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The Key to Left-Right Asymmetry

Clifford J. Tabin[1,*]
[1]Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
*Contact: tabin@genetics.med.harvard.edu
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Establishment of left-right asymmetry in vertebrates involves cilia as essential components in the breaking of symmetry, an asymmetric signaling cascade, and a midline barrier that helps to maintain asymmetry. A new study suggests that a reaction-diffusion mechanism also plays a key role.

The vertebrate body plan is inherently left-right asymmetric, with internal organs such as the heart, stomach, and intestines having both asymmetric structure and asymmetric positions within the body cavity. Strikingly, however, these gross anatomical asymmetries arise in early embryos that are bilaterally symmetrical along the mediolateral axis. A decade ago, nothing was known about the molecular or genetic underpinnings of left-right asymmetric morphogenesis, and no genes with left-right asymmetric expression had been identified. Since then, however, several discoveries have led to an emerging picture of how left-right asymmetry is initiated, stabilized, propagated, and translated into asymmetric organogenesis during development of vertebrate embryos.

An Asymmetric Cascade of Signals

The first breakthrough was the identification of a cascade of asymmetrically expressed signals present in the developing chick embryo during gastrulation, long before overt asymmetric morphogenesis (Levin et al., 1995). These signals included asymmetric expression of the gene *Sonic hedgehog* (*Shh*) on the left side of the chick embryo, which induced expression of *Nodal* (a TGFβ family member), first in a small domain adjacent to a structure known as Hensen's Node at the rostral end of the primitive streak and subsequently in a broad domain throughout the left lateral plate. The discovery of this pathway was a turning point and provided the first molecular marker for left-right asymmetry that could be used to follow left-right patterning prior to overt asymmetric morphogenesis. It also provided a broad outline of the process of left-right patterning: the establishment of asymmetric domains of gene expression during gastrulation in small regions near the node, followed by broad asymmetric signaling throughout the left lateral plate mesoderm. Crucially, this study also identified Nodal as a key left-sided signal.

A number of additional genes have been added to this general framework (see reviews by Levin, 2005; Raya and Belmonte, 2006; and references therein). Many of these have proven, rather surprisingly, to be specific to particular classes of vertebrates (Mammalia, Amphibia, Aves, etc.), including the asymmetric expression of *Shh* at the node in birds. Two key proteins, which are conserved in their expression and activated downstream of Nodal, are the highly divergent TGFβ family member Lefty (encoded by the two closely related genes *Lefty1* and *Lefty2*) and the transcription factor Pitx2. Neither is expressed in the *Nodal* domain adjacent to the node, but both are activated in response to Nodal signaling throughout the left lateral plate. *Lefty1* also is expressed on the left side of the midline in the floor plate of the neural tube.

The Importance of the Midline

If there are extensive domains in which signals are asymmetrically expressed on the right and left sides of the embryo, then there needs to be a mechanism for keeping them separate from one another. The idea that the midline acts as a barrier during left-right specification was first proposed by Danos and Yost (1996) to explain the results of experiments where the midline was experimentally manipulated or excised. Similar conclusions were reached by analysis of cross-signaling between twin embryos (Levin et al., 1996) and subsequently in experiments using mouse mutants where the midline is compromised (reviewed in Levin, 2005). Analysis of mice deficient in *Lefty1* gave molecular teeth to the midline barrier model and suggested that the barrier is formed, at least in part, by the activation of this gene (Meno et al., 1998).

Cilia and the Breaking of Symmetry

The fact that the earliest asymmetrically expressed genes are active at the node (Levin, 2005) drew attention to this structure as a location where the initial left-right decision might be made. Notably, the cells on the ventral surface of the mouse embryo node each have a monocilium projecting from their apical surface. A variety of genetic conditions in which the left-right orientation of internal organs is randomized have in common defects in cilia formation (Levin, 2005; Tabin, 2005; Raya and Belmonte, 2006; Shiratori and Hamada, 2006; Hirokawa et al., 2006). These convergent facts were put into a coherent context with the demonstration that the node cilia are motile (Non-

