Left-right asymmetry: Nodal points

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Summary
The striking left-right asymmetry of visceral organs is known to depend on left- and right-side-specific cascades of gene expression during early embryogenesis. Now, developmental biologists are characterizing the earliest steps in asymmetry determination that dictate the sidedness of asymmetric gene expression. The proteins and structures involved control fascinating physiological processes, such as extracellular fluid flow and membrane voltage potential and yet little is known about how their activities are coordinated to control laterality. By analogy with intercellular signalling in certain epithelial and endothelial cells, however, it is reasonable to speculate that at least three of these players, monocilia, gap junction communication and the Ca²⁺ channel polycystin-2, participate in a signalling pathway that propagates left-right cues through multicellular fields.

Key words: Embryo, Organogenesis, Development, Asymmetry, Monocilia, Polycystin

Introduction
Nearly all organs of the thorax and abdomen are left-right (LR) asymmetric in anatomy, placement and, in some cases, physiology. LR asymmetry itself is a highly conserved feature of chordates, even though certain details of the anatomical asymmetry, such as sidedness of the venous system, vary between species. Perhaps the most striking aspect of LR asymmetry is that all normal individuals of a given species have identically oriented visceral asymmetry. Despite normal health, only about 1 in 8500 people exhibit complete mirror-image reversal of visceral organs (situs inversus). Whereas visceral organs show the most obvious asymmetry, stereotypic LR asymmetry extends to the brain and nervous system in humans; thus, it is intriguing to consider that LR asymmetry determination has profound implications for behavior and cognition.

Conceptually, the generation of LR asymmetry can be considered to occur in three phases during early embryonic development (Fig. 1) (reviewed by Hamada et al., 2002; Mercola and Levin, 2001). During the first phase, the bilateral symmetry of the early embryo is broken and the LR axis becomes oriented with respect to the anteroposterior and dorsoventral body axes. This early pattern is processed and transduced into cascades of asymmetric gene expression that characterize the second phase. Although the asymmetric patterns of gene expression differ among species, a conserved feature in all animal species examined is the production of the secreted TGFβ-like factor Nodal (or a Nodal homologue) in the left lateral plate mesoderm. Asymmetric expression of a Nodal protein is critical as it provides a link to the third phase by inducing expression of the transcription factor Pitx2c. During the third phase, proteins such as Pitx2c control localized changes in cell migration, shape, proliferation and survival that render the stereotypic asymmetric anatomy of developing tissues.

Proteins and structures involved in early LR asymmetry determination
Several proteins or structures now appear to be components of the process that orients the axis of LR asymmetry (Fig. 2). Below, I focus on the proteins and structures that function upstream of asymmetric gene expression. Most of these are likely to act in the vicinity of the amniote node or analogous structures in amphibians and fish.

Monocilia
The mouse node is a structure at the distal tip of the E6.5 embryo consisting of a dorsal layer of ectoderm over a ventral layer of cells each with a single monocilium on their apical surface (Bellomo et al., 1996; Sulik et al., 1994) (Fig. 3). Although structurally different, the mouse node shares signalling properties with Hensen’s node in the chick, Spemann’s Organizer in amphibians and the shield in zebrafish. The defining characteristic of these structures is that, when grafted to an ectopic site, host tissue is induced to form a secondary body axis. The node forms at the anterior end of a furrow, known as the primitive streak, along which cells delaminate to form mesoderm. The line formed by the primitive streak and node correlates with the future dorsal midline of the embryo and lies along its axis of bilateral symmetry. The incidence of laterality defects correlates with immotile or absent node monocilia. Mutations in genes encoding dynein heavy chains in mice [Ind, also known as Iv (Okada et al., 1999; Supp et al., 1997)] or humans [Dnahe5 (Olbrich et al., 2002)] are associated with mirror-image reversals of internal organs and, in mice, lead to formation of immotile node monocilia. Similarly, loss of Polaris [also known as Tg737, a hypomorphic allele of which is orpk (Murcia et al., 2000)], which is required for epithelial polarity of ventral node cells, and targeted disruption of the kinesin genes Kif3α or Kif3β (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999) in mice cause an absence of node monocilia concomitant with LR defects. In what must be one of the most elegant studies in the field, Nonaka et al. visualized the rotational movement of node monocilia by placing a fluorescent microsphere in the extracellular space of...
leftward fluid flow dictates LR asymmetry rather than occurs as an unrelated consequence of monocilia chirality. It is important to bear in mind, however, that the genetic experiments and, more to the point, the reversal of asymmetry by pumping fluid do not indicate that fluid flow initiates asymmetry, but show only that monociliary movement is important. Nonetheless, using the inherent chirality of monociliary rotation to provide LR orientation is the most conceptually satisfying model for the initiating step in the mouse.

