REVIEW

Conserved and divergent mechanisms in left–right axis formation

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Vertebrates appear bilaterally symmetric but an internal left–right (L–R) axis is revealed by the placement of asymmetric organs about the midline. For example, the human heart is located to the left of the body cavity, whereas the liver is located to the right. Paired organs can also display L–R differences, as seen in human lungs, in which the right lung has three lobes and the left lung two. Alterations in L–R axis formation can lead to variations in the normal arrangement of organs, including complete inversion of the axis (situs inversus), randomized placement of organs (heterotaxia or situs ambiguus), and mirror image duplications of paired organs (isomerism). In the past 5 years important progress has been made in clarifying the embryological and molecular mechanisms underlying the development of laterality in different vertebrates. Surprisingly, however, it is still unclear if many of the strategies employed in one group of vertebrates are also applied in other groups. In this review, we compare the roles in L–R development ascribed to various molecules and embryological structures in the four common vertebrate model systems.

Overview

Conceptually, the development of the L–R axis can be divided into three phases: (1) initial break in symmetry; (2) establishment of asymmetric gene expression; and (3) transfer of positional information to developing organs. We will begin our discussion with phases 2 and 3, where several aspects of laterality development appear to be conserved. Namely, members of the Nodal and Lefty families of TGF β molecules and the transcription factor Ptx2 are expressed specifically on the left in all vertebrates. We will describe current models on how these genes are implicated in L–R development and discuss how the midline is involved in their regulation. We will then focus on the first phase, when the L–R axis has to be established with respect to the anterior–posterior (A–P) and dorsal–ventral (D–V) axes. A conserved mechanism

that establishes asymmetry has not yet been found, but we will describe three strategies that have been implicated in this process. First, the node in mouse and chick (corresponding to the organizer in frog) appears to confer laterality, and experiments in mouse suggest a leftward flow of molecules from the node, mediated by the rotation of cilia. Second, a localized L–R coordinator that acts as a global regulator of L–R development has been postulated in frog. Third, a role for gap junction communication has been proposed in frog and chick. In the last part of the review, we will describe the genetic cascade in chick that links phases 1 and 2, leading to the transfer of asymmetric gene expression from the node to the periphery. Signaling molecules such as Sonic hedgehog (Shh), Activin, FGF8, Retinoic acid (RA), and BMP have been implicated in this process. We will discuss the apparently divergent roles of many of these factors in other vertebrates.

Conservation of asymmetric gene expression

Nodal signaling

Nodal The expression and functions of members of the *nodal* gene family suggest a central conserved role for Nodal signaling in vertebrate L–R patterning (Fig. 1). Nodal signals are TGF β family members with essential roles in mesoderm and endoderm induction and neural patterning (for review, see Schier and Shen 2000). *Nodal* genes are expressed asymmetrically in the left lateral plate mesoderm (LPM) during somitogenesis in zebrafish, frog, chick, and mouse (Levin et al. 1995; Collignon et al. 1996; Lowe et al. 1996; Lustig et al. 1996; Rebagliati et al. 1998a; Sampath et al. 1998). Importantly, misexpression of Nodal in frog and chick can lead to altered situs including reversed heart looping (Levin et al. 1997; Sampath et al. 1997). Moreover, mutations in mouse and zebrafish that lead to situs abnormalities alter the asymmetric expression of *Nodal*, which correlates well with abnormal situs observed in these mutants (Collignon et al. 1996; Lowe et al. 1996; Heymer et al. 1997; Dufort et al. 1998; King et al. 1998; Melloy et al. 1998; Meno et al. 1998; Rebagliati et al. 1998a; Sampath

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et al. 1998; Gaio et al. 1999; Izraeli et al. 1999; Meyers and Martin 1999; Tsukui et al. 1999; Yan et al. 1999). Based on these results it has been suggested that Nodal signals are determinants for "left-sidedness" in all vertebrates.

Nodal is sufficient to change L-R laterality, but is it required? The early roles of *Nodal* in mesoderm induction and axis patterning preclude the exploration of L-R axis defects in mouse *Nodal* mutants. Surprisingly, no L-R defects have been reported in chimeric embryos that contain a large proportion of *Nodal* mutant cells (Beddington and Robertson 1999). This would suggest that *Nodal* is not required for L-R development or that a very small number of cells expressing *Nodal* is sufficient to confer L-R laterality in chimeric embryos. Compound *Hnf3 β /+;Nodal/+* or *Smad2/+;Nodal/+* mutants have L-R defects (Collignon et al. 1996; Nomura and Li 1998). Although these phenotypes are suggestive of a requirement for *Nodal* in L-R patterning, it has not been excluded that midline defects in these mutants lead to the observed L-R axis abnormalities (see below).

Studies of zebrafish *nodal* mutants have also failed to demonstrate unequivocally an essential role for *nodal* genes in L-R axis formation. The *nodal*-related gene *cyclops* (*cyc*) is expressed in the left lateral plate and left forebrain epithalamus (Rebagliati et al. 1998a). The *cyc*^{b16} allele has been reported to have effects on heart looping (Chen et al. 1997), but this is a deletion allele that may eliminate other genes involved in this process (Talbot et al. 1998). Two different point mutations in *cyc*, both thought to be null alleles, do not affect heart looping (Chen et al. 1997). However, the mutations affect the expression of *cyc* transcripts. In *cyc*^{m294} and *cyc*^{tf219} mutants, *cyc* expression is symmetric within the epithalamus (Sampath et al. 1998). In contrast, *cyc* transcripts in the LPM still appear predominantly restricted to the left side. In another report, however, symmetric expression of *cyc* in both the LPM and epithalamus was observed in *cyc*^{tf219} (Rebagliati et al. 1998b). The reasons for these discrepant results are unknown. Furthermore, it is unclear if the abnormal *cyc* expression in *cyc* mutants reflects a direct role of *cyc* in L-R axis formation or might be caused by midline defects observed in these mutants (Hatta et al. 1991).

EGF-CFC While the effects of zebrafish *cyc* mutations reported so far do not strengthen the argument for an essential role of Nodal signals in L-R patterning, another zebrafish mutation provides evidence for a requirement for Nodal signaling in L-R development. The *one-eyed pinhead* (*oep*) gene encodes an extracellular, membrane-attached protein belonging to the EGF-CFC family (Zhang et al. 1998; for review, see Shen and Schier 2000). Genetic studies have shown that *Oep* acts as an essential cofactor for Nodal signals during gastrulation (Gritsman et al. 1999). Expression of *oep* overlaps with *cyc* in the left LPM and midline and is also found in the right lateral plate. Strikingly, absence of late *oep* activity results in heterotaxia (Yan et al. 1999). In addition, the left-sided expression of *cyc*, *lefty*, and *pitx2* is not observed in

these mutants, suggesting that *oep* mediates Nodal signaling to establish left-sidedness (Fig. 1; Table 1). A similar phenotype is observed in mutants for the mouse EGF-CFC gene *Cryptic* (Gaio et al. 1999; Yan et al. 1999). Importantly, the integrity of the midline does not appear to be affected in *Cryptic* or late zygotic *oep* mutants, suggesting a direct and conserved role for Nodal signaling in establishing asymmetric gene expression in the LPM. As the loss of *cyc* has a less severe effect on L-R development compared to the loss of *oep*, an additional *nodal*-related gene or EGF-CFC-dependent molecule must be involved in L-R patterning in the fish.

