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Nodal Flow and the Generation of Left-Right Asymmetry

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The establishment of left-right asymmetry in mammals is a good example of how multiple cell biological processes coordinate in the formation of a basic body plan. The leftward movement of fluid at the ventral node, called nodal flow, is the central process in symmetry breaking on the left-right axis. Nodal flow is autonomously generated by the rotation of cilia that are tilted toward the posterior on cells of the ventral node. These cilia are built by transport via the KIF3 motor complex. How nodal flow is interpreted to create left-right asymmetry has been a matter of debate. Recent evidence suggests that the leftward movement of membrane-sheathed particles, called nodal vesicular parcels (NVPs), may result in the activation of the noncanonical Hedgehog signaling pathway, an asymmetric elevation in intracellular Ca^{2+} and changes in gene expression.

Introduction

Our bodies have left-right (LR) asymmetry. The heart, spleen, and pancreas reside on the left side, whereas the gall bladder and most of liver are on the right. Anatomically, LR asymmetry first becomes apparent with the orientation of the heart-tube looping (Kaufman, 1992), but LR asymmetry is already detectable at the stage of somitogenesis by the asymmetrical expression of several genes, such as *Lefty-1 (Leftb)*, *Lefty-2 (Ebaf)*, *Nodal*, and *Pitx2*. In most cases, asymmetric expression of genes at this stage is observed on the left side (Harvey, 1998; Yost, 1999; Levin, 2005; Hamada, 2002; Capdevila et al., 2000). However, the work by our lab and others has indicated that LR asymmetry has its origins at even earlier stages of development.

About half of the human patients with a genetic defect, called Kartagener's syndrome, have their organs in the reversed orientation. Affected individuals also have immotile sperm and defective cilia in their airway. Thus, this phenotype indicated that cilia may control LR asymmetry (Afzelius, 1976). What was unknown at that time was which cilia were relevant and at which stage. Results of many studies have suggested that the so-called "node," a transient midline structure formed during gastrulation (Figures 1A and 2), is important for LR determination (Harvey, 1998). This node arises after the dorsal-ventral (DV) and anterior-posterior (AP) axes have been defined. On the ventral surface of the node there is a ciliated pit (Figure 1A). The monocilia of ventral node cells are primary cilia that lack the central pair of microtubules and thus have 9 + 0 microtubule arrangement (Figure 1C), which differs from 9 + 2 microtubules of conventional motile cilia in normal ciliated cells (Figure 1C). Thus, based on their ultrastructure and from video-microscopic observations, nodal monocilia were once thought to lack motility (Bellomo et al., 1996). However, our discovery that nodal monocilia move rapidly to generate a leftward flow of extraembryonic fluid suggested new possibilities. In this review, we briefly summarize the discovery of nodal flow and discuss the mechanisms by which LR symmetry is broken by nodal monocilia.

The Discovery of Nodal Flow

Insight into the role of nodal cilia in LR determination has been obtained by studies on molecular motors of the kinesin superfamily (KIFs). Microtubules are regularly arranged in many kinds of cells and act as rails that transport membrane organelles and protein complexes over long distances. KIFs move various cargo, not only in polarized cells, such as neurons and epithelial cells, but also in many other cells (Hirokawa, 1998; Guzik and Goldstein, 2004; Hirokawa and Takemura, 2005). The KIF3 complex is composed of a heterodimer of the motor proteins KIF3A and KIF3B and an associated protein, KAP3, that binds to the tail ends of KIF3A and KIF3B. Studies on the *Kif3a* and *Kif3b* knockout mice revealed that roughly 50% of both *Kif3a*-deficient and *Kif3b*-deficient mice showed reversed heart loops, whereas the rest were normal. In order to determine the position of KIF3A and KIF3B in the signaling cascade for LR determination, the expression of *Lefty-2*, one of the earliest left-defining genes, was examined. Although *Lefty-2* was expressed exclusively at the left side of wild-type embryos, *Lefty-2* expression is either mostly bilateral or absent in *Kif3a*-deficient and *Kif3b*-deficient embryos (Nonaka et al., 1998; Takeda et al., 1999; Marszalek et al., 1999). These data suggest that both KIF3A and KIF3B act at an earlier step than *Lefty-2* in the LR-determination pathway.

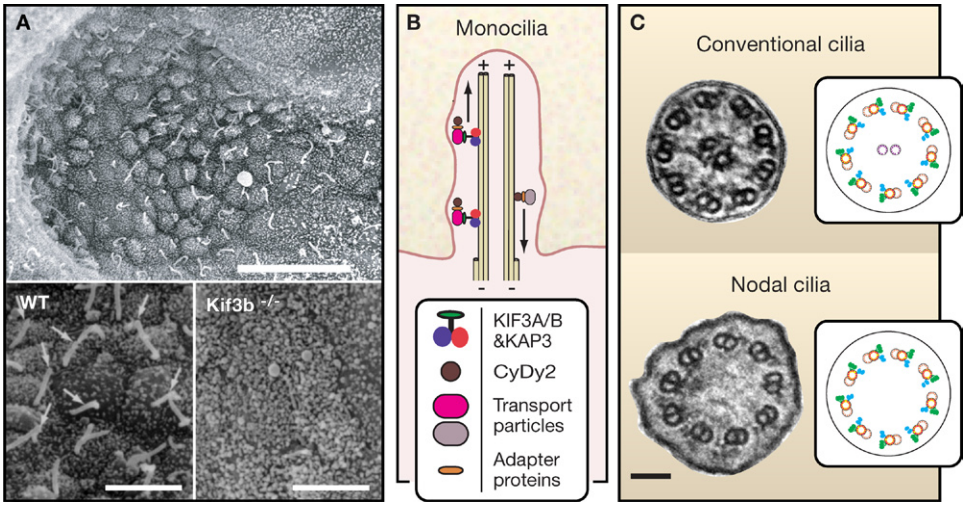

Figure 1. The Kinesin Motor KIF3 and Nodal Monocilia

(A) Scanning electron micrographs of the mouse ventral node in lower (upper panel) and higher (lower panels) magnification are shown. The surface is normally ciliated in wild-type (arrows, lower left panel), whereas the nodal cilia were absent in *Kif3b*$^{-/-}$ embryos (lower right panel). Bars, 20 μm in upper panel, 5 μm in the lower panels. These figures are modified from Nonaka et al. (1998).

(B) The KIF3 motor complex and the cytoplasmic dynein 2 (CyDy2) complex bidirectionally transport particles essential for the building and turnover of the monocilia.

(C) Shown is the ultrastructure of cilia (base to tip view). Electron micrographs and schematic representations (insets) of transverse sections of mouse tracheal cilia (upper panel) and mouse nodal monocilia (lower panel). Conventional cilia or flagella have a 9 + 2 arrangement of microtubules (upper panel). Nine doublet microtubules (inset panels, A-tubule, red; B-tubule, orange) with dynein arms (green and blue) are chirally oriented in a clockwise direction and are surrounded by a central pair of microtubules (purple). Nodal cilia lack the central pair of microtubules and thus have a 9 + 0 arrangement (lower panel). Bar, 0.1 μm.

