Cilia in Left–Right Symmetry Breaking

Kyosuke Shinohara¹ and Hiroshi Hamada²

¹Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Tokyo 184-8588, Japan

²Developmental Genetics Group, Graduate School of Frontier Biosciences, Osaka University, Osaka 565-0871, Japan

Correspondence: k_shino@cc.tuat.ac.jp; hamada@fbs.osaka-u.ac.jp

Visceral organs of vertebrates show left–right (L–R) asymmetry with regard to their position and morphology. Cilia play essential role in generating L–R asymmetry. A number of genes required for L–R asymmetry have now been identified in vertebrates, including human, many of which contribute to the formation and motility of cilia. In the mouse embryo, breaking of L–R symmetry occurs in the ventral node, where two types of cilia (motile and immotile) are present. Motile cilia are located at the central region of the node, and generate a leftward fluid flow. These motile cilia at the node are unique in that they rotate in the clockwise direction, unlike other immotile cilia such as airway cilia that show planar beating. The second type of cilia essential for L–R asymmetry is immotile cilia that are peripherally located immotile cilia. They sense a flow-dependent signal, which is either chemical or mechanical in nature. Although Ca²⁺ signaling is implicated in flow sensing, the precise mechanism remains unknown.

Visceral organs in all vertebrates are left–right (L–R) asymmetric with regards to their position, pattern, and size. Cilia, both motile and immotile in the mouse, play essential roles in L–R symmetry breaking. Four steps are required to generate such L–R asymmetric morphology in most of vertebrates including the mouse (Fig. 1): (1) Symmetry breaking by a leftward fluid flow (nodal flow) generated by the rotational movement of primary cilia at the node; (2) transmission of an asymmetric signal produced in or around the node (most likely Nodal activity) to the lateral plate mesoderm (LPM); (3) asymmetric expression of *Nodal* and the gene for its feedback inhibitor *Lefty2* in the left LPM; and (4) situs-specific organo-

genesis as a result of asymmetric expression of *Pitx2*, which encodes a transcription factor induced by Nodal signaling.

CILIA-DRIVEN FLUID FLOW DETERMINES LEFT–RIGHT ASYMMETRY IN THE MOUSE EMBRYO

L–R asymmetry of the mouse embryo is determined in the node cavity at 8 days after fertilization (E8.0 mouse embryo). In 1994, Sulik et al. reported the node cell harbors single cilium in the E8.0 mouse embryo, but the role of this cilium had remained obscure (Fig. 2). In 1998, Nonaka et al. discovered that the cilium is motile and generates unidirectional fluid flow

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Figure 1. Three step for L–R asymmetry. Three steps that contribute to the generation of L–R asymmetry are shown: (1) symmetry breaking, (2) differential patterning of the lateral plate mesoderm (LPM), and (3) asymmetric organogenesis. Scanning microscopic view of node cilia is shown for the symmetry-breaking step, whereas left-sided expression of *Nodal* and *Lefty* in the lateral plate is shown for the differential patterning step. For asymmetric organogenesis, three different mechanisms that can give rise to asymmetric anatomical structures are illustrated: differential branching, directional looping, and one-sided regression.

(Nonaka et al. 1998; Tabin and Vogan 2003; Hirokawa et al. 2006). This leftward flow is generated by the rotational movement of cilia, which are solitary motile structures that contain microtubules with dynein arms (Hirokawa et al. 2006). In general, there are two types of motile cilia. The 9+2 type cilia exist in the airway, brain, and oviduct, containing nine peripheral doublet microtubules with dynein arms, one pair of single microtubules at the center of the axoneme (central-pair), and radial spokes that bridge the peripheral microtubules to the central microtubules (Fig. 2). These cilia show planar beating and generate directional fluid flow. In contrast, the 9+0 type motile cilia present in the node cavity of the mouse embryo possess nine doublet microtubules with dynein arms but lack the central microtubules and radial spokes (Nonaka et al. 1998; Takeda et al. 1999; Hirokawa et al. 2006; Shinohara et al. 2015; Odate et al. 2016). The driving force of ciliary bending is generated by sliding of dynein arms between the peripheral doublet microtubules of