aka et al., 1998), and that they all rotate in a consistent clockwise direction generating a unidirectional leftward flow of extracellular fluid. The rotating cilia are able to generate fluid flow, in part, because they are all tilted toward the posterior. Mice unable to assemble cilia or with immotile cilia lack this flow and have randomized organ situs. Moreoever, artificial generation of directional flow was shown to be sufficient to specify downstream left-right asymmetric signaling and morphogenesis (Nonaka et al., 2002). The "nodal flow" hypothesis (Nonaka et al., 1998) emerging from these studies explains from first principles that a symmetric field of cilia, all rotating in the same direction, sets in motion a leftward flow of extracellular fluid, thereby breaking the bilaterally symmetric landscape of the early embryo.

A contentious question concerns the mechanism by which movement of fluid across the node is translated into asymmetric patterns of gene expression (see the contrasting reviews by Raya and Belmonte, 2006; Hirokawa et al., 2006; Shiratori and Hamada, 2006; Levin, 2005). Two alternative hypotheses have been proposed: asymmetric deformation of mechanosensory cilia, or the "two cilia" hypothesis, and the unidirectional transport of a morphogen. The two cilia model posits two classes of cilia, one generating a leftward flow and the second responding to it (Brueckner, 2001; Tabin and Vogan, 2003). Consistent with the idea of a second class of mechanosensory cilia that sense nodal flow, mutations in *PKD2*, a gene implicated in polycystic kidney disease, disrupt cilia-based mechanoreception in the mouse embryo and also disrupt early left-right signaling. *PKD2* appears to encode a cilia-gated calcium ion release channel. A subset of cilia at the node do indeed express PKD2, and moreover, intracellular calcium ion release can be detected at the left, but not the right, periphery of the node. However, mathematical modeling of fluid dynamics in the mouse embryo node suggests that, even in the presence of directional flow, the magnitude of shear stresses and flow velocities produced should be equivalent across the node and hence unable to asymmetrically deform cilia (discussed in Raya and Belmonte, 2006).

The alternative hypothesis of morphogen transport (suggested candidate morphogens include Fgf8, GDF1, and Nodal) has also been challenged on theoretical grounds, arguing that such small molecules would not be redistributed as demanded by the morphogen model. A variant on this hypothesis, which circumvents the biophysical issues, is based on the finding that Shh and retinoic acid are encapsulated in an Fgf-dependent process into membrane wrapped Nodal Vesicular Parcels (NVPs), which are indeed asymmetrically transported by the flow across the node (reviewed in Hirokawa et al., 2006). However, in spite of careful analysis, there is no hint of left-right asymmetric Shh or retinoid signaling at the node. Thus, this model requires the ad hoc, and so far unsubstantiated, proposition of a noncanonical vertebrate Hedgehog signaling pathway. Moreover, this model leaves unexplained the genetic identification of PKD2 and inversin, both expressed in node cilia, as early determinants of left-right asymmetric gene expression in the mouse embryo (reviewed in Tabin and Vogan, 2003; Tabin, 2005).

Other objections can be raised to each of these models (reviewed in Tabin, 2005), and, at this juncture, the issue of how nodal flow is interpreted remains unresolved. Nonetheless, genetic data strongly point to node cilia as the structures responsible for breaking symmetry in the mouse embryo. Similarly, it is striking that all known mutations producing *situs inversus totalis* in humans also implicate a ciliary mechanism for breaking symmetry, making it extremely likely that the nodal flow hypothesis, in one form or another, is the correct explanation for the initiation of left-right asymmetry in mammals.

Self-Enhancement and Lateral Inhibition

With the discoveries of nodal flow, the downstream signaling cascade, and the midline barrier, the overall scheme by which left-right patterning is set up in mammals seemed clear. Now, a new study by Nakamura et al. (2006) in this month's *Developmental Cell* forces us to change the way we think about each of these steps in light of the realization that Nodal acts through a modified reaction-diffusion mechanism to direct left-right asymmetry.