Lefty Further evidence for an important role of Nodal signaling in L-R axis formation comes from studies of *lefty* genes. Recent results have shown that these members of the TGF β family act as inhibitors of Nodal signaling during gastrulation (Bisgrove et al. 1999; Meno et al. 1999; Cheng et al. 2000; for review, see Schier and Shen 2000), most likely by blocking putative Nodal receptor(s) such as the Activin receptor ActRIIB (Meno et al. 1999). In all vertebrates studied so far, *lefty* genes are expressed in the midline and the left LPM, overlapping with *nodal* expression (Meno et al. 1996, 1997; Bisgrove et al. 1999; Rodriguez Esteban et al. 1999; Thisse and Thisse 1999; Yokouchi et al. 1999; Cheng et al. 2000; Ishimaru et al. 2000). Similar to *Nodal* expression, *Lefty* gene transcription is altered in mutant backgrounds that affect organ situs (Meno et al. 1996, 1998; Heymer et al. 1997; Dufort et al. 1998; King et al. 1998; Melloy et al. 1998; Nonaka et al. 1998; Gaio et al. 1999; Izraeli et al. 1999; Meyers and Martin 1999; Takeda et al. 1999; Tsukui et al. 1999; Yan et al. 1999; Constam and Robertson 2000). Importantly, mutations in mouse *Lefty1* lead to L-R defects, including symmetric expression of *Lefty2* in the LPM and left pulmonary isomerism (Meno et al. 1998). These defects are to some extent opposite to the ones in *Cryptic* mutants (loss of asymmetric gene expression, right pulmonary isomerism), supporting the idea that Nodal signaling requires EGF-CFC function to induce left-sidedness and is counteracted by *Lefty* inhibitors (Fig. 1; Table 1).

It has been proposed that during germ layer formation Nodal signals and EGF-CFC cofactors activate an Activin-like signaling pathway involving Activin receptors and transcription factors of the Smad and FAST families (Gritsman et al. 1999; Schier and Shen 2000). In turn, *Lefty* proteins are thought to act as feedback inhibitors to block *nodal* autoregulation by blocking Nodal receptors (Meno et al. 1999; Schier and Shen 2000). Similar regulatory interactions appear to take place during L-R development. Both mouse *Nodal* and *Lefty2* harbor left side-specific enhancers that contain essential binding sites for FAST2 (Saijoh et al. 2000). *Xenopus* animal cap experiments indicate that these enhancers are activated by Nodal in the presence of EGF-CFC proteins such as mouse *Cryptic*. These results suggest an autoregulatory feedback mechanism wherein *Nodal* maintains its own expression, while also activating *Lefty2*. *Lefty2* then acts as a feedback inhibitor to block both *Nodal* and *Lefty2*

Figure 1. Genetic cascades of L-R development. Green indicates conservation in at least two of the model organisms. Blue indicates conservation in more than two of the model organisms. Red indicates divergence among the model organisms. The dashed lines indicate the midline of the organism, with the left being to the reader's left. Asymmetric expression of genes is indicated. *Nodal* is not expressed in the right LPM in chick and thus is shaded in gray. The transient asymmetric *lefty* expression in the midline of the chick is indicated, although this gene later becomes symmetrically expressed in midline structures. When and where RA would act is not known and thus is not indicated in this figure. The ability of *Nodal* to induce *pitx2* in the LPM in zebrafish and mouse is inferred and thus is shown with a broken arrow. For clarity, not all potential gene interactions are indicated. (See text for details and references.)

expression on the left (Saijoh et al. 2000). Recent studies in *Xenopus* further support this model. Right-sided misexpression of the *nodal* gene *xnr1* induces *xatv*, a *Xenopus lefty* homolog. In contrast, left-sided misexpression of *xatv* suppresses expression of *xnr1* and *pitx2* (Cheng et al. 2000).

Mutations in the Activin receptor *ActRIIB* lead to right pulmonary isomerism, consistent with a role of this receptor in mediating Nodal signaling to promote left-sidedness (Oh and Li 1997). Surprisingly, however, expression of the extracellular domain of *ActRII* on the left or right side of chick embryos does not block expression of *pitx2*. Instead, it leads to the bilateral expression of *nodal* and *pitx2* and consequent abnormal situs (Ryan et al. 1998). It is conceivable that in this assay Nodal has already exerted an influence on the left side and that the *ActRII* extracellular domain might block Lefty activity, thus preventing repression of left side-specific genes on the right. Seemingly contradictory results such as these are surprisingly common for manipulations that affect L-R asymmetry and can complicate interpretations. Discrepant results may often reflect the stage-specific action of signals that establish the L-R axis. The different ef-

fects seen upon misexpression of Lefty at different times illustrate this point. For instance, misexpression of the *lefty* genes in chick have been reported to both inhibit and induce expression of *nodal* and *pitx2*. If Lefty is misexpressed on the right of the node at stage 4, *nodal* and *pitx2* are induced on the right (Rodriguez Esteban et al. 1999). If Lefty is overexpressed on the left or right of the node at stage 5, *nodal* and *pitx2* are repressed on the left (Yoshioka et al. 1998; Rodriguez Esteban et al. 1999). When Lefty is misexpressed later at stage 7 in the right LPM, *pitx2* is induced (Yoshioka et al. 1998). It is conceivable that at different stages in development, overexpression of Lefty could interfere with different TGF β signaling pathways. For example, at stage 4, Lefty could be interfering with endogenous Activin activity within the node. This allows the *nodal* inducer *sonic hedgehog* to be expressed on the right (see below), leading to the right-sided expression of *nodal* and *pitx2*. On the left of the node at stages 4 and 5, Lefty could act to repress Nodal signaling peripheral to the node and thus prevent the expression of left-side specific genes. Later at stage 7, misexpression of Lefty at nonphysiological levels in the right LPM may interfere with another TGF β signaling