Are node monocilia involved in asymmetry determination in other species? Using an antibody to acetylated tubulin, Essner et al. saw monocilia in chick ventral node cells, dorsal blastopore cells of *Xenopus* early neurula, and in dorsal forerunner cells in the zebrafish shield at the end of gastrulation (Fig. 4) (Essner et al., 2002). In each case, monocilia appear before asymmetric expression of the pertinent *Nodal* homologue, which suggests they have an evolutionarily conserved function. However, in contrast to the situation in the mouse, asymmetric expression of other genes in chicks and *Xenopus* occurs prior to the appearance of monocilia (Levin, 1998; Levin et al., 1995) and asymmetry in the distribution of H+/K+-ATPase mRNA in *Xenopus* or function in chick (see below). Moreover, transplant and regeneration studies revealed that nascent chick nodes receive LR asymmetry input from surrounding tissue and in the absence of these cues develop with abnormal asymmetry (Levin and Mercola, 1998a; Levin and Mercola, 1999; Pagan-Westphal and Tabin, 1998; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998). Therefore, although their presence is indeed conserved, monocilia are unlikely to initiate LR asymmetry in all species.

**Fig. 2.** Developmental stages when the various proteins and structures are likely to contribute to LR asymmetry determination. Animal species for which the components have been examined are listed in parentheses.

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embryos mounted in culture medium (Nonaka et al., 1998). They showed that the vortical movement of cilia creates a leftward fluid flow across the node and, on the basis of these data, proposed that the fluid flow increases the concentration of some diffusible determinant on the left side of the node. The direction of fluid flow is probably not due to the direction of ciliary diffusible determinant on the left side of the node. The direction proposed that the fluid flow increases the concentration of some show that the vortical movement of cilia creates a leftward embryos mounted in culture medium (Nonaka et al., 1998). They showed that the vortical movement of cilia creates a leftward fluid flow across the node and, on the basis of these data, proposed that the fluid flow increases the concentration of some diffusible determinant on the left side of the node. The direction of fluid flow is probably not due to the direction of ciliary diffusible determinant on the left side of the node. The direction proposed that the fluid flow increases the concentration of some show that the vortical movement of cilia creates a leftward fluid flow across the node and, on the basis of these data, proposed that the fluid flow increases the concentration of some diffusible determinant on the left side of the node. The direction of fluid flow is probably not due to the direction of ciliary diffusible determinant on the left side of the node.
Left-right asymmetry

Gap junctional communication in early chick or Xenopus embryos disrupts expression of normally unilaterally expressed genes and alters organ situs (Levin and Mercola, 1998b; Levin and Mercola, 1999). The endogenous spatial pattern of gap junctional communication appears critical, because injection of a dominant negative connexin into dorsal blastomeres of Xenopus embryos or wild-type connexins into ventral blastomeres (but not the reverse) affects laterality; this indicates that a signal is propagated through dorsal blastomeres and impeded at the ventral midline. The effect of these manipulations on early gene expression, as well as the temporal window when embryos are sensitive to the drugs, suggests that gap junctional communication is critical in early-streak-stage chick embryos and between cleavage and the gastrula stage Xenopus (Fig. 2). Gap junctions are composed of twelve transmembrane connexin proteins, six in each apposing cell. Numerous connexins exist and, of these, Connexin43 appears crucial for chick asymmetry: it is present in cells surrounding the node and its depletion by antisense oligonucleotides unbiases the sidedness of Sonic hedgehog (Shh) and Nodal expression (Levin and Mercola, 1999).

