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The left-right axis in the mouse: from origin to morphology

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The past decade or so has seen rapid progress in our understanding of how left-right (LR) asymmetry is generated in vertebrate embryos. However, many important questions about this process remain unanswered. Although a leftward flow of extra-embryonic fluid in the node cavity (nodal flow) is likely to be the symmetry-breaking event, at least in the mouse embryo, it is not yet known how this flow functions or how the asymmetric signal generated in the node is transferred to the lateral plate. The final step in left-right patterning – translation of the asymmetric signal into morphology – is also little understood.

Introduction

There are two key steps that contribute to the early establishment of left-right (LR) patterning in the mouse. The first step is the symmetry-breaking event that takes place in the node around embryonic day (E) 7.5 of mouse development (Fig. 1). In this step, an asymmetric signal(s) that is generated in the node is transferred preferentially towards the left side of the lateral plate mesoderm (LPM). (This mesoderm is located in the lateral region of the early-somite-stage mouse embryo and later contributes to the mesenchyme of various visceral organs.) The transfer of this signal results in the second step: the asymmetric expression of the gene *Nodal* in the left LPM (see Fig. 1A,B). Cells in the left LPM that receive Nodal signaling contribute to various visceral organs, such as the lung and heart, that develop left side-specific morphologies.

In this review, we discuss our current understanding of the mechanism of left-right (LR) patterning during development. In particular, we focus on genetic data from the mouse; we do not discuss finding from studies in other vertebrates, except where specifically mentioned. [For recent reviews of the similarities and differences in LR patterning between the mouse and other vertebrates, see Levin and Tabin (Levin, 2005; Tabin, 2005).]

Leftward fluid flow breaks LR symmetry

Although there is some controversy concerning the initiation of LR asymmetry in other vertebrates (see Tabin, 2005), at least in the mouse, the breaking of LR symmetry is most likely to be achieved by the unidirectional flow of extra-embryonic fluid in the node (the node is an embryonic structure that is located at the midline, at the anterior tip of the primitive streak in mouse embryos, see Fig. 1B and Fig. 2A). This fluid flow is referred to as nodal flow (Nonaka et al., 1998). This leftward laminar flow of extra-embryonic fluid in the node cavity occurs at a speed (visualized with fluorescent beads, see Fig. 2D) of ~15 to 20 μm/second and is generated by the rotational movement of 9+0 monocilia (these are cilia that have nine doublets of microtubules but that lack a pair of central microtubles), which protrude from cells located on the ventral side of the node into the node cavity (Sulik et al., 1994) (Fig. 2C). These 200-300 cilia rotate in the same direction (clockwise, as viewed from the ventral side) at a speed of 600 rpm (Nonaka et al., 1998). Nodal flow takes place for only a short period of time. It is, thus, first apparent at the one- to two-somite stage, persists for several hours and ends by the six-somite stage. The asymmetric expression of *Nodal* begins in the LPM at the two-somite stage and disappears by the six-somite stage. Nodal flow may therefore occur specifically to initiate *Nodal* expression on the left side of the LPM. Monocilia are also present in the notochordal plate (Nonaka et al., 1998), but they are reported to be immotile (Okada et al., 2005).

Nodal flow is essential for LR determination. Various mouse mutants that lack normal flow as a result of the absence or immotility of node cilia are reported to have abnormal LR patterning (Okada et al., 1999). The direct consequence of the absence of nodal flow appears to be the randomization of LR orientation, as is best exemplified by the *iv/iv* (inversus viscerum) mouse mutant, which possesses immotile cilia (Supp et al., 1999; Supp et al., 1997). However, most mouse mutants that lack node cilia exhibit complex phenotypes (typically, bilateral left sidedness, known as left isomerism) because they also have functional defects in the midline barrier. The best examples of such mutants are those that lack intraflagellar transport proteins, such as polaris (Ift88 – Mouse Genome Informatics), wimple (Wim; Ift172 – Mouse Genome Informatics) and Kif3 proteins (Garcia-Garcia et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Murcia et al., 2000; Nonaka et al., 1998).

Reversal of the direction of nodal flow by the imposition of an artificial flow leads to the reversal of LR patterning in mice (Nonaka et al., 2002), demonstrating that the flow per se directs subsequent LR patterning events. Recent evidence suggests that a similar mechanism may operate in other vertebrates (Okada et al., 2005). In zebrafish, for example, cilia have been detected in Kupffer's vesicle, the embryonic organizer equivalent to the mouse node (Essner et al., 2002; Essner et al., 2005; Kramer-Zucker et al., 2005). These cilia are motile and generate a unidirectional flow in the vesicle. Zebrafish mutants that lack the cilia in Kufpper's vesicle show impaired LR patterning (Essner et al., 2005; Kawakami et al., 2005; Kramer-Zucker et al., 2005), but the role of the flow has not been directly tested.

The origin of LR polarity

The mechanism of symmetry breaking must make use of the pre-existing positional cues: the anteroposterior (AP), dorsoventral (DV) and mediolateral axes. As Brown and Wolpert proposed in their conceptual F-molecule model (Brown et al., 1991), the origin of the LR axis must derive from AP and DV axis information. But how is AP and DV information translated into LR polarity? This question is now known to be equivalent to: how is such information translated into the generation of the leftward flow in the node? Furthermore, if all the node cilia rotate in the same clockwise direction, how can they generate a unidirectional flow?

The key to the answer to this last question was recently shown to lie in the rotation angle of the cilia (Okada et al., 2005; Nonaka et al., 2005). Hydrodynamic principles dictate that a simple rotational movement of cilia would generate a vortex only if the cilia protrude vertically from a surface. However, if the cilia are

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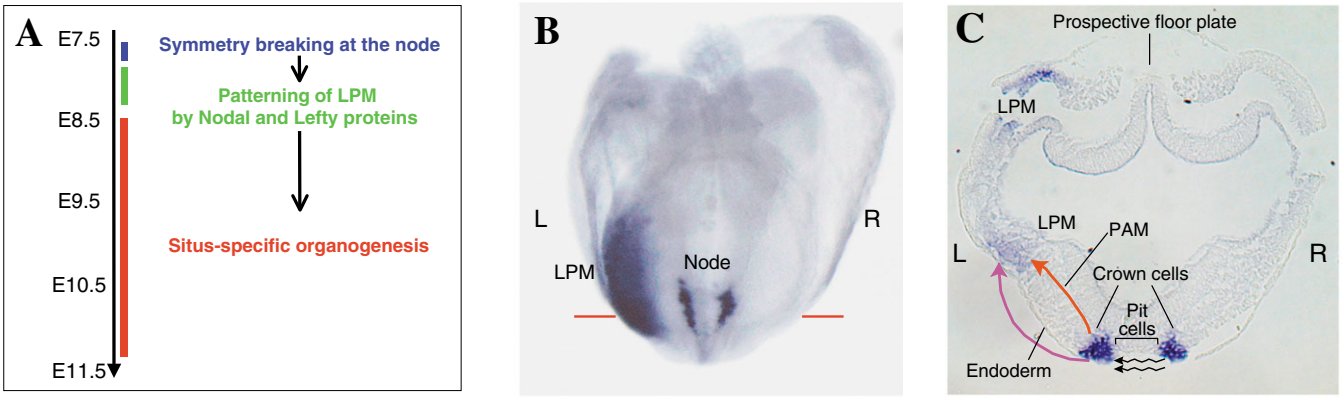