Surprising and more revealing findings have been obtained by the observation of the nodes. Although ventral node cells of wild-type embryos have monocilia composed of 9 + 0 microtubules on the ventral surface, cilia were either completely absent or were very short and only found sporadically in the ventral nodes of embryos lacking either KIF3A or KIF3B (Figure 1). In addition, immunocytochemistry shows that KIF3A and KIF3B are localized in the monocilia of the ventral node cells of wild-type embryos (Nonaka et al., 1998; Takeda et al., 1999). This indicates that KIF3A and KIF3B are essential for ciliogenesis in ventral node cells through the transport of protein complexes in the monocilia (Figure 1B).

However, it remained unclear why the lack of monocilia in ventral node cells relates to the randomness of LR asymmetry. Therefore, the behavior of monocilia both in wild-type and mutant embryos was observed in vivo by video-light microscopy. Surprisingly, we observed that the monocilia, which were once thought to be immotile, are in fact vigorously rotating (see Movies S1 and S2 in the Supplemental Data available with this article online). The rotational movement of nodal monocilia is unique—most motile cilia or flagella only move back and forth. Both the inner and outer dynein arms have been observed by electron microscopy, and the axonemal outer arm dynein was localized in the monocilia (Takeda et al., 1999). Although the unique rotational movement of monocilia could be generated by axonemal dynein, an explanation had been lacking for how the rotational movement relates to LR asymmetry. It was subsequently found that fluorescent beads, when added in the extraembryonic fluid at the node region in wild-type embryos, moved unidirectionally toward the left, a phenomenon referred to as "nodal flow" (Movie S1), whereas in mutant embryos lacking KIF3A or KIF3B the fluorescent beads showed only Brownian movement (Nonaka et al., 1998; Takeda et al., 1999). This led to the conclusion that monocilia are essential for left-right determination because their rotational movement produces the nodal flow required to establish a gradient of a putative morphogen in the extraembryonic fluid in the ventral node (Nonaka et al., 1998; Takeda et al., 1999).

This conclusion was also supported by the analyses of monocilia of ventral node cells in *inversus viscerum* (*iv*) mutant mice (Okada et al., 1999; Supp et al., 1999). *iv* is a spontaneous mouse mutant that results in the randomization of LR determination (Hummel and Chapman, 1959). A mouse axonemal dynein gene, *Left-right dynein* (*Lrd*), has been identified as the gene responsible for both *inversus vicerum* and *Legless* (*Lgl*), which also results in LR randomization (Supp et al., 1997). *Lrd* is a member of the dynein superfamily of genes, designated *Dlp11* (Tanaka et al., 1995), and is expressed exclusively by nodal cells at 7.5 days postcoitum (dpc) (Supp et al., 1997). In embryos heterozygous for *iv*, nodal cilia rotate as rapidly as wild-type embryos (~600 rpm; Okada et al., 1999). This rapid movement produces a fast (20–50 μm/s) and smooth leftward flow of the extraembryonic fluid in the ventral node. However, in *iv* homozygous embryos, the nodal cilia seldom moved and appeared

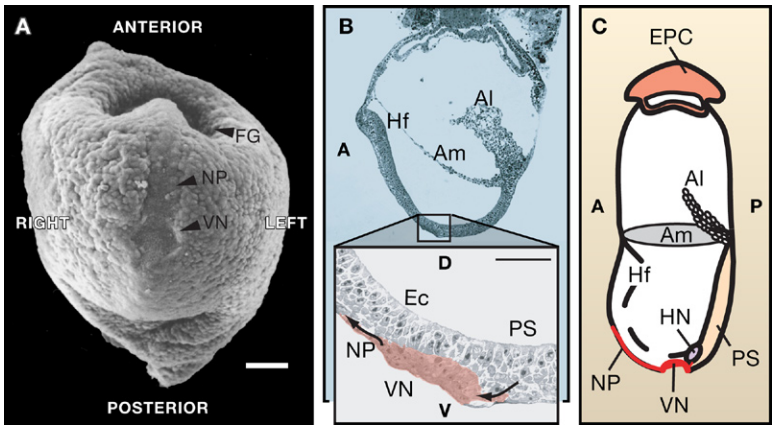

Figure 2. Anatomy of the Ventral Node

(A) A scanning electron micrograph shows the ventral view of a 7.5 dpc mouse embryo. VN, ventral node; NP, notochordal plate; FG, foregut; Bar, 100 μm.

(B) Shown is a 1 μm thick parasagittal section of a 7.5 dpc mouse embryo. The inside and outside of the egg cylinder become future dorsal (D) and ventral (V) sides, respectively. The boxed area is enlarged in the inset panel. Note that the columnar epithelium of the neuroectoderm (Ec) is dorsally adjacent to VN and the mesenchymal cells of the primitive streak are in the posterior region of VN. Hf, headfold; Am, amnion; Al, allantois. A group of cells that seemingly enters and exits the VN are colored together with the VN cells. Bar, 50 um.

(C) A schematic representation of (B) is shown. The ventral node is located at the most distal end of the embryo from the ectaplacental cone (EPC), which is the implantation site.

very rigid (Okada et al., 1999). Moreover, beads added to the extraembryonic fluid showed only Brownian movement (Okada et al., 1999). Consequently, nodal cilia in *iv* homozygous mice cannot rotate because of a mutation in *Lrd* and thus cannot generate the leftward nodal flow that is necessary for normal LR determination.

At this point, several important questions remained unanswered. Is leftward nodal flow the initial LR symmetry breaking event in mammals and in other vertebrates? Second, how does rotary movement of cilia generate linear leftward flow? Third, are secreted morphogens present in the ventral node, and if so, does nodal flow lead to the accumulation of putative morphogens on the left side?

The Ventral Node Is Distinct from Hensen's Node

Before discussing the breaking of symmetry by nodal flow, we should first define the ventral node. In this section, we review the anatomy of this LR signaling center of the postimplantation mouse embryo, especially in regard to its spatial relationship with the organizer, Hensen's node. As summarized in Figure 2, mouse embryos develop a cylindrical structure, whose inside is the dorsal side and is lined by the epiblast, and outside is the ventral side, which is covered with hypoblast. The anterior migration of the distal visceral endodermal cells from the most distal site from the ectaplacental cone defines the AP axis and the midline of the embryo. The primitive streak is located as a slit of epiblast on the posterior half of the midline. From the primitive streak, the epiblast cells make the gastrulation movement into deeper layers in the wall of the egg cylinder to determine their destination as definitive mesoderm or definitive endoderm. The most anterior portion of the primitive streak is called the primitive pit. A dorsal protrusion surrounding the primitive pit of the rabbit embryo became known as Hensen's node by his epoch-making paper (Hensen, 1876; reviewed by Viebahn [2001]). Hensen described a hillock-like structure, or "Knoten," which means "node" or "knot," in the center of the disc-like rabbit embryos. The structure looks like a knot of rope that is half-embedded into the blastodisc on the dorsal side. Thus, the functional and anatomical equivalent must be located inside of the mouse egg cylinder. Although this original definition of "Knoten" apparently describes a protruding structure (on the dorsal side), mouse embryologists including the late Rosa Beddington (Hogan et al., 1986) have expanded its definition to include the much broader area that encompasses the ventral structures because the mouse ventral node appears as a notch on the outside surface of midgestation embryos.

