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Cilia, KIF3 molecular motor and nodal flow

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The establishment of left–right asymmetry during development of vertebrate embryos depends on leftward flow in the nodal cavity. The flow is produced by the rotational movement of the posteriorly tilted nodal cilia. However, it remains poorly understood how the nodal cilia are tilted posteriorly, and how the directionality of the flow is translated into gene expression patterns in the embryo. Recent studies have identified signaling molecules involved in these processes. First, planar cell polarity signaling has been shown to be involved in the posterior positioning of the basal bodies of nodal cilia, which leads to the posterior tilting of their rotation axes. Second, identification of putative receptors and signaling molecules suggests a link between the signaling molecules delivered by the nodal flow, and downstream signaling in the cells surrounding the nodal cavity and the lateral plate mesoderm.

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Introduction

In humans, internal organs such as the heart, spleen and pancreas reside on the left side of the body, whereas the gall bladder and the majority of the liver are on the right. This left–right (LR) asymmetry first becomes apparent during heart loop orientation. However, LR asymmetry is detectable during the earlier stage of somitogenesis by the asymmetrical expression of several genes, such as *Lefty-1* (*Leftb*), *Lefty-2* (*Ebaf*), *Nodal*, and *Pitx2*. In most cases, the expression of these genes is observed on the left side of the embryo [1–3]. However, the work by our lab and others has indicated that LR asymmetry has its origins at even earlier stages of development. The organs of about half of the human patients affected by Kartagener's syndrome are reversed in orientation. Affected individuals also have immotile sperm and defective cilia in their airways. Thus, this phenotype indicated that cilia may control LR asymmetry [4], although which cilia are involved and at which stages of development were unknown until recently. Results of many studies have suggested that the 'node,' a transient midline structure formed during gastrulation (Figure 1a and b), is important for LR determination [1]. 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 node cells are primary cilia that lack the central pair of microtubules. Thus, node cells have a 9 + 0 microtubule arrangement, rather than the 9 + 2 microtubules of conventional motile cilia in normal ciliated cells. Therefore, based on their ultrastructure and videomicroscopic observations, nodal monocilia were once thought to lack motility [5]. However, our discovery that nodal monocilia move rapidly to generate a leftward flow (nodal flow) of extraembryonic fluid suggested new roles for nodal monocilia [3]. In this review, we briefly summarize the discovery of nodal flow, introduce recent advances and discuss the mechanisms by which LR symmetry is broken by nodal monocilia (Figure 1).

