which must become divided into two chambers with distinct, left–right features (Anderson, 1992; Min et al., 2000). In Xenopus, this process of division may be related to differences in atrial cells that derive from the left vs. right primary heart field (Gormley and Nascone-Yoder, 2003). Once differentiated, the morphological right atrium contains pectinate muscles in its atrial appendage and receives the IVC, and the morphological left atrium contains a trabeculated appendage that lacks pectinated muscle and receives the pulmonary vein. Failure to achieve one or the other of these lateralized differences during division of the common atrium is the basis of atrial isomerism.

Studies of the interatrial septum (IAS) suggest that the left–right differences that arise during lateralization of the common atrium also can be related to the existence of two different cell populations that are present in this region. The IAS is derived, in large part, from a myocardial infolding of the left atrial wall, followed by transformation of some IAS cells to mesenchyme (Wessels et al., 2000). Myocardial cells of the IAS share common characteristics, such as creatine kinase B and Pitx2c expression with cells in the left, but not right, atrial wall, indicating a molecular asymmetry that is present in the common atrium prior to its differentiation (Franco and Campione, 2003; Liu et al., 2002; Wessels et al., 2000). Because the IAS originates from the cell population specified for “leftness”, in instances where left-side signaling pathways are impaired (e.g., right isomerism), it would be predicted that structures derived from the “left” cell population will not form. Consistent with this prediction, there is a significant deficiency, if not complete absence, of the IAS in hearts of many individuals afflicted with right atrial isomerism (Bowers et al., 1996).

Whether lateralized processes can influence other aspects of cardiac septation has not been investigated; however, several features of cardiac cushion formation (progenitor valvuloseptal tissue) in the AVC region suggest that this is possible. Despite their dorsal and ventral anatomic positions in the looped heart, the original superior (dorsal) and inferior (ventral) AV endocardial cushions form from the original left and right sides of the AVC (Lamers and Moorman, 2002; Moreno-Rodriguez et al., 1997). Consistent with their initial left–right origins, the inferior and superior cushions exhibit distinct properties throughout septation morphogenesis, including differences in temporal proliferative rates, spatial distribution, and absolute amounts of mesenchymal tissue formed (Lamers and Moorman, 2002; Moreno-Rodriguez et al., 1997). Additionally, myocardium in the AV canal exhibits left, but not right, side Pitx2c expression (Campione et al., 2001). These types of cellular and molecular left–right differences suggest that similar to the common atrium, the AVC region also is lateralized. Laterality disturbances in the AVC would be predicted to affect endocardial cushion tissue formation, ultimately increasing risk for valvuloseptal defects. By analogy to the AVC and the common atrium, it is tempting to speculate that the AS and conotruncus similarly develop a cellular and molecular laterality that influences their subsequent division into the base of the outlet vessels. If so, this could represent a process that, if affected by abnormalities in left–right pattern, could result in OFT defects such as PTA and/or TGA.
Asymmetric tissue regression patterns the aortic arches and venous return

In addition to lateralization and looping, the third type of asymmetry that occurs during normal heart development is unilateral regression of blood vessels that connect with the inflow and outflow portions of the heart. This feature is the basis for the complex patterned regression and persistence of the six pairs of aortic arch (AA) arteries and the aorta (Davies and Guest, 2003). Initially, the AA arteries develop as a series of six bilaterally paired vessels that connect with the paired dorsal aortae (Fig. 2D). During AA artery remodeling, the first two pairs of AA arteries regress into capillary beds. The third pair of AA arteries persists and eventually becomes the paired common carotid arteries. In contrast to the symmetric fates of AAs 1–3, the left artery of the fourth pair of AA arteries contributes to the aortic arch (in mammals), and the right artery contributes to the right subclavian artery. The fifth pair of AA arteries completely regresses or fails to form, and the sixth pair of AA arteries persists only on the left side to contribute to the pulmonary artery and the truncus arteriosus. At the inflow region of the heart, similar mechanisms of regression/persistence operate to pattern the paired posterior cardinal, subcardinal, and supracardinal veins, which ultimately give rise to the right-side inferior vena cava (Fasouliotis et al., 2002) (Fig. 2E). Aberrant regression/persistence patterns in the AA arteries and the cardinal veins therefore can result in many types of AA anomalies or abnormal venous return, depending on which particular vessels are affected (Ruscazio et al., 1998).

Left–right patterning events upstream of cardiac development

Because cardiac laterality defects nearly always occur in conjunction with laterality defects in one or more other organs, this indicates that the causative perturbation is one that occurs early in development and that precedes organogenesis. A widely held view of left–right development is that it proceeds as a three “step” process. The first is left–right axis determination, which establishes initial asymmetry in the embryo that is in correct alignment with the other two body axes. Thereafter, left–right positional information emanating from this axis must propagate throughout the embryo over a wide range of developmental stages, so that this information becomes relayed to each emerging cell and tissue type. Finally, once organogenesis begins, cells then must interpret and respond to the global left–right “blueprint” in order to translate this information into anatomical asymmetries. Errors in any of these three steps can result in cardiac (and other) laterality defects. The nature of the upstream signaling molecules that convey left–right patterning information in each of the three “steps” remained for the most part unknown until the pivotal 1995 discovery that Sonic Hedgehog and Activin can function as left–right asymmetry genes in the chick (Levin et al., 1995). Since this time, the field of vertebrate left–right development has rapidly grown to recognize dozens more genes, as well as many types of cell–cell signaling interactions, that act in concert to establish left–right asymmetries in the embryo. Molecules and inductive signaling processes that are central to current models of vertebrate left–right development are discussed below.