The ciliated cells on the ventral side of the node are at the most posterior part of the notochordal plate, which is a primordium of the notochord (Kinder et al., 2001). From the late-streak to early-somite stage of development, these axial mesoendodermal cells form an epithelial sheet that is exposed to the ventral midline surface and is surrounded by sheets of endoderm on both sides. Although the dorsal surface of the ciliated cells is directly associated with epiblast or ectodermal cells, there is a smooth boundary between those germ layers and the ventral node such that it is not likely that the ventral node is a part of the primitive streak (Figure 2B). Conversely, we could identify a group of mesodermal cells in parasagittal sections of 7.5 dpc embryos (Figure 2B), which would represent the invaginating cells that are derived from the Hensen's node located at regions of the embryo more toward the posterior and dorsal end. Interestingly, ciliated primordial cells in the ventral node are very closely packed and look like a longitudinal section of an onion bulb, supporting a "toothpaste" model of notogenesis (Sausedo and Schoenwolf, 1994), in which primordial cells for the notochord rush into the ventral node from the posterior side and remain there before exiting from the anterior side toward the notochordal plate. Thus, although cells of the ventral node are originally derived from the Hensen's node, the ventral node is a totally distinct structure. This difference is much clearer in other vertebrates such as the rabbit, in which Hensen's node was originally designated. In rabbit embryos, the ventral structure that corresponds to the "ventral node" of

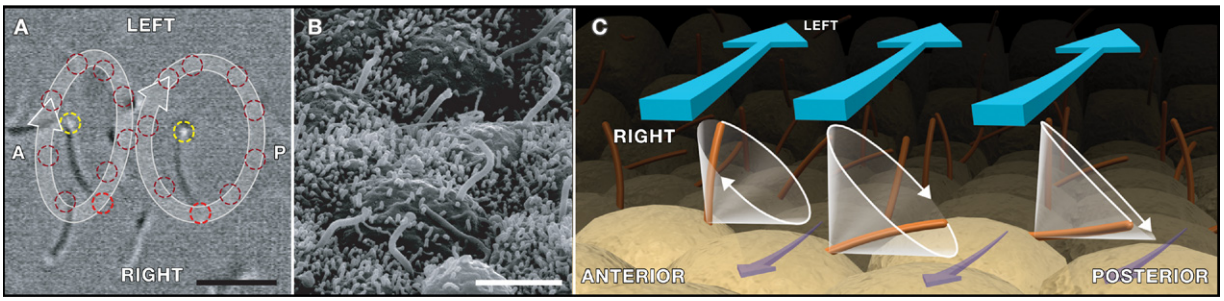

Figure 3. Leftward Flow Is Generated by the Posterior Tilt of Nodal Cilia
(A) The trajectory of the tips of nodal cilia (red circles on the white ellipse) is shifted toward the posterior when compared to the root of the cilia (yellow circles). See also Movie S2.
(B) A scanning electron micrograph of ciliated cells of the ventral node of a rabbit embryo (at the presomite stage) is shown. The root of a cilium is most frequently found toward the posterior of a cell. The dome-like curvature of the apical plasma membrane helps explain the posterior tilt of the cilia. Bar, 5 μm.
(C) A hydrodynamic mechanism generates leftward flow. Due to a gradient of shear resistance, a cilium cannot efficiently drive the extraembryonic fluid when it makes a rightward movement in the proximity of the surface. These figures are modified from Okada et al. (2005).

the mouse is found on the posterior notochord, which is distant from the original Hensen's node on the dorsal side (Okada et al., 2005). However, for the sake of consistency and convenience with already established terms such as "nodal flow" and "nodal cilia," we will use the term "ventral node" for this ventral ciliated pit.

Nodal Flow Breaks LR Symmetry in Mouse Embryos

From here, we focus on the initial events that establish LR asymmetry in the mouse. Although nodal flow is conserved between fish, rabbits, and mice, we will begin by discussing alternative symmetry breaking event(s) at earlier stages of development. Theoretically speaking, the DV and AP axes and the chirality of the system are required to break the LR symmetry in a stereotypic manner. Or to put it another way, laterality can only be established once the AP and DV axes are determined based on the given chirality of the system. Thus, the key issue in the initial breaking of LR symmetry is how the specification of the AP and DV axes and the chirality of the system relates to the specification of the LR axis. To address this question, we first discuss when these axes are determined during development.

In many lower vertebrates and invertebrates, eggs are asymmetrical even before fertilization (as in *Drosophila*, [Gilbert, 2003]). In some organisms, such as fish and frog, the DV and AP axes are determined at fertilization by the distribution of the yolk and the entry position of the sperm (Gilbert, 2003). Although it remains controversial in mammals (Gardner, 1997, Hiiragi and Solter, 2004), experimental manipulations of mammalian embryos before implantation have little effect on axis formation after implantation (Alarcon and Marikawa, 2003).

The determination of the AP axis in mammalian embryos should be reorganized after implantation because anterior specification involves the proximal-distal information from the implantation site (ectoplacental cone), which may be determined irrespective of the polarity in the inner cell mass (ICM) of the blastocysts before implantation. Following implantation, mouse embryos are thought to be initially cylindrically symmetrical. Subsequently, the AP axis is determined by newly emerging signaling centers along the future midline of the embryo, which induce the headfold (to the anterior) and the primitive streak (to the posterior; Beddington and Robertson, 1999). As LR determination occurs with respect to the midline, it must strictly follow the formation of the midline. This explains why the laterality of the mouse embryo that might exist before implantation cannot persist after implantation. Assuming that the anterior side is randomly selected, as hypothesized, from the 360° of the egg cylinder (Alarcon and Marikawa, 2003), any preexisting asymmetry of LR determinants should be cancelled at that stage. If it were not, when the anterior position is specified at 0° versus 180°, the laterality would be inverted.

This is consistent with the findings that gene expression with LR asymmetry is first determined at the early-somite stage. Patrick Tam and colleagues have described markers that are transiently expressed on either side of the mouse embryo before this stage (Tsang et al., 1999), although no significant tendency in the laterality of expression was observed, supporting the hypothesis that mouse laterality is first determined when nodal flow occurs.

Our observations of living wild-type and mutant mice embryos (such as *Kif3b*$^{-/-}$, *Kif3a*$^{-/-}$, *iv*, and *inv* [inversion of embryonic turning]) by videomicroscopy revealed an event (nodal flow) with LR asymmetry prior to the asymmetrical gene expression in 7.5 dpc embryos at the early-somite stage. The clockwise movement of monocilia produces a localized net right-to-left fluid flow on the surface of the ventral node (Movie S1; Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999). Abnormal nodal flow in mutant mice precedes the disruption of LR asymmetry, which suggests that the direction of the flow of the extraembryonic fluid determines the subsequent LR asymmetry of the mouse embryo. This idea was later confirmed by culturing mouse embryos under conditions of artificial flow (Nonaka et al., 2002).