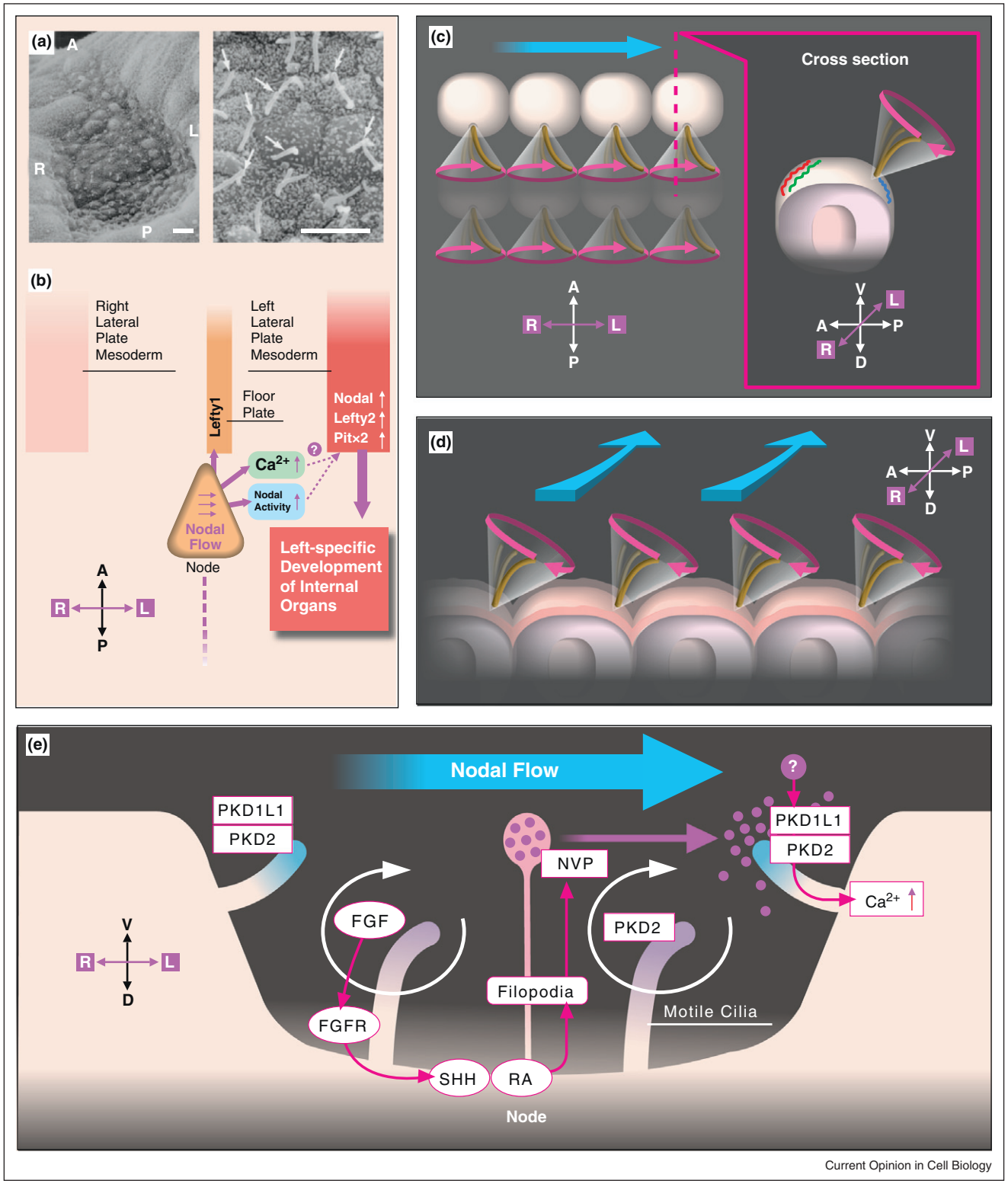
How the nodal flow was discovered

The nodal flow was discovered through molecular genetic studies of the kinesin superfamily (KIFs) KIF3 motor. KIFs mainly transport various cargoes such as membranous organelles, protein complexes and mRNA along microtubules within cells [6].

The KIF3 complex is composed of a heterodimer of the motor proteins, KIF3A and KIF3B, and an associated protein, KAP3. Studies with *Kif3a* and *Kif3b* knockout mice revealed that roughly 50% of both *Kif3a*-deficient and *Kif3b*-deficient mice showed reversed heart loops, the first visible sign of LR asymmetry, whereas the rest were normal. Our analyses suggested that both KIF3A and KIF3B act at an earlier step than *Lefty-2*, the most upstream gene in the LR-determination pathway (Figure 1b). Studies of the node then unexpectedly revealed that nodal cilia were lacking in *Kif3*$^{-/-}$ mutants.

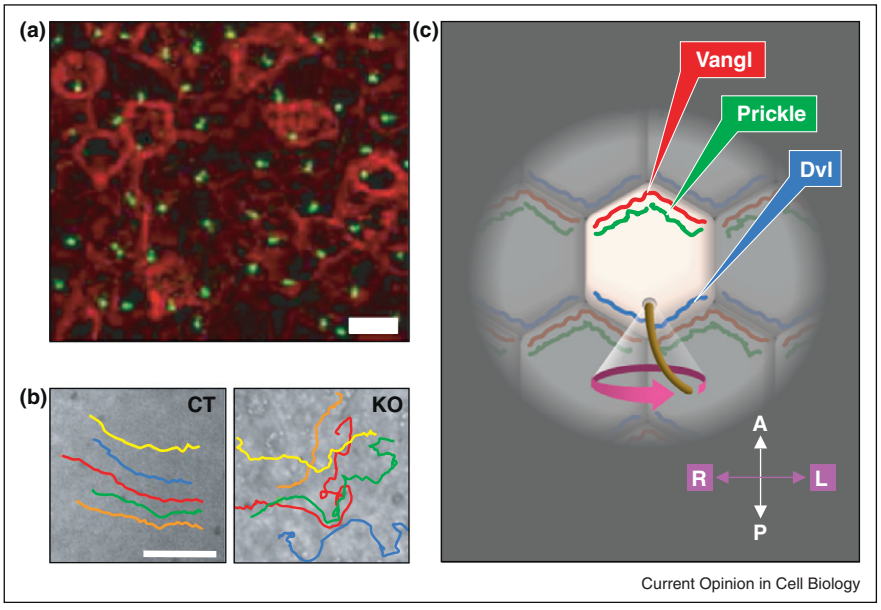
Previously, the nodal cilia were believed to be immotile in wild-type mice [5]. However, we surprisingly found that they rotate vigorously at approximately 600 rpm. Furthermore, these rotating cilia generate leftward flow of fluid in the node cavity (nodal flow) [7••,8,9]. In *Kif3*$^{-/-}$ mutant mice, the nodal flow was not generated because of the lack of nodal cilia. Further studies by our group and others demonstrated that the directionality of this nodal flow is necessary and sufficient for the determination of the LR axis [9,10].

