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Establishment of left–right asymmetry in vertebrate development: the node in mouse embryos

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Abstract

Establishment of vertebrate left–right asymmetry is a critical process for normal embryonic development. After the discovery of genes expressed asymmetrically along the left–right axis in chick embryos in the mid 1990s, the molecular mechanisms responsible for left–right patterning in vertebrate embryos have been studied extensively. In this review article, we discuss the mechanisms by which the initial symmetry along the left–right axis is broken in the mouse embryo. We focus on the role of primary cilia and molecular mechanisms of ciliogenesis at the node when symmetry is broken and left–right asymmetry is established. The node is considered a signaling center for early mouse embryonic development, and the results we review here have led to a better understanding of how the node functions and establishes left–right asymmetry.

Keywords

Cell cycle; Cilia; Growth factor signaling; Left–right determination; Node

Introduction

Despite superficial bilateral symmetry along the left–right axis, vertebrate bodies have internal left–right asymmetry. For example, the heart resides on the left side, while the liver is on the right side [12, 23]. Although the leftward looping of the heart-tube is the earliest morphological event exhibiting left–right asymmetry [14], the underlying molecular cascades are initiated much earlier. Thus, one of the central questions is to identify the genetic cascades and molecular mechanisms responsible for the establishment of left–right asymmetry in vertebrates (Fig. 1).

In 1995, Dr. Tabin’s laboratory demonstrated that Sonic hedgehog (*Shh*) and *cNR-1* (related to the mouse gene *Nodal*; a member of the TGF- β superfamily) are specifically expressed in the left side of the Hensen’s node (anterior portion of the primitive streak in the chick developing gastrula that is structurally homologous to the mouse node) and within the lateral plate mesoderm (LPM, located in the lateral region of the early somite-stage’s embryo) in chick embryos [39]. Soon afterwards, both Dr. Robertson’s and Dr. Kuehn’s groups identified left-side-specific *Nodal* expression in mouse embryos [20, 41]. Independently, Dr. Hamada’s laboratory discovered that a new TGF- β superfamily gene, *Lefty2*, is expressed within the left LPM in early mouse embryos [45]. Shortly after these discoveries, multiple

groups found that *Nodal* induces expression of the bicoid-type homeobox transcription factor, *Pitx2*, which is necessary to support asymmetric morphogenesis in the developing visceral organs [13, 32, 40, 78]. Furthermore, mouse genetic studies revealed that expression of *Lefty1*, which like *Lefty2* also belongs to TGF- β superfamily, is required for proper midline function. *Lefty1* produces a signal antagonizing gene expression on the right side of the mouse embryo, indicating that *Lefty2* and *Nodal* encode signals for leftness [46]. These studies suggest that subsequent asymmetric signals of *Nodal*, *Lefty1/2*, and *Pitx2* are required for the establishment of left–right asymmetric patterning. However, the cellular and molecular functions triggering the initiation of left–right asymmetric gene cascades remained unclear.

In 1998, Dr. Hirokawa's laboratory provided critical evidence explaining how situs-specific gene cascades are activated while studying the function of molecular motors in the kinesin superfamily (KIFs). They found that loss of function of KIF3B led to heart loop reversal and bilateral or absent *Lefty2* expression in roughly 50 % of *Kif3b*-mutant mouse embryos [54]. Their finding suggests that KIF3B functions upstream of the *Nodal-Lefty* genetic cascades. Importantly, they found that in wild-type mouse embryos, primary cilia (a microtubule-based antenna like structure lacking the central pairs of microtubules) at the node called “nodal cilia” [68] rotate in a clock-wise direction generating leftward fluid flow (i.e., “nodal flow”) in the node cavity [54]. In the *Kif3b*-deficient mouse embryos, no primary cilia developed at the node, so no consistent nodal flow was generated. These data suggest that nodal flow is the earliest event breaking symmetry along the left–right axis in the mouse embryo. To demonstrate the contribution of nodal cilia motility in breaking left–right symmetry, they showed that loss of ciliary motility within the node results in aberrant left–right patterning using the *situs inversus* (*iv*) homozygous mutant mouse embryo [56]. Consistent with these findings, imposing artificial rightward fluid flow reverses the left–right patterning of mouse embryos [53]. These results strongly support the idea that generation of nodal flow is a critical trigger in the establishment of the left–right asymmetry. While nodal flow is not responsible for breaking the left–right symmetry in all vertebrates, this discovery lead researchers to find that it is the initial event for establishing of left–right asymmetry in numerous species [8, 28, 57, 69].