Breaking bilateral symmetry: models of left–right axis determination

In Xenopus, there is a patch of tissue located in the dorsal part of the blastopore, called the dorsal lip, that is capable of initiating gastrulation and directing complete secondary axis formation when transplanted to a ventral region in a host embryo. Because of the unique inductive properties of this tissue, the dorsal lip is historically known as the “organizer”. Functionally equivalent structures exist in mammals (the node), avians (Hensen’s node), and zebrafish (shield). Implicit in the patterning properties observed in grafted organizer/node tissue is that the organizer/node is a source of positional information for the different cell types that it induces. Direct evidence for node involvement in left–right development was first derived from studies in chick and mouse, in which a series of grafting and extirpation experiments indicated that the node is both necessary and sufficient to direct left–right asymmetry of the body plan (Davidson et al., 1999; Pagan-Westphal and Tabin, 1998). In chick, the node is not the first source of left–right asymmetry information; but rather, tissues adjacent to the node impart left–right pattern that is in turn relayed by the node to surrounding tissues as development proceeds (Pagan-Westphal and Tabin, 1998). In the mouse embryo, node ablation during late gastrulation results in embryos with normal anterior–posterior and dorsoventral development, but abnormal left–right development, highlighting its important function in propagating left–right patterning information (Davidson et al., 1999).

The earliest molecular aspect of left–right axis determination that is clearly conserved among all vertebrates is the asymmetric, left-side expression of Nodal. Nodal is a TGFβ family member that is expressed in the left half of the node, followed by the onset of a wide domain of expression in the left, but not right, lateral plate mesoderm. Normal, left-side only Nodal expression is required for normal left–right development in all species thus far examined (Collignon et al., 1996; Hyatt et al., 1996; Levin et al., 1995; Lohr et al., 1997; Lowe et al., 1996; Rebagliati et al., 1998b; Sampath et al., 1997). Bilateral, absent, or right-side Nodal expression patterns are observed in iv/iv mice, a classic animal model of heterotaxy (Collignon et al., 1996; Lowe et al., 1996). In inv/inv mice, which exhibit situs inversus, inverted (right-side) Nodal expression is observed (Collignon et al., 1996). Consistent with these findings, direct perturbation of Nodal expression in mouse results in situs ambiguus, indicating that left–right development of the heart and visceral organs requires restricted, left-side nodal activity (Brennan et al., 2002). Studies of Nodal homologs and components of the nodal signaling pathway in Xenopus and zebrafish have corroborated its central role in establishing vertebrate left–right asymmetries (Ahmad et al., 2004; Hashimoto et al., 2004; Lohr et al., 1997, 1998; Rebagliati et al., 1998a,b; Sampath et al., 1997; Schier, 2003; Schier and Shen, 2000).
Experimental evidence derived from mouse, chick, and *Xenopus* has led to a number of models that seek to explain the steps of left–right axis determination that operate upstream of nodal expression. In the mouse, “nodal flow,” a mechanism that involves ciliary function in node cells, is the prevailing model. Null mutations in genes that are necessary for cilia formation and/or function (e.g., *iv* (a.k.a. *left–right dynein*), *inv*, *Kif3-A*, *Kif3-B*, *HFH-4*, *RFX3*, *D2LIC*) result in pronounced laterality defects (Bonnafe et al., 2004; Brody et al., 2000; Chen et al., 1998; Marszalek et al., 1999; Morgan et al., 1988; Nonaka et al., 1998; Rana et al., 2004; Supp et al., 1997; Watanabe et al., 2003). A role for ciliary genes was not necessarily unexpected, since much earlier studies of Kartegener’s syndrome (a situs inversus phenotype) had revealed an association between human laterality defects and ultrastructural defects in cilia (reviewed by Palmblad et al., 1984). Stunning experiments performed with video microscopy have shown that motile cilia present in the center of the mouse node propagate directional fluid flow, and furthermore, that this flow is abnormal in several mouse models bearing null mutations in ciliary motor proteins (Nonaka et al., 1998, 2002; Okada et al., 1999; Watanabe et al., 2003). One widely held interpretation is that nodal flow directs left–right axis formation by causing asymmetric accumulation of a diffusible morphogen that, in turn, launches widespread asymmetric gene expression of factors such as *nodal*. In support of this model, it has been shown that nodal cilia in mouse, rabbit, zebrafish, and medaka fish exhibit a posterior tilt that is thought to result in much deeper ciliary contact with nodal fluid in the right-to-left portion of ciliary rotational beating, a phenomenon that in turn could account for asymmetric deposition of components swept by nodal flow (Kramer-Zucker et al., 2005; Okada et al., 2005). Direct evidence for the ability of nodal cilia to generate an asymmetric gradient was demonstrated by the introduction of fluorescently conjugated dextran particles (comparable to protein ~40 kDa in size), which were found to distribute preferentially on the left side of the rabbit node (Okada et al., 2005), and FGF, SHH, and retinoic acid are factors that are proposed to be involved in generating the morphogen gradient in the mouse node (Tanaka et al., 2005). However, as detailed elsewhere (Hornstein and Tabin, 2005; Levin, 2004; Wagner and Yost, 2000), there are some inconsistencies between predictions of the nodal flow morphogen model and the nature of the laterality defects present in mice null for genes that are needed for ciliary formation or function. With the discovery of a second population of nodal cilia, the so-called “mechano-sensory cilia,” an alternative model has been put forth. As reported by Brueckner and colleagues (McGrath et al., 2003), the mouse node also contains non-motile cilia that are located on its periphery and that detect flow generated by the central, motile cilia (Fig. 4A). The net result of this detection is a transient spike in intracellular Ca\(^{2+}\) levels in cells to the left of the node, which in turn culminates in left-side nodal expression (Fig. 4B). In this alternative, “two-cilia” model, defects in either or both types of cilia would cause defective left–right axis determination. Consistent with this, mice null for *polycystin-2*—a gene that is mutated in polycystic kidney disease (Mochizuki et al., 1996) and that also is expressed by the non-motile, sensory cilia in the node (McGrath et al., 2003)—exhibit abnormal left–right development in addition to renal and pancreatic cysts (Pennekamp et al., 2005).