Figure 1



Initial symmetry breaking process in vertebrates.**(a)** Scanning electron micrographs of wild-type mouse nodes in the ventral view. Note that the node is a small triangular concave structure located at the midline of early somite stage embryos. Arrows, monocilia. Bars, 5 μm. Upper, anterior (A); lower, posterior (P); left of the figure, right (R); right of the figure, left (L) in **(a)**–**(c)**.**(b)** Readouts of nodal flow. According to the direction of the nodal flow, the downstream side (the left half) of the floor plate expresses *Lefty1* and left lateral plate mesoderm expresses *Nodal*, *Lefty2* and *Pitx2*, possibly through Ca elevation and nodal activation on the left periphery of the node.**(c and d)** Generation of leftward flow by tilted cilia rotation, represented by ventral

Figure 2



Planar cell polarity signaling and cilia positioning.
(a) Posterior positioning of the cilia represented by immunofluorescence microscopy. Red, cell margin; Green, cilia. Reproduced from [11]. Upper, anterior (A); lower, posterior (P); left of the figure, right (R); left of the figure, right (L); throughout this figure. Bar, 10 μm.
(b) Disorganization of the nodal flow in a mouse mutant for PCP genes. CT, control; KO, *Vangl2*$^{\Delta/\Delta}$; *Vangl1*$^{gt/gt}$ (reproduced from [23••]). Bar, 20 μm.
(c) Schematic representation of PCP signaling in nodal pit cells. Vangl (Red) and Prickle (Green) in the anterior pole and Dishevelled (Dvl; Blue) in the posterior pole may polarize the nodal pit cells along the anterior–posterior axis, resulting in posterior positioning of the monocilia (modified from [55•]).

PCP, cilia positioning and leftward flow generation

One of the remaining key questions was how the direction of the nodal flow is determined. Experimental and theoretical studies revealed a simple geometrical mechanism. High temporal resolution observation of nodal cilia movement (500 frames per second [fps]) demonstrated that the rotating axis of the nodal cilia is posteriorly tilted, and theories of fluid mechanics confirmed that this tilted rotation produces directional flow (Figure 1c and d) [11–16]. This result clearly explains how the LR axis is determined *de novo* in a manner consistent with the preexisting dorsoventral (DV) and anteroposterior (AP) axes.

However, the mechanism by which the AP axis information generates the posteriorly tilted arrangement of nodal cilia is still unclear. We have noticed that the basal body, the root of the cilia, is mostly located at the posterior side of the node cell [11,12] (Figure 2a). The convex curvature of the apical plasma membrane of the node cells may explain that the posterior positioning of the basal body will lead to posterior tilting of the cilia. In earlier developmental stages, the nodal cilia are located near the center of the node cell, where they can produce only a weak vortex. The nodal cilia gradually move to the posterior side and then produce strong leftward flow [9,17••]. This scenario is similar to the planar cell polarity (PCP) pathways that govern the coordinated positioning and orientation of bristles in *Drosophila*, and of sensory cilia in vertebrate hair cells [18]. We thus proposed that PCP is the putative mechanism that mediates the posterior positioning and tilting of the nodal cilia [11], and recent studies confirmed this hypothesis.