Polycystin-2

Polycystin-2, the gene product of PKD2, is mutated in autosomal dominant polycystic kidney disease and is a Ca\(^{2+}\)-permeable cation channel (Cai et al., 1999; Gonzalez-Perret et al., 2001; Koulen et al., 2002). In addition to causing cardiac and kidney malformations, targeted disruption of PKD2 in mice causes hallmarks of LR disturbance that include randomization of embryo turning, right pulmonary isomerism (e.g. left lung exhibits lobation typical of the right lung), anomalous heart looping (including dextrocardia) and gut coiling defects (Pennekamp et al., 2002). Importantly, the normally left-sided nodal, lefty2 and Pitx2 expression in anterior lateral plate mesoderm is absent in PKD2\(^{-/-}\) embryos, which indicates that polycystin-2 acts upstream of asymmetric gene expression. Consistent with this possibility is the fact that polycystin-2 mRNA is expressed strongly in and near the node, notochord and floorplate (Pennekamp et al., 1998) (R. D. Trelles, M. Levin and M. Mercola, unpublished).

It is not yet clear how polycystin-2 influences laterality. Interestingly, elevated Ca\(^{2+}\) levels prevent calmodulin from interacting with INV (Morgan et al., 2002; Yasuhiko et al., 2001), a protein that when mutated in mice causes mirror-image organ reversals and results in slow and turbid fluid flow across the node (but does not reverse flow direction). Other

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**Fig. 3.** The mouse node and monocilia. Scanning electron micrographs (provided by Daisuke Watanabe and Hiroshi Hamada, Osaka University) showing the node at the distal tip of an E8.0 mouse embryo (A). Higher magnification views show the ventral node cells (B) and individual monocilia (C). Anteroposterior body axes (A, P) and direction of flow (arrow in B) to left side (L) are indicated. Magnifications: ×200 (A); ×700 (B); ×7000 (C).

**Fig. 4.** Conservation of node monocilia. mRNA encoding endogenous left-right dynein (LRD), a protein involved in node monocilia in the mouse, is seen in Hensen’s node in the chick, dorsal blastopore cells of the early neurula stage Xenopus, and the zebrafish shield (A,D,G,J; arrows). Monocilia are revealed on the apical surfaces of mouse ventral node cells and in cells of the related structures in chick, Xenopus and zebrafish embryos by immunostaining with anti-acetylated tubulin (B,E,H,K; arrows); a schematic representation is also shown (C,F,I,L). Reproduced, with permission, from the Nature Publishing Group (http://www.nature.com) (Essner et al., 2002).
than acting as a potential regulator of INV, polycystin-2 has not been linked to other proteins involved in LR asymmetry. Mutation of either polycystin-2 or INV, however, contributes to abnormal kidney tubule and duct growth, leading to formation of cysts. Monocilia on the surface of the tubule and duct epithelial cells are thought to act as sensors of tube diameter and trigger an intercellular Ca\textsuperscript{2+} wave that negatively regulates cell proliferation and lumen expansion (Praetorius and Spring, 2001; Praetorius and Spring, 2003; Schwartz et al., 1997). The idea that a similar mechanism might operate to regulate asymmetric gene expression near the node has been proposed as an alternative to the fluid flow model (Brueckner, 2001) and is considered in more detail below.