the axoneme (Summers et al. 1971; Fox et al. 1987). Motile cilia harbor two types of dynein arm complexes: outer and inner dynein arms. Dynein arm consists of heavy chain, intermediate chain, light chain of dynein, and accessory proteins (Inaba 2007). The outer dynein arm contains two or three types of heavy chains, whereas inner dynein arm consists of seven types of heavy chains in *Chlamydomonas* (Bui et al. 2008; Yagi et al. 2009). In transmission electron micrographs (TEMs) described previously, mouse node cilia seem to contain outer dynein arms with two heads, but the inner dynein arm was not identified (Takeda et al. 1999; Caspary et al. 2007; Shinohara et al. 2015).

Allelic mouse mutations *inversus viscerum* (*iv*) caused loss of motility of node cilia and left–right patterning defect in mice (Supp et al. 1997, 1999). Positional cloning identified its causative gene *Lrd*, which had a single amino acid substitution in the *iv* mutant allele at a conserved position of *Lrd* protein. *Lrd*/Dnah11 is thought to be a heavy-chain protein of outer

Figure 2. Node cilia in the E8.0 mouse embryo. Cells harbor one single cilium inside the node cavity in the E8.0 mouse embryo. The length of cilium is between 2 to 6 μm . Scale bar, 10 μm . A, Anterior; P, posterior; L, left; R, right.

dynein arm of motile cilia (Horani et al. 2014). Furthermore, mutation in DNAH5 (*Dnah5*), another putative heavy-chain protein of outer dynein arm, also causes left–right patterning defects in humans and mice (Ibanez-Tallon et al. 2002; Olbrich et al. 2002; Hornef et al. 2006). Although the role of inner dynein arm remains obscure, it seems that outer dynein arm plays a critical role in motility of mouse node cilia.

The direction of the flow is determined by a combination of two features of node cilia: their posterior tilt and clockwise rotation. Clockwise rotation of posteriorly tilted cilia thus generates a leftward effective stroke and rightward recovery stroke near the surface of the cell (Cartwright et al. 2004; Okada et al. 2005; Nonaka et al. 2005). Rotational movement of the cilia would generate slow rightward fluid flow near the cell surface and fast leftward flow in the middle of the node cavity (Cartwright et al. 2007). The precise mechanism of the tilt of node cilia is mentioned in the latter part of this review.

COMBINATION OF PLANAR CELL POLARITY AND ROTATIONAL MOVEMENT OF CILIA INDUCE LEFTWARD FLUID FLOW

Planar Cell Polarity Governs Posteriorly Tilted Rotational Axis of the Mouse Node Cilia

According to hydrodynamics, if the node cilia stand vertically to the apical surface of the node

cells, they would generate the vertical flow inside the node cavity. However, the rotational axis of node cilia is actually tilted toward the posterior direction of the E8.0 mouse embryo (Nonaka et al. 2005; Okada et al. 2005; Hashimoto and Hamada 2010; Hashimoto et al. 2010; Song et al. 2010). Clockwise rotation of posteriorly tilted cilia generates a leftward fluid flow by effective stroke far from the surface of the cell and rightward recovery stroke near the surface (Cartwright et al. 2004). To create the tilted axis, the basal body position of node cilia has to be tightly regulated, because the apical surface of the node cell is curved/spherical (not flat) (Hashimoto and Hamada 2010).