The initial study that confirmed the hypothesis investigated the interaction between Inversin (Inv) and Dishevelled (Dvl) [19]. *Inv* mutant mice developed randomly

(Figure 1 Legend Continued) (**c**, left) and lateral (**c**, right; **d**) views of the node. Owing to the expression of planar cell polarity (PCP) genes in the respective anterior and posterior poles of the cells (red, blue, and green lines in the inset of **c**), the cilia are positioned on the posterior ends of the node cells, resulting in the clockwise-rotating axes being tilted posteriorly. Accordingly, the surface shear resistance influences the left-to-right cilia sweep more than the right-to-left sweep, to generate net leftward flow of the extracellular fluids. V, ventral; D, dorsal.**(e)** NVP hypothesis of signal propagation in the node. FGF signaling in the cilia may activate sonic hedgehog (SHH) and retinoic acid (RA) signals that release nodal vesicular parcels (NVPs) from the cell surface with the help of filopodia-like cellular protrusions. NVPs are transported to the left by the nodal flow and elevate intracellular Ca on the left side, possibly through chemical activation of PKD1L1/PKD2 receptors on the sensory cilia on the left margin of the node.

oriented nodal cilia that resulted in abnormal LR patterning [9,11]. Dvl is one of the core PCP components and is localized near the basal body of cilia [20]. The link between Dvl and LR determination was further suggested by the study of Bicaudal C (BicC) mutant mice [21•]. The posterior positioning of the nodal cilia and the leftward nodal flow was disrupted in BicC mutant mice. In the same study, BicC was shown to interact with Dvl and was suggested to be involved in the regulation of Dvl-mediated PCP signaling. More direct results were reported last year. Dvl mutant mice were generated that showed defective LR patterning and random positioning of the basal body of nodal cilia [17••]. It was also shown that Dvl-GFP was enriched at the posterior edges of node cells (Figure 2c).

Other PCP core proteins were also shown to be involved in nodal cilia positioning. Van Gogh like (Vangl) and Prickle were shown to localize to the anterior edges of node cells [22••] (Figure 2c). Knock out of Vangl in mice disrupted the posterior positioning of the nodal cilia, resulting in abnormal nodal flow (Figure 2b) and defective LR patterning [22••,23••]. Vangl2 knockdown in *Xenopus* and zebrafish produced similar phenotypes [22••,24••].

These recent studies strongly suggested that PCP pathways might play critical roles in the posterior positioning of the nodal cilia. However, it remains unclear how the PCP signaling pathway locates the basal body of the nodal cilia. It is also unclear how AP axis information reflects the localization of PCP core proteins.

