Cilia are at the heart of vertebrate left–right asymmetry
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Handed asymmetry of the shape and position of the internal organs is found in all vertebrates, and is essential for normal cardiac development. Recent genetic and embryological experiments in mouse embryos have demonstrated that left–right asymmetry is established by directional flow of extraembryonic fluid surrounding the node, which is driven by motile monocilia.

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Abbreviations
e   embryonic day
FGF   fibroblast growth factor
IFT   intraflagellar transport
LR   left–right
lrd   left–right dynein
MT   microtubule

Introduction
Formation of the three embryonic axes defines the vertebrate body plan. The last axis to arise divides the embryo into asymmetric left and right halves. For decades, the only useful tools to study left–right (LR) development were the rare occurrences of spontaneous or insertional mutational events in the mouse or human. With the advent of targeted mutagenesis, an ever-increasing number of genes have been found to play a role in LR development. With more refined dissection of the function of these genes, in all or only a subset of cells of the embryo, we should soon know when and how the left and right sides of the body diverge. In this review, we focus on how cilia found at the node are instrumental in developing vertebrate LR asymmetry.

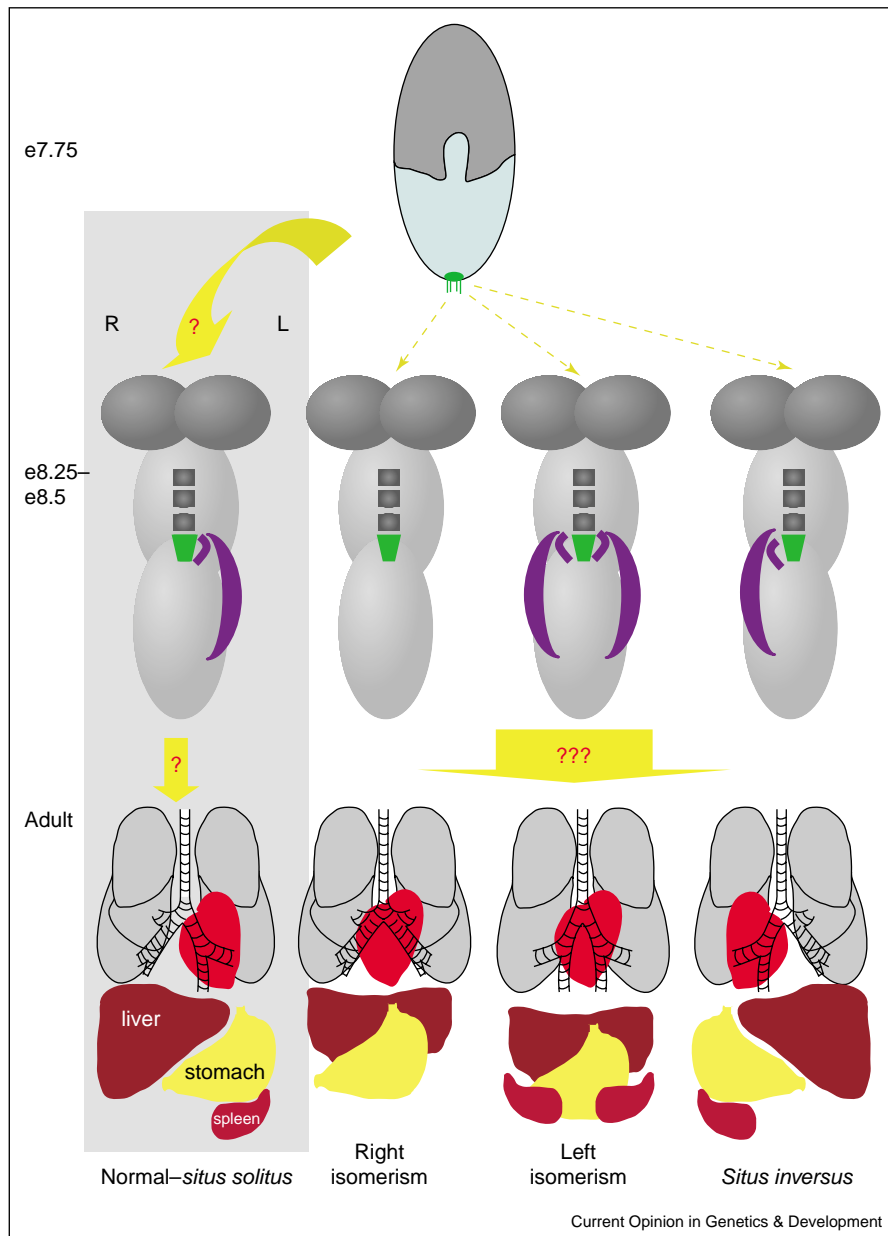## Bilateral symmetry to handed asymmetry to asymmetric gene expression
The development of LR asymmetry requires several distinct developmental processes, summarized in Figure 1.