The Direction of Nodal Flow Is Determined by Hydrodynamics

Since the initial discovery of the leftward nodal flow, how the rotating cilia generate unidirectional flow has been resolved by our observation at a high temporal resolution of nodal monocilia (Movie S2; Okada et al., 2005). We have carefully observed ciliary movement and determined the axes of rotation in three dimensions. Unlike conventional beating cilia, the monocilia in the node have a clockwise rotational motion (Figure 3A; Movie S2). The axis of rotations does not show a lateral bias from the midline but is tilted 40° ± 10° to the posterior from the vertical angle (Figures 3A and 3C). As a consequence, the cilia make a leftward swing away from the surface and a rightward sweep near the surface. According to hydrodynamics, a stationary surface retards the movement of fluids by shear resistance. Thus, the rightward sweep is less effective than the leftward swing in generating fluid movement (Figure 3C).

The posterior tilt for the axis of rotation has been modeled in a study by Cartwright et al. (2004). According to their model, rightward flow along the surface of the ventral node should be the same speed as the leftward flow. However, this model does not take into account shear resistance and is not consistent with experimental observation. Nodal flow has a Reynolds number (a value commonly used in hydrodynamics that characterizes fluid flow) on the order of 10^{-3}, indicating that viscosity dominates over inertia. Thus, the viscous drag or the shear resistance from the cell surface is not negligible. Our recent theoretical study explicitly takes this factor into consideration and quantitatively supports the idea that shear resistance of the cell surface is essential for the generation of leftward flow (Buceta et al., 2005). This new hydrodynamic model is consistent with experimental results with *inv* mutant mice (Okada et al., 2005) and with an artificial experimental model system (Nonaka et al., 2005).

Brown and Wolpert (1990) have proposed that a chiral structure aligned with respect to the AP and DV axes is needed to generate LR laterality. Cilia in the ventral node that are tilted toward the posterior represent exactly this kind of structure. The structural properties of nodal cilia determine the direction of flow, without relying on any a priori LR asymmetry (Figure 3C). The clockwise rotation of cilia will reflect the chiral architecture of the nodal monocilia, in which the dynein arms are aligned in a clockwise manner on the side of each doublet microtubule (Figure 1C; Brokaw, 2005). Interestingly, the basal body or the root of each monocilia was positioned at the cell posterior, possibly reflecting planar cell polarity (PCP) of the ventral node cells toward the AP axis (Okada et al., 2005). The convex curvature of the ventral node cells might explain how this posterior localization contributes to the tilting of the rotation axes (Figure 3B).

This mechanism for generating laterality is conserved in rabbits and fish. Rabbits, like humans and chicks, develop flat, disc-like embryos that differ from mouse embryos, which are more like cup-shaped cylinders. Leftward fluid flow develops on the ventral surface of the posterior notochordal plate. The expression of *Nodal* demonstrates that this region in the rabbit embryo corresponds to the ventral node of mouse embryos, although it is anterior to Hensen's node (Okada et al., 2005). One or two primary cilia project from the posterior side of the ventral surface of each cell of the notochordal plate. As in the mouse embryo, these cilia rotate around an axis tilted to the posterior.

Similar events are observed in the Kupffer's vesicle (KV) of zebrafish and medakafish, which also corresponds to the ventral node of mouse (Essner et al., 2005; Kawakami et al., 2005; Okada et al., 2005). The optical transparency of medakafish allowed the direct observation of the primary cilia (rotating on a posteriorly tilted axis) on the ventral surface of KV (Okada et al., 2005). The body axes of lower vertebrates including amphibians and fish are determined at much earlier stages than in mammals, which might reflect the difference between oviparity (development in an egg) and viviparity (having live birth). Thus, in fish, LR asymmetry is determined prior to the period of gastrulation and is perturbed by the inhibition of H^+/K^+-ATPase using drugs such as omeprazole (Levin et al., 2002). However, leftward flow is normal in KVs even after the preexisting LR asymmetry is disrupted (Kawakami et al., 2005). This strongly supports the notion that the directionality of nodal flow in KV is autonomously determined without requiring any preexisting LR asymmetry.

Generating Asymmetric Gene Expression by Nodal Flow

Two alternative models, which are not mutually exclusive, were proposed to explain how the information generated by leftward nodal flow is interpreted: the first model is based on the formation of chemical gradients, whereas the other model invokes physical stimulation by nodal flow. The chemical gradient model came first. It assumes that the directional flow will produce a concentration gradient of a secreted morphogen in the cavity of the ventral node (Nonaka et al., 1998; Okada et al., 2005). The physical stimulation model was proposed as a critique of the chemical gradient model, and it poses that physical stimulation by the flow itself is mechanically sensed by the cells in the ventral node (McGrath et al., 2003). Some nodal cilia, especially those in the peripheral region of the ventral node, are immotile. Thus, it has been suggested that these immotile cilia could act as a sensor for the direction of flow. This physical stimulation model is now often referred to as the "two-cilia hypothesis," in which one type of cilia generates flow and the other senses it (Tabin and Vogan, 2003).

The two-cilia hypothesis was proposed to explain the differences between the phenotypes observed in mutant embryos that lack cilia from those mutants with immotile cilia. Mutant mice with immotile cilia exhibit a complex pattern of expression for genes that are normally spe-

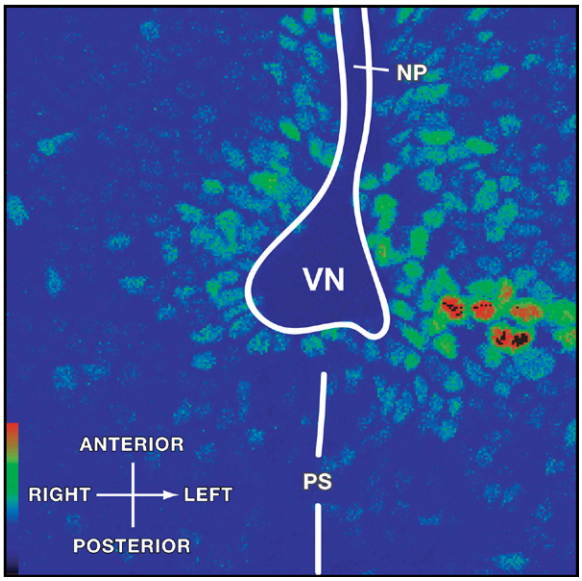

Figure 4. Ca^{2+} Is Elevated on the Left Side of the Node
A ventral view of a 7.5 dpc mouse embryo stained by Fluo3-AM (to detect calcium) is shown in a pseudocolor image. NP, notochordal plate; VN, ventral node; PS, primitive streak. VN, NP, and PS are traced by white lines. This figure is modified from Tanaka et al. (2005).

cific to the left side (Supp et al., 1999). In contrast, mice without nodal cilia have a greater tendency to exhibit bilateral patterns of gene expression in the lateral plate mesoderm (Nonaka et al., 1998; Marszalek et al., 1999; Takeda et al., 1999; Murcia et al., 2000). A key foundation for the two-cilia hypothesis is the idea that some nodal cilia would have a sensory function. This does not necessarily imply that the nodal cilia sense mechanical stimulus by flow but that they may serve as the sensor for chemical molecules like the cilia of the nasal epithelium. The strongest support for the mechanical-sensation model is the finding that early LR signaling is disrupted by mutations in human polycystic kidney disease (PKD) genes that are involved in mechanosensation by the monocilia of renal epithelial cells (Murcia et al., 2000, Pennekamp et al., 2002).