FGF, Shh and Ca signaling in the node

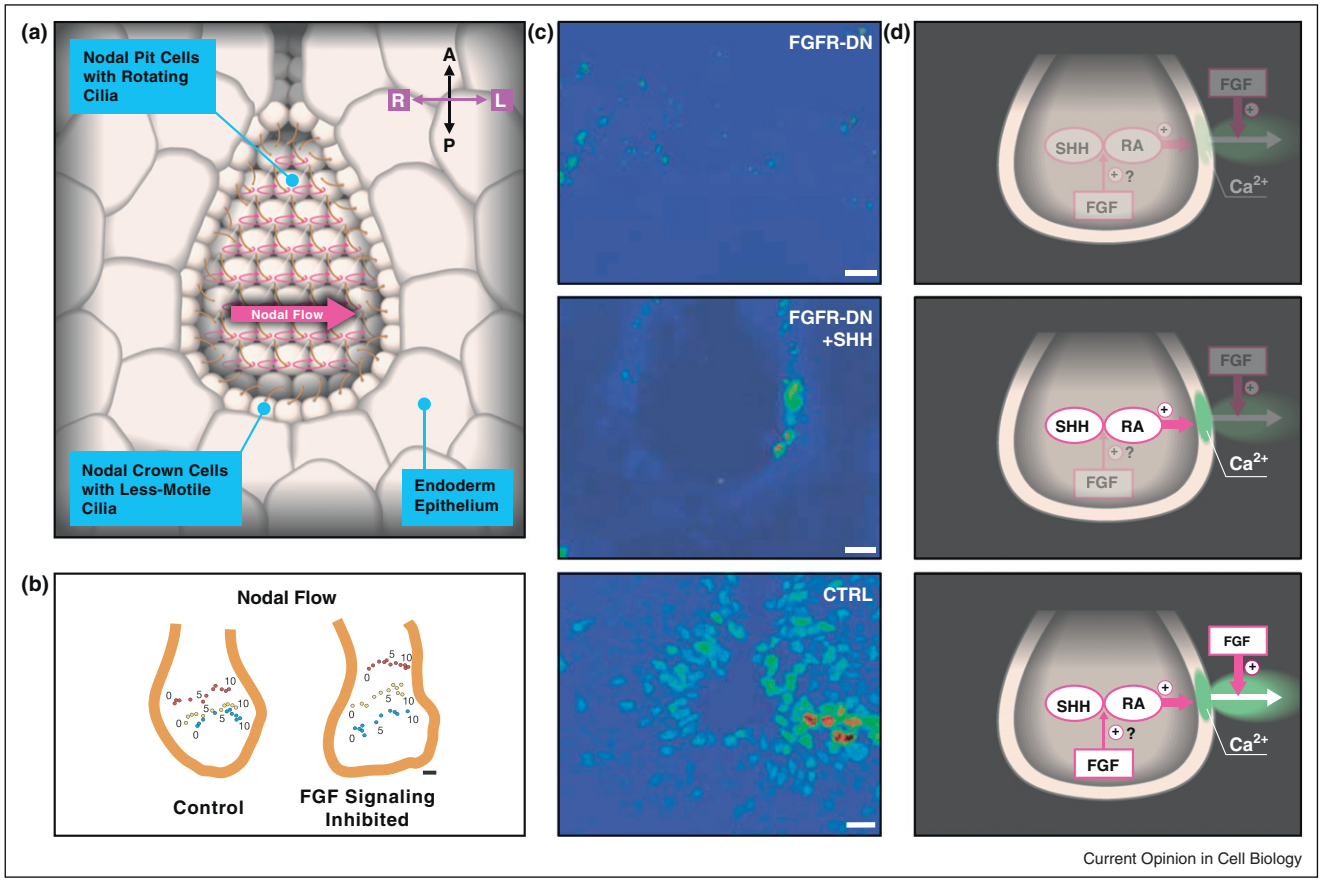
What mechanism links leftward nodal flow and left-specific expression of left-determination genes in the left lateral plate mesoderm (LPM) to enable asymmetrical development of the body? Although the precise mechanism of sensation remains controversial, two major readouts of the flow on the left margin of the node have been described. These are the elevation of Nodal activity and intracellular Ca (Figure 1b). These two mechanisms may not be mutually exclusive, but rather may run in parallel or work synergistically with each other.

Nodal is a secreted TGFβ morphogen that is expressed in both sides of the perinodal region in chick, mouse, *Xenopus*, zebrafish and rabbit [25]. Because the Nodal co-receptor, Cryptic, is effective only in the LPM, and because cell surface sulfated glycosaminoglycans are indispensable for Nodal signaling [26], direct transport of Nodal from the perinodal region to the LPM through the extracellular space is likely to be one of the signal propagation mechanisms. Furthermore, injection of Nodal into the mesoderm layer, but not its simple addition to the extraembryonic medium, affected the left-determination pathway [26], suggesting that Nodal was a morphogen that acts within the mesoderm layer but not on the superficial endoderm layer. This will need further confirmation at the cellular level.

Although a somewhat asymmetrical transcription pattern of Nodal has been described in the chick [2], the lateral difference in the mouse and *Xenopus* in particular is considered to be too subtle and transient by itself to fully explain the drastic asymmetry of the signaling [25]. The Nodal inhibitor, Coco, was recently found to be downregulated specifically on the left side of the *Xenopus* gastrocoel roof plate, which is an organ equivalent to the node [27•]. Coco is homologous to mouse *Cerberus-like-2* (*Cerl2*), chick *Caronte* and fish *Charon*. The downregulation of Coco is dependent on the nodal flow. Genetic evidence suggests that Coco works essentially through the Nodal pathway, although it may also inhibit the Wnt and BMP signaling pathways. Thus, left-specific downregulation of this Nodal inhibitor probably enables Nodal to freely diffuse within the mesoderm layer toward the LPM only on the left side. The upstream mechanistic link between flow sensation and downregulation of Coco transcription will be investigated in the future.