The first of these is a mechanism to create handed asymmetry in a previously bilaterally symmetric embryo. The second signals this small-scale molecular asymmetry across larger regions of the embryo. Finally, regional molecular asymmetry must be converted into asymmetric organogenesis. Over the past decade, much progress has been made elucidating asymmetrically expressed molecules that comprise a pathway that signals small-scale LR asymmetry across the embryo (reviewed in [1]). In mouse, the first molecular asymmetry arises when the *nodal* gene, initially expressed throughout the node, develops markedly asymmetric expression at the left side at embryonic day (e) 8.5 (Figure 1) [2–4]. In the presence of the *nodal* co-factor *cryptic*, the limited domain of asymmetric *nodal* expression at the left of the node then expands to the left lateral plate mesoderm [5], where *lefty-2* is also expressed. *lefty-1* is expressed in the left side of the neural floorplate where it may function as part of a midline barrier. Overall, asymmetric gene expression begins at the node, and asymmetric expression of *nodal* and other lateralized molecular markers then proceeds both laterally and caudally. Expression of *nodal* at the left edge of the node is essential for LR patterning [6]. It is important to note, however, that asymmetric expression of a signaling molecule like *nodal* cannot arise *de novo*, and requires preexisting LR positional information. Here, we focus on emerging evidence that bilateral symmetry is broken by a novel mechanism: motile embryonic cilia generate directional flow of extraembryonic fluid surrounding the node.

Mouse mutants affecting both cilia and left–right development
To date, at least 27 independent mouse mutations affecting LR development have been identified (reviewed in [7•]). Some are known to be required for the formation of the embryonic node itself, such as FGF8 and *nodal*, whereas others are in conserved signaling pathways, such as *notch1*, *notch2* and *Delta1* [8–10], or in secreted growth factors, of which several are asymmetrically expressed such as *lefty2*. However, surprisingly about one-third (9/27) of the known LR asymmetry mutations affect genes that have a role in ciliary biogenesis and function (Table 1). For example, defects in the genes *KIF3A* and *KIF3B*, encoding components of heterotrimeric kinesin, and defects in the gene encoding TG737 (polaris), all result in embryos with a complete absence of cilia *and* abnormal development of LR asymmetry [11–14]. Both heterotrimeric kinesin and TG737 are essential components of the machinery required for intraflagellar transport (IFT) [15,16]. IFT is required for ciliary biogenesis in organisms ranging from *Chlamydamonas*, to *Caenorhabditis elegans* to vertebrates, and IFT components are also highly

Figure 1

Summary of the development of mammalian LR asymmetry. The embryo is apparently symmetric at e7.75. The node, located at the tip of the embryo (shown in green) is ciliated and generates leftward nodal flow. At e8.25 (0–3 somites), the previously symmetric perinodal expression of the growth factor *nodal* becomes asymmetric (shown in blue). Shortly thereafter at e8.5, a large region of asymmetric *nodal* expression appears in the left lateral plate mesoderm (shown in blue). Several other genes also become asymmetrically expressed at this time, including the transcription factor Pitx2, which is expressed in a more posterior region of lateral plate mesoderm abutting the heart primordia, and eventually in the heart itself. At e8.5, the embryo turns, the heart forms its characteristic rightward (D) loop, and molecular asymmetry is converted to asymmetric organogenesis of the lungs, heart, spleen and gut. Normal LR development results in situs solitus, and is highlighted by the gray box at the left of the figure. When LR development does not proceed normally, a range of outcomes is possible. Mirror-image reversal of all the organs is called situs inversus, and is usually not associated with significant pathology. Complete failure to break bilateral symmetry is thought to result in left isomerism (and the development of bilateral multi-lobed lungs and bilateral spleens) or right isomerism (bilateral bi- or uni-lobed lungs and absent spleen). The isomerisms have associated severe complex cardiac malformations.