Reaction-diffusion systems were first conceived to provide a theoretical mechanism to create stable positional values across a responsive developmental field. A reaction-diffusion system comprises two diffusible molecules: an activator that positively regulates production of both molecules and a feedback inhibitor that negatively regulates the activator but does not regulate itself (Turing, 1952). Under these conditions, self-enhancing and laterally inhibiting properties emerge that reinforce initial discontinuities in a homogeneous system to yield sharp, discrete patterns. The left-side determinant Nodal is capable of inducing its own expression, whereas its downstream target Lefty acts as a feedback inhibitor of Nodal activity (see reviews by Raya and Belmonte, 2006; Levin, 2005). Based on this information, several groups realized that Nodal and Lefty could, in principle, constitute a reaction-diffusion system (Saijoh et al., 2000; Chen and Schier, 2002; Hamada et al., 2002). For a reaction-diffusion system to be effective, the inhibitor must diffuse more quickly than the activator. Based on studies using tagged exogenous proteins, it does indeed appear that the Lefty protein spreads farther than Nodal (Sakuma et al., 2002; Nakamura et al., 2006), although the actual relative speeds of diffusion remain to be verified in real time.

Nodal and Lefty most accurately fit the formulation of a reaction-diffusion system in the left lateral plate where Nodal induces itself and its

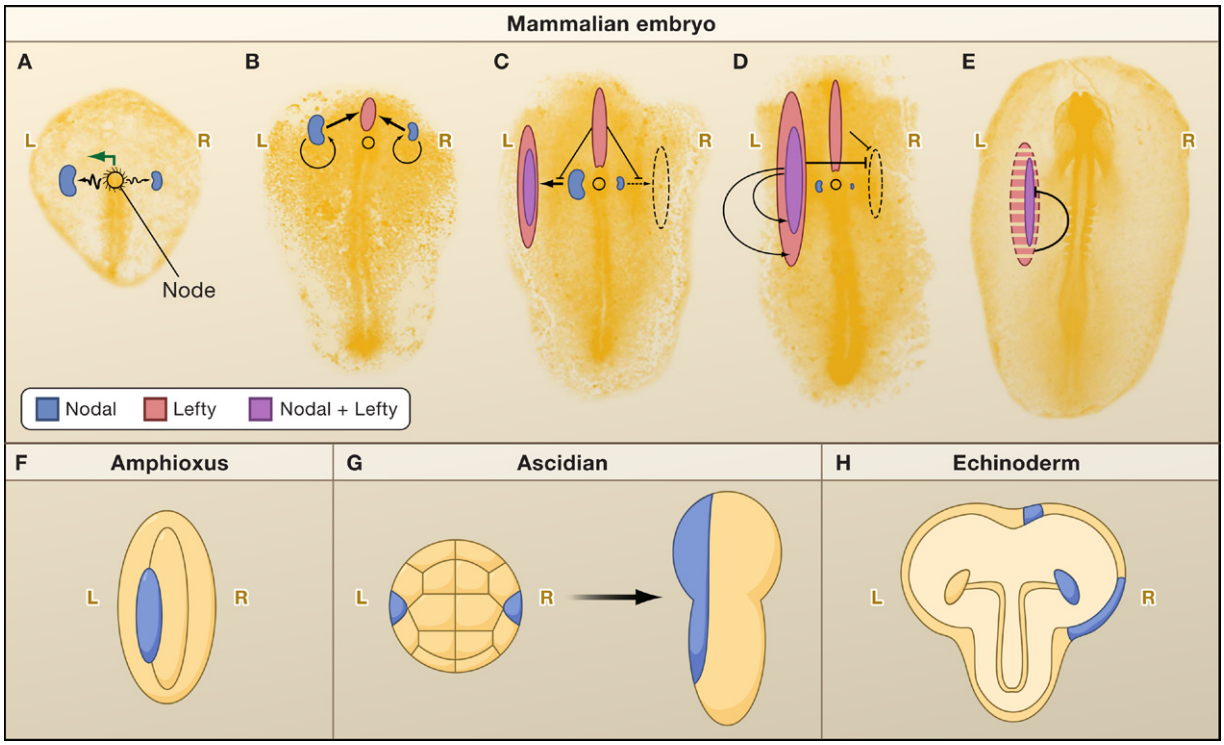

Figure 1. A Reaction-Diffusion System Generates Left-Right Asymmetry

Expression of the *Nodal* and *Lefty* genes forms a modified reaction-diffusion system that establishes left-right asymmetry in chordate embryos.