How is the position of basal body determined? Recent studies have shown that planar cell polarity (PCP) signaling positions the basal body at the posterior side of the node cells (Hashimoto et al. 2010; Song et al. 2010). PCP is a mechanism that governs the cell orientation within an epithelial sheet in multicellular organisms (Lawrence et al. 2007). The large majority of PCP signaling components and pathways have been discovered in *Drosophila* and vertebrates. It is known that two pathways mainly control PCP signaling. One system consists of atypical Cadherin Dachsous, Fat, and Four-jointed. The other system consists of transmembrane proteins Frizzled, Celsr, Vangl, and membrane proteins Dishevelled and Prickle. In both systems, the core proteins show asymmetric localization at the apical membrane of the epithelium through a feedback loop (Lawrence et al. 2007). In E8.0 mouse embryo, it is suggested that Dishevelled (Hashimoto et al. 2010), Prickle (Antic et al. 2010), and Vangl (Antic et al. 2010; Song et al. 2010) are required for planar polarization of node cilia. A mouse genetics-based mosaic assay reveals that Dishevelled protein is localized to the posterior side of the apical membrane of node cells (Hashimoto et al. 2010). Vangl and Prickle seem to be localized to the anterior side (Fig. 3) (Antic et al. 2010). Vangl is critical also for left–right determination in zebrafish and *Xenopus* embryos (Antic et al. 2010; Borovina et al. 2010), suggesting that the role of PCP signaling in basal body positioning is evolutionarily conserved among vertebrates.

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Figure 3. Ultrastructure of mouse motile cilia. (A) Two types of motile cilia in mammals. 9+2 type cilia have one central pair of microtubule and radial spokes at the center of the axoneme, whereas 9+0 type cilia do not contain any central structure. (B) Node cilia basal body. Scale bar, 200 nm. A, A-tubule; B, B-tubule; C, C-tubule.

Clockwise Rotation of the Mouse Node Cilia

Node cilia show the clockwise rotation, which is another reason why they can generate the leftward flow. However, how node cilia are able to sustain stable clockwise rotation has remained unknown. One can address this question into two essential questions: (1) Why do they rotate instead of beating planarly? and (2) Why do they rotate into the clockwise direction?

To address the first question, we should examine the role of regular arrangement of doublet microtubule found in mouse node cilia. A structural data-driven computer simulation of mouse node cilia suggests that the regular circular arrangement of doublet microtubules is essential for the stable rotational movement of node cilia (Shinohara et al. 2015). Furthermore, this work reported that absence of radial spoke is also critical for rotational movement of node cilia. To explore the role of radial spoke in mice, the investigators generated mice that lack the *Rsp4a* gene, an ortholog of *Rsp4*, which encodes the head of the radial spoke in *Chlamydomonas* (Shinohara et al. 2015). In *Rsp4a*

knockout mice, airway cilia showed clockwise rotation as well as mouse node cilia, suggesting that the radial spokes translate ciliary motion pattern from the clockwise rotation to the planar beating. Although the mechanism of switching of ciliary motion pattern remains unknown, this work proposes the mouse node cilia need to lose the radial spoke to acquire rotational movement. In the mouse embryo, the dorsoventral (DV) axis and the anteroposterior (AP) axis of the embryo are already established until E8.0. Described above, the node cells recognize the AP axis and move the basal body to the posterior side. Thus, the mouse embryo acquires the LR axis based on the AP axis (Hashimoto and Hamada 2010). The planar beating of ciliary motion makes unidirectional fluid flow in the airway, brain, and oviduct of mice (Fliegauf et al. 2007). If the node cilia harbored the planar beating, they have made fluid flow along the AP axis. To translate AP polarity to LR polarity, the clockwise rotation rather than the planar beating of ciliary motion pattern is indispensable and we speculate that this is the reason why the mouse node cilia have lost radial spokes

during evolution. A very recent study reports the presence of minor population of cells in the mouse node containing cilia of 9+2 structure (Odate et al. 2016). It is tempting to propose that the mouse embryo might still be evolving toward the loss of 9+2 arrangement.