In the two-cilia model, each ciliated cell should detect the directionality of flow using the primary monocilium as the sensor. However, the directionality of the flow is local and does not convey global information on laterality. In other words, because the shape of the ventral node is symmetrical, nodal cells on both sides should similarly sense the flow from the right. Further information such as communication with surrounding cells would be required to know whether they are on the left side of the node or on the right side. This conceptual issue remains unanswered for the two-cilia hypothesis.

In contrast, the chemical-gradient model is simple and straightforward. Chemical morphogen(s) are secreted into the cavity of the ventral node and are transported to the left side by nodal flow. Thus, the chemical morphogen(s) accumulate only on the left side. In this model, cells can tell whether they are on the left side of the embryo simply by sensing this morphogen. The phenotypic differences between mutants that lack cilia and mutants with immotile cilia can be explained if some (or all) nodal cilia serve as the sensor for this chemical morphogen. Thus, the biggest difficulty of the chemical gradient model has been whether a concentration gradient of a secreted chemical morphogen is actually generated by nodal flow. Because the ventral node is a closed or semiclosed cavity, it might be assumed that the leftward fluid flow at the bottom surface would be balanced by a rightward counterflow elsewhere. A potential consequence of counterflow might be that secreted molecules end up uniformly distributed. As expected, leftward nodal flow in the mouse is compensated by a rightward counterflow, although the counterflow is much slower and further above the surface of the ventral node (Okada et al., 2005). A caged fluorescently labeled protein was introduced into the cavity to establish whether a concentration gradient is generated. Labeled protein was locally activated by UV irradiation to simulate continuous or intermittent secretion. Interestingly, only proteins with a size of 20–40 kDa generated a stationary concentration gradient. This size matches that of candidate proteinaceous morphogens such as FGF8 and Nodal. Theoretical analyses can explain this size dependency. The rate of diffusion for proteins of this size lies between the fast nodal flow to the left and the much slower counterflow to the right. This balance is also conserved in rabbit, despite the difference in the size of the ventral node. These results suggest the possibility that those molecules can spontaneously generate a chemical gradient in the ventral node. Alternatively, as is discussed later, our group has discovered much bigger extracellular particles that are secreted and transported to the left. In this case, unique cell biological mechanisms ensure the unidirectionality of movement.

Perinodal Ca^{2+} Elevation Initiates Left-Specific Signaling

As an important clue linking nodal flow to left-specific cell signaling, Martina Brueckner and colleagues have identified an elevation in the intracellular concentration of Ca^{2+} on the left periphery of the ventral node (McGrath et al., 2003). We have also observed this phenomenon and have established that the Ca^{2+} elevation is stable and long lasting (Tanaka et al., 2005; Figure 4). Nodal flow first leads to Ca^{2+} elevation at the left periphery of the ventral node, which is then propagated laterally to fix the identity of the left side. This event may affect the balance of Lefty/Nodal TGFβ signaling to establish the "leftness" of the lateral plate mesoderm by inducing the left-specific gene cascades. However, the detailed molecular mechanism of these signal transduction events is still being investigated. From a recent study in zebrafish, the lateral propagation of elevated Ca^{2+} can be achieved by diffusion of IP6 through gap junctions (Sarmah et al., 2005), although it is not known whether

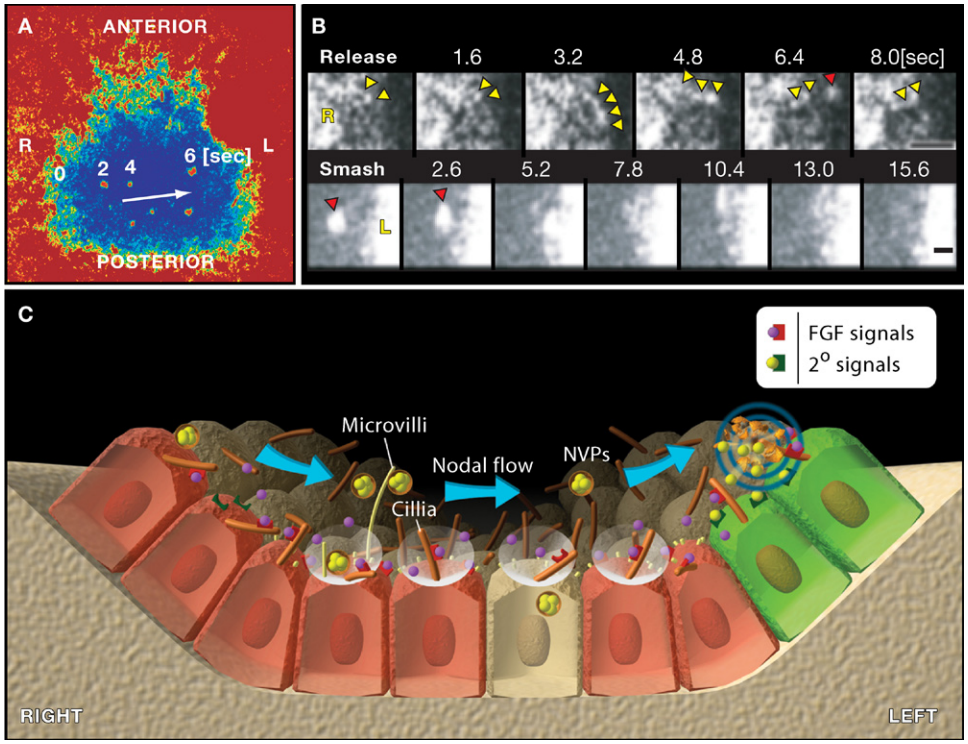

Figure 5. The Flow of NVPs

(A) A merged image from time-lapse microscopy is shown in pseudocolor. The trajectory of two nodal vesicular parcels (NVPs) flowing from the right (R) to the left (L) is shown in 2 s intervals. The left side (the right in the figure) is brighter than the right side. See Movie S3.

(B) Time-lapse images show both the release and smash phases for NVPs. NVPs are released from a whip-like cell protrusion and then are fragmented nearby at the left wall of the ventral node. See Movies S4 and S5.