---

**Fig. 4.** Nodal flow models proposed for mouse and zebrafish. In mouse (A), there are two populations of cilia located in the node. The centrally located cilia, which express *left–right dynein* (*lr*; green) and *polycystin-2* (*red*), propagate directional fluid flow that is detected by peripherally located cilia that express *polycystin-2* but not *left–right dynein*. In response to the directional fluid flow, Ca\(^{2+}\) levels become elevated in cells located to the left of the node, which in turn is proposed to regulate left-side nodal expression (B). In zebrafish (C), dorsal forerunner cells express *lr*. Genetic mutations (*oep*, *sur*, *ntl*) that inhibit *lr* expression cause left–right defects, as do mutations in genes that are necessary for these cells to form Kupffer’s vesicle (*npl*, *spt*). Once organized into Kupffer’s vesicle, these cells develop cilia that propagate directional fluid flow, which in turn, is proposed to regulate asymmetric gene expression (*nodal*, *lefty*, *pitx2*) in the lateral plate mesoderm. Panels A and B courtesy of M. Brueckner (McGrath et al., 2003) and panel C courtesy of H. J. Yost (Essner et al., 2005).
Exactly how left–right differences in Ca\textsuperscript{2+} signaling regulate nodal asymmetry still needs to be defined, and it has been suggested that decreased levels on the right side of the node might function to repress nodal expression on this side (McGrath et al., 2003). The recent findings that inversin (the protein encoded by inv) blocks canonical Wnt signaling and that fluid flow can cause increased levels of inversin in ciliated cells suggest that important roles for these two factors in ciliary function and ultimately, regulation of nodal asymmetry, might also be found (Simons et al., 2005).

As details unfolded with the nodal flow model in the mouse, efforts were made to determine whether this mechanism operates in other vertebrates. “Nodal” cilia were soon discovered in chick, frog, and zebrafish (Essner et al., 2002). Functional studies in zebrafish indicate that similar to the nodal cilia models in mouse, directional fluid flow is a critical aspect of left–right asymmetry determination in this species (Essner et al., 2005; Kramer-Zucker et al., 2005). In the zebrafish, a small population of cells known as dorsal forerunner cells migrate at the leading edge of the shield (“node”). Near the end of gastrulation, these cells involute and form a structure of undefined function called Kupffer’s vesicle. Defects in dorsal forerunner cell migration or interference with Kupffer’s vesicle formation causes left–right defects (Amack and Yost, 2004) and ciliated cells in Kupffer’s vesicle express LRD, which is necessary for their ability to generate directional fluid flow in this region (Essner et al., 2005) (Fig. 4C). As in mouse, it is proposed that unidirectional fluid flow (in Kupffer’s vesicle) regulates asymmetric nodal expression in lateral tissue via directed accumulation of an unknown, left-side determinant (Essner et al., 2005).

In *Xenopus*, there is abundant evidence that molecular asymmetry is established at stages of development that precede detection of “nodal” cilia (Fig. 5A). In the cleavage stage *Xenopus* embryo, two molecular asymmetries are present: a fusicoccin receptor, called the 14-3-3E protein, is expressed in right, but not left, blastomeres at the 2–4 cell stage (Bunney et al., 2003), and an H+/K+-ATPase pump is transiently asymmetrically expressed in the right, but not left, ventral cell of the 4-cell stage embryo (Levin et al., 2002). Whether there is functional overlap between these two proteins is not clear (14-3-3 proteins control a variety of H+ pumps and ion channels in other systems); but, regardless, assays employing inhibitors of either the 14-3-3E protein or the H+/K+-ATPase pump result in situs ambiguous as well as abnormal nodal expression patterns (Bunney et al., 2003; Levin et al., 2002). The asymmetric expression of the H+/K+-ATPase pump results in differential left–right pH and voltage gradients; these gradients are proposed to set up left-side accumulation of small molecule morphogen(s)—serotonin is a recently identified candidate (Fukumoto et al., 2005)—via unidirectional movement through gap junctions, which have been shown to be necessary for normal left–right patterning in *Xenopus* (Levin and Mercola, 1998). One possible role for motor proteins such as lrd, inv, and Kif3-B is that they function as cytoplasmic transporters to localize proteins and miRNAs involved in establishing left–right asymmetry, e.g., the 14-3-3E protein and the H+/K+-ATPase (Levin, 2004). Implicit in this model is that the cytoskeleton is oriented in alignment with the future left–right axis—a phenomenon that is possible given that left–right organ reversals have been linked with disturbances in the microtubule-

---

![Fig. 5. Models of axis initiation in *Xenopus* and chick.](image)

(A) A summary of processes and genes that function upstream of left-side nodal expression in *Xenopus*. Beginning with the one-cell stage embryo, cortical rotation affects formation of both the dorsoventral and left–right axes. During subsequent cleavages, various proteins and physiological processes become restricted in expression and/or activity to the left or right side of the embryo. Nodal expression in the left lateral plate mesoderm is regulated by left-side Vg1 signaling, and an opposing, right-side BMP signaling pathway represses right-side nodal expression. Arrows denote functional relationships but do not necessarily imply direct interactions; question marks indicate possible functional interactions. See text and references cited within for details.