(A) The action of cilia in Hensen's node in the mammalian embryo generates a leftward flow of fluid (green arrow), leading to increased signaling to the left of the node compared to the right (black squiggly arrows). This results in unequal activation of *Nodal* expression (blue).

(B) Nodal positively regulates its own expression and also induces the expression of its inhibitor, *Lefty* (red), in the midline.

(C) Nodal expression adjacent to the node induces *Nodal* (purple) and *Lefty* (red) in the lateral plate mesoderm. This only happens on the left because, in a reaction-diffusion mechanism, the inhibitory influence of Lefty produced in the midline is relatively stronger than the inductive signal on the right but weaker than the inductive signal on the left.

(D) Nodal in the lateral plate mesoderm induces both *Nodal* and *Lefty* expression, leading to a rapid expansion of both domains. Lefty from the left lateral plate and the midline acts on the contralateral side to prevent activation of *Nodal* expression on the right.

(E) Inhibitory activity of Lefty in the left lateral plate mesoderm contributes to the transient nature of *Nodal* expression by preventing its maintenance by autoinductive Nodal signaling.

(F) *Nodal* expression in the cephalochordate *Amphioxus* is restricted to the left side of the larva in all three germ layers. *Lefty* is similarly expressed.

(G) *Nodal* expression in the urochordate ascidian (sea squirt) is at first bilaterally symmetrical in the prospective endoderm, epidermis, and trunk lateral cell lineages. Subsequently, it is expressed only in the left epidermis. *Lefty* is similarly expressed.

(H) In echinoderms, *Nodal* is expressed in the right oral ectoderm and a subset of the cells in the right coelomic pouch. (Drawings show the sequence of signaling events but are not to scale. Domains of gene expression are not to scale.)

inhibitor Lefty. The self-enhancing autoinduction component of this system can explain the rapid spread of Nodal throughout the lateral plate (Figure 1D). Moreover, the transient nature of Nodal expression in the lateral plate can potentially be explained by the subsequent diffusion of the inhibitor, Lefty2, through the tissue (Figure 1E). The longer range diffusion of Lefty2 from the left lateral plate to the right side also may explain the block in the contralateral activation of downstream left-side targets such as the transcription factor *Pitx2*.

The effectiveness of this feedback system in maintaining unilateral signaling is potentiated by mediolateral differences in the responsiveness of different tissues to Nodal signaling. The intermediate mesoderm does not respond at all to Nodal signaling, creating a spatial gap that puts distance between high levels of Nodal and Lefty in the left lateral plate and the potentially responsive lateral plate on the right side. Given that Lefty diffuses further than Nodal, inhibition over activation is favored on the contralateral side (Figure 1D). The perinodal region is only capable of inducing *Nodal* in response to Nodal signaling. Conversely, *Lefty1* but not *Nodal* is induced along the entire midline in response to Nodal. The localized induction of the inhibitor, but not the activator, deviates from a pure reaction-diffusion mechanism. However, it creates a source of inhibitor between the activator and the contralateral tissue that acts as a midline barrier, enhancing the fidelity of the system (Figure 1B).