The H\textsuperscript{+}/K\textsuperscript{+}-ATPase

A biological screen of small molecules known to affect ion channels and pumps for the ability to induce defects in heart looping, gut coiling and gall bladder position in early Xenopus embryos revealed that several compounds that cause laterality defects inhibit the H\textsuperscript{+}/K\textsuperscript{+}-ATPase, a pump that exchanges H\textsuperscript{+} and K\textsuperscript{+} across membranes (Levin et al., 2002). The window of drug sensitivity places the H\textsuperscript{+}/K\textsuperscript{+}-ATPase as the earliest known component of LR asymmetry determination in both Xenopus and chicks (Fig. 2). In addition to causing organ reversals, inhibition or misexpression of the H\textsuperscript{+}/K\textsuperscript{+}-ATPase ubiases asymmetric gene expression, including that of Shh, fgf8 (encoding fibroblast growth factor-8) and Wnt8c (encoding a secreted protein of the of the Wingless/Wnt family) in the chick node. Surprisingly, H\textsuperscript{+}/K\textsuperscript{+}-ATPase \( \alpha \) mRNA itself becomes selectively depleted on the left ventral side of Xenopus embryos by the 4-cell stage, well before the onset of embryonic transcription (which occurs at the mid-blastula transition when the embryo has about 1000 cells), making it the earliest known asymmetric mRNA and revealing that LR patterning is present during the first cell cycle.

In chick embryos, H\textsuperscript{+}/K\textsuperscript{+}-ATPase mRNAs are first apparent adjacent to the primitive streak and developing node, but expression is symmetrical. Nonetheless, imaging studies suggest that the H\textsuperscript{+}/K\textsuperscript{+}-ATPase functions on the right, but not left, side of the streak and node to maintain cell membrane potentials. A domain of depolarized cells can be detected by raised fluorescence of the potentiometric dye DiBAC\textsubscript{4}(3) on cell membranes (Levin et al., 2002). Cell-cell contact appears to have a causative role in depolarization, as blocking antibodies to N-cadherin and claudin are also essential for normal LR asymmetry (Sanderson, 1995) and might also influence earliest LR asymmetry determination at the node. Inhibition or misexpression of the H\textsuperscript{+}/K\textsuperscript{+}-ATPase unbiases asymmetric gene expression, including that of Shh, fgf8 and Wnt8c (a similar model has been proposed recently (Tabin and Vogan, 2003)). From the involvement of monocilia, gap junctions and polycystin-2 in LR asymmetry and expression in the vicinity of the node, evidence for the model comes from studies on autosomal dominant polycystic kidney disease, which is known to involve each of these proteins and structures. In normal kidney tubules and ducts, monocilia located on the apical surface of epithelial cells

Epithelial structural proteins

N-cadherin and claudin are also essential for normal LR asymmetry. N-cadherin is asymmetrically expressed in the chick node: it is highly expressed in the anterior right and posterior left margins of the node, corresponding to the asymmetric shape of the node itself (Garcia-Castro et al., 2000). Importantly, asymmetric expression of N-cadherin precedes asymmetric expression of Shh (left anterior node) but is also influenced by Shh. Addition of a blocking antibody to N-cadherin at stage 3-4, but not later, affects heart morphogenesis and Pitx2 expression. Curiously, Nodal and lefty expression are unaffected; thus, N-cadherin seems to function in a pathway parallel to that involving these proteins. Claudins are important cell adhesion molecules of tight junctions that are directly involved in intercellular sealing. Overexpression of either an intact protein or a C-terminally truncated version in Xenopus caused heterotaxia and bilateral expression of the Nodal homologue XN\textit{t}1 (Brizuela et al., 2001). Notwithstanding that these experiments were done in different species, the importance of N-cadherin and claudins highlight the importance of epithelial integrity in normal LR asymmetry determination.

Syndecan-2

Yost and colleagues have shown by western analysis that a cytoplasmic domain of the heparin-sulphate proteoglycan syndecan-2 is phosphorylated in animal cap (presumptive ectoderm) cells on the right, but not left, half of the Xenopus embryo during gastrulation [at stage 11 (Kramer et al., 2002)]. Phosphorylation is thought to affect the ability of syndecan-2 to confer LR patterning on the mesoderm that comes into contact with the ectoderm during gastrulation. How syndecan-2 might pass signals to the mesoderm is unclear but it might serve to localize a growth-factor-like protein within a signalling complex at the cell surface (Bernfield et al., 1999). The idea that syndecans mediate the transfer of LR information from ectoderm to mesoderm is particularly interesting and a critical question is whether or not a similar mechanism operates in species other than Xenopus.