These critical findings provided researchers an opportunity to address the molecular pathogenesis of Kartagener's syndrome, which is classified as one of numerous cilia-related dysfunctions in humans, also called “ciliopathies” [1, 37, 62, 79]. Kartagener's syndrome patients have multiple cilia-related defects including immotile sperm and defective cilia in their airway. Importantly, around 50 % of patients with Kartagener's syndrome develop *situs inversus*, in which the positions of major visceral organs are reversed along the left–right axis. This is an important phenotypic link demonstrating how normal ciliogenesis and cilia function are critical in governing the location of visceral organs [1].

In this review, we first discuss the cellular events required for generating nodal flow at the node of the mouse embryo. Next, we summarize our current understanding of how nodal flow is sensed. Finally, we describe the molecular mechanisms by which primary cilia are exclusively formed at the node when symmetry is broken along the left–right axis in mouse embryos. Together, these findings provide a basis to develop our molecular and cellular understanding of how left–right asymmetry is established, a phenomenon that has fascinated developmental biologists for decades.

Generation of nodal flow

In mouse embryos, the fully formed node is located at the distal tip of the cylindrical embryo, which corresponds to the anterior end of the primitive streak (Fig. 2a) [7, 38, 68].

The node consists of two cell layers, within which 200–300 cells contain cilia that project from the ventral surface (Fig. 2b) [25, 28, 68]. Initially, these cilia were thought to be motile [68], but soon after, their “9 + 0” microtubular structure and lack of dynein arms, typical characteristics of immotile cilia, led researchers to believe that cilia at the node were immotile [6]. However, in 1998, conclusive work demonstrated that cilia at the node are motile and rotate in a clockwise direction generating a unidirectional leftward liquid flow, i.e., the “nodal flow” [54]. Interestingly, nodal flow is a very temporally restricted biological phenomenon during early mouse embryogenesis. It becomes apparent at the early head-fold stage and is no longer visible by the six or seven-somite stage [56]. It has remained unknown whether every cilium at the node generates the motility necessary for nodal flow. However, a study has shown that left–right dynein containing monocilia, which are centrally located in the node, may be capable of generating flow, while nodal cilia in the peripheral region of the node are immotile [44]. Consistent with this, it has been reported that the mouse node contains two types of cilia: “9 + 2” monocilia (motile cilia) and “9 + 0” monocilia (immotile cilia) [17].

Because various mouse mutants with left–right patterning defects have abnormal nodal flow due to either the lack of nodal cilia or the loss of their motility [42, 43, 54, 56, 71, 75], nodal flow is considered to be critical in generating typical left–right asymmetry. Numerous studies have demonstrated that similar mechanisms utilizing nodal flow to break the symmetry along with the left–right axis are conserved in many but not all vertebrate embryos (e.g., chick embryos) [7, 21, 35, 57, 61, 69].

One of the key questions arising from the results described above was how the rotation of nodal cilia leads to unilateral leftward flow in the nodal cavity (Fig. 2c). Theories of fluid mechanics suggest how the posterior tilt of nodal cilia account for conversion of rotational motion to directional leftward laminar flow [16]. Also, three-dimensional computer models suggest that wall-induced drag in tilted cilia produces a laminar flow [11]. Indeed, two experimental studies showed evidence indicating that posterior tilted nodal cilia can generate consistent leftward flow [55, 57]. Several groups further substantiated the importance of ciliary wall and membrane effects for generation of nodal flow through additional computer simulations [15, 50, 65, 66].