The Nakamura et al. (2006) study focuses on an additional property

of reaction-diffusion systems: the ability to convert small differences in signaling between two distinct regions into a robust difference through local activation and long-range inhibition. The temporal expression patterns of Nodal show that, initially, this key signal is activated almost as strongly on the right as on the left in the perinodal region (as judged by in situ hybridization). This suggests that the activity of cilia (whether mediated by a transported morphogen or by a mechanosensory mechanism) may not yield unilateral signaling but rather may produce signaling that is only slightly biased toward one side (Figure 1A). Only the left side triggers subsequent expression of *Nodal* in the lateral plate mesoderm. To explain the conversion of this initial bias in signaling to robust left-side determination, Nakamura et al. (2006) developed a self-enhancement, lateral inhibition in silico model of Nodal and Lefty behavior. In this model, the slight difference in *Nodal* expression across the node is translated into a transient induction of *Nodal* in both the left and right lateral plate mesoderm. Quickly, however, autoinduction in the lateral plate mesoderm and lateral inhibition across the entire embryo produces robust unilateral expression of Nodal on the left side. The induction of the inhibitor *Lefty1* in the midline is an important component in achieving this. Mathematical modeling indicates that Lefty1 is more important in this regard than Lefty2 produced in the left lateral plate mesoderm. The simulations carried out by Nakamura and colleagues indicate that an initial difference in the intensity of Nodal signaling as small as a 3:2 ratio between the left and right sides would be sufficient to generate asymmetric gene expression (Figure 1C).

If such a reaction-diffusion mechanism is really responsible for amplifying the initial difference in left and right perinodal expression of *Nodal*, then there are a number of testable predictions. For example, increasing the level of Nodal initially expressed on the right side, increasing the amount of the inhibitor Lefty produced on the left, or simply removing the left lateral plate mesoderm should all reverse the bias and result in right-sided expression of *Nodal* in the right lateral plate while blocking *Nodal* expression in the left. These predictions were experimentally tested and the outcomes were indeed as predicted (Nakamura et al., 2006). Moreover, while surgical removal of the left-side source of Nodal signaling resulted in activation of *Nodal* expression in the right lateral plate mesoderm, it failed to do so if the node itself was also removed, verifying that the initiation of *Nodal* expression requires signaling from the node itself. Appreciation of the role of reaction-diffusion and the regulation of gene expression in the lateral plate mesoderm mechanistically explains the phenotypes of several mouse mutants that had been confusing. These include the phenotypic differences between mice deficient in the ability to produce cilia (such as Kif3a, Kif3b, Polaris, and Winn mutants) and those with immotile cilia (such as the iv mutant).

That Nodal and Lefty form a reaction-diffusion system changes how we view previous advances in the field. For instance, although the midline is certainly involved in preventing inappropriate expression of left-sided genes on the right, it should probably not be called a "barrier." More accurately, the midline is essential for allowing lateral inhibition to reach from the left lateral plate mesoderm to the right. In its absence, it is not Nodal leaking across from the left lateral plate that triggers right-side gene expression, but rather it is intrinsic right-side Nodal activity that is not inhibited. The midline plays this transduction role both actively (by expressing Lefty1 but not Nodal, thereby amplifying inhibitory activity) and passively (in the sense that long-range Lefty2 activity cannot reach the contralateral side if the midline tissue is physically disrupted).

Our view of the fluid flow produced by rotating cilia must also change, as it is now apparent that this mechanism is very inefficient, yielding a very small difference in Nodal activity. It is through the reaction-diffusion system that broad, distinct asymmetric gene expression patterns are produced that convey asymmetric specification. Without reaction-diffusion, the cilia would be ineffectual and the two are therefore inexorably linked into a single mechanism for generating asymmetry.

The Critical Event in Establishing Asymmetry

For years, the overriding goal has been to identify the mechanism by which symmetry is first broken. However, the focus on breaking symmetry as the key issue with downstream events being viewed as of secondary importance reflects our intellectual conceit that we could intuit the most critical step of a process before even knowing the nature of the pathway. It is now apparent that the mechanism by which bilateral symmetry is broken is not, in fact, conserved, and at least from an evolutionary standpoint the initial breaking of bilateral symmetry within the embryo does not appear to be the key event.

In many taxa, molecular left-right asymmetries are observed well before cilia are present (reviewed in Tabin, 2005; Levin, 2005). For example, in chick embryos, there is asymmetric expression of an activin receptor on the right side of the primitive streak long before markers of ciliary motility are expressed. Even more striking, in the frog *Xenopus*, left-right asymmetric expression of an H^+/V-ATPase occurs at the four-cell stage. Similarly, there is asymmetric phosphorylation of Syndecan-2 by PKC-γ, and evidence for asymmetric gap junction signaling prior to cilia formation in *Xenopus.* Importantly, all of these early, pre-cilia molecular asymmetries regulate later morphological asymmetry in amphibians.