conserved between species [17]. A second group of genes affecting cilia encode proteins that have a role in ciliary motility. Defects in left–right dynein (lrd) and DNAH5 result in paralyzed cilia as a result of defective axonemal dynein motors [18–20]. Dyneins are large multi-subunit ATP-dependent motor proteins that move cargo towards

Table 1

Correlation of ciliary phenotypes and LR phenotypes.

Mutation	Gene	Organism	Cilia	Nodal flow	*Nodal* expression in LPM	LR phenotype
Random asymmetry						
iv [14,15,24,30]	*lrd*	Mouse, human	Paralyzed	–	Left, right, bilateral or absent	Random asymmetric
DNAH5 [16]	*Axonemal dynein heavy chain*	Human, mouse	Paralyzed (adult)	?	?	Random asymmetric
DNAI1 [25]	*Dynein intermediate chain*	Human	Paralyzed (adult)	?	?	Random asymmetric
hfh4 [20,21]	*Hepatocyte nuclear factor/fork-head homologue 4*	Mouse	Present, fn. unknown	?	Left, right bilateral or absent	Random asymmetric
Bilateral symmetry						
Tg737 [10,35••]	*Polaris*	Mouse	Absent	?	Bilateral	Symmetric (random, abnormal heart loop; no turning)
KIF3A [9]	*Heterotrimeric kinesin*	Mouse	Absent	–	? (*lefty* bilateral or absent)	Symmetric (like Tg737)
KIF3B [8]	*Heterotrimeric kinesin*	Mouse	Absent	–	? (*lefty*, bilateral or absent)	Symmetric (like TG737)
Other						
Pkd2 [33]	*Polycystin*	Mouse	Unknown	?	Absent	Symmetric? Bilateral? Right
inv [17,18,19,30]	*Inversin*	Mouse	Present	Slow, leftward	Right	Non-random reversal

LPM, lateral plate mesoderm.

the minus end of microtubules (MTs), or generate ATP-dependent bending of cilia and flagella. Finally, several mutations affect proteins that are associated with cilia but whose function is less well understood. Inversin, an ankyrin-repeat containing protein of unknown function, is necessary for normal LR development and has been shown to localize to node cilia [21,22,23•]. The transcription factor Foxj1 is also required for LR development in addition to the development of cilia in the brain and trachea, and although embryonic cilia are present in Foxj1-deficient embryos, their function may be abnormal [24,25].