Fig. 1. Mechanisms of left-right (LR) patterning in mouse embryo. (**A**) Three steps in the generation of LR asymmetry: a symmetry-breaking event in the node, the patterning of the lateral plate mesoderm (LPM) and asymmetric organogenesis. The black arrow on the left represents a time course during development, from earlier embryonic (E7.5) stages to later ones (E10-11.5). (**B**) Posterior view of a mouse embryo at E8.0 showing *Nodal* expression in the node and left LPM. (**C**) A transverse section taken at the level indicated by the red lines in B. The location of pit cells, crown cells, paraxial mesoderm (PAM), LPM, endoderm and prospective floor plate are shown. Arrows indicate how signals are transferred during LR patterning. Asymmetric signal(s) generated by the leftward flow in the node (black wavy lines) might be transferred to the left LPM, either through the endoderm (pink line) or through the PAM (red line). According to the determinant-transporting model, an unknown molecule produced in the node/perinodal cells is secreted into the node cavity and transported towards the left side, where its signal may be transduced by the endoderm and finally by the LPM (pink line). Alternatively, cells in or near the node may sense the mechanical stress and send an unknown signal(s) to the left LPM thorough the PAM (red line). It is also possible that the perinodal (crown) cells respond to the chemical determinant and send a signal via the red route.

tilted towards a specific direction, it is possible for them to generate a unidirectional flow (Cartwright et al., 2004; Nonaka et al., 2005; Okada et al., 2005). Thus, when a cilium moves closer to the surface, the movement of fluid near the surface will be restricted as a result of the so-called no-slip boundary effect [according to this effect, fluid contacting a solid wall will not move; owing to its viscous force, this static fluid layer would prevent the fluid that overlies it from being dragged by the cilia (Liron, 1996)]. Conversely, when a cilium moves away from the surface, it induces the movement of the neighboring fluid more effectively. If cilia are tilted toward the posterior side, they would be moving towards the right when they come close to the surface and towards the left when they are far from the surface (see Fig. 3). Hydrodynamics therefore predict that a leftward flow can be generated if rotating cilia are tilted towards the posterior side. High-speed microscopic observations have revealed that the node cilia are indeed tilted posteriorly, at an average angle of 30° (Nonaka et al., 2005; Okada et al., 2005).

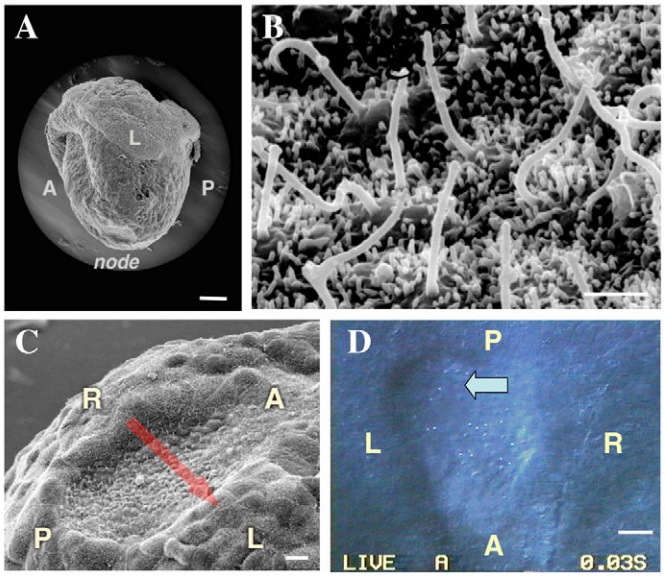

Fig. 2. Leftward fluid flow generated by rotational movement of node cilia. (**A**) Lateral view of a mouse embryo at 8.0 dpc. Scale bar: 100 μm. (**B**) Monocilia in the node of a mouse embryo shown at high magnification. Scale bar: 1 μm. (**C**) Ventral view of the node cavity. Anteroposterior (AP) and left-right (LR) orientations are indicated. Red arrow indicates the direction of nodal flow. Scale bar: 10 μm. (**D**) Ventral view of the node cavity, with fluorescent beads used to visualize fluid flow. The beads move towards the left side of the node (arrow). Scale bar: 10 μm. Image in D courtesy of Shigenori Nonaka (National Institute for Basic Biology, Japan).

The direction of nodal flow, then, is determined by the pre-existing AP and DV axes, and by the unidirectionality of ciliary rotation (Fig. 3). The AP axis influences the tilt of the node cilia, whereas the DV axis is represented by their ventral protrusion. The origin of the LR axis is thus, indeed, dependent on AP and DV positional information. The node cilium thus corresponds to the hypothetical F-molecule proposed by Brown and Wolpert (Brown et al., 1991).

How does AP information dictate the positioning of each node cilium in a posteriorly tilted manner? A careful examination of the node cilia (Nonaka et al., 2005; Okada et al., 2005) has revealed that most of them protrude from the posterior region of node pit cells (cells on the ventral surface of the node that have monocilia, see Fig. 1C and Fig. 3). The basal body of each cilium is also positioned posteriorly within the cell (Fig. 3). Node pit cells are roughly spherical. As such, the protrusion of cilia perpendicular to the cell membrane and the posteriorly shifted localization of the basal body may explain the tilt of the cilia toward the posterior side. This scenario is similar to the planar cell polarity (PCP) pathway that is responsible for the coordinated localization and orientation of hairs in *Drosophila* cuticles and of sensory hairs in the vertebrate inner ear (Klein and Mlodzik, 2005). Similarly, DV positional information may determine the positioning of the cilia according to apicobasal