Table 1. Mutations affecting L-R development

Gene	Protein	Organism	Phenotype	Effects on Gene Expression	References
Mutations affecting the node					
<i>Brachyury ; T</i>	Transcription factor	mouse	Node defects; lack notochord; cardiac looping randomized	<i>nodal</i> absent (100%) from LPM and node; <i>lefty1</i> & 2 absent	King 1998
<i>Hfh-4</i>	Transcription factor	mouse	Assumed nodal flow defects due to loss of LRD; heart and viscera positions randomized	<i>lrd</i> exp not detected by RT-PCR.	Chen 1998
<i>HNF3 β</i>	Transcription factor	mouse	Loss of node and notochord; turning and heart looping fail to occur	<i>nodal</i> absent 100%; <i>lefty</i> absent or bilateral	Collignon 1996; Dufort 1998
<i>inv</i>	Novel ankyrin repeat protein	mouse	Abnormal nodal flow; near 100% <i>situs inversus</i>	<i>nodal</i> right; <i>pitx2</i> right; <i>lefty2</i> right; <i>lefty1</i> right in midline; <i>nodal</i> in node symmetrical or stronger on right	Yokoyama 1993; Collignon 1996; Lowe 1996; Meno 1996; Mochizuki 1998; Morgan 1998; Ryan 1998; Okada 1999
<i>iv lrd</i>	Axonemal dynein	mouse	Immotile node monocilia; abnormal nodal flow; <i>situs inversus</i> and heterotaxy	<i>nodal</i> complex ³ ; <i>pitx2</i> complex; <i>lefty</i> right or left	Hummel 1959; Layton 1976; Lowe 1996; Meno 1996; Piedra 1998; Ryan 1998; Yoshioka 1998; Campione 1999; Okada 1999; Supp 1999
<i>Kif3A</i>	Kinesin superfamily member	mouse	Lack node monocilia; heart looping randomized	<i>pitx2</i> bilateral; <i>lefty2</i> bilateral or absent	Marszalek 1999; Takeda 1999
<i>Kif3B</i>	Kinesin superfamily member	mouse	Lack node monocilia; heart looping randomized; embryos that turn are randomized	<i>lefty2</i> bilateral or absent	Nonaka 1998
Mutations affecting the midline					
<i>HNF3 β +/-; nodal +/-</i>	Transcription factor; TGFβ molecule	mouse	Cyclopic; defect in heart and viscera positions	<i>nodal</i> bilateral 100%	Collignon 1996
<i>No turning (nt)</i>	N.D.	mouse	Notochord degeneration; lack HNF3β exp in notochord; heart looping randomized; failure to turn	<i>nodal</i> complex; <i>lefty</i> bilateral or absent; <i>lefty</i> absent from midline	Melloy 1998
<i>Shh</i>	hedgehog family member	mouse	Lack floor plate; left pulmonary isomerism; heart looping randomized (Meyers 1999); turning randomized	<i>lefty1</i> absent from midline and LPM 100%; <i>pitx2</i> bilateral (near 100%); <i>nodal</i> and <i>lefty2</i> left or bilateral	Chiang 1996; Izraeli 1999; Marszalek 1999; Meyers 1999
<i>SIL</i>	Early response gene	mouse	Gaps in notochord; heart looping randomized	<i>nodal</i> and <i>pitx2</i> bilateral 100%; <i>lefty2</i> right or bilateral; <i>lefty1</i> absent from midline	Izraeli 1999
<i>floating head (fih)</i>	Transcription factor	zebrafish	Notochord absent; discontinuous fp; heart looping and gut placement randomized	<i>cyc</i> bilateral in LPM and epithalmus	Danos 1996; Talbot 1995; Chen 1997; Rebagliati 1998; Sampath 1998; Schilling 1999
<i>no tail (ntl)</i>	Transcription factor	zebrafish	no differentiated notochord, heart looping and gut placement randomized	<i>cyc</i> bilateral in LPM and epithalmus	Halpern 1993; Danos 1996; Chen 1997; Rebagliati 1998; Sampath 1998; Schilling 1999
Mutations of Nodal pathway components					
<i>ACVR2B (ActRIIB)</i>	TGFβ receptor type II	human	Variety of L-R defects		Kosaki 1999b
<i>LEFTY A</i>	Divergent TGFβ molecule	human	Variety of L-R defects		Kosaki 1999a
<i>ActRIIB</i>	TGFβ receptor type II	mouse	No midline defects reported; right pulmonary isomerism; asplenia or hyposplenia; cardiac looping and turning randomized		Oh 1997
<i>Cryptic</i>	EGF-CFC protein	mouse	No midline defects reported; right pulmonary isomerism; asplenia or hyposplenia; cardiac looping and turning randomized	<i>nodal</i> , <i>lefty1</i> & 2, <i>pitx2</i> absent (100%); asymmetric <i>nodal</i> in node maintained	Gaio 1999; Yan 1999
<i>Lefty-1</i>	Divergent TGFβ molecule	mouse	No midline defect reported; left pulmonary isomerism	Complex <i>nodal</i> ; <i>lefty2</i> left or bilateral	Meno 1998
<i>Pitx2</i>	Transcription factor	mouse	No midline defects reported; right pulmonary isomerism; heart positioning defects; hyposplenia	<i>nodal</i> and <i>lefty</i> exp unaffected	Gage 1999b; Kitamura 1999; Lin 1999; Lu 1999
<i>Smad2 +/- nodal +/-</i>	Downstream effector of TGFβ signaling; TGFβ molecule	mouse	No midline defects reported; right pulmonary isomerism; abnormal heart looping		Nomura 1998
<i>cyclops (cyc)</i>	<i>Nodal</i> -like TGFβ molecule	zebrafish	Cyclopia; fp absent; only deletion allele has high incidence of randomized heart looping and gut placement	<i>cyc</i> left or bilateral	Hatta 1991; Chen 1997; Rebagliati 1998b; Sampath 1999; Schilling 1999
<i>LZoe</i>	EGF-CFC protein	zebrafish	No midline defects reported; heart looping and pancreas placement randomized	<i>cyc</i> , <i>lefty</i> , and <i>pitx2</i> absent (100%)	Yan 1999
Other mutations					
<i>Connexin43</i>	Gap junction protein	human	Variety of L-R defects		Britz-Cunningham 1995
<i>ZIC3</i>	Transcription factor	human	X-linked <i>situs inversus</i>		Gebbia 1997
<i>Fgf8 neof</i>	Fibroblast growth factor	mouse	Hypomorph; right pulmonary isomerism; heart looping randomized	<i>nodal</i> and <i>pitx2</i> absent ⁴	Meyers 1999
<i>Furin (fur)</i>	Serine protease	mouse	Unlooped hearts; fail to turn	Increase in <i>pitx2</i> levels; <i>pitx2</i> and <i>lefty2</i> left or bilateral	Roebroek 1998; Constam 2000
<i>Fused toes (Ft)</i>	N.D.	mouse	Heart looping and turning randomized	<i>nodal</i> and <i>lefty</i> bilateral (100%)	van der Hoeven 1994; Heymer 1997
<i>Smad5</i>	Downstream effector of TGFβ signaling	mouse	Hearts that loop are randomized; embryos fail to turn	<i>nodal</i> , <i>lefty2</i> , and <i>pitx2</i> bilateral; asymmetric <i>nodal</i> in node maintained; <i>lefty1</i> absent or reduced in midline	Chang 2000
<i>Mgat-1</i>	GlcNAc transferase	mouse	Heart looping randomized; embryos that turn are randomized		Metzler 1994

1. Only defects relating to classification in this table are noted. Not all reported left-right defects are included. See references for details.