(B) A summary of processes and genes that function upstream of left-side nodal expression in chick. Asymmetric depolarization of cells to the left of the node drives a transient, left-side increase in extracellular calcium, which in turn activates left-side notch signaling. Notch signaling, in addition to left-side sonic hedgehog expression, is required for left-side nodal expression; however, the relationship between these two pathways, if any, is not defined. A cascade of both left-side and right-side pathways, as well as midline influences, reinforce/repress nodal expression in the lateral plate mesoderm. Arrows denote functional relationships but do not necessarily imply direct interactions. See text and references cited within for details.
dependent, cortical rotation of the first cell cycle in *Xenopus* (Yost, 1991). Although much remains to be tested, it is clear that molecular asymmetry in *Xenopus* is established at least within the first few cell cycles, well before the formation of the Organizer and the appearance of “nodal” cilia. Thus, if cilia do play a role in left–right development in *Xenopus*, it is very likely that they participate in relaying left–right patterning cues, rather than initiating the left–right axis.

As in *Xenopus*, studies in chick do not indicate a role for nodal flow in establishing left–right asymmetry beyond the circumstantial identification of cilia present in Hensen’s node. In fact, a previous study implicated an earlier source of left–right asymmetry signals in the embryo by showing that tissues lateral to the node inductively interact with the node to specify its left–right identity (Pagan-Westphal and Tabin, 1998). Although the nature of the signal(s) that induce node left–right identity is not known, they probably are involved in regulating pH and voltage gradients, which as in *Xenopus*, are required to set up early asymmetry in chick (Fig. 5B). In the chick, there are distinct boundaries of gap junctional communication surrounding the node as well as asymmetric activities in the H+/K+-ATPase pump that result in left–side depolarization of cells to the left of the node (Levin et al., 2002). The H+/K+-ATPase driven depolarization causes a transient spike in left-side extracellular Ca$^{2+}$ levels (as opposed to intracellular Ca$^{2+}$ levels that are regulated by nodal flow), which preferentially activates left-side notch signaling (Raya et al., 2004). The latter occurs through modulating the affinity of notch for its ligands, Dll1 and Srr1, an interaction that is temporally influenced by locally expressed waves of lunatic fringe (Przemeck et al., 2003; Raya et al., 2004). Notch signaling is required for asymmetric nodal expression (Raya et al., 2004); whether this regulation is direct or through activation of other factors that are clearly involved in mediating nodal expression (e.g., activin, sonic hedgehog) remains to be determined. It is alternatively possible that notch acts in a parallel pathway to these other factors in controlling nodal expression. In mouse, notch-mediated induction of nodal expression is direct, although how this interaction relates to other processes of left–right axis determination in this species, particularly nodal flow, is not known (Krebs et al., 2003; Raya et al., 2003). In zebrafish, both notch signaling and H+/K+-ATPase activity function in left–right development prior to the formation of Kupffer’s vesicle (and hence, asymmetric nodal expression), suggesting that the role of nodal cilia in this species is to relay previously established asymmetric pattern (Kawakami et al., 2005).

In addition to asymmetric H+/K+-ATPase activity in *Xenopus*, there is abundant evidence that left–right development in the frog also requires TGFβ-related signaling mediated by a pathway comprised of Vg1, ALK4 (a type I Vg1 receptor), and syndecan-2 (a co-factor that facilitates Vg1-ALK4 signaling) (Chen et al., 2004; Hyatt and Yost, 1998; Kramer and Yost, 2002) (Fig. 5A). Ectopic activation of this pathway, either through overexpression of mature Vg1 ligand or constitutively active ALK4, results in a population of embryos exhibiting predominately right-side or bilateral nodal expression and situs inversus. Interruption of this pathway, either through interference with Vg1-ALK4 signaling or syndecan expression or function, causes abnormal nodal expression and situs inversus (Chen et al., 2004; Kramer and Yost, 2002; Kramer et al., 2002; Ramsdell and Yost, 1999). Elegant studies performed with temporally regulated dominant-negative forms of syndecan-2 pinpointed the necessity of this pathway during gastrulation (Kramer and Yost, 2002), which is the same developmental window in which ALK4 expression is detected in the organizer of the frog embryo (Chen et al., 2005). Consistent with these findings, syndecan-2 is asymmetrically expressed to the right side of Hensen’s node (Fukumoto and Levin, 2005), suggesting conservation of TGFβ signaling function downstream of the initial symmetry-breaking events. Since the discovery of a role for Vg1 and components of its signaling pathway in mediating left–right axis determination in *Xenopus*, it has been unclear though, how this pathway might be mechanistically linked to the other processes upstream of nodal expression. The recent discovery that ALK4 signaling in *Xenopus* can induce expression of notch and its ligands, delta-1 and delta-2 (Abe et al., 2004), raises the possibility that the Vg1-ALK4 pathway could function to link asymmetric pH and voltage gradients with notch pathway activation. Because null mutations in the mouse Vg1 orthologue, Gdf1, illustrate that this pathway also is necessary for left–right axis determination in this species (Wall et al., 2000), studies aimed at coupling the Vg1/Gdf1 pathway with activation of notch signaling are logical and potentially very interesting directions for future investigation.