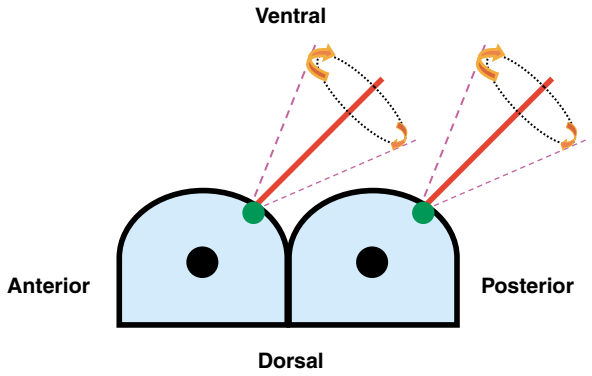

Fig. 3. De novo generation of left-right (LR) asymmetry via three sources of positional information. Node pit cells of E8.0 mouse embryo are illustrated. Each cilium (red bar) protrudes from a node pit cell (light blue) towards the ventral side of the mouse embryo. The cilium is also posteriorly tilted, most probably because the position of the basal body (green) is posteriorly shifted within each cell. Each cilium rotates in a clockwise direction when viewed from the ventral side. The posterior tilt of the ciliary rotation axis generates a leftwards flow instead of a vortex. LR asymmetry is thus formed de novo through a combination of dorsoventral information, anteroposterior information and the unidirectionality of ciliary rotation.

polarity of the epithelium. It appears likely that a mechanism similar to PCP operates to correctly position the basal body of each cilium in the node pit cells.

How does fluid flow break LR symmetry?

Nodal flow is the event that most probably breaks LR symmetry during development, at least in the mouse, but the mechanism by which it achieves this effect has been subject to debate. Several models have been proposed, but there are two principal hypotheses (Fig. 4).

Does nodal flow transport a determinant?

This is an obvious possibility that was initially proposed by Nonaka et al. (Nonaka et al., 1998). According to this model (Fig. 4A), a molecule that acts as the LR determinant is transported by nodal flow towards the left side. If the molecule is secreted by node cells (such as pit cells located in the node cavity or perinodal crown cells) into the node cavity, it would be readily transported by the fluid flow. Indeed, recent evidence (Tanaka et al., 2005) indicates that node pit

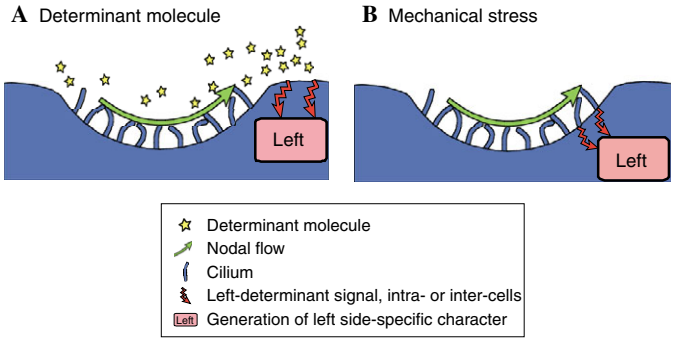

Fig. 4. Two models for the mechanism of action of nodal flow. (**A**) The transportation of a left-right (LR) determinant or (**B**) the generation and sensing of mechanical stress by leftward flow at cilia. Modified, with permission, from Nonaka et al. (Nonaka et al., 1998).

cells actively secrete vesicles, called nodal vesicular parcels (NVPs), that appear to contain Hedgehog (Hh) protein and retinoic acid (RA) into the node cavity. However, the identity of such a transported molecular determinant of LR polarity remains to be established, Furthermore, the identity of the cells on the left side that receive the determinant signal is unknown. Several candidates have been proposed for the putative LR determinant, such as sonic hedgehog (*Shh*) and RA (Tanaka et al., 2005), but none seems to fulfill all the required criteria. According to these criteria, a candidate for the LR determinant should be produced in or near the node, and its loss should result in the lack of *Nodal* expression in the lateral plate.

Shh is asymmetrically expressed and plays an important role in LR determination in avian species (Levin et al., 1995) but not in other vertebrates, including the mouse. Shh is expressed in the midline, including the node, and $Shh^{-/-}$ mice exhibit LR defects (Meyers and Martin, 1999). However, $Shh^{-/-}$ embryos show left isomerism as a result of impaired midline function. The LR decision is initially normal in the *Shh*-null embryos, but they subsequently develop bilateral *Nodal* expression in the lateral plate because of the midline abnormality (see below for a discussion of the function of the midline). Another Hedgehog family protein, Indian hedgehog (Ihh), is also implicated in LR patterning. Mice lacking both Shh and Ihh, similar to smoothened (*Smo*)-null mice, fail to develop asymmetric *Nodal* expression in the lateral plate (Zhang et al., 2001). However, the LR defects of these animals are most likely to be caused by the associated lack of *Gdf1* expression, which is required for asymmetric *Nodal* expression in the LPM (Rankin et al., 2000). Moreover, an examination of the expression of Hh target genes, such as *Ptch1,* failed to reveal any asymmetry in Hh signaling in normal mouse embryos (Zhang et al., 2001). These observations suggest that, in the mouse, Hh signaling is required for the formation of a functional midline, including the node, but that it is not directly involved in LR determination.

RA is synthesized in regions near the node by the enzyme retinaldehyde dehydrogenase 2 (RALDH2). RA signaling, as revealed by expression of an RA-responsive transgene, has also been detected in the perinodal region (Vermot et al., 2005). However, mice that lack RALDH2, the only RA-synthesizing enzyme expressed near the node, exhibit normal LR patterning in the lateral plate; that is, asymmetric *Nodal* expression is maintained (Vermot et al., 2005). It is, thus, unlikely that RA, which was shown to be contained in NVPs (Tanaka et al., 2005), regulates the LR decision at the node. Instead, RA is required for the maintenance of bilateral symmetry during somite formation (Kawakami et al., 2005; Vermot et al., 2005; Vermot and Pourquie, 2005).

Fibroblast growth factor 8 (Fgf8) has also been considered as a candidate for the LR determinant that is transported by nodal flow. Mice conditionally deficient in *Fgf8* lack *Nodal* expression in the LPM and exhibit right isomerism (Meyers and Martin, 1999). Superficially, Fgf8 appears to be required for determination of the left side. Experiments with an Fgf inhibitor (SU5402) suggest that Fgf8 may be necessary for the secretion of NVPs (Tanaka et al., 2005), but it remains uncertain exactly when and where Fgf8 plays a role in LR patterning. Fgf8 may thus function in the node or in the LPM to maintain the competence of the LPM to respond to the Nodal signal, for example. The site-specific ablation of *Fgf8* will be necessary to clarify the precise site of Fgf8 function.

Does nodal flow generate mechanical stress?