2. Expression refers to LPM expression except where noted.

3. Complex refers to a combination of patterns including left, right, bilateral, or absent.

4. Lack of expression seen in a proportion of homozygotes presumably due to hypomorphic nature of the allele.

Abbreviations: exp = expression, fp = floorplate, N.D. = not determined

pathway, perhaps BMP (Meno et al. 1997), leading to de-repression of *pitx2* transcription (see below). These experiments illuminate the critical need to determine exactly when and where the signaling pathways involved in establishing L–R asymmetries are acting in order to properly interpret misexpression results.

In summary, the results described above suggest that the Nodal signaling pathway used during gastrulation is also employed during L–R patterning: Nodal signals, in the presence of EGF–CFC proteins and via Activin receptors, and Smad and FAST transcription factors, induce left-sidedness and are counteracted by inhibitors belonging to the Lefty family. This pathway appears to be conserved in humans as well. Mutations in *LEFTY A* and *ActRIIB* are associated with laterality defects in humans (Kosaki et al. 1999a,b). It is worth noting, however, that it remains unclear exactly how the components of the Nodal signaling pathway interact at the molecular level, where in the embryo (e.g., midline or lateral plate) they are required to fulfill their role, and when their influence is essential.

Downstream of Nodal signaling: *Pitx2* and *Snail*

The transcription factors Snail and *Pitx2* appear to be downstream targets of Nodal signaling. They are thought to act at the earliest steps in the third phase of L–R patterning, when positional information is transferred to the organs. The events that occur at later stages of phase three are not well understood at present. We thus focus here only on the expression and functioning of these early factors.

Pitx2 *Pitx2* is expressed asymmetrically in the left LPM in all vertebrates examined (Logan et al. 1998; Meno et al. 1998; Piedra et al. 1998; Ryan et al. 1998; St Amand et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Tsukui et al. 1999; Yan et al. 1999). Nodal overexpression can induce *pitx2* transcription and overexpression of the *lefty* genes can inhibit *pitx2* expression in the LPM (Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Cheng et al. 2000). In addition, *pitx2* transcription in the LPM is not established in *Cryptic* or late zygotic *oep* mutants, in which *nodal* signaling is attenuated (see above). *Pitx2* expression is altered in mutants that have L–R axis defects (King et al. 1998; Meno et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Gaio et al. 1999; Izraeli et al. 1999; Marszalek et al. 1999; Meyers and Martin 1999; Tsukui et al. 1999; Yan et al. 1999; Constam and Robertson 2000), and misexpression of *pitx2* can affect organ situs. For instance, heart looping is altered upon ectopic expression of *pitx2* in frog and chick (Logan et al. 1998; Ryan et al. 1998; Campione et al. 1999). Interestingly, different protein isoforms of *Pitx2* exist due to alternative splicing and the use of different promoters. Only certain isoforms are expressed asymmetrically and have the ability to affect situs in misexpression studies (Gage et al. 1999a; Kitamura et al. 1999; Essner et al. 2000; Schweickert et al.

2000). These results have led to the postulate that *Pitx2* isoforms act as downstream mediators for “leftness.” Unexpectedly, however, *Pitx2*-null mutant mice have hearts that loop correctly, and body turning, when initiated, is to the correct side (Gage et al. 1999b; Kitamura et al. 1999; Lin et al. 1999; Lu et al. 1999). The *Pitx2*-mutant mice have L–R defects such as right pulmonary isomerism which might be expected for the loss of a left determinant; however, the rather mild effect overall on situs argues against an essential global role of *Pitx2* as a downstream effector of leftness in Nodal signaling.

Snail To date there is only one gene reported to be asymmetrically expressed in more than one vertebrate on the right. The zinc finger transcription factor Snail is expressed more intensely within the right LPM in both chick and mouse (Isaac et al. 1997; Sefton et al. 1998). *Snail* transcription is repressed by Nodal signaling and thus represents a “right” side factor (Patel et al. 1999). *Snail* is involved in defining organ situs as shown by antisense oligonucleotide treatment against *snail* in chick (Isaac et al. 1997). Interference with *snail* activity leads to the expression of *pitx2* on the right side, suggesting that *snail* represses *pitx2* transcription in chick (Patel et al. 1999). However, the induction of *pitx2* in these experiments was observed only in severe cases where the midline was also disrupted and thus the effect may be indirect (see below). Other studies have failed to demonstrate potential interactions between *snail* and *pitx2* (Logan et al. 1998; Ryan et al. 1998). Characterization of *snail* family members in zebrafish and frog is needed to demonstrate a conserved role for this right determinant.

A midline barrier or repressor?

The asymmetric expression patterns described above are dependent on an intact midline (Fig. 2). The midline is in part derived from organizer tissue (the shield in fish and the node in chick and mouse) and separates the left and right sides of the embryo. Embryological experiments and mutant analysis in frog, zebrafish, and mouse indicate that an intact midline is crucial for the development of L–R asymmetry. For instance, extirpation of midline tissues including the notochord in *Xenopus* leads to randomization of heart looping and gut coiling which correlates with symmetric expression of the *nodal*-related gene *xnr-1* in both left and right LPM (Danos and Yost 1996; Lohr et al. 1997). In addition, zebrafish and mouse mutants affecting notochord development [e.g., *no tail* and *floating head* in zebrafish (Danos and Yost 1996; Rebagliati et al. 1998a; Sampath et al. 1998), *No turning* and *SIL* in mouse (Melloy et al. 1998; Izraeli et al. 1999)] have randomized heart looping and express *nodal* genes symmetrically. These results suggest that the role of the midline is to maintain asymmetry in the embryo by preventing the right lateral plate from acquiring left-sided identity.