The next logical step was to identify the molecular mechanisms necessary to generate the posteriorly tilted arrangement of nodal cilia. Planar cell polarity (PCP) signaling was initially identified in the fruit fly, where it controls the uniform orientation of hairs and bristles on the body [33, 80]. Recently, several groups demonstrated that PCP governs the coordinated positioning and orientation of nodal cilia in mouse embryos. That is, PCP determines the position of the basal body, orienting the tilt of nodal cilia to generate unidirectional nodal flow. Several core components of PCP including *Vangogh* (*Vangl*) and *Dishevelled* (*Dvl*) genes, which are conserved from *Drosophila* to vertebrates, were knocked out in mice [27, 42, 67]. The *Vangl* gene deletion in mice (*Vangl1^{gt/gt} Vangl2^{Δ/Δ}*) displayed lateral defects including abnormal heart looping, lung lobe sidedness and bilateral expression of left-side-specific marker genes such as *Nodal*, *Lefty2*, and *Pitx2*. Interestingly, *Vangl1^{gt/gt} Vangl2^{Δ/Δ}* mutant embryos develop primary cilia at the node that are morphologically comparable to control mouse embryos, but the basal bodies in the nodal cilia are not appropriately positioned at the posterior on pit cells at the node. The mutant embryos do not generate consistent nodal flow resulting in loss of left-side-specific Ca^{2+} release (discussed in detail in the next section). These results suggest that PCP is not essential for ciliogenesis, but is critical for the posterior localization of cilia essential for generating nodal flow [67]. Consistent with this observation, knockdown of *Vangl2* in both *Xenopus* and zebrafish embryos exhibited altered left–right patterning and disruption of the posterior localization of cilia in the gastrocoel roof plate in *Xenopus* and Kupffer’s vesicle in

zebrafish [3, 10]. Interestingly, compound mutant mice lacking *Dvl* genes showed that positioning of basal body in node cells and nodal flow were impaired, further supporting the notion that posterior positioning of the basal body is controlled by PCP [27].

Sensing mechanisms of nodal flow

It appears that the establishment of the left–right asymmetry through nodal flow is a conserved mechanism in many vertebrate embryos. However, it remains unknown how nodal flow is directly sensed in order to break the symmetry along the left–right axis at the node. So far, two models have been proposed (Fig. 3) [28, 64, 70]. In the “two-cilia model”, immotile cilia at the node that express polycystin-2 (*Pkd2*), which encodes a Ca^{2+} permeable cation channel, function as mechanosensors in cells that relay nodal flow directional information by inducing left-side-specific Ca^{2+} signaling (Fig. 3a) [44]. Two research groups proposed that *Pkd1*-related locus *Pkd1l1* is a critical binding partner for *Pkd2* and is required for left–right patterning in both medaka fish and mouse embryos [22, 30]. On the other hand, it has also been suggested that factors transported by nodal flow may trigger the initiation of left-side-specific gene regulation (Fig. 3b) [28, 72]. In this model, FGF signaling triggers secretion of membrane-sheathed particles termed nodal vesicular parcels (NVPs) that carry SHH and retinoic acid. NVPs are transported by nodal flow and eventually fragment close to the left wall of the node, thus initiating left-side-specific gene cascades. This model suggests that NVP flow is probably the mode of extracellular transport that forms a left–right gradient of morphogens [72]. These two models are not mutually exclusive and may together account for the biological responses at the node when left–right symmetry is broken. However, it is now clear that nodal flow-sensing mechanisms are critical for the break in symmetry. Results in *Pkd2*-deficient mice demonstrate that nodal flow is sensed by cilia of peri-nodal crown cells through ciliary-localized PKD2 [77]. This group further established three types of transgenic mice expressing *Pkd2* specifically in node/notochord, crown cells, and pit cells at the node to examine whether the left–right abnormalities seen in *Pkd2*-deficient mice were rescued. They found that *Pkd2* is required only in crown cells at the node to correct left–right determination [77]. Importantly, they showed that restoration of primary cilia in crown cells in *Kif3a*-deficient mouse embryos (*Kif3a*-deficient embryos lack all cilia at the node) [71] rescues the response to artificial flow, demonstrating that nodal flow is sensed by cilia in crown cells located at the edge of the node. Despite these advances, it is still unclear which of the two described models (Fig. 3) correctly describes how cilia in nodal crown cells sense flow. However, there is strong evidence suggesting that these cilia indeed sense mechanical forces because in both kidney cells and vascular endothelial cells PKD2 mediates mechanosensation in primary cilium [26, 29]. Nonetheless, as little as two rotating cilia at the node are sufficient for establishing the left–right asymmetry [63], suggesting there is a highly sensitive system in the node to detect flow upon left–right symmetry breakage in mouse embryos.