An alternative to the hypothesis that nodal flow transports an LR determinant is that it generates mechanical stress that is sensed by node cells, either pit cells or crown cells (Fig. 4B) [crown cells are

peri-nodal cells of endoderm origin that express *Nodal*, *Gdf1* and *Cerl2* (*Dand5* – Mouse Genome Informatics); see Fig. 1B]. There are several examples of cells that sense flow-generated mechanical stress (for a review, see Orr et al., 2006), perhaps the best known of which is the sensing of blood flow by vascular endothelial cells. The magnitude of mechanical stress generated by a flow depends on several factors, including the speed of the flow and the viscosity of the fluid. As mentioned above, the speed of nodal flow, as visualized with fluorescent beads, is ~15 to 20 μm/second, which is much slower than that of blood flow in peripheral arteries (~10 mm/second). The precise viscosity of the extra-embryonic fluid present in the node cavity is unknown, but the viscosity of bovine amniotic fluid is 3 to 5 mPa·s, which is similar to that of blood (14 mPa·s). The Reynold number (*Re*, which is the ratio between inertial force and viscous force and is very low for microscopic phenomena) for ciliary rotation is only $\sim 5 \times 10^{-4}$ (Cartwright et al., 2004), suggesting that the associated inertial force is negligible. Therefore, the shear stress generated by nodal flow might be too small to be sensed by the cell surface.

The mechanical stress experienced by a cell may also depend on which subcellular organelle is responsible for its detection. Shear stress can be detected by the cell membrane (as in vascular endothelial cells), as well as potentially by nonmotile cilia (McGrath et al., 2003). If the latter is the case, a small physical force generated by the slow nodal flow might be amplified by the bending of the cilia. Alternatively, if the mechanical stress is sensed by the membrane of node cells, the signal may be amplified within the cell by signaling cascades.

Recently, a family of mechanically gated channels called TRP channels has been implicated in mechanotransduction in sensory systems, including hearing and touch sensitivity (Pedersen et al., 2005). Interestingly, some of the TRP family members contain ankyrin repeats, which may serve as a molecular spring to amplify a small level of shear stress (Lee et al., 2006). It may be interesting to search for the expression of TRP members in or near the node.

Role of a Ca^{2+} signal

Two lines of evidence indicate a role for Ca^{2+} in LR determination downstream of nodal flow, which possibly favor the mechanosensory model. First, asymmetric Ca^{2+} signaling has been detected at the left margin of the node (McGrath et al., 2003). The asymmetric elevation of Ca^{2+} and its lateral propagation have also been reported (Tanaka et al., 2005). Second, a putative Ca^{2+} channel has been implicated in LR decision making. *PKD2*, a causative gene for autosomal recessive polycystic kidney disease in humans, encodes a protein that functions as a Ca^{2+} release channel in cultured cells (Luo et al., 2003). *Pkd2* mutant mice develop LR defects that are consistent with impaired mechanosensation. They, thus, possess morphologically normal and motile cilia but fail to develop asymmetric expression of *Nodal* in the LPM (Pennekamp et al., 2002). They also exhibit a low level of bilateral *Pitx2* expression in the posterior lateral plate (LP) [Pitx2 is a transcription factor, the expression of which is induced in left LPM by Nodal signaling (Shiratori et al., 2001)]. However, this is probably due to a decrease in the level of the signal that is required to activate *Nodal* expression in the LPM, given that a similar phenotype is apparent in conditional *Foxh1* mutants (Yamamoto et al., 2003) and can be simulated by a theoretical model known as the reaction-diffusion system (T. Nakamura, N. Mine, E. Nakaguchi, M. Yamamoto, K. Yahsiro, C. Meno and H.H., unpublished). Foxh1 is a transcriptional factor that mediates Nodal signaling (Hoodless et al., 2001; Yamamoto et al., 2001) and is required to upregulate *Nodal* and *Lefty2* expression in the left LPM (Saijoh et al., 2000; Yamamoto et al., 2003). Recent observations support the notion that Pkd2 acts as a Ca^{2+} channel that is gated by mechanical stress. First, *Pkd2*$^{-/-}$ embryos do not manifest asymmetric Ca^{2+} signaling in the perinodal endoderm (McGrath et al., 2003). Second, kidney epithelial cells derived from *Pkd2*$^{-/-}$ embryos fail to respond to mechanical flow, while wild-type ones do so (Nauli et al., 2003; Nauli and Zhou, 2004). Although Pkd2 may be a component of a mechanosensor, the precise role of Pkd2 in LR determination remains to be clarified. Given that *Pkd2* is expressed ubiquitously, Pkd2 may potentially function during LR patterning at any site between, and including, the node and LPM. The subcellular localization of Pkd2 has also been controversial, as it has been detected in association with a variety of organelles, including the plasma membrane, Golgi apparatus (Scheffers et al., 2002), mitotic spindle (Rundle et al., 2004) and cilia (Yoder et al., 2002). If it is localized to the surface of node cilia, Pkd2 may serve as a mechanosensory, as proposed by the two-cilia model (McGrath et al., 2003; Tabin and Vogan, 2003), which proposes that two kinds of node cilia exist in the node: motile cilia that are positive for a dynein called Lrd (Supp et al., 1997), which generate the flow; and immotile Lrd-negative cilia, which presumably act as mechanosensors.

Hints from kidney cilia

Epithelial cells of the kidney collecting duct also possess 9+0 monocilia, but, unlike the node cilia, they are immotile. Many of the genes that cause polycystic kidney, including *Pkd2*, also play a role in LR patterning, indicating that there may be a similarity in function between the cilia of the kidney and those of the node. Polaris is a protein that contributes to intraflagellar transport, and its deficiency results in polycystic kidney, as well as in LR defects characterized by bilateral *Nodal* expression in LPM and left isomerism (Murcia et al., 2000). As mentioned above, *inv* is a rare mutation that gives rise to situs inversus, but it also results in polycystic kidney in homozygotes (Yokoyama et al., 1993). The Inv protein localizes to both node cilia and kidney cilia (Watanabe et al., 2003).

Recent evidence suggests that renal cilia function as flow sensors, as removal of the primary cilium from Madin-Darby canine kidney (MDCK) cells abolishes flow sensing (Praetorius and Spring, 2003). Mechanical stress also stimulates Ca^{2+} signaling in kidney epithelial cells. Mechanical bending of the cilium of MDCK cells either with a micropipette or by artificial flow induces an increase in the intracellular Ca^{2+} concentration (Praetorius and Spring, 2001). Kidney epithelial cells in primary culture also manifest intracellular Ca^{2+} signaling in response to artificial flow. However, such cells deficient in Pkd2 fail to respond to flow (Nauli et al., 2003).