These results have led to the proposal that the midline serves as a barrier to block the spread of signals from the

Figure 2. Models for the role of the midline. Left identity is abbreviated with an L and the right-sided repressor is abbreviated with a R. Bold lettering indicates left identity. Gray lettering indicates that left identity is repressed. Thick solid lines indicate intact midlines. Midline defects resulting from extirpation experiments or midline mutations are indicated by thick broken lines. The question mark indicates that although this effect is predicted by the model, it does not occur in extirpation experiments or in midline mutants. The left twin in the conjoined twins is shaded. (See text for details and references.)

left to the right (Fig. 2). How would the midline function as a barrier? It is conceivable that extracellular matrix (ECM) proteins in the midline may act to restrict the passage of signals (or cells) across the midline, thus restricting their activity to one side. The inhibition of proteoglycan synthesis or disruption of fibronectin networks alters organ situs in *Xenopus* embryos much like loss of the midline can (Yost 1990, 1992). Furthermore, mutation of a mouse glycosylation enzyme *Mgat-1* leads to a loss of glycosaminoglycan molecules in the ECM and L-R patterning defects (Metzler et al. 1994).

Lefty expression has been observed in the notochord and/or floorplate in all vertebrates examined (see above) and thus provides another intriguing possibility for a molecular midline barrier. In this model, *Lefty* expressed at the midline would bind to Nodal receptor(s) and prevent Nodal signaling from propagating across the midline (Meno et al. 1998). In the absence of the midline or in *Lefty1* mutants, Nodal signaling could propagate across the mesoderm to the right side.

The midline barrier model would also explain the L-R defects observed in conjoined twins (Fig. 2). In chick, *Xenopus*, and human twins, in which the midlines are not well separated, one twin will often have correct situs while the other twin will have randomized situs (Hyatt et al. 1996; Levin et al. 1996, 1997; Nascone and Mercola 1997). The twin with randomized situs is often found on the right suggesting that the left twin (designated in gray in Fig. 2) can have a negative effect on the right twin. Furthermore, right twins often do not express the left genes (Levin et al. 1996; Nascone and Mercola 1997; Hyatt and Yost 1998). These twin studies can be interpreted according to the midline barrier model. For instance, the midline of the left twin might act as a barrier for a left-sided global determinant (see below). It could be that the right twins in these cases were created from tissue that never received a left determinant and thus were unable

to express left-sided genes. However, such a global determinant in chick has not been identified and there is evidence against a prepattern in the chick blastoderm (see below).

An alternative version of the midline barrier model postulates that the midline may act as a barrier for a repressor located on the right side (Levin et al. 1996). This repressor would inhibit leftness, but is prevented from acting on the left by the midline. In conjoined twins, a right-sided repressor, such as *Activin* (see below), produced by the left twin could act on the adjacent twin, preventing expression of left side-specific genes (Fig. 2). This model would predict that loss of the midline barrier should allow the right-sided repressor to inhibit left side-specific genes. However, in midline extirpation experiments and in midline mutants, the opposite effect is seen. The right sided repressor model does therefore not account for all roles assigned to the midline.

An alternative model also involves a repressor, but postulates that the midline is the source of the repressor (Fig. 2). In this scenario, extirpation experiments and midline mutations would result in the loss of the right-side repressor allowing left side-specific gene expression to occur on the right side. The twin results are also consistent with a midline repressor model. It is conceivable that the midline of the left twin could be actively suppressing gene expression in the adjacent left LPM of the right twin, leading to randomization of situs. The most direct support for the midline repressor model comes from explant assays. In *Xenopus* explants, the notochord can block expression of nodal in the left LPM (Lohr et al. 1998). These observations are consistent with a midline repressor model, but it is unclear how the midline repressor would specifically influence the right, but not the left side. Taken together, both the midline barrier and midline repressor models are consistent with most

of the current results and further studies are required to distinguish between these models.

Early events and the initial break in symmetry

No unified picture has emerged for how lateral symmetry is broken and how the L-R axis is established with respect to the A-P and D-V axes. We will describe three mechanisms that have been implicated in these early events (Fig. 3): Nodal flow in mouse, a L-R coordinator in frog, and gap junction communication in chick and frog.

The node promotes left-sided gene expression

In contrast to the apparent role of the midline in restricting gene expression, midline progenitors in the chick node are thought to promote gene expression on the left at earlier stages. For instance, induction of node-like structures in lateral plate mesoderm explants leads to

expression of *nodal* (Levin and Mercola 1998a). Furthermore, rotation of a stage 5 node, placing the left side of the node closer to the right side of the embryo, can induce right sided *Nodal* expression in the LPM (Pagan-Westphal and Tabin 1998). Additional evidence for a role of the node in promoting left-sided gene expression comes from mouse mutants such as *HNF3 β* and *Brachyury* (Dufort et al. 1998; King et al. 1998). Mutant mice have no nodes or reduced or abnormal nodes and have been reported to lack *Nodal* expression in the LPM. Node ablation experiments in the mouse partially support the proposed role of the node in promoting left-sidedness as a proportion of the resulting embryos lack *Nodal* expression (Davidson et al. 1999). Interestingly, however, node ablation in mouse can also result in symmetric expression of *Nodal*. This may be another example of the importance of timing in embryological experiments (see *lefty* genes above). The loss of *Nodal* expression in node-ablation experiments may reflect the loss of an early acting “left-sided” inducer from the node. By contrast, the bilateral *Nodal* expression seen in these experiments could result from ablations that occurred after the induction of left-sided identity, and instead may reflect the later loss of midline structures derived from the node as described above. Further support for temporally changing roles of the node comes from experiments in chick. Stage 5 nodes are capable of inducing *nodal* expression, but extirpation of a stage 5 chick node and replacement with a stage 4 node results in absence of *nodal* gene expression in the LPM. Although further studies will be necessary to determine the timing involved in establishing asymmetry, these results taken as a whole support the role of the node in promoting left-sided gene expression.

The nodal-flow hypothesis

How does the node promote L-R laterality? Although chick data suggest that regions outside the node are important for establishing the L-R axis (see below), a role inherent in the node has been suggested in mouse. A single monocilium is found on each cell in the ventral cell layer of the mouse node (Sulik et al. 1994; Bellomo et al. 1996; Nonaka et al. 1998). Monocilia generally have been thought to be immotile, but recent reports demonstrate the motility of such cilia on the mouse node. The rotation of the monocilia can lead to a net leftward flow of material across the node by what has been termed nodal flow (Nonaka et al. 1998). The nodal-flow hypothesis postulates that a left determinant is asymmetrically distributed by the monocilia in the node to the left side, leading to asymmetric gene expression in the developing embryo.