(C) A dynamically protruding microvillum picks up an NVP and releases it into the nodal flow. This event is stimulated by FGFR signaling. The NVP is transported to the left (the right of the figure) by the fluid flow and is hit by the ciliated surface on the left periphery of the node, releasing its contents onto the surface of nodal crown cells. Movie S6 presents an animated model of this process. These figures are modified from Tanaka et al. (2005).

this is also true in mice. It is also controversial whether this is an evolutionary counterpart of the left-specific elevation in extracellular Ca^{2+} reported in chick downstream of Notch (Raya et al., 2004).

Polycystins are membrane proteins that may also be involved in this prolonged Ca^{2+} elevation (reviewed by Nauli and Zhou [2004]). Polycystin-1 (PC1 or PKD1) and Polycystin-2 (PC2 or PKD2) interact with each other and are essential for kidney development due to their role in the mechanosensation of tubular flow (Schwartz et al., 1997; Delmas, 2004). The mechanosensory function for PC1 is thought to involve an extracellular PKD domain, which could be involved in a homophilic interaction with another PC1 molecule in order to sense the distance between them. In response to mechanical stimuli, the C terminus of PC1 is cleaved and translocates to the nucleus, where it transactivates AP-1 (Chauvet et al., 2004). PC2 is thought to be a Ca^{2+} channel that is localized in both the plasma membrane and ER. PC2 may be stimulated by PC1 through an interaction between the C termini of each molecule (Delmas, 2004). Embryos of PC2-deficient mice lack elevated Ca^{2+} at the left periphery of the ventral node (McGrath et al., 2003), fail to express *Lefty-2* and *Nodal* in the lateral plate mesoderm and have random determination of the left-right axis (Pennekamp et al., 2002). On the contrary, PC1-deficient mice have normal left-right determination but develop progressive polycystic kidney disease (Nauli and Zhou, 2004), suggesting that Ca^{2+} elevation in left periphery of the ventral node is triggered by a different mechanism than the mechanosensation that is mediated by PC1/PC2 in renal tubules. As PC2 has multiple sites of action (Delmas, 2004), the perinodal elevation in Ca^{2+} is not necessarily associated with mechanosensation, as is predicted by the two-cilia model (Tabin and Vogan, 2003). Brueckner's group also finds PC2 in all nodal cilia including those that are motile; thus, it is unlikely that PC2 is only involved in Ca^{2+} elevation at the periphery of the ventral node (McGrath et al., 2003). In the motile cilia, PC2 might directly regulate coordination of motility of the cilia by changing local Ca^{2+} levels during each rotation (Sleigh and Barlow, 1982). As described in detail in the next section, we have also shown that pharmacological treatments can eliminate and rescue the perinodal elevation in Ca^{2+} without perturbing the fluid flow (Tanaka et al., 2005), suggesting that the mechanical

element of fluid flow is not sufficient by itself to elicit the Ca^{2+} elevation. Future identification of the precise role of PC2 in the ventral node will help elucidate the molecular mechanisms underlying Ca^{2+} elevation.

Ca^{2+} Elevation Can Be Modulated by the FGF/SHH/RA System

Our group has recently identified that specific inhibitors of fibroblast growth-factor receptors (FGFRs) can almost completely disrupt the increase in Ca^{2+} at the left periphery of the ventral node in mouse embryos (Tanaka et al., 2005). FGF receptors 1–3 are expressed on the cell surface of the ventral node and at its periphery, as well as on the cilia of ventral node cells. FGFR inhibitors do not perturb the fluid flow within the node. This indicates that flow by itself is insufficient for left-specific Ca^{2+} elevation, contrary to previous reports (McGrath et al., 2003). Interestingly, treatment with either the N terminus of sonic hedgehog (SHH-N), retinoic acid (RA), or the N terminus of Indian hedgehog (IHH-N) reverses the inhibition of the Ca^{2+} elevation by the blockade of FGFR signaling. SHH-N treatment promotes an elevation in Ca^{2+} in small cells on the left periphery of the node, whereas RA elevates Ca^{2+} in a broader area on the left half of the node. In comparison, IHH-N stimulates an elevation in Ca^{2+} over a broader area bilaterally. As all three of these morphogens have been implicated in the signaling cascades leading to left specification from previous studies (Levin, 2005), it was conceivable that these molecules could directly or indirectly affect the pathway for Ca^{2+} elevation. Moreover, the spatial differences in the effects of these morphogens might reflect different roles for each morphogen in the pathway. For instance, it was intriguing that SHH or RA restored Ca^{2+} elevation with a significant asymmetry toward the left side. How exogenous SHH-N or RA elicited the asymmetrical elevation in Ca^{2+} was a fascinating mystery that motivated further exploration.

NVPs Are Membrane-Sheathed Particles Transported to the Left

In order to look at the direct consequences of FGFR suppression on the cells in the ventral node, we labeled the surface lipids of embryos by a fluorescent lipophilic dye and made observations in time lapse with a confocal laser scanning microscope. In addition to fine movements of microvilli and cilia, we detected materials with diameters of 0.3–5 μm that were transferred toward the left by nodal flow (Tanaka et al., 2005; Figure 5; Movies S3–S6). Occasionally, the labeling intensity of the left side was brighter than that of the right side, suggesting a massive transfer of lipids toward the left. We named these transported materials nodal vesicular parcels (NVPs) and characterized them further using pharmacological and morphological methods. Electron microscopy revealed the presence of many prereleased NVPs adhering to the surface of the ventral node. Also, the diameter of NVPs observed in this analysis is approximately 1–2 μm, consistent with the observations made by fluorescent labeling. Each NVP contains multiple smaller electron-lucent droplets ensheathed in an outer membrane. These droplets seem like lipoprotein particles, although their precise chemical nature is still largely unknown. NVPs are frequently associated with long cellular processes, which, because of the apparent absence of microtubules, may be dynamically protruding microvilli. Burst NVPs (that is, NVP membrane sheaths that lack interior contents) have also been observed (Y.T. and N.H., unpublished data).

How does such a big particle avoid circling within the ventral node? The answer likely relates to the mechanisms by which NVPs are released and then caught (Tanaka et al., 2005). The release of NVPs is apparently an active process that takes approximately 10 s. The initial direction of the NVPs, which emerge from all regions of the ventral node, is random and is sometimes against the direction of nodal flow. NVPs appear as if they are leaving from the tip of a bending rod (perhaps a microvillium) much like a whip (Movie S4). Release puts NVPs into the middle of laminar fluid flow several micrometers above the ciliated surface, where the flow rate is fastest. Thus, NVPs avoid being hit by rotating cilia in the initial phase. During the next 10 s, NVPs are transported to the left side by the nodal flow. NVPs then hit the ciliated surface, burst, and are fragmented (Movie S5). In a final phase (approximately 10 s long), NVPs are absorbed by the surface of nodal crown cells on the left side. Bursting may keep the NVP particles from flying back to the right side and may also promote the release the active contents. A physical interaction between an NVP and a cilium, respectively containing SHH and its receptor Smoothened (Smo), is possibly essential for the burst because the turnover of NVPs appears to be delayed in *Kif3a* mutants that lacked cilia compared with the *iv/iv* mutant that has immotile cilia (Tanaka et al., 2005). Because an expansion of the volume in NVPs appears to precede bursting from our observations, the bursts might be triggered by an elevation in the internal pressure of NVPs through the opening of a membrane channel on their surface. The precise molecular mechanism of NVP bursting is open to future research.