Ciliogenesis at the node

Although little is known about the fundamental mechanisms that regulate ciliogenesis at the node, several transcription factors were shown to be involved in the formation of nodal cilia in mouse. For example, *Foxj1*-deficient mouse embryos displayed normal cilia length at the node, but these cilia appear to be immotile, resulting in left–right patterning abnormalities [19, 81]. *Rfx3*-deficient embryos formed nodal cilia, but they reached only half the length of wild-type nodal cilia resulting in situs inversus [9]. Embryos lacking the homeobox transcription factor *Noto* display short and abnormal nodal cilia leading to randomized situs organogenesis [5]. Importantly, the expression of both *Foxj1* and *Rfx3* at the node is down-regulated in *Noto* mutants, suggesting that *Noto* acts upstream of *Foxj1* and *Rfx3* to govern

ciliogenesis at the mouse node [5]. Recently, *Noto* was recognized as a key regulator important for the posterior localization of cilia [2].

While growth factors including FGF, NOTCH, and WNT are required for the establishment of left–right patterning [36, 47, 51, 59], bone morphogenetic protein (BMP) signaling regulates molecular cascades that generate left–right asymmetry in the vertebrate embryo. In early stage chick embryos, BMP signaling suppresses the expression of *Nodal* and thus initiates a right-side molecular cascade resulting in right side identity in LPM [60, 76]. Similar observations were made in *Xenopus* embryos using a constitutively active form of a BMP type I receptor, *Acvr1* [58]. Two novel type II receptors for BMPs are also required to establish left–right asymmetry in the zebrafish [49]. In mice, lack of *Smad5*, one of the intracellular transducers for BMP signals [73], results in left–right patterning abnormalities [18]. *Bmp4* is required in the formation of the node and coordination of left–right asymmetry in the mouse [24]. In addition, BMP antagonists including *Chordin* and *Noggin* are important for both the node and LPM to establish left–right axis [48]. Compound mutants for *Noggin* and *Chordin* demonstrate lateral abnormalities, further suggesting that BMP signaling contributes to the establishment of left–right asymmetry [4]. However, molecular details underlying the role of BMP signaling in this process, including the mechanism to initiate ciliogenesis at the node, remain unclear.

The molecular mechanisms that initiate the formation of transient primary cilia at the node are largely unknown, however, it is clear that the cell cycle in the ventral surface of mouse node cells (where nodal cilia are formed) is quiescent when nodal cilia begin to develop [6]. This is a very interesting observation as cells in other locations at this stage of embryonic development are highly proliferative (Fig. 4). Since the ventral surface of the node is the only place where nodal cilia are formed, the question arises as to whether any links exist between growth factor signaling and cell-cycle progression that regulate ciliogenesis at the node. We recently addressed this question through the targeted deletion of one of the BMP type I receptors (*Acvr1*). Since ACVR1 is produced in the ventral surface of the node [31], BMP signaling through ACVR1 may induce cellular quiescence allowing the development of nodal cilia. After screening, many cell-cycle regulators including cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) using control and *Acvr1*-deficient mouse embryonic fibroblast cells (MEF), we found that BMP signaling positively regulates the stabilization of p27^{Kip1} through phosphorylation on Ser¹⁰ keeping MEFs in the quiescent phase and allowing the formation of primary cilia [34]. The next critical question was whether both p27^{Kip1} and p27^{Kip1} phospho-Ser¹⁰ are specifically produced in the ventral surface of nodes. As shown in Fig. 5, p27^{Kip1} and p27^{Kip1} phospho-Ser¹⁰ are present exclusively at the ventral surface of the node in wild-type mouse embryos, supporting the idea that stabilization of p27^{Kip1} via phosphorylation on p27^{Kip1} Ser¹⁰ is required for quiescence and subsequent formation of nodal cilia. Importantly, protein levels of both p27^{Kip1} and p27^{Kip1} phospho-Ser¹⁰ were significantly reduced on the ventral surface of the node in the *Acvr1* mutant embryos as compared to wild-type embryos [34]. Quiescence at the node seems to be specifically regulated by p27^{Kip1}, since the other members of the Cip/Kip family including p21^{Cip1} and p57^{Kip2} were not present at the node in wild-type mouse embryos [34]. These data suggest that BMP signaling is required for the induction of quiescence by stabilizing the p27^{Kip1} protein, which is a prerequisite for development of the nodal cilia. While FGF signaling is critical for ciliogenesis in zebrafish and *Xenopus* embryos [52], it remains unclear how BMP or other growth factors interact with FGF signaling to regulate cilia formation. Further studies are necessary to understand the interplay of growth factor signaling that governs ciliogenesis at the node.