Support for the nodal-flow hypothesis comes from the analysis of mouse mutants. Mutations in the dynein motor components KIF3A or KIF3B prevent the formation of node monocilia (Nonaka et al. 1998; Marszalek et al. 1999; Takeda et al. 1999). These mutants also have defects in L-R patterning suggesting a correlation between the loss of nodal flow and the establishment of L-R

Figure 3. Models for establishing asymmetry. The localization of a left determinant is indicated in red. In the nodal-flow model, the motility of monocilia and the shape of the node create a net leftward flow resulting in the asymmetric localization of a determinant to the left. The L-R coordinator would result from asymmetric activation of Vg1 on the left. In the gap junction communication model, the left determinant would flow unidirectionally through gap junctions to the left side. This determinant would be prevented from crossing to the right side by a coupling of unidirectional transport with a zone of isolation where gap junctions cannot function. The gap junction communication model is based on Levin and Mercola (1999). (Refer to the text for details and references.)

asymmetry. Nodal flow is also altered in the mouse mutant *iv* (Okada et al. 1999). Mutations in *iv* result in 50% situs inversus in the affected pups (Hummel 1959; Layton 1976). The *iv* locus encodes L–R dynein (LRD) which belongs to the family of axonemal dyneins (Supp et al. 1997). Importantly, the only defects seen in targeted deletions of LRD are inverted situs and loss of nodal flow, further underscoring the correlation of nodal flow with L–R patterning and confirming a role for LRD in the motility of node monocilia (Supp et al. 1999).

Further evidence for a link between cilia and L–R defects comes from the study of human patients with Kartagener's syndrome. This disorder results in situs inversus and is accompanied by lack of ciliated cells such as those found in the respiratory and reproductive epithelium (Afzelius 1976). These primary cilia are very different from the monocilia in the mouse node and mouse mutations in LRD do not affect primary cilia (Supp et al. 1999). However, mutations in the mouse gene *Hfh-4* appear to link these two cilia populations together. In *Hfh-4* mutants ciliated cells are absent similar to that seen in the human syndrome. In addition, the expression of LRD is abolished (Chen et al. 1998). It may be that *Hfh-4* controls the transcription of a number of dyneins involved in both normal cilia and node monocilia motility. The effect of *Hfh-4* mutations on nodal flow is not yet known, but the lack of LRD suggests that nodal flow will resemble that of *iv* mutants.

Another mouse mutation that affects the L–R axis is not as easily explained by the nodal-flow hypothesis. The recessive *inv* mutation results in situs inversus in nearly all homozygous individuals (Yokoyama et al. 1993). The gene affected by the *inv* mutation encodes Inversin, a novel protein containing ankyrin repeats (Mochizuki et al. 1998; Morgan et al. 1998). Because a lack of nodal flow (*iv* mutations) results in randomization of the axis, the prediction is that nodal flow should be reversed in *inv* mutants to invert the axis. Nodal flow is abnormal in *inv* mutants, but is severely slowed and not reversed. The *inv* mutation clearly argues against the nodal-flow hypothesis in its simplest form. Rather complex models might explain how slowing nodal flow could invert the axis (see discussion in Okada et al. 1999). For instance, reduced nodal flow might lead to the accumulation or activation of a putative left determinant on the right side, leading to a reversal of the L–R axis. The test of this model has to await the isolation of the molecule(s) that induce leftness. No evidence in support of the nodal-flow hypothesis has been provided in organisms other than mouse. In particular, node monocilia have not been reported in other organisms, so it is not clear if nodal flow could be a common mechanism in the establishment of the L–R axis. In addition, LRD is expressed in cells seemingly devoid of cilia and thus may function in other cellular processes such as transport of cargo molecules along microtubule tracks (Supp et al. 1997). Therefore, it should be noted that although the correlation between node monocilia motility and L–R defects is strong in some cases, it does not formally prove a cause and effect relationship.

Induction of asymmetry by signals outside the node?

The nodal-flow hypothesis suggests that the initial break in asymmetry occurs within the node and this information is transferred to the periphery. This model seems to contradict results in chick that indicate that areas outside of the node establish L–R asymmetry within the node. The asymmetric expression of the signaling molecule *shh* in the left region of the node is essential for induction of genes on the left (Levin et al. 1995). In particular, misexpression of Shh on the right side of the node leads to ectopic expression of *nodal* on the right. In addition, Shh blocking antibodies result in absence of *nodal* expression on the left (Pagan-Westphal and Tabin 1998). Two lines of evidence suggest that the asymmetric expression of Shh on the left side of the node might be induced by neighboring cells. First, extirpation studies show that a newly regenerated node has normal expression of *shh* on the left (Psychoyos and Stern 1996). Second, rotation of a stage 4 node results in normal expression of *shh* at stage 5 (Pagan-Westphal and Tabin 1998). These results indicate that there is no prepattern in the node to activate *shh* on the left. Instead, there might be a prepattern in neighboring tissues that then induces *shh* on the left. However, the observed experimental outcomes could also be explained by the nodal-flow hypothesis. If nodal flow exists in the chick, it is possible that regenerated nodes or reversed nodes in the experiments described above could re-establish proper nodal flow resulting in correct L–R patterning. The mouse nodal-flow model suggests that the shape of the node is important in generating a net leftward flow from the motions produced by the motile cilia (Nonaka et al. 1998; Okada et al. 1999). As rotation or regeneration would not change the clockwise rotation of monocilia, the regenerated or reversed chick nodes would have to adjust their shape to re-establish proper leftward nodal flow. While the nodal-flow hypothesis is quite attractive in linking the A–P and D–V axis to the induction of laterality, it will be crucial to test if nodal flow occurs in vertebrates other than mouse and to isolate the L–R determinant transported by nodal flow.

A L–R coordinator?

Twin studies in chick suggest that there is no prepattern for L–R asymmetry before streak formation. When streaks are oriented 180° away from each other, each twin develops a properly oriented L–R axis independent of the twin axis. This argues against a prepattern and suggests that if the twin axes are well separated, each can establish its own L–R axis properly. The mouse nodal-flow hypothesis also argues against a prepattern. In *Xenopus*, however, experiments suggest that the L–R axes may be established as early as the one cell stage (Fig. 3). UV treatment of *Xenopus* embryos at this stage disrupts microtubules and prevents cortical rotation. Such embryos can be rescued by tilting of the embryos, re-establishing the D–V and A–P axes, but the L–R axis is now randomized with respect to heart looping and gut

coiling (Yost 1995). This suggests a prepattern in the *Xenopus* embryo that can be disrupted by loss of microtubules.