Role of Invs

The *inv* mouse mutation induces situs inversus (a malformation in which the LR asymmetry of the viscera is completely reversed), instead of LR randomization, in virtually all homozygotes (Yokoyama et al., 1993). Nodal flow in *inv* mutant mice is slow and turbulent, but its direction is still leftwards (Okada et al., 1999). A leftward artificial flow is able to correct situs inversus in such mutant embryos (Nonaka et al., 1998; Watanabe et al., 2003), suggesting that the endogenous flow is abnormal. However, examination of the node cilia of *inv/inv* embryos has failed to reveal obvious anomalies in their structure or movement (Watanabe et al., 2003), but a recent report (Okada et al., 2005) showed that a small proportion of the node cilia are abnormally tilted. The *Invs* gene encodes a protein that contains ankyrin repeats (Mochizuki et al., 1998; Morgan et al., 1998) and is preferentially localized to 9+0 cilia, including those of

the node (Watanabe et al., 2003). *Invs* is expressed ubiquitously, however, and the encoded protein is present in both the cytoplasm and nucleus. Biochemical assays have shown that the Invs (inversin) protein interacts with a variety of other proteins, including calmodulin (Yasuhiko et al., 2001), dishevelled 1 (Dvl1) (Simons et al., 2005), β-cadherin and N-cadherin (Nurnberger et al., 2002), and a component of the anaphase-promoting complex (Morgan et al., 2002). The precise function of Invs remains unknown.

Chemosensory versus mechanosensory models

Additional observations may be relevant to the role of nodal flow. It has been thought that data obtained with artificial flows are inconsistent with the transport model because such fast flows would be expected to disperse a soluble determinant molecule. However, this conclusion might be incorrect. Although a fast flow was imposed on embryos in the study by Nonaka et al., the effective flow speed in the node cavity (20 μm/second) was within the range of physiological flows (Nonaka et al., 2002). Therefore, depending on which cells receive a determinant molecule, these data do not completely exclude the transport model. It has also been argued that the phenotypic difference between mutants with immotile cilia and those lacking cilia favors the mechanosensory model. Thus, whereas *iv* mutant mice, which possess immotile node cilia, show randomized *Nodal* expression in the LPM, embryos without node cilia (such as those deficient in Kif3a, Kif3b, polaris and wimple) exhibit bilateral *Nodal* expression (Nonaka et al., 1998; Murcia et al., 2000; Huangfu and Anderson, 2005). However, such a difference in *Nodal* expression might simply be due to the presence or absence of midline defects. The *iv* mutant does not have a midline defect, whereas the latter mutants all do (they all lack *Lefty1* expression). In our view, the difference in phenotype between these two types of mutant does not support one model over the other.

In summary, some observations favor the chemosensory model over the mechanosensory model, whereas others do the opposite. No evidence obtained to date provides a conclusive answer to this problem, which remains a fascinating example of how a signal is transferred during development.

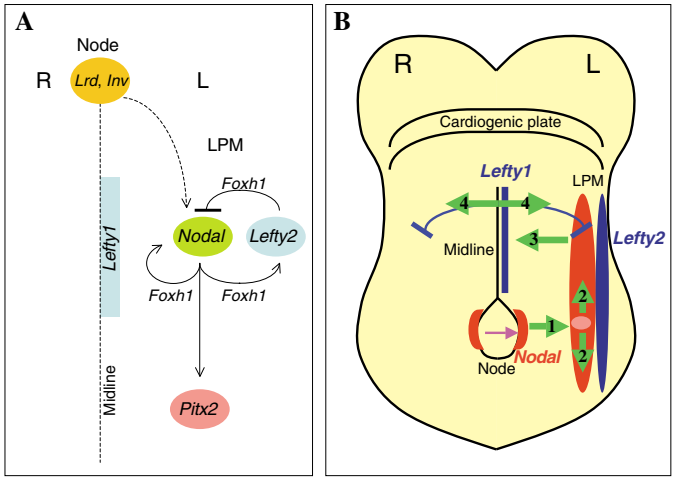

Fig. 5. Genetic pathway for, and signal transfer during, left-right (LR) patterning. (**A**) A genetic pathway for LR patterning. Only major components are shown. An asymmetric signal generated in the node initiates *Nodal-Lefty1-Lefty2* regulatory loops in the left lateral plate mesoderm (LPM). Nodal activity induces *Pitx2* expression in the left LPM. The broken line represents the midline. Lrd and Inv are required to generate asymmetric signal in the node. Foxh1 is a component of the Nodal-Lefty loops. (**B**) Signal transfer during LR patterning. A ventral view of an E8.0 mouse embryo. At least four steps of signal transfer take place during LR patterning: (1) from the node to the left LPM; (2) within the LPM; (3) from the left and right LPM to the midline; and (4) from the midline to the LPM. The Nodal signal is transferred in the second and third steps, but the identity of the signal that is transferred from the node to the LPM is unknown. In the fourth step, a midline signal (Lefty1) negatively regulates *Nodal* in the LPM. Pink arrow, the nodal flow; pink oval, a small region of LPM that initially receives the signal from the node. The expression domains of *Nodal* (red), and *Lefty1* and *Lefty2* (blue) are indicated.

Signal transfer from the node to the lateral plate

The asymmetric signal (or signals) generated in or near the node, whether it is mechanical stress or a molecular determinant, must be transferred to the lateral plate, where it induces the asymmetric expression of *Nodal* (Fig. 1, Fig. 5). Several important questions remain unanswered about this process. How and through which route is the signal transferred to the LPM? How does the signal activate *Nodal* expression in the left LPM? What is the nature of the signal that reaches the left LPM and activates *Nodal* expression there? Asymmetric elevation of Ca^{2+} may be an intermediate event between the node and the LPM, but how is it related to the asymmetric *Nodal* expression in LPM? These questions do not address separate issues, but rather are interrelated.

Route of signal transfer

Whether the cilia-derived asymmetric signal is a molecule or mechanical stress, the topology of the mouse embryo is an important determinant of its transfer. Several potential routes can be envisioned for the transfer of a signal generated in or near the node to the LPM (Fig. 1C). This route may include: the node pit cells with their rotating cilia; node cells with immotile cilia, as suggested by the two-cilia model (McGrath et al., 2003; Tabin and Vogan, 2003); crown cells in the perinodal region; or endoderm cells distantly located from the node.

Is the signal from the node to the LPM relayed or directly transferred?

How is the LR signal transferred from the node to the lateral plate? Signaling molecules expressed in the node are essential for correct LR patterning of the lateral plate, and they may play a role in the transfer of the LR signal. *Nodal* is bilaterally expressed in the node (in perinodal crown cells) before its expression begins in the left LPM (see Fig. 1C). Furthermore, genetic evidence has established that *Nodal* expression in the node is essential for subsequent *Nodal* expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). The specific ablation of *Nodal* expression in the perinodal region has been shown to prevent *Nodal* expression in the left LPM (Brennan et al., 2002). *Cerl2*, which encodes an antagonist of Nodal, is also expressed in the perinodal region before *Nodal* expression begins in the left LPM (Marques et al., 2004). *Cerl2* is expressed in an asymmetric manner, with the level of expression on the right side being substantially higher than that on the left side (Pearce et al., 1999). Mice that lack Cerl2 show bilateral or right-sided expression of *Nodal* in the LPM (Marques et al., 2004), suggesting that this Nodal antagonist produced in the node regulates the asymmetric expression of *Nodal* in the LPM. These observations thus indicate that Nodal may play a role in signal transfer from the node to left LPM.

