## Review

# Molecular and cellular basis of left–right asymmetry in vertebrates

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**Abstract:** Although the human body appears superficially symmetrical with regard to the left–right (L-R) axis, most visceral organs are asymmetric in terms of their size, shape, or position. Such morphological asymmetries of visceral organs, which are essential for their proper function, are under the control of a genetic pathway that operates in the developing embryo. In many vertebrates including mammals, the breaking of L-R symmetry occurs at a structure known as the L-R organizer (LRO) located at the midline of the developing embryo. This symmetry breaking is followed by transfer of an active form of the signaling molecule Nodal from the LRO to the lateral plate mesoderm (LPM) on the left side, which results in asymmetric expression of *Nodal* (a left-side determinant) in the left LPM. Finally, L-R asymmetric morphogenesis of visceral organs is induced by Nodal-Pitx2 signaling. This review will describe our current understanding of the mechanisms that underlie the generation of L-R asymmetry in vertebrates, with a focus on mice.

**Keywords:** chirality, cilia, left–right asymmetry, Nodal

## 1. Introduction

The bodies of bilaterian animals possess three axes: anterior–posterior (A-P), dorso–ventral (D-V), and left–right (L-R). These axes are determined early during development, with the L-R axis being the last to be established. Most visceral organs of vertebrates including humans are L-R asymmetric in terms of their size, shape, or position (Fig. 1). In normal conditions (*situs solitus*) in humans, the stomach and spleen are located on the left side, whereas the liver is on the right. In addition, the left and right sides of the lung have two and three lobes, respectively. The heart also manifests L-R asymmetries. The apex of the heart is thus located on the left side, and the connection of the great arteries to the ventricles depends on L-R information, with the pulmonary artery and aorta being connected to the right ventricle and left ventricle, respectively. Vertebrates manifest marked diversity in morphological asymmetries of their organs. For instance, there are four lobes of the lung on the right and one on the left in the mouse, female birds have an ovary only on the left side,[1)] and many snake species possess only a right lung.[2)]

The normal laterality pattern is altered in various different ways in pathological conditions (Fig. 1). For organs that exist singly in the viscera such as the heart and stomach, their position may be normal, reversed, or ambiguous. For bilateral organs such as the lung, their morphology may be normal or reversed, or show right isomerism or left isomerism. At the level of the whole organism, the laterality of all organs may be completely reversed in a mirror image (*situs inversus*). Alternatively, visceral organs might be altered in a discordant manner (heterotaxy), with some organs being unaffected and others showing an ambiguous or reversed position. Two types of heterotaxy are often encountered in humans: heterotaxy with right isomerism and heterotaxy with left isomerism. The former is usually recognized by a bilateral trilobed lung, a large symmetric liver, and the absence of a spleen, whereas the latter is characterized by a bilateral bilobed lung and multiple spleens.

The heart may be the organ most sensitive to laterality defects. Heart anomalies often associated with such defects include transposition of the great arteries, in which the pulmonary artery and aorta are

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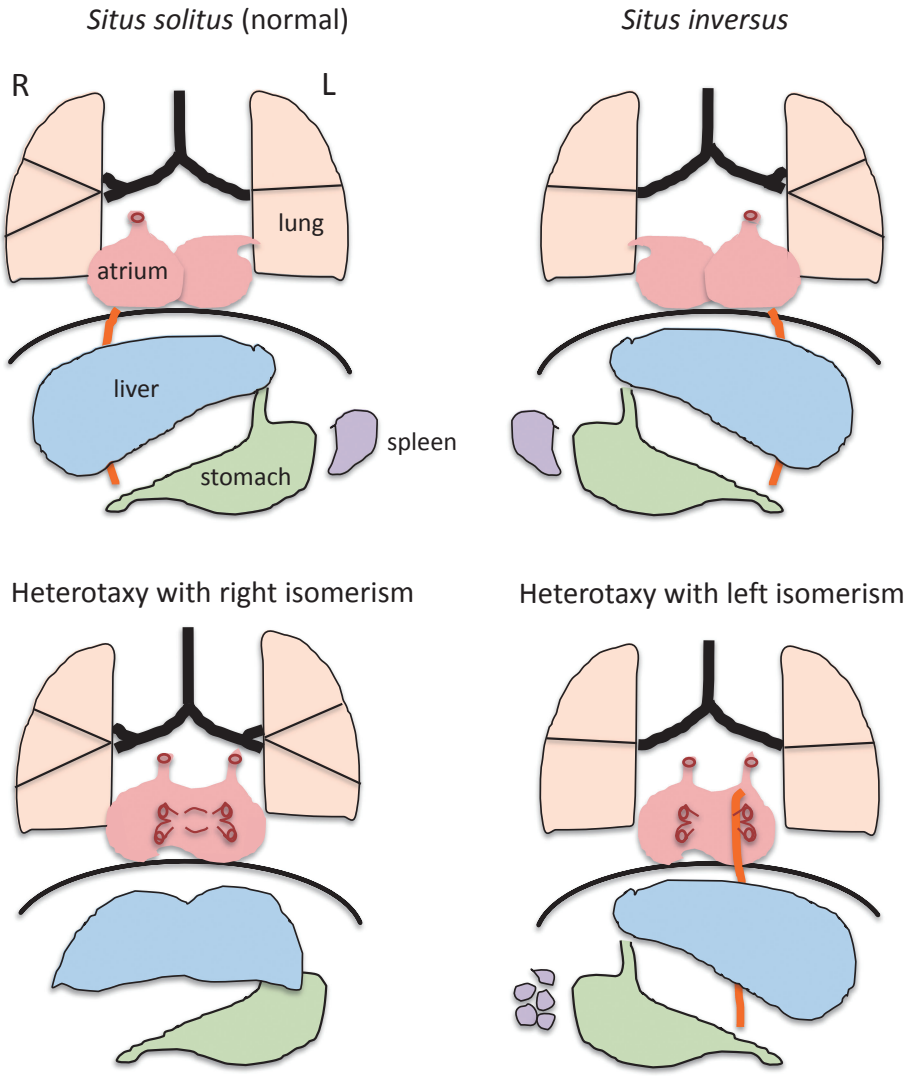