The existence of a L-R coordinator peripheral to midline structures has also been inferred by the ability of an active form of Vg1, a TGF β family member, to completely invert the axis when injected in specific blastomeres on the right (Hyatt and Yost 1998). This suggests that Vg1 normally acts on the left, presumably in descendants of the L3 blastomere, to orient the L-R axis with the D-V and A-P axes. The L3 blastomere gives rise to structures outside of the axial mesoderm, including LPM, suggesting the coordinator acts outside of the midline (Dale and Slack 1987; Moody 1987). The ability of Vg1 to invert the axis cannot be mimicked by other TGF β members tested, suggesting a specificity for Vg1 in this process. However, many TGF β members converge upon common downstream effectors such as Smad2 and thus could functionally substitute for one another in such injection experiments (Gritsman et al. 1999). Since no active or processed Vg1 protein has been detected in *Xenopus* embryos, Vg1 may be mimicking the effect of another related TGF β family member that has not yet been tested in this assay. Furthermore, when and where this coordinator functions is not clear. Vg1 plasmid injections can also orient the L-R axis suggesting this process could occur after mid-blastula transition (Hyatt and Yost 1998).

A role for gap junction communication

One process that may function early in the establishment of the L-R axis upstream of the role of the node has been shown to be conserved in both frog and chick. In *Xenopus* gap junction communication (GJC) shows an early asymmetry with GJC being more active on the dorsal versus the vegetal halves of the embryo (Levin and Mercola 1998b). Inhibition of GJC in *Xenopus* by pharmacological agents or misexpression of connexin constructs leads to an increase in heart looping defects (Levin and Mercola 1998b). Likewise antibodies or antisense oligonucleotide treatment against connexin subunit Cx43 in chick can inhibit GJC communication, cause bilateral expression of *shh* and *nodal*, and thus effect normal heart situs (Levin and Mercola 1999). These treatments also prevent nodes that regenerate after ablation from establishing proper asymmetric gene expression of *shh* or *nodal*, suggesting GJC functions in transferring L-R information to the node (Levin and Mercola 1999). Mutations in the connexin subunit Cx43 have been found in some human patients displaying heterotaxia (Britz-Cunningham et al. 1995, but also see Gebbia et al. 1996), and expression of the mutant human Cx43 in *Xenopus* can induce heterotaxia, suggesting GJC may be important in establishing the human L-R axis as well (Levin and Mercola 1999). Cx43 mutant mice have not been reported to have L-R defects, but this could be due to the ability of other connexins to functionally substitute for the loss of Cx43 (Lo 1999).

It is not clear how GJC functions in L-R axis estab-

lishment (Fig. 3). It is conceivable that gap junctions allow unidirectional transport of small molecules, resulting in the accumulation of a L-R determinant on one side. For GJC to be asymmetric in the embryo, there must be a barrier, or "zone of isolation" to prevent GJC from traveling back from one side to the other. In *Xenopus* this is achieved by preventing GJC communication from occurring in the ventral blastomeres (Levin and Mercola 1998b). In chick GJC there is a lack of gap junctions in the midline, thus acting as the zone of isolation (Levin and Mercola 1999). If the model proposed above is correct, it implies that the initial break in symmetry must occur upstream of GJC to result in asymmetric transport of the L-R determinant. To clearly establish the role of GJC in L-R patterning, it will be crucial to isolate the potential L-R determinant transported via gap junctions.

Molecular conservation and divergence

Although the initial mechanisms that lead to a break in symmetry remain mysterious, a detailed molecular pathway involved in transferring positional information from node to the periphery has been described in chick (Fig. 1). A number of these factors have been shown to have effects only in chick or have conflicting roles in different organisms.

A signaling cascade transfers L-R asymmetry in chick from node to lateral plate

An Activin-like signaling pathway has been shown to act upstream of *shh* and *nodal* in the chick node. Expression of *activinBB* and its receptor *ActRIIA* is restricted to the right side of the node (Levin et al. 1995, 1997). Activin signaling leads to the restriction of *shh* expression to the left of the node and induces *FGF8* on the right side of the node (Levin et al. 1995; Boettger et al. 1999). *shh* then activates *nodal* lateral to the node, whereas *FGF8* acts to inhibit *nodal* expression on the right (Levin et al. 1995; Boettger et al. 1999). Subsequent transfer of positional information downstream of the node to the left lateral plate appears to require paraxial mesoderm (Pagan-Westphal and Tabin 1998) and the expression of *caronte* (*car*). *car* is induced by *shh* on the left and is expressed asymmetrically on the left in the paraxial mesoderm and the left LPM. *car* expression is blocked on the right by *FGF8*. Misexpression of *Car* on the right induces *nodal*, *lefty*, and *pitx2* expression and can lead to abnormal situs (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999; Zhu et al. 1999).

How does *Car* act to induce *nodal* expression in the LPM? *Car* is a member of the DAN family of extracellular inhibitors. *Car* binds both BMPs and *Nodal* in vitro (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999), but seems to activate *nodal* expression in the left LPM by repressing BMP activity. BMPs are expressed symmetrically in the LPM at the time of *car* expression, and BMP addition to the left can inhibit *nodal* expression (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999). Noggin, which can also bind and inhibit BMPs, is able to induce

nodal expression when expressed on the right (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999; Zhu et al. 1999). As Nodal can signal in the right LPM when mis-expressed, without inducing *car* in the LPM (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999; Zhu et al. 1999), the main role of *Car* is to allow Nodal expression.

Several aspects of the cascade operating in chick might not be conserved in other vertebrates

The genetic cascade for L–R laterality in chick has been elegantly established, but several observations suggest that aspects of it may not be conserved in other vertebrates.

Activin *Activin* β B and *ActRIIA* are not asymmetrically expressed in mouse, and mutations in these genes do not affect the L–R axis (Matzuk et al. 1995a,b). Although *ActRIIB* mouse knockouts do have L–R defects, this phenotype is likely to be caused by blocking Nodal signaling (Oh and Li 1997). Moreover, blocking of Activin signaling by dominant-negative *ActRIB* does not change L–R laterality in *Xenopus* (Ramsdell and Yost 1999).

Shh Although *shh* plays a major role in the chick, *shh* is not expressed asymmetrically in other vertebrates examined (Collignon et al. 1996; Sampath et al. 1997; Schilling et al. 1999). The *Shh* mutant in mouse has some alterations in the L–R axis, most notably pulmonary left isomerism, which has led to the idea that *Shh* acts on the right in mouse (Izraeli et al. 1999; Meyers and Martin 1999; Tsukui et al. 1999). However, a Hedgehog pathway plays a role in lung development, perturbation of which can lead to underdeveloped unlobed lungs (Bellusci et al. 1997; Pepicelli et al. 1998). In addition, *Shh* mutant mice have midline defects that make interpretation of their phenotype problematic (Chiang et al. 1996). In particular, the loss of floor plate development in these mutants results in absence of *Lefty1* expression. The L–R defects that are observed in *shh* mutants might thus simply be a secondary effect of loss of *Lefty1* function. While misexpression of *shh* can affect situs in *Xenopus* and zebrafish (Sampath et al. 1997; Schilling et al. 1999), the *shh* mutation *sonic you* in zebrafish does not have adverse effects on L–R patterning (Chen et al. 1997). It is possible that other *hedgehog* genes serve the role in L–R development that *shh* does in the chick. *Xenopus* *banded hedgehog* seems to be more potent in affecting the L–R axis, supporting this idea (Sampath et al. 1997), but it remains unclear if Hedgehog signaling is directly involved in L–R axis formation in vertebrates other than chick.