Nodal is currently the only signaling molecule whose function in the node has been established by genetic means to be essential for *Nodal* expression in the LPM (Brennan et al., 2002; Saijoh et al.,

2003). Any model for LR patterning must therefore take this fact into consideration. But, what is the precise role of Nodal produced in the node? The Nodal signal might be relayed to the LPM. Nodal produced in the node may thus act on cells that are located between the node and the LPM (such as perinodal cells, endodermal cells distantly located from the node, paraxial mesoderm cells or intermediate mesoderm cells) and induce in them a secondary signal that travels to the LPM and activates *Nodal* expression. In the chick embryo (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), Shh produced in the node activates the expression of *Caronte* in the paraxial mesoderm, which encodes an inhibitor of bone morphogenetic protein (BMP). Caronte, in turn, induces *Nodal* expression in the left LPM. However, it is not known whether a similar signaling mechanism operates in other vertebrates. A BMP antagonist corresponding to Caronte has not been identified in the mouse or zebrafish genomes. Nevertheless, a BMP signal may negatively regulate *Nodal* expression in the LPM of the mouse embryo, given that, in the absence of the BMP effectors Smad1 and Smad5 (Chang et al., 2000), *Nodal* is expressed bilaterally in the LPM.

An alternative is that Nodal itself is transported from the node to the left LPM. Several lines of circumstantial evidence support this possibility. First, *Nodal* expression in the LPM can be induced by Nodal itself. Thus, the ectopic introduction of a Nodal expression vector in the right LPM induces the expression of endogenous *Nodal* (Yamamoto et al., 2003). Second, a search for transcriptional regulatory sequences that control asymmetric *Nodal* expression has identified two enhancers, both of which are able to confer asymmetric gene expression in the left LPM (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 2000). Importantly, both enhancers possess binding sequences for the transcription factor Foxh1 that are essential for enhancer activity and Nodal responsive (Saijoh et al., 2005). However, the transport of Nodal from the node to the left LPM remains to be directly demonstrated.

GDF1 is a transforming growth factor β (TGF-β)-related protein that is expressed in the node and that plays a role in LR patterning. Like Nodal, GDF1, which shares sequence similarity with Vg1 in *Xenopus*, is bilaterally expressed in the perinodal region. Mice that lack GDF1 do not manifest asymmetric *Nodal* expression in the LPM and exhibit right isomerism of the visceral organs (Rankin et al., 2000). Similarities in *Gdf1* and *Nodal* expression domains and their respective mutant phenotypes indicate that GDF1 may play a role in transferring a laterality signal from the node to the LPM by interacting with Nodal. However, evidence suggests that GDF1 may also play a different role in LR patterning. First, *Gdf1* is expressed not only in the perinodal region but also in the LPM at the early somite stages, indicating that GDF1 renders the LPM competent to respond to the Nodal signal. Second, GDF1 alone can activate signaling by Nodal signaling components, as well as by a Nodal responsive reporter when overexpressed in frog embryos or cultured cells (Cheng et al., 2003; Wall et al., 2000), suggesting that GDF1 may play a role in LR patterning independently of Nodal.

It thus remains unknown how the LR signal travels from the node to the lateral plate. Clarification of this issue will require us to understand the precise role of Nodal (and of GDF1) produced in the node.

Lateral plate asymmetric patterning by positive and negative signaling loops

The asymmetric patterning of the lateral plate is perhaps the best understood step of the establishment of the LR axis. Nodal, Lefty1 and Lefty2 play a central role in this process (Capdevila et al., 2000; Hamada et al., 2002). Genetic evidence has revealed that Nodal acts as a left-side determinant. Cells in the left LPM that have received the Nodal signal contribute to the left side-specific morphology of various visceral organs (as discussed in more detail below), whereas cells in the right LPM, which do not receive the Nodal signal, contribute to right side-specific morphology. Thus, in the absence of Nodal signaling, bilaterally asymmetric visceral organs, such as the lungs, adopt right isomerism.

Lefty1 is a feedback inhibitor of Nodal that restricts the area of Nodal signaling and the duration of *Nodal* expression. Mammals possess two Lefty genes, *Lefty1* and *Lefty2*, which are expressed in the midline and left LPM, respectively (Fig. 5). Their expression is induced by Nodal signaling (Yamamoto et al., 2003). In the absence of Lefty1 or Lefty2, asymmetric *Nodal* expression in the LPM begins normally, but the Nodal signal subsequently leaks to the right side, resulting in bilateral *Nodal* expression (Meno et al., 1998; Meno et al., 2001).

The expression of *Nodal* and Lefty genes is dynamic and transient. In the mouse embryo, *Nodal* expression in the LPM begins in a small region on the left at the level of the node and subsequently expands within the left LPM along the AP axis (Fig. 6). Nodal produced in left LPM induces *Lefty2* expression in the left LPM (Fig. 5). Nodal is also thought to travel to the midline, where it induces *Lefty1* expression (Yamamoto et al., 2003). Expression of *Nodal* (as well as that of *Lefty1* and *Lefty2*) then starts to decrease and has completely ceased by the six-somite stage. Asymmetric expression of *Nodal* in LPM thus persists for only ~6 hours. Such dynamic expression of *Nodal* and Lefty genes is achieved by positive and negative regulatory loops that are mediated by Nodal and the Lefty proteins. Thus, the expression of both *Nodal* and *Lefty2* is regulated by the Nodal responsive enhancer ASE (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 1999; Saijoh et al., 2000). This system ensures the presence of the Nodal signal at the correct time and place.