**Maintenance of asymmetric nodal expression and relay of left–right positional information to developing tissues**

Once established, nodal expression in the perinodal area is believed to activate left-side nodal expression in the lateral plate mesoderm, and the latter domain of nodal asymmetry is maintained by complex interactions among a number of positive- and negative-acting regulators (Fig. 5B). This regulatory network is best characterized in chick, where sonic hedgehog induction of nodal expression initiates an autoregulatory loop that also requires the action of BMPs and caronte, a member of the Cerberus/DAN family of BMP antagonists (Monsoro-Burq and Le Douarin, 2000, 2001; Piedra and Ros, 2002; Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). BMP2 expression in the lateral plate mesoderm is symmetric (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), and its role in regulating nodal expression is to induce CFC-cripto, a co-factor that is necessary for cellular responsiveness to nodal (Fischer et al., 2002; Fujiwara et al., 2002; Schlake et al., 2002). Caronte also is induced by BMP2; while the function of caronte in this cascade is not certain, it might be to limit (indirectly) the extent of nodal autoinduction (Piedra and Ros, 2002). Two important targets of nodal expression include Pitx2, a bicoiid-related transcription factor that is discussed in detail below, and the homeobox gene *NKX 3.2* (also termed Bapxi). In chick, *NKX 3.2* is detected in the left, but not right, lateral plate mesoderm, where its expression is positively regulated by nodal BMP2 (Schlage et al., 2002; Schneider et
al., 1999). Despite its inverted expression pattern in mouse (Schneider et al., 1999), null mutations nevertheless are associated with defects in laterality of the spleen and pancreas (Hecksher-Sorensen et al., 2004). In addition to nodal, lefty-1 and lefty-2 are two other TGFβ family members which are involved in relay of left–right pattern and which are expressed in the left half of the prospective floor plate, and the left lateral plate mesoderm, respectively (Bisgrove et al., 1999; Meno et al., 1996, 1997, 1998). Both lefty proteins function as nodal antagonists to prevent the spread of left-side signals to the opposite side of the embryo (Branford and Yost, 2004; Cheng et al., 2000; Meno et al., 1998; Thisie and Thissie, 1999). As observed for nodal, lefty-2 expression is similarly altered in iv/iv and inv/inv mice, and a number of experimental perturbations of nodal activity also cause abnormal lefty-1 and lefty-2 expression patterns (Meno et al., 1996, 1997). On the right side of the node, a series of signaling pathways is necessary for repression of right-side nodal expression and for induction of cSnur, a transcription factor that is normally restricted to the right lateral plate mesoderm (Isaac et al., 1997). Beginning with right-side activin signaling, which is necessary for BMP4 expression, BMP4 in turn induces FGF8 and PCL2 expression, the latter which functions to represses right-side sonic hedgehog (Boettger et al., 1999; Wang et al., 2004). BMP2 signaling also is active on the right side of the embryo, where it cooperatively functions with the FGF8 pathway to induce cSnur expression (Boettger et al., 1999).

The importance of BMP, FGF, and nodal antagonists in maintaining left-side specific nodal expression is suggested by functionality of these pathways in other vertebrate species. In mouse, BMP4 is required for left-side nodal expression (Fujiwara et al., 2002) and null mutations in Cerl-2, which encodes a novel nodal antagonist, result in situs ambiguous (Marques et al., 2004). In zebrafish, functional inhibition of the nodal antagonist charon leads to bilateral nodal-related gene expression and reversed heart looping (Hashimoto et al., 2004). Null mutations in mouse ACRVRI (also termed ALK2), which encodes a type I BMP receptor, result in situs ambiguous and bilateral nodal expression, the latter probably due to loss of lefty-1 expression in the midline (Kishigami et al., 2004). Gain-and loss-of-function studies of ALK2 in Xenopus also indicate that this pathway is necessary for normal left–right development of the heart and visceral organs (Ramsdell and Yost, 1999). It should be emphasized that even though many of the same signaling pathways that are active in chick, frog, and fish are employed in mouse, this species differs from other vertebrates in that some of its left–right signaling molecules either act on the contralateral side of the embryo (e.g., FGF8 is a left-side determinant in mouse), or are expressed in a symmetric fashion while nevertheless being involved in left–right patterning (e.g., sonic hedgehog) (Meyers and Martin, 1999). Other genes that affect nodal expression and heart and visceral organ asymmetries in mouse include rotatin (Faist et al., 2002), cited2 (Bamforth et al., 2004), PKD2 (Pennekamp et al., 2002), and BP1/PLUNC (Hou et al., 2004).

In conjunction with left- and right-side regulatory networks, gene expression in the midline itself is important in regulating asymmetric nodal expression. In addition to lefty-1, another important midline gene is Zic3, a member of the GLI transcription factor family that is normally present in midline mesoderm and that is induced by activin/Vg1 signaling in Xenopus (Kitaguchi et al., 2000, 2002; Nagai et al., 1997; Purandare et al., 2002). Misexpression of either wild type or deletion mutant forms of Zic3 in Xenopus embryos causes situs ambiguous and predominantly bilateral or right-side nodal expression (Kitaguchi et al., 2000). In mouse, Zic3 null mutations result in a failure to maintain asymmetric nodal expression in the node and situs ambiguous (Purandare et al., 2002). Zic3 is thought to function in left–right development by controlling formation of midline tissues such as the notochord.

One of the main consequences of maintaining a midline barrier, via midline gene expression such as Zic3 or lefty-1, is that cell populations positioned on opposite sides of the midline propagate left–right patterning information independently of one another. Clues to how the midline operates on a cellular level to prevent left–right signals from reaching the contralateral side come from notable studies in which cell death in the primitive streak (Kelly et al., 2002) and a novel population of ventral foregut cells derived from Hensen’s node (Kirby et al., 2003) are shown to contribute to left–right patterning activity. Further investigation of these two newly identified midline cell populations and how they interface with left–right signaling cascades should greatly increase our understanding of the specific mechanisms employed at the embryonic midline.

It should be noted that concomitant with the development of left–right asymmetry, there are paired tissues located to either side of the midline (e.g., presomitic mesoderm) that must undergo symmetric morphogenesis. Exactly how this coordination occurs, and particularly how these tissues remain refractory in response to opposing left- and right-side lateralizing influences, has been a longstanding question. A series of very recent studies demonstrate that, in chick, mouse, and zebrafish, somite formation is labile to left–right signaling pathways, but that under normal conditions, retinoic acid mediates synchronized left and right side somitogenesis by preventing presomitic mesoderm from responding to left–right asymmetric signaling (Kawakami et al., 2005; Vermot et al., 2005; Vermot and Pourquie, 2005).