To address the second question, we should consider the origin of microtubule polarity in the cilia node. Cilia harbor doublet microtubules that consist of complete A-tubules and incomplete B-tubules (Fig. 4). Previous studies revealed that the outer dynein arms are always attached to the A-tubules via dynein-docking complex in *Chlamydomonas* flagella (Takada et al. 1994; Koutoulis et al. 1997). Thus, the clockwise rotation of node cilia is probably originated from the orientation of A-B-tubules and sliding direction of outer dynein arms. During ciliogenesis, it is thought that formation of the doublet microtubules occurred by the template of the basal body. Basal bodies contain nine pairs of triplet microtubules that consist of A-, B-, and C-tubules (basal bodies of mouse node cells in Fig. 4) (Li et al. 2012). Because the basal body originally acquires the arrangement of A-B-C-tubule in order, A-tubules locate at the most central position, whereas C-tubules locate at the most peripheral side of the basal body. Thus, the order of each tubule is originated from the order of triplet tubules found in the basal body. To understand how the triplets acquire this order, we should further examine and determine the fine structure of basal bodies found in motile cilia and in the mouse node cilia.

ACTION OF THE FLUID FLOW FOR L–R SYMMETRY BREAKING

Immotile Cilia Act as Flow Sensor

In addition to motile cilia that generate the flow, there are immotile cilia at the edge of the node (Yoshida et al. 2012; Yoshida and Hamada 2014). Those cells with immotile cilia are often called crown cells, and express three signaling molecules required for correct L–R patterning, Nodal, Gdf1, and Cer12. Why are they immotile? Presumably these immotile cilia are immotile because they lack outer dynein arms like other immotile cilia, but this has not been addressed experimentally.

Genetic evidence has shown that the immotile cilia located at the periphery of the node are required to sense the flow (Fig. 5) (Yoshida et al. 2012). Thus, *Kif3a*^{−/−} mouse embryo that lacks all the cilia are unable to respond to the flow. However, when *Kif3a* expression was rescued in crown cells by the crown-cell-specific enhancer, such embryos now contain immotile cilia and can respond to flow (strictly speaking, these results still leave a possibility that motile cilia act as flow sensors, because cilia rescued by the crown-cell-specific enhancer are mainly immotile but most likely contain a small number of motile cilia as well).

It is still unknown what exactly immotile cilia sense. They may sense a chemical molecule(s) that is transported toward the left side by the nodal flow and functions as the left-side determinant. Such a molecule should fulfill the

Figure 4. Planar cell polarity (PCP) of node cells. The PCP core proteins are localized at the apical membrane of the node cells: Dishevelled is localized at the posterior side of the apical membrane and controls positioning of the node cilia basal body. Scale bar, 2 μ m.

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Figure 5. Immotile cilia sense the flow. Two types of cilia found in the node are shown: motile cilia located in the central region of the node generate the flow, while immotile cilia located peripherally (pink) sense the flow. Flow sensing involves a ciliary localized Pkd111–Pkd2 complex with Ca^{2+} channel activity. Flow-mediated signals lead to degradation of *Cerl2* mRNA. In this model, an immotile cilium on the *left* side is bent in response to the flow. However, this has not been confirmed in vivo.

following criteria: (1) It is an extracellular molecule that can be transported by the flow, (2) its absence would lead to the loss of left-sided *Nodal* expression in the lateral plate, and (3) its bilateral/ubiquitous presence would induce bilateral *Nodal* expression in the lateral plate. Up to now, there is no candidate molecule that satisfies all of these criteria. Alternatively, immotile cilia may sense the mechanical force produced by the flow. It has been suggested that some immotile cilia can sense mechanical forces. While this may represent a more likely mechanism, it is difficult to envisage how immotile cilia on both sides sense mechanical forces differently. Currently, there has been no direct evidence either supporting or discarding either possibility.