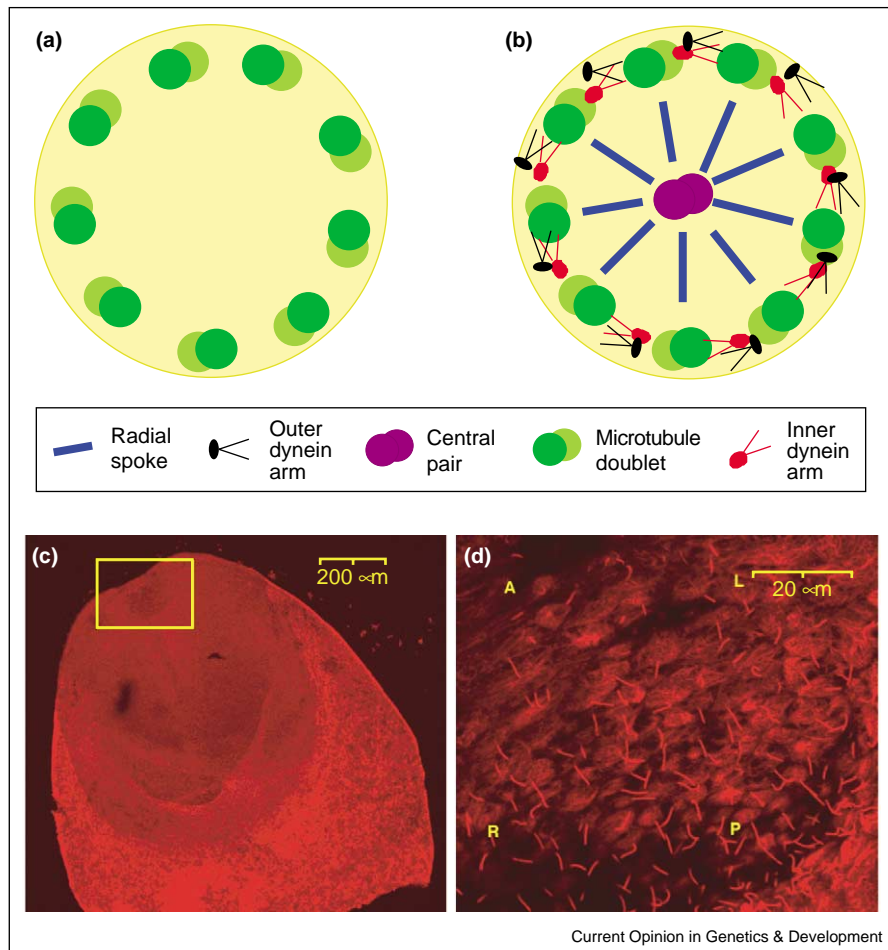## Kartagener syndrome: an additional link between cilia and left–right development

Kartagener syndrome is a human syndrome characterized by recurrent respiratory infections, chronic sinusitis and male infertility [26]. Most cases are sporadic and may represent recessive inheritance, but occasional dominant transmission has been reported [27]. One-half of patients with Kartagener syndrome have complete situs inversus. Mutations in both dynein intermediate chains and dynein heavy chains have been demonstrated in some families with Kartagener syndrome, providing the molecular link between the structural ciliary defects in Kartagener syndrome and specific ciliary proteins [20,28,29]. Analysis of cilia from affected patients shows a range of defects, including absent outer or inner dynein arms or defective radial spoke structures. These defective cilia are immotile, and provide a ready explanation for the respiratory disease and male infertility in Kartagener patients. The link with abnormal development of LR asymmetry, how-

ever, is less apparent. As early as 1976, Afzelius postulated "that cilia on the embryonic epithelia have a certain position and a fixed beat direction and that their beating is somehow instrumental in determining the visceral situs" [26], but many years elapsed before any cilia, and in particular motile cilia, were found in embryos at a time in embryogenesis consistent with a role in the development of LR asymmetry.

The structure and function of cilia

Cilia are large complex organelles that protrude up to 20μM beyond the cytoplasm. They are composed of a MT skeleton comprising 9 MT doublets surrounded by the ciliary membrane (Figure 2a). This basic structure has been adapted for many distinct cellular functions. Some cilia cover the apical surface of epithelial cells in the trachea, choroid plexus or oviduct. Here, thousands of individual cilia arise from basal bodies located beneath the cell membrane of a single cell. Most (if not all) of these cilia contain a central pair of MTs, linked to the outer doublets by radial spokes (Figure 2b). A combination of outer and inner arm dynein motors, along with the associated dynein-regulatory proteins, hydrolyze ATP to generate ciliary movement. The concerted action of large numbers of closely spaced motile cilia transport surrounding fluid, such as tracheal secretions or cerebrospinal fluid. In contrast to the highly specialized ciliated epithelia, almost all cells — with the exception of a few myeloid and lymphoid lines — can carry monocilia, so-called because they are a single cilium per cell, arising from the centriole. Like the cilia found in ciliated epithelia, monocilia are

Structure and distribution of node monocilia. **(a)** The MT scaffold found in all cilia is built from nine MT doublets, each consisting of an A-tubule and a B-tubule. All cilia are chiral, with the chirality based on the structure of the underlying centriole or basal body. **(b)** The structure of a 9+2 tracheal cilium. A central pair of MT is connected to the outer MT doublets by radial spokes. Inner and outer arm dynein arms link the outer doublets to each other to generate ATP driven sliding movement of the axoneme. **(c)** Mouse embryo at e7.75. The node is a bowl-shaped structure that is 50–150μM across (indicated in this figure by the yellow square). It is located at the anterior end of the primitive streak, which has begun to regress from the ventral tip of the embryo towards the posterior end. **(d)** Close-up view of node monocilia, labeled with anti-acetylated tubulin antibody. There is a single cilium on every node cell, with a total of 200–300 cilia at the fully developed node. Each cilium measures 7–10μM in length, and the cilia are spaced 5–10μM intervals.