Ca elevation is considered to be another readout of the nodal flow. In the mouse node [28••,29••] and zebrafish Kupffer's vesicle [30], intracellular Ca is observed to be elevated on the left side of these flow-generating midline organs. Consequently, CaMKII is specifically activated on the left side: this CaMKII activity was shown to be essential for left-specific gene expression and correct determination of the organ laterality by a morpholino study in zebrafish [31•]. Interestingly, this elevation correlates with the developmental stages in which the nodal flow is expressed (2–3 somite stage in mouse) and requires a normal nodal flow [28••,29••]. According to our previous pharmacological study, the elevation of Ca could be divided into two successive steps [29••] (Figure 3). The first step occurs on the left boundary of the node (Figure 3a), as one or a few small foci of Ca elevation. These focal Ca transients could be eliminated in mouse embryos by acute suppression of FGF signaling with dominant negative FGFR peptide or an FGFR specific inhibitor. The Ca transients were rescued by supplementing the medium with SHH-N peptide or retinoic acid (RA) (Figure 3c and d). Because the FGF antagonists were added after the nodal flow was established, the nodal flow was not disturbed by this treatment (Figure 3b). Thus, this action of FGF was considered to be distinct from its actions in the development of the node itself via gastrulation [32,33] or the development of cilia [34]. Furthermore, because SHH-N or RA could rescue the Ca transients, the role of FGF for this first step in Ca elevation may be indirect. The action of FGF may be upstream or in parallel to the action of SHH-N or RA.

Figure 3



Left-specific Ca elevation is dependent on FGF/SHH/RA signals.
(a) Two-cilia theory, illustrated in the ventral view of the node. Although the monocilia of nodal pit cells are mostly motile, those of nodal crown cells are mostly immotile owing to the lack of left–right dynein (LRD). The readouts of the nodal flow will be propagated through the endoderm epithelium and the mesoderm located underneath. A, anterior; P, posterior, L, left; R, right, throughout this figure.
(b) Nodal flow tracked by the bead location every second in the absence (left trace) or presence (right trace) of an FGF inhibitor. Note that transient inhibition of FGF signaling does not apparently affect the nodal flow. (Modified from [29••].) Bar, 10 μm.
(c and d) Pharmacological ablation of Ca elevation on the left margin and in the periphery of the node **(c)**, and its interpretation **(d)**. (Modified from [29••].) Bars, 20 μm.

The second step of Ca elevation occurs in the lateral region between the node and LPM, which may be downstream of the initial focal Ca elevation. This step could be also eliminated by FGFR inhibition, but could not be rescued by SHH-N [29••]. This suggested that the propagation of Ca elevation from the node to the LPM was highly dependent on FGF signaling. Because FGFR1–3 were expressed on all cilia within and around the node [29••], the timing of LR signaling may be developmentally regulated by transient concentrations of FGFs at the node level. This Ca elevation could facilitate signaling through transcriptional regulation and/or morphogen transcytosis, which has been reported to be related with Ca signaling [35], to activate the downstream left-specific signaling cascades [31•].