BMP It is not yet clear if the role of BMPs in L–R patterning is conserved in other organisms. In zebrafish *BMP4* is expressed asymmetrically in the heart and symmetric expression has been shown to correlate with abnormal heart jogging and looping (Chen et al. 1997). This proposed role for BMP is later than that postulated in chick. Moreover, asymmetric BMP expression in other

organisms has not been reported. A role for BMP signaling in L–R development has been suggested in *Xenopus*. Dominant-negative BMP receptors (ALK2) change situs and induce bilateral *nodal* expression in half of the injected embryos (Ramsdell and Yost 1999). This result would be consistent with the proposed role for BMP signals as repressors of left-sidedness. However, expression of an activated BMP receptor on the left did not result in the repression of *nodal* expression as expected from the chick model (Ramsdell and Yost 1999). Furthermore, no *caronte* homolog expressed on the left has been identified yet in other vertebrates. In support of a conserved role for BMP signaling in L–R patterning, mice homozygous mutant for the BMP signaling component *Smad5* have heart looping defects and fail to turn. Furthermore *Nodal*, *Lefty2*, and *Pitx2* expression is bilateral in these embryos (Chang et al. 2000). This can be interpreted as a BMP signaling pathway functioning to suppress Nodal signaling as demonstrated in the chick. However, *Lefty1* expression is severely reduced or absent in the midline and LPM of these embryos. Thus there may be two roles for BMP and *Smad5* signaling in L–R patterning in the mouse, one early to inhibit Nodal signaling in the LPM and an additional role in inducing *Lefty1* expression in the midline.

FGF8 *Fgf8* appears to play roles on the left in mouse as opposed to its role on the right in chick. *Fgf8* is not expressed asymmetrically in the mouse but a conditional mouse knockout of *Fgf8* displays loss of *Nodal* and *Pitx2* expression, implicating *Fgf8* as a left determinant (Meyers and Martin 1999). The conditional knockouts also have defects in heart looping and right pulmonary isomerism, further suggesting the left side was not properly specified. Importantly, FGF8 beads induce *Nodal* on the right in the mouse, the opposite to what is expected from the chick model.

Nkx3.2 *Nkx3.2* was shown to be downstream of Nodal signaling on the left and repressed by the Activin/FGF pathway on the right in chick (Rodriguez Esteban et al. 1999; Schneider et al. 1999). However, *Nkx3.2* is expressed on the right side in mouse (Schneider et al. 1999) and symmetrically in *Xenopus* initially, only becoming asymmetric at later stages (Newman et al. 1997). Moreover, mutations in mouse *Nkx3.2* do not display any obvious defects in L–R development (Lettice et al. 1999; Tribioli and Lufkin 1999).

RA In contrast to the factors described above, RA might have a conserved role in all vertebrates. Embryos from quail deprived of Vitamin A are deficient in RA and 65% of these embryos display defects in turning and heart looping, suggesting an endogenous need for RA in these events (Dersch and Zile 1993; Twaal et al. 1995). Although RA treatment of mouse embryos can affect the midline (Wasiak and Lohnes 1999), misexpression studies suggest a more direct role for RA in the induction of left side genes. Ectopically applied RA can induce expression of *nodal*, *lefty*, and *Pitx2* resulting in abnormal situs in all vertebrates examined (Chazaud et al. 1999; Tsukui

et al. 1999; Wasiak and Lohnes 1999). Importantly, inhibitors of RA abolish the expression of these genes suggesting that the effect of RA on their induction is direct and not via loss of the midline barrier (Chazaud et al. 1999; Tsukui et al. 1999). It is intriguing that an asymmetric enhancer of mouse *Nodal* contains retinoic acid response elements (Norris and Robertson 1999) and that *Lefty* expression is induced by RA in P19 cells (Oulad-Abdelghani et al. 1998). RA is found in the nodes of both chick and mouse and thus is present at the right time and place to play an endogenous role in initiating gene expression on the left (Chen et al. 1992; Hogan et al. 1992).

Conclusions and prospects

Studies of L-R patterning have revealed a number of components (Nodal signals, EGF-CFC factors, *Pitx2*, RA) and embryonic regions (midline and node) that are very likely to have roles conserved among all vertebrates. However, it has also become clear that a number of molecular processes might not be strictly conserved (Activin, FGF8, Shh). It is interesting to note that a *Ptx* gene with similarity to *pitx2* has been cloned from Chinese lancelets and is expressed asymmetrically on the left (Yasui et al. 2000). In addition, the only *shh* ortholog in amphioxus is also expressed asymmetrically (Shimeld 1999). This argues that the origins of asymmetry may be found in the ancestors of the chordates and thus some aspects should be conserved in all vertebrates. But why are many of the molecules employed in the establishment of L-R laterality in chick not used or used differently in other vertebrates? It is conceivable that functional conservation occurs at the level of generating asymmetries in organs and their placement, not at the level of gene expression patterns or side of action. For example, the stomach in avians seems to generate asymmetry by differential growth, whereas rotation generates asymmetries in mammals (see discussion in Schneider et al. 1999). A gene thus could be expressed on a different side between birds and mammals but play the same role in each. More likely, however, the divergent strategies used in different vertebrates underscores evolution's inventiveness, indicating that only some of the mechanisms underlying L-R laterality are universal.

Many questions remain to be answered in order to obtain a more thorough understanding of L-R asymmetry: Is left-sidedness generated within the node and/or transferred to the node? How does the midline regulate laterality? How many of the genes and functions are conserved or divergent between organisms? What are the molecular interactions and additional components in the L-R pathway? A group of zebrafish mutants isolated in the large-scale Tübingen and Boston screens have defects in L-R patterning as shown by examining heart looping and jogging (Chen et al. 1997). An additional screen in zebrafish has isolated mutations that affect L-R patterning of the heart and viscera (J.N. Chen and M.C. Fishman, pers. comm.). Further exploration of these mutants and identification of the affected gene products will add

to our current understanding of L-R patterning in the zebrafish. Such studies will complement those ongoing in other vertebrates and will help determine which genes and pathways are conserved.

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