constructed on a scaffold comprising 9 MT doublets. Unlike the stereotypical arrangement of MTs, motors and structural proteins found in ciliated epithelial cilia, the contents of monocilia are extremely varied. By displaying a variety of receptors, these cilia are adapted to function as 'cellular antennae', reaching out beyond the cell surface to capture signals ranging from light in the photoreceptor in the retina, to mechanical and chemical stimuli in the ciliated cells found at the anterior tip of *C. elegans* (reviewed in [30]). Although most monocilia are immotile, some carry dynein motors and beat.

The node is a ciliated structure

The mouse node, or organizer, develops at the time of gastrulation in a region at the distal tip of the embryo where ectodermal and endodermal cells contact each other, separated only by a basement membrane. The endodermally derived node cells are able to induce a partial secondary axis when transplanted to other regions of the embryo, indicating that these cells posses 'organizer' capabilities and are the mammalian equivalent of Hensen's node in the chick [31]. As gastrulation progresses, node cells coalesce to form an organized bowl-shaped structure, measuring up to 100μM across, and containing 200–300 cells (Figure 2c). When the primitive streak regresses, the node moves towards the posterior end of the embryo, and ingresses after somitogenesis. Node cells are mitotically inactive, and after gastrulation populate only the notochord [32,33]. Each node cell carries a monocilium (Figure 2d). Because of the tight packing of the central

node cells, the node monocilia are fairly closely spaced: each cilium is 5–10μM long, and separated from its neighbor by only 5μM. It remained unclear whether node monocilia were functional, or whether they were merely signposts indicating a mitotically inactive cell. Structural and videomicroscopic analyses of node monocilia provided conflicting results: some studies showed ciliary movement [33], others showed immotile cilia [32]; further, transmission EM data showed 9+0 cilia with none of the dynein arms required for motility.
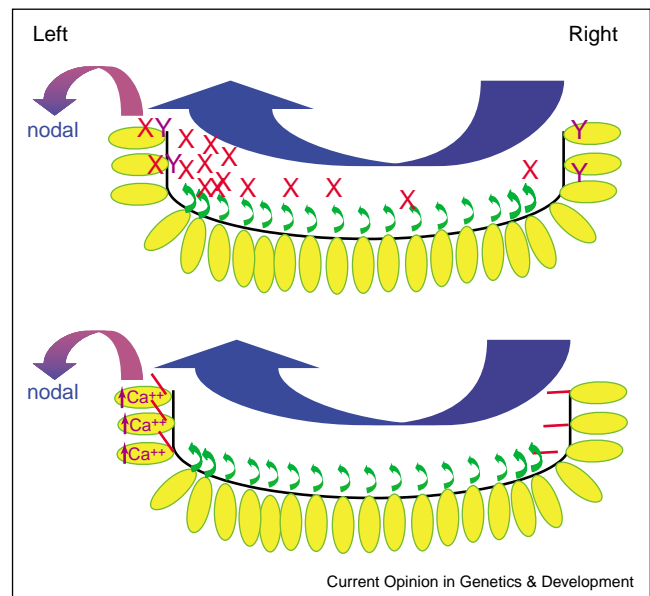
Nodal flow initiates left–right asymmetry