Mechanosensation versus chemosensation for Ca entry

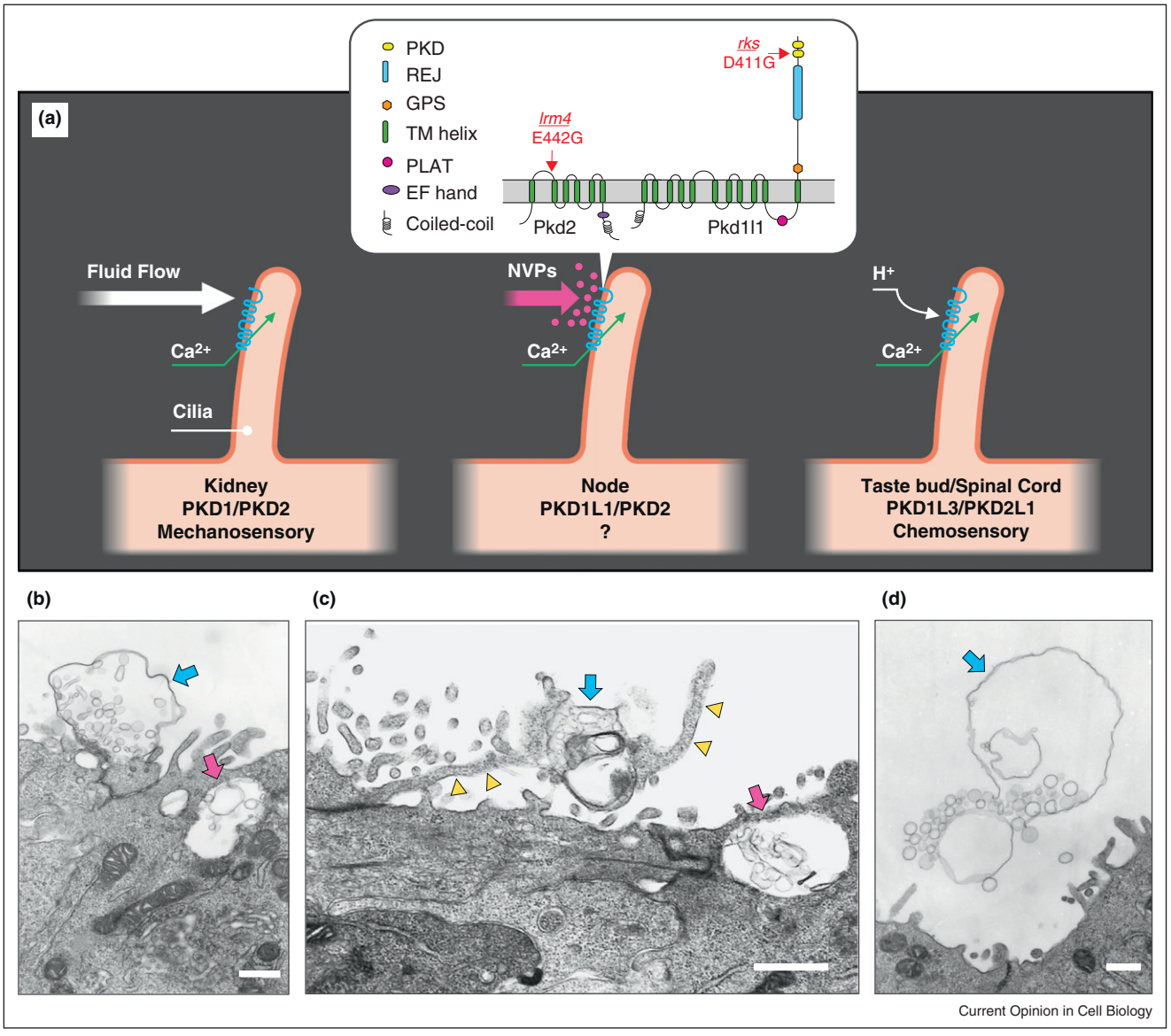
The elevation of Ca appears to be dependent on the peripheral cilia of the node because, first, the peripheral cilia are less motile than the central cilia owing to lack of left–right dynein (LRD), and second, the elevation of Ca in LRD or PKD2 mutant animals is impaired [28••] (Figure 3a). The central concept of the so-called 'two-cilia theory,' that peripheral cilia are mostly sensory and the central cilia are mostly motile, has become widely accepted [36]. The peripheral cilia do not express LRD, while all cilia express PKD2 [28••].

However, it is still debated whether the flow sensation mechanism of the peripheral cilia occurs via direct

mechanosensation of the hydrodynamic force [36] or chemosensation of morphogens concentrated by the leftward fluid flow ('nodal vesicular parcel' [NVP] hypothesis) [29••].

The PKD1L1/PKD2 complex has been suggested to be a good candidate for the ciliary receptor (Figure 4a) for two reasons. First, PKD2 is a Ca channel responsible for polycystic kidney [37], and second, fish or mice with mutant PKD1L1 exhibited right pulmonary isomerism and randomized laterality of abdominal organs with impaired Ca elevation and left-specific gene expressions [38••,39••], similar to those for PKD2 [38••,40–42]. In the case of kidney epithelium, the related PKD1/PKD2 protein complex senses fluid flow in order to raise intracellular Ca, which is important for normal kidney development and to prevent polycystic kidney syndrome [37]. PKD1 is not expressed in nodal cilia, and PKD1 mutant embryos do not exhibit laterality defects [43]. Instead, the PKD1-like protein, PKD1L1, forms a complex with PKD2 on nodal cilia. Although mechanical stimulation by the flow opens Ca channels in cilia that express PKD1/PKD2 [37], it remains unclear whether this PKD1L1/PKD2 channel is mechanosensory