“Translating” left–right axis information into anatomical asymmetry

The nodal \(\rightarrow\) Pitx2c pathway

The connection between asymmetric nodal expression in the lateral plate mesoderm and cardiac asymmetry is the regulation of downstream genes that have direct roles in heart development. One such gene that is induced to be expressed in the left lateral plate mesoderm by nodal and that is required for normal heart development is Pitx2c (Fig. 6). Pitx2c is one of three Pitx2 isoforms (Pitx2a, Pitx2b, and Pitx2c) that belong to the bicoid group of paired homeobox transcription factors. Pitx2 is a homolog of the human RIEG gene, whose mutation
results in Rieger syndrome, a haploinsufficiency condition characterized by dental hypoplasia, craniofacial dysmorphism, umbilical stump protrusions, and ocular anomalies (Amendt et al., 2000). Less frequently, defects in cardiac, limb, and pituitary development are observed (Amendt et al., 2000).

Once induced by nodal (Logan et al., 1998; Long et al., 2003; Piedra et al., 1998; Shiratori et al., 2001; Yoshioka et al., 1998), Pitx2c expression in the left lateral plate mesoderm is maintained by nkx2.5 (Shiratori et al., 2001). Similar to nodal, a combination of right side only, bilateral, and absent expression Pitx2c patterns is observed in experimentally induced cases of situs ambiguous in mouse, chick, and frog (Bamforth et al., 2004; Bigs Grove et al., 2000; Boettger et al., 1999; Brandford et al., 2000; Chen et al., 2004; Piedra et al., 1998; Sch Lange et al., 2001; St Amand et al., 1998; Yoshioka et al., 1998). The observation that null mutations in many different left–right axis genes result in a similar defective left–right phenotype can be attributed to the downstream effects of these genes on the nodal → Pitx2c pathway. For example, null mutations that result in absent Pitx2c expression include cryptic, FGFR8, gdf1, and nodal and the predominant phenotype in these mice is right isomerism. Null mutations that cause bilateral Pitx2c expression include lefty-2 and ACRIa/ALK2 and these mice predominantly exhibit left isomerism. Midline genes that are necessary for left–right development also converge at the nodal → Pitx2c pathway; shh and lefty-1 are two examples that cause abnormal nodal (and hence Pitx2c) expression in null mutants. Mice that are null for all three Pitx2 isoforms (Pitx2abc) exhibit defective body wall closure, gut malrotation, right pulmonary isomerism, and the spectrum of cardiac defects that are usually associated with heterotaxy, including DORV, TGA, common AV canal, ASD/VSD, and anomalous venous return (Gage et al., 1999; Kitamura et al., 1999; Liu et al., 2001, 2002). Right atrial isomerism, PTA, and aortic arch anomalies such as right aortic arch or double aortic arch are also sometimes present. Interestingly, despite the numerous cardiac defects caused by Pitx2abc null mutation, all of these can be rescued with hypomorphic Pitx2c alleles except right atrial isomerism, which requires a higher dosage of Pitx2c expression/activity (Liu et al., 2001). Consistent with these findings, analysis of Pitx2c function in Xenopus has shown that Pitx2c antisense oligonucleotides cause OFT, AVC, and atrial septation defects (Dagle et al., 2003).

\[ Pitx2c \text{ is implicated in regulating multiple aspects of cardiac left–right development}\]

Unlike nodal, Pitx2c is expressed during stages of heart tube formation when it is detected exclusively in the left, but not right, side of the developing heart (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; St Amand et al., 1998; Yoshioka et al., 1998) (Fig. 7). Cardiac Pitx2c expression persists through looping stages, where it is present in the entire left side of the heart, spanning from the left atrium to the most distal part of the outflow tract, reaching into the left side of the SANF and the left pharyngeal arch mesenchyme (Liu et al., 2002) (Fig. 7). In addition, Pitx2c plus two other Pitx2 isoforms (Pitx2a and Pitx2b) are expressed in migrating cardiac neural crest (Hamblet et al., 2002; Kioussi et al., 2002). During later stages, Pitx2c expression is observed in the left atrioventricular canal, left atrium, interatrial septum, and the left caval vein (Franco and Campione, 2003).

Initial studies showed that right side ectopic Pitx2c expression in chick and Xenopus causes reversed heart looping (Campione et al., 1999; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). These findings, coupled with the observations that Pitx2c is expressed by components of other organs that undergo looping, suggested that Pitx2c mediates looping morphogenesis (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998). However, Pitx2abc and Pitx2c null mutant mice do not exhibit reversed cardiac looping (Gage et al., 1999; Kitamura et al., 1999), suggesting that Pitx2c is dispensable for this aspect of the looping process. In Xenopus, Pitx2c loss-of-function causes abnormal (though not necessarily reversed) shifting of the OFT, confirming that although Pitx2c can mediate later aspects of looping, it is not involved in controlling initial directionality of looping. Errors in the leftward shifting and/or rotation of the OFT are causatively linked with DORV, TGA, and VSDs; therefore, the abnormal OFT looping that is associated with impaired Pitx2c function could account for these types of CHDs.