Pharmacology connects NVP release and Ca^{2+} elevation. Treatment with an inhibitor of FGFR completely suppresses both NVP release and Ca^{2+} elevation, without perturbing the leftward fluid flow. Respectively, RA or SHH-N rescue both NVP release and Ca^{2+} elevation (either completely or partially), thereby preserving a left dominance. Although IHH could not facilitate NVP release, the bilateral elevation in Ca^{2+} following IHH treatment suggests that it acts downstream of NVP release and may in fact be transported together with NVPs or might also be secreted from the left side of the ventral node by a different mechanism. These findings suggest that the release of NVPs is regulated by morphogens such as FGF, SHH-N, and RA and that NVPs carry the signal for Ca^{2+} elevation. Although RA and SHH are transported by NVPs, signaling by NVPs is distinct from

the canonical signaling pathway for the following reasons: first, when added in excess, SHH generally does not have the effect of elevating Ca^{2+} bilaterally (Y.T. and N.H., unpublished data). Second, knockout of either the *Shh* or *Raldh2* gene (involved in RA synthesis) in mice does not apparently perturb the initial LR determination (Tsukui et al., 1999). And finally, both RA and SHH can rescue the Ca^{2+} elevation on the left side.

Consequently, the discovery of NVP flow provides the first experimental evidence that morphogens are actually transported by nodal flow, and the existence of NVPs expands our understanding of the extracellular transport of morphogens. The evidence correlating the leftward transport of NVPs to a left-specific elevation in Ca^{2+} suggests that NVP signaling is indispensable for the initial left-right determination process in the mouse ventral node.

Noncanonical Hedgehog Signaling by NVPs

Is NVP-mediated signaling unique to LR determination in the mouse ventral node? SHH is contained in NVPs and can also activate their release (Tanaka et al., 2005). Currently, two hedgehog (Hh) signaling pathways are considered: the canonical short-range pathway that is dependent on Patched/Smo/Gli and the noncanonical and long-range pathway (Ingham and McMahon, 2001; Torroja et al., 2004).

Canonical short-range Hh signaling might be functional in the ventral node because Smo is localized on the nodal cilia (Corbit et al., 2005). However, its action is apparently symmetrical; thus, it is not likely to be mediated by NVP flow. The components downstream of the canonical Shh pathway, *Ptch1*, *Gdf1*, and *Cryptic*, are expressed in a symmetrical manner within or on the periphery of the ventral node. This pathway is indeed essential for correct LR determination (Tsukui et al., 1999; Yan et al., 1999; Rankin et al., 2000; Zhang et al., 2001), probably via *Lefty-1* expression on the midline (Meno et al., 1998). Because of the symmetrical nature of its signaling, it may be mediated by a freely diffusible form of SHH, called SHH-Np (Zeng et al., 2001).

Recently, a candidate for an evolutionary counterpart of NVP signaling has been identified in a noncanonical Hh signaling pathway of *Drosophila*. From the analysis of the imaginal disc of *Drosophila* larva, a lipid-mediated long-range pathway for noncanonical Hh signaling has been revealed (Panáková et al., 2005). The imaginal disc is a primordial structure that gives rise to legs and other organs and has a clear A-P boundary whose posterior side has an expression domain of Hh. Cells within 10 μm of the boundary respond to Hh by activating the transcription of *collier* and *patched* by the canonical pathway, whereas cells further away respond by differentially activating the transcription of *decapentaplagic* (*dpp*). Interestingly, the latter long-range signaling is shown to be transmitted by extracellular lipoprotein particles called argosomes (Greco et al., 2001) containing apolipophorins I and II (ApoLI and ApoLII), Wingless (Wg), Hh, and GPI-linked proteins. RNAi knockdown of lipophorin reduces the range and strength of the long-range signal and conversely enhances the strength of the short-range signal, suggesting that these two modalities of Hh signaling are mutually exclusive. Both NVPs and argosomes could be the same type of membrane-sheathed lipoprotein particles, although the utrastructure of argosomes has yet to be determined. Recent evidence suggests that a lipoprotein-mediated mechanism of signaling could also function in the regulation of the immune system (van den Elzen et al., 2005).

Thus, signaling by NVPs likely mediates a long-range noncanonical Hh signaling pathway that elevates Ca^{2+} on the left side by an unresolved molecular mechanism. As RA can synergistically enhance the release of NVPs with SHH-N, these two signaling pathways converge on NVPs. Recently, a putative lipid binding/transfer protein Lplunc1 protein has been found to be expressed bilaterally on the nodal crown cells of 1-somite-stage embryos (Hou et al., 2004). Its expression gradually comes to have a left-side dominance as a consequence of nodal flow. This may suggest the existence of an asymmetrical mechanism for the turnover or sensing of lipids that have been transferred to the left side by the NVP flow.

The Ventral Node, an Evolutionary Showcase

Thus, the leftward flow of NVPs in the ventral node is a good candidate for the initial mechanism that breaks the LR symmetry of mouse embryos. However, this mechanism is not apparent in some other organisms (reviewed by Levin [2005]). Although the monocilia in the ventral node or its equivalent structures seem to be evolutionarily conserved (Essner et al., 2002), the leftward nodal flow might not be the initial determinant for the LR asymmetry in some other vertebrates, such as frogs, birds, and fish. In the frog, the LR axis is established extremely early in development and is linked to the formation of the DV axis (Yost, 1991). Maternal mRNA of the H^+/K^+-ATPase is already localized asymmetrically during the initial two cleavages of fertilized eggs, and this localization is essential for left-right determination (Levin et al., 2002). Thus, the laterality has already been generated 2 hr after fertilization in frog eggs. The Hensen's node of the chick is morphologically tilted before the neurulation stage (Kölliker, 1879). It is obvious that these LR asymmetries are established before the formation of monociliated cells on the ventral midline. Furthermore, although the nodal monociliated cells in frogs and chicks do express left-right dynein that is an essential motor for the motility of nodal monocilia (Essner et al., 2002), the cilia do not face into a cavity but instead are embedded in a dense array of cells (Essner et al., 2002; N.H., unpublished data). Thus, it is quite unlikely that these monocilia produce a laminar fluid flow in either frog or chick embryos.