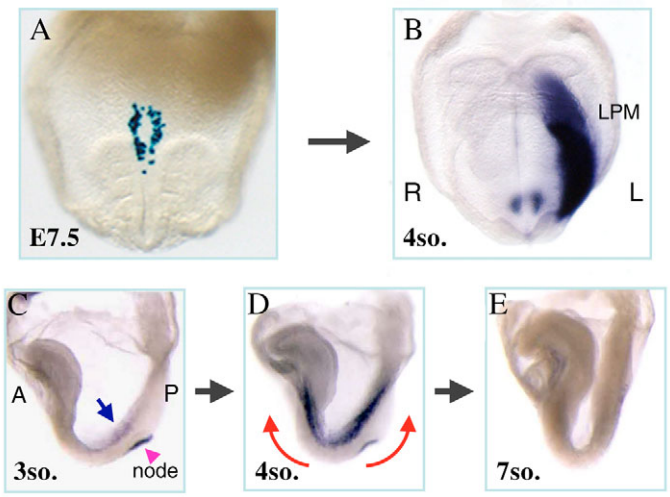

Fig. 6. Dynamic expression pattern of *Nodal*. (A,B) Ventral (A) and posterior (B) views of mouse embryos. (**A**) *Nodal* expression begins in the node at E7.5 and (**B**) develops asymmetrically a few hours later (four-somite stage) in the lateral plate mesoderm (LPM). (C-E) Lateral views of mouse embryos. (**C**) *Nodal* expression in the left LPM begins in a small region at the level of the node (purple arrow) at the two- to three-somite (s) stage at E8.0. (**D**) It then expands rapidly within the left LPM along the AP axis (red arrows indicate the expansion of Nodal expression), and (**E**) disappears by the seven-somite stage (red arrow indicates). Figure courtesy of Chikara Meno (Kyushu University, Japan).

Given the operation of the positive and negative regulatory loops mediated by Nodal and Lefty proteins, we and others (Chen and Schier, 2002; Hamada et al., 2002; Juan and Hamada, 2001; Saijoh et al., 2000; Branford and Yost, 2004) have suggested that these secreted factors constitute a reaction-diffusion system, a theoretical model that is able to convert a small difference between two separated regions into a robust difference through local activation and long-range inhibition (Turing, 1990; Meinhardt and Gierer, 2000). This notion is further supported by: the dynamic expression patterns of *Nodal* and both Lefty genes; the ability of Nodal and both Lefty proteins to act over a long distance; and the construction of a mathematical model that can simulate in vivo data for wild-type and various mutant embryos (T. Nakamura, N. Mine, E. Nakaguchi, M. Yamamoto, K. Yahsiro, C. Meno and H.H., unpublished). It is, thus, possible that nodal flow generates only a small difference, which is converted to robust asymmetry by a reaction-diffusion system (Fig. 7).

Despite such progress, important questions related to asymmetric patterning in the LPM remain unanswered. First, LPM on both sides is connected at the posterior end. Given that the introduction of a Nodal expression vector into the right LPM activates expression of endogenous *Nodal* (Yamamoto et al., 2003), left LPM and right LPM are equally competent to respond to the Nodal signal. What then is the mechanism that prevents the Nodal signal from leaking across the midline at the posterior end? In the absence of Lefty2 in the LPM, the Nodal signal (as revealed by the expression of *Pitx2*) extends to the posterior region of right LPM (Meno et al., 2001). Is the inhibitor Lefty2 sufficient to prevent leakage of the Nodal signal, or is there an additional mechanism that restricts Nodal signaling? The autonomous positive-negative regulatory loops mediated by Nodal and Lefty proteins may operate to reduce the level of *Nodal* expression as it approaches the posterior midline, but such a mechanism would not be expected to be error free.

It is also not clear how the midline barrier functions. The midline structures, such as the floor plate and notochord, are essential for establishing the asymmetric *Nodal* expression in the LPM and to prevent the Nodal signal that originated in the left LPM from crossing the midline. In the absence of a functional midline barrier, *Nodal* expression in the LPM becomes bilateral. Lefty1 is the major component of the midline barrier in the region anterior to the node (Meno et al., 1998). Theoretical simulation (Meinhardt and Gierer, 2000) also suggests that a diffusible inhibitor produced at the midline (Lefty proteins) establishes asymmetric *Nodal* expression in the LPM by negatively regulating *Nodal* over a long distance (Fig. 5B, step 4). However, is Lefty1 alone sufficient for barrier function? What is the nature of the midline barrier in the posterior region where *Lefty1* expression is absent? The mechanism that restricts the Nodal signal exclusively to the left side is thus not fully understood.

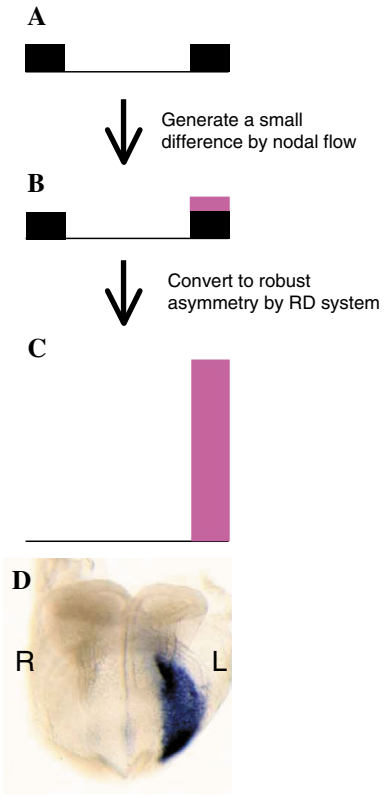

Fig. 7. Two steps in the generation of robust left-right (LR) asymmetry in the lateral plate mesoderm (LPM). In this model, robust LR asymmetry (asymmetric *Nodal* expression) in the LPM is generated in two steps. (**A**,**B**) First, nodal flow generates a small difference (pink) between the two sides of the LPM. (**C**) This small difference is then converted to a robust asymmetry by a reaction-diffusion (RD) system comprising Nodal and Lefty proteins. (**D**) Photomicrograph illustrating this asymmetry. A RD system requires that both an activator and inhibitor travel over a long distance and that an inhibitor diffuses faster than an activator does. Circumstantial evidence supports that Nodal and Lefty fulfill these requirements (Chen and Schier, 2001; Chen and Schier, 2002; Meno et al., 2001; Sakuma et al., 2002).

Situs-specific organogenesis: the final step of LR signal interpretation

Less clearly understood is the final step of LR patterning, situs-specific organogenesis. It is not currently known how LR asymmetric information is conveyed to give rise to asymmetric organogenesis. Anatomic asymmetries become recognizable in

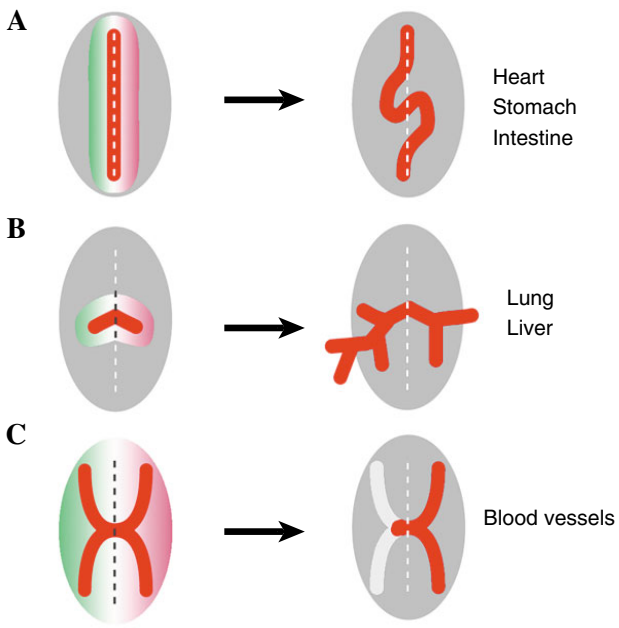

Fig. 8. Generation of anatomical asymmetries. (A-C) Three different mechanisms for the generation of morphological asymmetries: (**A**) directional looping, (**B**) differential branching and (**C**) one-sided regression. Examples of anatomical structures generated by each mechanism are shown. Figure courtesy of Yukio Saijoh (University of Utah, USA).