Involvement of Ca^{2+}

Although it is not clear what exactly the immotile cilia of crown cells sense, many lines of evidence suggest the involvement of Ca^{2+} in flow sensing. Most convincing evidence is the essential role of Pkd2 and Pkd111, a Ca^{2+} channel complex, in L–R symmetry breaking at the node (Pennekamp et al. 2002; Field et al. 2011;

Kamura et al. 2011). Pkd2 is required in crown cells, those cells with immotile cilia, acting upstream of *Cerl2*, a target gene of the fluid flow. In particular, Pkd2 protein is localized at the immotile cilia node and its ciliary localization is indeed required for correct L–R establishment (Yoshida et al. 2012). Furthermore, treatment of mouse embryos with various Ca^{2+} signaling inhibitors perturbs L–R asymmetry at the node (Yoshida et al. 2012). Inhibition of IP3 signaling in *Xenopus* embryos is known to randomize the L–R pattern (Hatayama et al. 2011).

When Ca^{2+} influx was directly examined with Fluo4- or Ca^{2+} -sensing fluorescent protein, L–R asymmetry in Ca^{2+} oscillation was indeed detected at the node of the mouse embryo (Takao et al. 2013) and at the Kupffer's vesicle of zebrafish embryo (Yuan et al. 2015).

Asymmetry in *Cerl2* mRNA as the Readout of the Flow Sensing

Crown cells respond to the fluid flow when their immotile cilia sense it. The most immediate gene that responds to the nodal flow is *Cerl2* (Schweickert et al. 2010; Shinohara et al. 2012). *Cerl2*, encoding a Nodal antagonist, is

asymmetrically ($R > L$) expressed in crown cells at the node. In the mouse embryo, expression of *Cerl2* is initially symmetric, but it becomes $R > L$ as the nodal flow increases its velocity, with expression on the left side being down-regulated (Kawasumi et al. 2011; Shinohara et al. 2012). L–R asymmetry in the level of *Cerl2* mRNA is determined not at the transcriptional level but at a posttranscriptional level (Nakamura et al. 2012). Thus, *Cerl2* mRNA in crown cells on the left side undergoes degradation via its 3′ untranslated region. Preferential decay of *Cerl2* mRNA on the left is initiated by the leftward flow and further enhanced by the *Wnt*–*Cerl2* interlinked feedback loops, in which *Wnt3* up-regulates *Wnt3* expression and promotes *Cerl2* mRNA decay, whereas *Cerl2* promotes *Wnt* degradation.

L–R asymmetry of *Cerl2* mRNA at the node is the earliest molecular L–R asymmetry at least in the mouse embryo, and plays an essential role in symmetry breaking (Marques et al. 2004). *Cerl2* protein inhibits Nodal activity, most likely by interacting with Nodal protein. The level of *Nodal* mRNA (and also Nodal protein) is bilaterally symmetric, but the $R > L$ expression of *Cerl2* renders Nodal activity in crown cells higher on the left side (Kawasumi et al. 2011). $R < L$ pattern of Nodal activity will eventually be transmitted to the lateral plate and induces left-sided *Nodal* expression there (Shiratori and Hamada 2006; Yoshida and Hamada 2014).

PERSPECTIVES AND REMAINING QUESTIONS

We have reviewed the generation and sensing of fluid flow by cilia during L–R determination in the mouse embryo. The node cilia make leftward fluid flow driven by the axonemal dynein arm complex. PCP and motion pattern of cilia determine the direction of fluid flow via the positioning of the basal body and the clockwise rotation of cilia. Although previous studies provide a lot of knowledge on the mechanism of L–R determination, several essential questions still remain to be solved: (1) How cilia sense fluid flow during L–R determination: do motile cilia act as mechanosensors or chemosensors?

(2) How the node cilia rotate in the clockwise direction? (3) What is the positional cue that polarizes node cells along the anteroposterior axis? We expect that advanced technologies, including CRISPR-mediated genetics, live imaging, and electron microscopy, will address these questions in the future.

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