Figure 4



Putative chemosensory components in the node.**(a)** Comparison of PKD family cation channels. Inset, mutations in the PKD1L1/PKD2 complex that caused laterality defects in the fish. (Modified from [39••].)**(b–d)** Electron micrographs of nodal vesicular parcels (NVPs, blue arrows) in the mouse node on day 7.5 postcoitum (dpc). Small electron-lucent particles are enclosed in a membrane sheath, which is occasionally associated with cellular protrusions (yellow arrowheads). Pink arrows, possibly nascent NVPs. **(c)** Modified from [29••]. Bars, 500 nm.

or chemosensory [38••,39••]. The PKD complex in some cilia could serve as the chemosensory channel. For example, its family member PKD1L3/PKD2L1 channel was shown to act as a pH sensor in taste bud cells [44] and in the central canal of the spinal cord [45].

In parallel to these findings, our group has used laser scanning confocal microscopy and electron microscopy to observe flowing materials of 0.3–5 μm in diameter that are secreted from the node cells of DiI-soaked embryos (Figure 4b–d). We have named these materials 'nodal vesicular parcels' (NVPs) [29••]. NVPs are released from slowly growing filopodia-like processes into the leftward flowing extracellular fluid, conveyed to the left, and fragmented and absorbed by the left margin of the node. Interestingly, NVP secretion appeared to strictly follow the pharmacological characteristics of the initial focal Ca elevation (Figure 3c and d). Inhibition of FGFR signaling eliminated NVP secretion, while addition of SHH-N or RA reversed the secretion. LM-level and EM-level immunohistochemistry suggested that SHH and RA were localized to putative secreting vesicles. These findings suggested that FGF/SHH/RA signaling facilitates the secretion of NVPs, which are transported to the left side to activate chemosensory receptors (possibly PKD1L1/PKD2 complex) and to elevate intracellular Ca.

Recently, many examples of long-range transport of morphogens by extracellular particles and/or long cellular processes have been described, which include Ca-response-evoking putative NVP cargoes. First, SHH accumulates at the surface of nodal crown cells specifically on the left [29••]. Hedgehog proteins are carried by lipoprotein particles [46,47]. A Hedgehog-dependent pathway in the LPM is involved in LR asymmetry [48], and a noncanonical Hedgehog signaling elevates the Ca response in developing spinal cords [49]. Second, Wnt3a is another candidate morphogen because it is involved in LR asymmetry [50], is carried by lipoprotein particles [51] and evokes Ca response in hippocampal neurons [52]. Third, PKD proteins themselves can be carried by exosomes [53]. These morphogens may sometimes be carried by cytoneme-like cellular processes over a long distance [54]. Future biochemical research will be awaited to identify the ligands of PKD receptors, which are probably transported by the NVPs toward the left.

Conclusion

Following the initial discovery of the nodal flow, it has been established that the leftward nodal flow generated by the rotation of cilia that are posteriorly tilted on node cells is the central process in LR determination. These cilia are built by transport via the KIF3 motor complex. Recent studies focusing on PCP components such as Dvl, Vangl, and Prickle strongly suggested that the PCP pathway may play critical roles in the posterior positioning of the nodal cilia. However, it remains unclear how the PCP signaling pathway locates the basal body of the nodal cilia. It is also unclear how AP axis information is reflected in the localization of PCP proteins.

Recent evidence suggests that nodal flow creates LR asymmetry by the leftward movement of membrane-sheathed particles, called NVPs. NVPs may then activate the noncanonical Hedgehog signaling pathway to cause an asymmetric elevation in intracellular Ca^{2+} and changes in gene expression. Although NVP release is likely to be upstream of Ca elevation, its cellular signaling cascade remains elusive. In this regard, chemosensation by PKD receptors on the peripheral cilia of the node is the most likely process, involving the long-range transport of unknown ligand morphogens by the nodal flow.

Acknowledgements

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