various visceral organs only after asymmetric *Nodal* expression in the LPM has disappeared. Macroscopically, at least three different mechanisms are responsible for generating asymmetric structures (Fig. 8). The first is directional looping: organs that are initially formed as a tube, including the heart and gut, undergo a series of looping, bending and rotation steps that result in their correct positioning within the body (Fig. 8A). In the second, a pair of primordial organs that form symmetrically on both sides subsequently develop differences in their size or branching pattern, as is the case for the lungs (Fig. 8B). And in the third, one side of a symmetric structure, such as a blood vessel, undergoes regression and disappears, leaving only the other side (Fig. 8C).

The cellular basis of asymmetric organogenesis is not clear, but each organ primordium seems to interpret LR information differently. It is likely that differential cell death contributes to one-sided regression of blood vessels. Branchial arch arteries undergo complex remodeling that includes asymmetric regression. In the case of the sixth branchial arch artery, for example, the right component disappears while the left counterpart persists (Navaratnam, 1963). LR signaling may induce differential cell death in developing arteries, but how the LR signal is interpreted by the arteries is unknown. By contrast, directional looping of the gut in the chick embryo is thought to be mediated by differential cell elongation (Muller et al., 2003). Looping of the zebrafish gut, however, results from the asymmetric migration of left and right LPM (Horne-Badovinac et al., 2003). The left LPM thus migrates dorsally to the endoderm, while the right LPM migrates ventrolaterally, resulting in a shift of the developing intestine to the left. Genetic evidence indicates that asymmetric LPM migration is dependent on LR signaling (Horne-Badovinac et al., 2003), but it remains unknown how the direction of cell migration is regulated. Cardiac looping, the first visible indication of asymmetric morphogenesis, has received much attention because impaired looping results in congenital heart malformations in humans. However, the cellular and biophysical bases of cardiac looping remain unknown. The looping may be achieved by forces intrinsic to the heart tube, such as those generated by changes in myocardial cell shape (Manasek et al., 1972) or in the arrangement of intracellular actin bundles (Itasaki et al., 1991; Itasaki et al., 1989). Alternatively, the looping may be driven by external forces, such as that exerted by the splanchnopleure (Voronov et al., 2004). The splanchnopleure is the rudiment of the spleen that forms as a single condensation of mesenchyme along the left side of the mesogastrium dorsal to the stomach. The formation of this rudiment, which comprises a group of cells positive for the transcription factors Hox11 and Nkx2.5, depends on a columnar mesoderm-derived cell layer known as the splanchnic mesodermal plate (SMP) (Hecksher-Sorensen et al., 2004). The SMP is bilateral at early stages, surrounding the prospective stomach located at the midline. Under the control of the LR signal, however, the left SMP grows laterally while the right SMP disappears. Differential cell proliferation is at least one of the mechanisms responsible for the asymmetry in the SMP (Hecksher-Sorensen et al., 2004). The SMP may induce the formation of both the splenic mesenchyme and the pancreas bud.

At the molecular level, the main player that regulates asymmetric organogenesis downstream of the Nodal signal is the transcription factor Pitx2 (Logan et al., 1998; Yoshioka et al., 1998). Like *Nodal* and *Lefty2*, *Pitx2* is expressed asymmetrically in left LPM, but asymmetric expression of *Pitx2* persists until much later stages than does that of these other two genes. An analysis of transcriptional regulatory elements has indicated that asymmetric *Pitx2* expression is induced by Nodal and is maintained by Nkx2 in the absence of the Nodal signal (Shiratori et al., 2001). Mice that lack Pitx2 (specifically, Pitx2c, the isoform that is asymmetrically expressed) exhibit laterality defects in most visceral organs (Liu et al., 2001). LPM-derived cells that express Pitx2 activity develop left-side morphologies. Thus, in the absence of Pitx2, bilateral organs, such as the lungs, exhibit right isomerism. However, certain laterality events, such as cardiac looping and embryonic turning, take place normally in the absence of Pitx2, suggesting that both Pitx2-dependent and -independent mechanisms are operative. How can Pitx2 induce seemingly different cellular events, such as increased or decreased cell proliferation, cell death and cell migration? It may regulate distinct sets of genes in different organs, so that the readout of Pitx2 activity also differs. Currently, no target gene of Pitx2 that is relevant to asymmetric morphogenesis has been identified. Pitx2 stimulates cell proliferation in the pituitary gland by regulating cyclin genes (Kioussi et al., 2002). However, it is not known whether a similar mechanism operates in LPM-derived cells.

Perspectives

The field on LR asymmetry has developed rapidly since the discovery of LR asymmetrically expressed genes, such as *Shh* and *Nodal* in the chick (Levin et al., 1995), and *Nodal* and *Lefty1* in the mouse (Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996). We now know that a large numbers of genes are involved in generating LR asymmetric organs. The systematic screening of LR mutants will further identify those genes that are required for normal LR patterning. Although LR asymmetry is an interesting scientific problem to solve, it has a practical application as well, as it is likely that many human congenital cardiac malformations are due to defective LR patterning. The knowledge obtained from animal models will therefore certainly contribute to our understanding of such abnormalities.

As is clear from this review, LR asymmetry is still a challenging topic! Many key questions remain to be answered despite recent progress. Key challenges in the immediate future are to clarify how nodal flow works, to know the nature of the asymmetric signal generated in the node and how it is transferred to the LPM, and to understand the cellular basis of asymmetric morphogenesis. To address these outstanding issues, it may be necessary to develop and integrate new techniques including imaging, in vitro culture and time-lapse observation systems, theoretical modeling, and more sophisticated genetic manipulations. In addition, there are many more issues that are taken for granted but that need to be examined in the near future (such as the exact role of the midline; the behaviors of secreted Nodal and Lefty proteins; and how and where BMP signaling acts during LR patterning). They may look less challenging but are certainly important issues. With steady progress and a breakthrough or two, we should be able to understand the whole process of LR asymmetry in the near future.

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