The first evidence for a function of node monocilia in initiating LR asymmetry came from study of mice with a mutation affecting KIF3B, an essential component of IFT. The KIF3B knockout mice have defective development of LR asymmetry, along with midgestational lethality and a complete absence of node monocilia. This observation led to detailed videomicroscopic analysis of the node in wild-type mouse embryos. Strikingly, wild-type node monocilia were noted to be motile. Further, fluorescent beads placed in the fluid bathing the node demonstrated that the perinodal fluid moved by rapid (10–20μM/second) laminar flow from the right side of the node to the left side of the node. This directional fluid movement is called 'nodal flow' [11]. Nodal flow occurs only during a few hours during the late neural plate stage of development, immediately before the formation of the first somite, and before there is any molecular evidence of LR asymmetry [34]. In mice with mutations in the axonemal dynein lrd, the node monocilia are paralyzed, and there is no nodal flow [19,34]. These observations strongly suggested that node monocilia have a central role in LR development, but they did not absolutely prove that the cilia themselves created LR asymmetry. The possibility remained that the paralyzed cilia in the *lrd* mutant embryos were instead merely correlative to a defective node cell. The final proof that nodal flow itself initiates mouse LR asymmetry came from elegant experiments on the effect of artificial nodal flow on cultured mouse embryos [35••]. After removal of the membranes surrounding the node, live embryos were fixed in position and exposed to the flow of culture medium. Rapid rightward (opposite to the normal leftward) nodal flow applied to wild-type embryos resulted in reversal of the direction of observed nodal flow, and reversed LR development. Notably, application of normal leftward nodal flow was sufficient to completely rescue the LR phenotype of mice with slow nodal flow as a result of mutations in inversin [23•], and mice with paralyzed node monocilia as a result of mutation in lrd [35••], indicating that both inversin and lrd function in LR development by generating leftward nodal flow.

How does nodal flow lead to asymmetric gene expression?

Net leftward flow of the extracellular fluid surrounding the node generated by directional movement of node

Two models for the mechanism by which nodal flow establishes LR asymmetry. **(a)** Morphogen gradient model. Nodal flow (indicated in blue) generated by motile node monocilia (indicated in green) moves a soluble morphogen, indicated by the X, towards the left side of the node. This establishes a concentration gradient of X, which then interacts with its receptor Y at the left border of the node, leading to asymmetric expression of genes such as *nodal*. **(b)** Mechanosensory cilia model. Motile cilia (indicated in green) generate leftward nodal flow (indicated in blue). The direction of nodal flow bends mechanosensory cilia (indicated in red) on the left border of the node against their cell bodies — whereas cilia at the right border of the node are extended away from the cell bodies. The bend of the node monocilium against its cell body triggers an increase in intracellular calcium in cells at the left margin of the node, which subsequently results in asymmetric expression of genes such as *nodal* at the left border of the node.

monocilia is the event that breaks embryonic bilateral symmetry in the mouse. How this flow activates asymmetric gene expression at the node and beyond, however, remains a mystery. Nodal flow could establish a gradient of a soluble morphogen at the left side of the node, whereupon it interacts with its receptor and sets up the cascade of asymmetric gene expression that results in, for example, eventual left-sided *nodal* and *PitX2* expression (Figure 3a). Recent experiments demonstrate that artificial leftward nodal flow can establish normal LR development; notably, however, normal LR development was entirely independent of both the volume and flow-rate of the fluid surrounding the embryo [35••]. This observation is somewhat puzzling because a stable gradient of a soluble morphogen would depend on an extremely tight coordination between the kinetics of morphogen production, rate of nodal flow and kinetics of morphogen binding and degradation. Several secreted molecules found at the node have a role in LR development and are possible candidates for the morphogen(s): GDF1 (growth and

differentiation factor 1), *nodal*, and *indian/sonic hedgehog*, to name a few. A second possibility is that directional fluid flow activates sensory cilia located on the left side of the node (Figure 3b), triggering an intracellular signal that results in left-sided secretion of signaling molecules such as nodal.