Conclusions

Studies elucidating the mechanisms that establish left–right asymmetry have led to some of the most elegant advances in the field of embryology over the last decade. This field has facilitated the development of novel concepts including the rotation of subcellular structures at the node triggering symmetry breakage along with the left–right axis. As usual, one answer gives rise to numerous new questions. While we demonstrated that node-specific BMP signaling might be involved in one initial step in breaking the symmetry at the node, it is unclear why this happens exclusively at the node. Another unanswered question is how BMP signaling negatively regulates cell-cycle progression at the node whereas it stimulates proliferation in other contexts. In vitro, ciliogenesis occurs when the cell cycle is in G0 phase [74]. Consistent with this discovery, we found that the cell cycle is quiescent in ventral side of the node that allowing ciliogenesis to trigger the breaking of left–right symmetry. This is a unique developmental phenomenon since the cell cycle is specifically arrested in the ventral surface of node where nodal cilia are formed. The quiescence of the ventral cells of the node seems to be very important for ciliogenesis. However, it is not clear how growth factor signaling through factors such as BMP controls cell cycle quiescence and vesicular trafficking in ciliary extensions at the node. These questions need to be addressed in the future to more fully appreciate the mechanisms underlying the development of embryonic left–right asymmetry.

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Fig. 1.

Establishment of left–right asymmetry during early mouse embryogenesis. Whole-mount view of wild-type mouse embryos from embryonic day (E) 7.75–E10.5. Around E7.75–E8.0, left–right symmetry is broken at the node (*left panel*, red marked on the ventral side of embryo). Next, *Nodal-Lefty2-Pitx2* gene cascades are initiated to develop the patterning of lateral plate mesoderm (*middle panel*, showing *Pitx2* gene expression). Finally, situs-specific organogenesis is seen in heart looping at E10.5 (*right panel*). *A* anterior, *P* posterior, *L* left, *R* right

Fig. 2.

Generation of leftward fluid flow at the node in mouse embryos. **a** Scanning electron microscopic analysis of the node at E8.0. Wild-type mouse embryos show a cup-shaped node with evenly distributed ciliated cells when viewed from the ventral side. **b** High magnification images from (**a**). Around 3–4 µm length of primary cilia at E8.0 are developed in each node cell. **c** Model illustrating the posterior tilting of nodal cilium in pit cell. Nodal cilia are posteriorly tilted to generate a leftward laminar flow (**a**, **b**) were reproduced from [34]

Fig. 3.

Two hypothetical models to sense the nodal flow. **a** Nodal cilia expressing PKD2/PKD1L1 complex function as a mechanosensors unit that relays the information of nodal flow direction by inducing left-side-specific Ca²⁺ signaling. **b** Nodal flow transports chemicals that trigger the initiation of left-side-specific gene regulation. Nodal vesicular parcels (NVPs) that carry SHH and retinoic acid are transported by nodal flow

Fig. 4. Importance of cell-cycle arrest in ventral surface of node cells. Cell-cycle progression was examined by Ki-67 immunohistochemistry in E8.0 mouse embryos. Note that Ki-67 is not detectable in cells of the ventral surface of node where nodal cilia are developed

Fig. 5. Cell quiescence is required for nodal cilia formation and is controlled by BMP signaling. Ventral node was stained by Ki-67 (red) (a–f), p27^{Kip1} (green) (a, c, e) and p27^{Kip1} phospho-Ser¹⁰ (green) (b, d, f) in wild-type (WT) and *Acvr1* cKO embryos. A anterior, P posterior (c–f) were reproduced from [34]