If cilia are not responsible for breaking symmetry in all vertebrates, and the data, especially from *Xenopus*, make it clear that they are not, then what mechanisms are used instead? A few ingenious alternatives have been proposed based on the action of gap junctions and ion transporters (Levin, 2005), but these seem somewhat ad hoc in their required assumptions. An attractive possibility might involve the action of directional motor proteins, such as those involved in ciliogenesis, working on chiral intracellular cytoskeletal components polarized with respect to the orientation of the cell itself. Indeed, there is some evidence for the importance of microtubule and actin cytoskeletal organization and microtubule-dependent motor proteins acting as obligate determinants of left-right asymmetry early in *Xenopus* development (Qiu et al., 2005). Another possibility is that the molecular determinants of left-right asymmetry are maternally inherited in *Xenopus* and some other vertebrates. A precedent for this comes from the snail *Limnaea peregra*, where the snail embryo coils either left or right based on the genotype of its mother (Freeman and Lundelius, 1982).

Frogs and birds are closer phylogenetically to mammals than are fish, and yet the evidence supporting the cilia mechanism in both mammals and fish is compelling. This leaves only two possibilities: the cilia mechanism evolved independently in fish and mammals, which seems extremely unlikely, or the cilia mechanism operated in a common vertebrate ancestor but was subsequently lost in the amphibian and avian lineages and replaced by nonciliary mechanisms. Regardless, it is clear that left-right asymmetry can be achieved in some vertebrates without using cilia and *Nodal* flow.

In contrast to the apparent diversity in symmetry-breaking mechanisms in vertebrates, there is a striking conservation in the role played by Lefty and Nodal. The small bilateral perinodal domains of Nodal, the midline domain of Lefty, and the left lateral plate domains of both of these genes are present in frog and chick embryos even though the initiation event appears to differ. Moreover, the expression of these genes is conserved not only in vertebrates but in all deuterostomes including cephalochordates (amphioxus), urochordates (ascidians), and echinoderms (reviewed in Duboc and Lepage, 2006). Interestingly, the tissue layer and even the side of the embryo where Nodal and Lefty are active can vary, but the general pattern of broad unilateral expression is universal (Figures 1F–1H). These data suggest that the reaction-diffusion mechanism produced by the coupled regulation of Nodal activation and the Lefty feedback inhibitor is evolutionarily conserved. It seems to be at the root of left-right asymmetry in deuterostomes, enabling broad left-right specification after a variety of symmetry-breaking mechanisms induce an initial small left-right bias in signaling.

Notably, the small bilateral domains of Nodal seen in the vertebrate perinodal region and similarly observed in the early ascidian embryo (Figure 1G) are not found either in amphioxus or in echinoderms. Similarly, these species do not display midline expression of *Lefty*. Hence, in these taxa, the lateral-inhibition self-enhancement mechanism apparently does not amplify a small initial asymmetry. Thus, the reaction-diffusion mechanism first may have evolved in the context of a robust symmetry-breaking mechanism to promote the spread of Nodal throughout a unilateral domain while blocking its spread contralaterally. Once this primary role was established, the properties of the system were such that they could subsequently be co-opted for amplifying small differences. In evolutionary terms, the system was "preadapted" for this second task, allowing other less robust mechanisms of symmetry breaking, such as cilia-generated nodal flow, to be used as a trigger while still ensuring a robust outcome.

The discovery of the cilia mechanism has been important for explaining the etiology of situs defects in patients with immotile cilia (Kartagener's syndrome). However, from a developmental perspective, the goal is to understand how nature is able to produce organisms with distinct left and right anatomies. We now know that there are multiple ways of getting asymmetry rolling. It was the evolutionary innovation of the Nodal-Lefty reaction-diffusion system that was the key step in making our asymmetric body plan possible.

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