A model uniting monocilia, gap junction communication and polycystin-2 into a signalling pathway

How (or, indeed, do) these very diverse proteins and structures work together to process or propagate LR asymmetry information? The fluid flow model suggests that monociliary action propels an extracellular determinant. Another possibility is that monocilia initiate a signal, such as a Ca\textsuperscript{2+} wave, that spreads through the sheet of cells surrounding the node. Fig. 5 depicts how monocilia, gap junctions and polycystin-2 could function coordinately to propagate an intercellular Ca\textsuperscript{2+} wave. Intercellular Ca\textsuperscript{2+} waves are well known [for instance in endothelial, glial, and hepatic epithelial cells (for a review, see Sanderson, 1995)] and might also influence earliest asymmetric gene expression in and around the chick node, such as Shh, fgf8 and Wnt8c. How (or, indeed, do) these very diverse proteins and structures work together to process or propagate LR asymmetry information?
appear to sense lumen diameter by detecting changes in fluid dynamics (Nauli et al., 2003; Praetorius and Spring, 2001; Praetorius and Spring, 2003). Bending of the monocilia triggers Ca\(^{2+}\) influx through ciliary or nearby channels, which in turn causes Ca\(^{2+}\) release from inositol 1,4,5-trisphosphate [Ins(1,4,5)\(P_3\)]-sensitive intracellular stores. The signal propagates as a wave to surrounding cells by diffusion of a second messenger [such as Ins(1,4,5)\(P_3\)] through gap junction channels. Polycystin-2 co-localizes to kidney monocilia (Pazour et al., 2002; Yoder et al., 2002), where it might be the channel that permits entry of extracellular Ca\(^{2+}\). Localization of polycystin-2, however, is still a matter of debate, and it has been proposed to function both as a Ca\(^{2+}\) channel on the plasma membrane (Hanaoka et al., 2000) and as an Ins(1,4,5)\(P_3\)-sensitive intracellular release channel on the ER (Koulen et al., 2002).

In polycystic kidney disease, a lack of either polycystin-2 or monocilia causes focal proliferation of epithelial cells that eventually dilate into cysts. When applied to LR asymmetry determination at the node, the model makes several predictions: (1) a polycystin-2-dependent Ca\(^{2+}\) wave should be visible in the vicinity of the node and might be asymmetric; (2) propagation of the Ca\(^{2+}\) signal should be dependent on gap junctions and monocilia; and (3) the Ca\(^{2+}\) signal should affect asymmetric gene expression. Of these, only monocilia are firmly linked to asymmetry determination and the remainder need to be substantiated. The model does not predict whether the Ca\(^{2+}\) signal is propagated leftwards, rightwards or both. A sided pattern of propagation provides the simplest link to downstream gene expression but, if this is the case, what provides the orientation? Tabin and Vogan recently proposed that two types of node monocilia exist in the mouse node: one set to generate the leftward fluid flow and a second to sense it (Tabin and Vogan, 2003). This view, in which inherent asymmetry in node monocilary structures generates embryonic asymmetry, might apply to the mouse, where the leftward nodal fluid flow is the earliest known asymmetry. However, it is difficult to reconcile the conclusion that inherent properties of the mouse node generate the embryo’s handed asymmetry with the observations that the developing chick node receives LR pattern from surrounding tissues (as discussed above). Thus, it is reasonable to propose, at least for the chick, that any sidedness to a monocilia/polycystin-2-dependent Ca\(^{2+}\) signal might be dictated by an upstream signal, perhaps one dependent on the H\(^+/K^+\)-ATPase activity that maintains cell voltage potential on the right side of the node and streak at a developmental window that overlaps the time when monocilia are detected. Control over the propagation of a Ca\(^{2+}\) signal could also be achieved by regulating some feature of epithelial structure or physiology required to receive or propagate the second messenger. It might be relevant, therefore, that Cx43 mRNA is asymmetrically expressed on the right side of the chick node and might therefore impose directionality, but its role in this process is poorly understood beyond the published observation that antisense depletion of Cx43 mRNA affects asymmetry of downstream gene expression (Levin and Mercola, 1999).