Sensory cilia have a role in polycystic kidney disease and LR development

Sensory cilia have recently been implicated in the pathogenesis of human polycystic kidney disease. Human autosomal dominant polycystic kidney disease presents with renal failure during the 4th to 5th decade of life due to multiple renal cysts. 15% of cases are due to mutation in the *Pkd2* gene encoding the cation channel polycystin-2 [36]. Curiously, mice with polycystic kidneys due to targeted mutation in *Pkd2* also have abnormal LR development [37•]. Polycystic kidneys are also found in several of the mouse mutants with ciliary abnormalities (Table 1), including *Tg737* and *inv* [14,22]. Observations that Polycystin-2, and the associated polycystin-1, localize to monocilia extending from the apical surface into the lumen of kidney tubules [38,39] suggested that cilia were the possible link between polycystic kidney disease and LR development. In *C. elegans*, the homologue of *Pkd2* is similarly expressed in the cell bodies and sensory cilia of the male-specific mating organ [40]. Two lines of evidence suggest that monocilia in the kidney function as mechanosensors. Bending of the monocilia on cultured MDCK cells generates a release of intracellular calcium [41,42]. The application of flow to ciliated renal tubular cells results in increased intracellular calcium, which is dependent on the presence of both intact cilia and polycystin-2 [43••]. In the kidney, it is postulated that cilia function as mechanosensors sensing fluid flow in the renal tubule. Polycystin-2 on the ciliary membrane transduces the flow signal into a calcium signal, which may lead to normal differentiation of renal tubular cells.

Node monocilia generate and sense nodal flow

Thus far, it has been demonstrated clearly that there are motile node monocilia, and that nodal flow is essential for LR development. If the production of nodal flow were the only role for node monocilia in LR development, one would expect that the LR phenotype of mice with immotile node monocilia would be identical to that observed in mice with absent node monocilia. This, however, is not the case. Mutations resulting in paralyzed cilia (in rows 1–4 in Table 1) generate random asymmetry: asymmetrically expressed molecular markers such as *nodal* and *lefty* are still asymmetrically expressed, and there is asymmetry of the final position of the organs; it is the handedness of the asymmetry that becomes random. By contrast, mutations that result in the failure to assemble node monocilia (rows 5–7 in Table 1), such as *KIF3A*, *KIF3B* and *Tg737*, result in maintenance of

bilateral symmetry. Here, normally asymmetric molecular markers are expressed symmetrically, becoming either bilateral or absent. On the basis of the detailed LR phenotype of mouse and human mutations affecting ciliary structure and function (see Table 1), it becomes apparent that there is a striking correlation between the ciliary phenotype and the LR phenotype: absent nodal flow due to absent monocilia results in a complete failure to break bilateral symmetry, whereas mutations that have no nodal flow due to immotile monocilia have random asymmetry. We envision that the dichotomy between random asymmetry versus retained bilateral symmetry may be explained by postulating that cilia have function(s) other than motility in the development of LR asymmetry. Some monocilia could be mechanosensors that sense leftward nodal flow, or chemosensors that detect a subtle morphogen gradient. These cilia would lack the necessary intraciliary machinery for motility but, for example, directional bending of this population would initiate a signal transduction pathway in a manner analogous to that observed in renal cilia. The existence of two node cilia populations, one motile, one sensory, should be easily testable and, if true, will provide a plausible link as to how nodal flow initiates LR development.

Conclusions

In the development of handed LR asymmetry, the mouse embryo takes advantage of the inherent asymmetry found in cilia and centrioles to generate directional nodal flow. Cilia may both generate and sense nodal flow. The two most unique features of this mechanism are that it is based almost entirely on mechanical forces, and that it occurs outside of the embryo itself. Some evidence supports that this mechanism is conserved across vertebrates, as fish, frogs and chicks all have nodal cilia equivalents, and all express lrd homologs [44•]. Interestingly, *Xenopus* may have another mechanism for the development of LR asymmetry, as it has been reported that there is asymmetry in mRNA localization of the α subunit of H^+/K^+ ATPase at the 4-cell stage, which is long before the appearance of cilia and lrd [45•]. It is possible that different timing during *Xenopus* development requires both mechanisms, one at early stages before the onset of zygotic transcription, and the other at later developmental stages. Alternatively, the eventual position and orientation of the centrioles that will determine monocilia location, and hence direction of nodal flow, may coincide with mRNA location of the H^+/K^+ ATPase at the 4-cell stage. Whether that colocalization is causative or coincidental remains to be seen.

Although recent insights have given us a glimpse as to how LR development unfolds, many questions remain unanswered. How are cilia oriented relative to the anterior–posterior axis, and how do cilia at the node coordinate their beat to generate laminar nodal flow? How does an asymmetric signal generated by nodal flow lead to asymmetric

expression of genes such as *nodal*? Finally, how does asymmetric gene expression lead to asymmetric organogenesis?

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