The mechanism by which Pitx2c regulates OFT looping is undefined. During looping, the outlet region of the heart undergoes lengthwise growth due to the addition of myocardium derived from the SANF (Brand, 2003; Mjaatvedt et al., 2001; Waldo et al., 2001). Thus, one possible function of Pitx2c in OFT looping is that its expression in the SANF may...
somehow be involved in the recruitment of cells from the SANF to the conotruncus. Alternatively, Pitx2c might mediate OFT looping by affecting the cardiac neural crest. In Pitx2abc null mice, the population of neural crest cells that contributes to the heart appears decreased (Kioussi et al., 2002; Liu et al., 2002). Although Pitx2 does not directly regulate migration of neural crest cells, null mutations in Pitx2abc do reduce the number of neural crest cells that accumulate in the OFT by inhibiting proliferative expansion of this cell population (Kioussi et al., 2002). This inhibition requires the upstream activation of the Wnt/Dvl/β-catenin pathway that is responsible for inducing Pitx2 expression (Kioussi et al., 2002). In other mouse mutants (e.g., FGF8−/−, BMP4−/−), decreased numbers of neural crest cells in the OFT have been associated with defective OFT looping (Abu-issa et al., 2002; Liu et al., 2004). It has been proposed that the link between neural crest cells and altered OFT looping is that neural crest cells somehow regulate the factors that mediate the addition of SANF cells to the outflow myocardium to the heart (Farrell et al., 1999, 2001; Yelbuz et al., 2002, 2003). In turn, it has been proposed that when cardiac neural crest cells are ablated, the impaired recruitment of SANF cells to the OFT causes the OFT to develop a shortened stature that impedes its ability to normally loop (Farrell et al., 2001; Yelbuz et al., 2002, 2003). Whether this model is correct is somewhat controversial because it is difficult to reconcile that a shortened OFT can simultaneously occur with PTA, a defect that is unequivocally and causally linked to neural crest cell ablation. Thus, if the function of Pitx2 in the neural crest is required for normal OFT looping, it is alternatively possible that the neural crest plays a role unrelated to SANF cell recruitment. Besides Pitx2, other left–right asymmetry genes—ALK2, ALK4, and FGF8—are expressed in the cardiac neural crest, suggesting that neural crest might be subject to left–right specification prior to or during its migration (Abu-issa et al., 2002; Chen et al., 2005; Kaartinen et al., 2004; Kishigami et al., 2004). However, whether expression of Pitx2 or other laterality genes by cardiac neural crest cells is induced as part of the left–right signaling cascade that specifies the midline remains to be determined. With the availability of neural crest mutants in mouse and zebrafish, as well as the ease of experimentally ablating neural crest in chick and Xenopus, the different potential roles for neural crest cells in cardiac looping could be tested using a variety of molecular, embryological, and genetic approaches.

In addition to OFT looping, Pitx2c might play a role in regulating septation in the IFT and OFT (Fig. 8). The myocardium in the AVC exhibits left, but not right, side Pitx2c expression (Campione et al., 2001), which is required for migration of cells into cardiac cushions (Liu et al., 2002). The origin of the Pitx2c expressing cells within the cushions is probably the AV myocardium, which together with epicardial-derived cells, is required to complete septation and valve formation in the heart (Lamers and Moorman, 2002). Pitx2 also appears to be required for migration of a small subset of myocardial cells from the left to the right side of the OFT (Liu et al., 2002); although the significance of this migratory process is unknown, it does suggest that Pitx2 may be involved in conferring cellular and molecular asymmetry to this portion of the heart. With respect to the OFT, Pitx2abc null mice exhibit PTA, a condition in which the conotruncus and AS are not divided properly into the base of the aorta and pulmonary artery by the APS (Kitamura et al., 1999; Lu et al., 1999; Liu et al., 2001). This suggests that Pitx2 is necessary for septation of the OFT. Finally, the expression of Pitx2c in cells that will form the IAS suggests it too plays a direct role in atrial septation, perhaps by affecting competency of cells to transform to mesenchyme, as appears to occur in the AVC region. Consistent with this idea, IAS defects are frequently present in Pitx2c null mice (Liu et al., 2001).

**Pitx2c and aortic arch anomalies**

Aortic arch anomalies are frequently present in heterotaxy patients, indicating that patterned regression and persistence of vessels are governed by the left–right axis (Bowers et al., 1996).
During vascular remodeling involves recruitment of cells that respond to the same set of left–right patterning signals, isomerism suggest that vascular endothelial cells are competent to respond to the same set of left–right patterning signals, regardless of which side of the midline they are located (Bowers et al., 1996; Ruscazio et al., 1998). Moreover, vessel persistence during vascular remodeling involves recruitment of cells that interact with the endothelium to promote its survival and differentiation. Failure to recruit support cells or disrupted interaction between support cells and the endothelium would result in vessel regression. Therefore, with respect to asymmetric AA remodeling, laterality gene expression in pharyngeal arch mesenchyme may be important. Pitx2c is expressed in the left, but not right, pharyngeal arch mesenchyme (Liu et al., 2002), and it has been suggested that Pitx2c null mutations could impair recruitment, maintenance, or cross-talk between supporting cells and endothelial cells in this region. Because Pitx2c is expressed only by left-side AAs, the absence of Pitx2c expression would be predicted to result in abnormal patterning on this side, consistent with the right-side aortic arch, right-side ductus arteriosus, and left inominate arteries that frequently occur in Pitx2c null mice.

Genes in addition to Pitx2 that regulate cardiac left–right morphogenesis

In addition to Pitx2c, BMP4 and flectin are two other genes that play important roles in cardiac left–right morphogenesis. In zebrafish and Xenopus, BMP4 is asymmetrically expressed in the nascent heart tube (Chen et al., 1997), and transgenic studies in Xenopus using cardiac-targeted inhibitors of BMP signaling demonstrate that BMP4 is required for looping morphogenesis, independent of Pitx2c function (Breckenridge et al., 2001). Flectin is an extracellular matrix protein that is asymmetrically expressed in chick, with predominant expression observed in the left heart fields (primary and anterior), the left dorsal mesocardium, and the left side of the heart tube during looping stages (Linask et al., 2003; Tsuda et al., 1996, 1998). Ectopic Pitx2c expression causes a rightward shift in flectin expression in the heart fields and the dorsal mesocardium, coincident with reversed or absent heart looping (Linask et al., 2002). Treatment of embryos with CFC antisense oligonucleotides, which likewise cause reversed or absent looping, also inverts the asymmetry of flectin expression (Linask et al., 2003). However, this treatment does not alter normal Pitx2c expression, indicating either that flectin is a downstream target of Pitx2c that is directly mediating looping, or that flectin acts in a pathway parallel to the nodal → Pitx2 pathway. In addition to the role of flectin in looping, these studies also showed that Pitx2c could alter the position of the developing foregut relative to the embryonic midline. As shown in these studies and in the previously mentioned study of node-derived ventral midline cells (Kirby et al., 2003), this finding is significant because the orientation of heart looping is somehow regulated by cells of the ventral floor of the foregut. Given that CFC antisense treatment also causes a rightward shift of the ventral foregut relative to the midline, this suggests that the ventral foregut may be a primary tissue target in the relay of left–right axis signals.