**Conclusions and perspectives**

The past few years have seen remarkable progress towards understanding how LR asymmetry is patterned and the next few should be as impressive. Clearly, the speculative and incomplete model that associates monocilia, gap junctions and polycystin-2 into a signalling pathway needs to be evaluated and refined. It is important to learn if (and how) upstream signals, such as generated by the H\(^+/K^+\)-ATPase, are coupled to signalling at the node and whether such signalling operates only in chick and *Xenopus* but not in the mouse. Indeed, a major challenge is to resolve whether early LR patterning information is initiated and processed similarly in different species. Differences in the timing of axial patterning, early...
cleavage and gastrulation, and the anatomy of key signalling centers, such as the node or analogous structures, make the possibility of diverse mechanisms quite likely. Divergent chick, frog, fish and mouse evolution might have provided selective advantages that favored the orientation of LR asymmetry as early as the first cell cycle in animals such as the frog. Similarly, whether the node functions to initiate LR patterning, as monociliary motion might do in the mouse, or respond to earlier cues, as might occur in chick, could reflect divergent architectures of the node (Dathe et al., 2002), and this possibility needs to be addressed functionally. The application of recent advances in reverse genetics, such as RNAi, is certain to provide answers to these fascinating questions.

Another longstanding issue to be resolved is why nearly 100% of homozygous inv mutant mice exhibit LR inversions (Yokoyama et al., 1993). The mutation deletes nearly the entire coding region of the cytoplasmic INV protein (Mochizuki et al., 1998; Morgan et al., 1998) and, in its presumed absence, nodal fluid flow has been described as slowed, but not reversed (Okada et al., 1999), thus frustrating attempts to couple INV to monocular function. Perhaps related, therefore, are the observations that homozygous inv mutant embryos exhibit abnormal epithelial thickening on the edges of the node (Okada et al., 1999) and develop kidney cysts resembling those in polycystic kidney disease (PKD) (Mochizuki et al., 1998; Morgan et al., 1998; Yokoyama et al., 1993), which suggests that disturbances in epithelial character might affect the ability to propagate signalling downstream of monociliary function. Nonetheless, why these alterations would lead to reversals remains puzzling.

Finally, it is important to distinguish between when LR asymmetry information is first generated and when it can be regenerated. The mammalian embryo, for instance, has a considerable ability to reconstitute axial pattern after experimental intervention, such that removal or addition of blastomeres of pre-implantation embryos can lead to perfectly normal animals. This plasticity has been misinterpreted as meaning that axial patterning occurs relatively later than in more ‘mosaic’ embryos, such as those of the frog, as a result of interactions between blastomeres. Careful studies, however, have indicated that the animal-vegetal axis in the fertilized mouse egg, defined by the location of the polar body, correlates with the future long axis of bilateral symmetry in the blastocyst and, if left undisturbed, presages the anteroposterior body axis of the fetus (Gardner, 2001; Johnson, 2001). Any LR information laid down this early and perturbed experimentally might also be reconstituted during subsequent development. An example of this plasticity is the apparent de novo generation of asymmetry in Xenopus embryos by manipulations that act at multicellular stages. Fertilized Xenopus embryos normally acquire LR asymmetry by the two- to four-cell stage, as revealed by the selective depletion of H+/K+-ATPase α mRNA from the ventral left blastomere (Levin et al., 2002). Yet previous studies showed that forced expression of the transcription factor Siamois (which mimics endogenous dorsal Siamois after the 1000 cell stage) induces secondary body axes on the ventral side of the embryo that are oriented ventral to ventral with respect the primary axis (belly-to-belly twins) and that these secondary axes can have normal LR asymmetry (Nascone and Mercola, 1997). The observation that manipulated embryos can bootstrap LR information well after the initial orientation event normally occurs suggests that multiple mechanisms might exist to discriminate left from right in the early embryo.

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