In zebrafish, the situation is more complicated. Nodal flow does exist in Kupffer's vesicle (KV) as mentioned above, and is essential for the left-side Ca^{2+} elevation

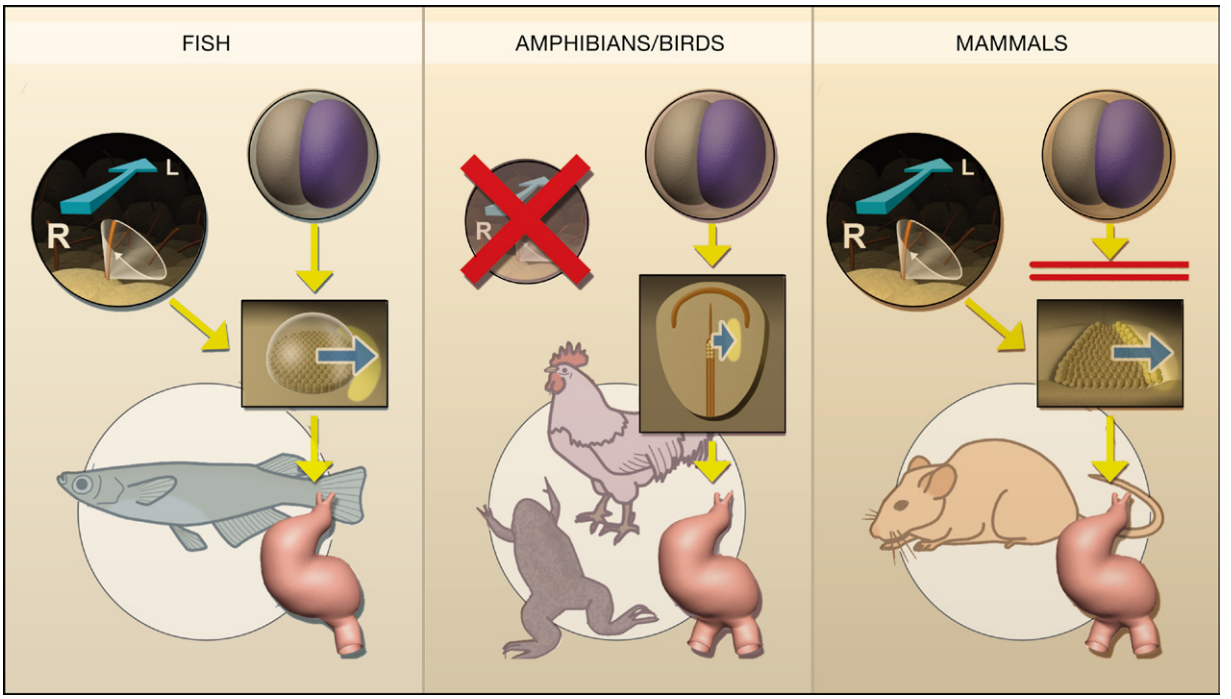

Figure 6. The Breaking of LR Symmetry in Vertebrates
Fish might be the best archetype for the breaking of LR symmetry in vertebrates because they have two independent sources of LR asymmetry: the first occurs with the initial cleavage of the fertilized egg, and the second is created by the leftward nodal flow. The ventral node serves as the "knot" tying these two sources of information together and is necessary for relaying information to the left lateral plate mesoderm (LPM), where a well-conserved LR signaling pathway for asymmetrical development takes place. Nodal flow might have retrogressed in the amphibian and avian lineages but could have reevolved in viviparous animals, such as mammals, to compensate for the loss of the information from the asymmetry of early cleavage due to implantation.

during the neurulation stage and for the subsequent LR asymmetry of development (Kramer-Zucker et al., 2005; Essner et al., 2005). However, treatment of eggs with an H^+/K^+-ATPase inhibitor during the cleavage period results in LR patterning defects without disturbing the leftward flow in KV (Kawakami et al., 2005). These results suggest the presence of at least two different and independent symmetry-breaking processes in the course of fish development, one that is dependent on the differential activity of H^+/K^+-ATPase in early-cleavage-stage embryogenesis and another requiring nodal flow at neurulation. The leftward direction of nodal flow is autonomously defined without referring to the LR asymmetry at the early-cleavage stage, whereas nodal flow is not sufficient for the subsequent development of LR asymmetry of the visceral organs such as the heart, liver, and gut. Thus, the earlier symmetry breaking might be the principal determining event. The ventral node might be required for the downstream signaling pathway that may amplify or relay the LR information established during the early-cleavage stage to properly accomplish the subsequent developmental processes in the mesoderm (Raya et al., 2004).

It is probable that the nodal cilia also have roles other than the generation of mechanical flow, especially in Hh signaling. In addition to the signaling mediated by NVPs, nodal cilia might also contribute to the canonical Hh signaling pathway. There are many proteins localized on dot-like structures in the nodal cilia, such as FGFRs, Smo, and PC2. Some of these might be essential for Smo-dependent processing of Gli3 in the canonical Hh signal-transduction pathway. Thus, the transport of protein complexes in the cilia by the KIF3 motor might be required for Hh signaling (Huangfu et al., 2003; Huangfu and Anderson, 2005; Liu et al., 2005; Corbit et al., 2005). Nodal flow may have been lost during the evolution of both birds and amphibians because of the redundancy in the processes that break symmetry. Instead, the cilia in these species may propagate preexisting information concerning laterality, possibly through Hh signaling, which is also essential for LR determination in those animals (Levin et al., 1995; Sampath et al., 1997).

However, in mammals, the determination of body axes occurs at a much later stage. Polarity information prior to the implantation of the embryo may have little influence on the body axis formation after implantation. To date, no biased asymmetrical events have been found in mouse embryos earlier than the stage of nodal flow. In fact, an experiment in mice using artificial flow (Nonaka et al., 2002) demonstrates that the breaking of symmetry by nodal flow dominates the earlier polarity information. Hence, we speculate that nodal flow might have become

the principal determination process with the appearance of viviparity, which might entail the need of a more robust strategy for orienting LR asymmetry from the cues present at the time of gastrulation. Joseph Yost has proposed a "zootype" hypothesis for LR determination (Yost, 1999), in which expression of left-specific genes are evolutionarily conserved only in a phylotypic stage and that the expression patterns, regulation, and functions of genes in earlier or later stages of development are not necessarily conserved among vertebrates (reviewed in Palmer [2004]). Our new hypothesis poses that the ventral node is a "knot" of redundant symmetry-breaking processes. This provides an intriguing explanation of the apparent differences between the earliest events in left-right determination among species (Figure 6).

Concluding Remarks

As discussed in this review, nodal flow is generated by cilia that are built by KIF3 molecular motors. Nodal flow is an autonomous symmetry-breaking process. The posterior tilt and clockwise rotation of nodal cilia in viscous fluids generates leftward flow. Although there are redundant symmetry-breaking processes in lower vertebrates, nodal flow is probably essential and sufficient for the determination of the laterality of visceral organs in mammals. NVP flow is a newly identified mechanism of morphogen transport by nodal flow, which may activate Ca^{2+} elevation specifically on the left side. This may be one of the first examples of a lipoprotein-mediated noncanonical Hh signaling pathway in mammals. These points provide a framework for studying the biophysical and biochemical properties of signal transduction in the ventral node.

Supplemental Data

Supplemental Data include six movies and can be found with this article online at http://www.cell.com/cgi/content/full/125/1/33/DC1/.

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