Some “isolated” CHDs might actually be subtle laterality defects

Although the majority of human laterality defects are thought to be sporadic, it is clear that left–right defects also
can arise from environmental perturbations and from heritable modes of transmission (Kuehl and Loffredo, 2002). Clinical analyses of familial cases indicate three modes of inheritance for laterality defects: X-linked, autosomal dominant (usually with incomplete penetrance), and autosomal recessive (Belmont et al., 2004; Kosaki and Casey, 1998). In many cases, specific mutations have been mapped and include ZIC3, ACVR2B, CRYPTIC/CFC1, LEFTYA, and NKX2.5 (Bamford et al., 2000; Belmont et al., 2004; Gebbia et al., 1997; Kosaki et al., 1999a,b). Because these genes also have been identified or implicated in animal models of heterotaxy, this confirms that our current models are (and presumably will continue to be) of great value in understanding the etiology of human laterality disease.

Observations made in animal models of heterotaxy on the role of the midline in left–right development (reviewed by Yost, 1998) have prompted investigators to look for clinical correlations between human midline defects and laterality disturbances (Goldstein et al., 1998; Morelli et al., 2001). Intriguingly, in one of these studies, a significant association was observed not only between laterality defects and midline defects, but also between laterality defects and “isolated” cardiac defects (Morelli et al., 2001). The latter association has been observed in both animal and human studies, leading to the suggestion that some instances of isolated cardiac defects may actually represent subtle, “forme fruste” laterality defects (Goldmuntz et al., 2002). For example, in the cited-2 null mouse, which lacks Pitx2c expression in the left lateral plate mesoderm and in the heart, embryos develop the cohort of CHDs that is typical of heterotaxy (i.e., defects in OFT, septal, and AA remodeling) in addition to abnormal body turning, right atrial and pulmonary isomerism, and hypoplastic spleen (Bamforth et al., 2004; Weninger et al., 2005). Comparison of these mice with cited-2 null mutations made on a mixed genetic background gave surprising results (Bamforth et al., 2004). In the latter group, Pitx2c expression was present in the left lateral plate mesoderm but absent or reduced specifically in the OFT of the heart. These mice exhibited DORV and VSD but laterality of other organs was not affected. These results suggest that there are genetic modifiers that can influence the cited-2 null phenotype by directly or indirectly regulating Pitx2c expression. Moreover, the occurrence of CHDs in the mixed background mutant embryos suggests that some CHDs actually may result from perturbations of left–right axis genes, even in instances of otherwise normal body situs. As a direct test of this hypothesis in a clinical setting, a population of patients with TGA (but no overt laterality defects) was examined for mutations in CFC1, a gene that is expressed in the OFT of the embryonic mouse heart (Dono et al., 1993) and that is required for cellular responsiveness to nodal signaling in the lateral plate mesoderm, probably by functioning as a co-receptor for nodal (Fischer et al., 2002; Fujiwara et al., 2002; Schlange et al., 2002). A subset of patients afflicted with TGA (but not laterality defects) was found to indeed harbor CFC1 mutations, indicating for the first time, a common genetic etiology between human TGA and laterality disease (Goldmuntz et al., 2002). Because many laterality genes identified in animal models are known also to participate in processes of cardiac morphogenesis, it is predicted that many more seemingly “isolated” cardiac defects will be found in situs solitus individuals that represent a mutation or other perturbation in left–right axis gene(s).

Prospects

The past decade has brought immense progress in deconstructing the complexities of left–right axis determination and its relationship to cardiac development while at the same time illuminating areas in which further investigation is much needed. Studies in mouse, chick, frog, and zebrafish reveal that establishing the left–right axis involves a number of intriguing physiological processes that extend beyond the conventionally studied, growth factor-mediated signaling pathways. Whether processes of “nodal” flow, gap junctional communication, and membrane voltage potential are unique for axis determination in each species is an important but unresolved issue. Relatively more is known about the role of the midline in left–right patterning, but despite unraveling many of the complicated genetic interactions, the specifics of which cell types are engaged in relay of left–right signals are less well defined. With regard to asymmetric morphogenesis, work on Pitx2c has accelerated our understanding of the types of cardiac defects that can arise from impaired function of a single laterality gene. Yet, how this one gene actually regulates the three different endpoints of cardiac left–right patterning (looping, regression/persistence, and lateralization) is by no means definitively answered, and is limited, in part, by our current understanding of fundamental, morphogenetic processes that drive normal cardiac development. Finally, there is exciting interplay between clinical observations and experimental observations in animal models of laterality disease, indicating that some seemingly “isolated” congenital cardiac defects might actually arise from disturbances in left–right signaling pathway(s). Future progress in this latter area is anticipated to be especially significant, as elucidating common genetic etiologies will greatly advance our understanding of the causes of many types of deleterious and life-threatening congenital heart malformations.

Acknowledgments

Thanks to colleagues near and far for enjoyable discussions of cardiac left–right development and to R. Markwald, D. Bernanke, and C. Drake for comments on the manuscript. Special thanks to Sue Tjepkema-Burrows for drawing the schematic figures. Cardiac asymmetry research in the author’s lab is supported by HL73270 (to A.F.R.) and a SC COBRE for Cardiovascular Disease P20-RR-1634 (to Roger R. Markwald).

References