Fig. 1. Schematic illustration of L-R asymmetry of human visceral organs. Normal L-R asymmetry (*situs solitus*) and three laterality defects that affect the lung, heart, liver, stomach, and spleen are shown. Heterotaxy with right isomerism is usually associated with a bilateral trilobed lung, a large symmetric liver, and the absence of a spleen. Heterotaxy with left isomerism often manifests as a bilateral bilobed lung, multiple spleens, and a pulmonary vein that drains into both the right and left atria.

incorrectly connected to the left ventricle and right ventricle, respectively, and double outlet right ventricle, in which both great arteries connect to the right ventricle. Such heart defects in humans are life threatening and usually require immediate medical attention after birth.

## 2. Historical view

Disorders of L-R asymmetry result in randomization (heterotaxy) or complete reversal (*situs inversus*) of visceral organs. Humans with laterality defects were described by physicians as long ago as the 16th century. An individual showing the complete mirror-image reversal of visceral organs was recorded in the 18th century.[3] Some of the described individuals also manifested bronchiectasis, with their condition being named Kartagener syndrome in the early 20th century[4] (Table 1) and later renamed primary ciliary dyskinesia.

The notion that L-R asymmetry is genetically determined became obvious when a mutant mouse (*situs inversus viscerum*, or *iv*) with randomized L-R asymmetry was identified in 1959 (Table 1).[5] This spontaneously occurring mutation caught the attention of biologists, but the causative gene remained unknown for decades. Another mutant mouse (*inv*)

Table 1. Genetic control of L-R asymmetry. Human disorders and mutant animals that provided breakthroughs in our understanding of the mechanisms underlying the development of L-R asymmetry

| Animal | Disorder or mutation | Defects | Year reported | Ref. |
|---|---|---|---|---|
| Human | Kartagener syndrome (ciliary proteins) | *Situs inversus*/ heterotaxy, chronic respiratory infection, infertility | 1789<br>1933 | 3<br>4 |
| Mouse | *Iv* (Dnah11) | L-R is randomized | 1959 | 5 |
| | *Inv* (Inversin) | L-R is reversed | 1993 | 6 |
| *Drosophila* | Myo31DF (myosin 1D) | Looping of gut and testes is reversed | 2006 | 13, 14 |
| Snail | | Direction of coiling: sinistral *vs.* dextral | 1923 | 15 |

was described in 1993.[6] In contrast to the *iv*/*iv* mutant, L-R asymmetry is always reversed in *inv*/*inv* mutant mice. In the mid-1990s, the identification of *Nodal* and *Lefty* as L-R asymmetrically expressed genes in chick[7] and mouse[8),9] embryos provided a breakthrough in the study of the molecular and genetic mechanisms underlying the development of L-R asymmetry. The causative gene for the *iv* mutation was found to encode an axonemal dynein protein in 1997,[10] which provided support for the idea that L-R asymmetry requires motile cilia. The gene that harbors the *inv* mutation was also identified and found to encode a large protein designated Inversin.[11] Inversin plays a role in cilia (immotile cilia), but its precise function remains elusive. Although a link between laterality and cilia had been suggested earlier, the discovery of motile cilia and a leftward fluid flow at the node of the mouse embryo in 1998 clearly established the role of cilia in L-R asymmetry for the first time.[12]

Unlike many other topics in developmental biology, molecular and genetic analysis of L-R asymmetry was initiated by studies of vertebrates such as the mouse and chick before being expanded to include other vertebrates (*Xenopus*, zebrafish) and various invertebrates. In particular, the first *Drosophila* mutant with L-R defects was described in 2006.[13),14] Snails, which are characterized by directional coiling of their shell, might be the most beautiful example of L-R asymmetry in animals, and genetics has shown that L-R asymmetry in these animals is determined by a single gene.[15)–17]

## 3. Symmetry breaking at the L-R organizer

A vertebrate embryo is L-R symmetric when it undergoes gastrulation and forms the primitive streak. The L-R organizer (LRO), at which L-R symmetry is broken, is formed at the anterior tip of the primitive streak and corresponds to the ventral node in the mouse embryo, Hensen's node in the chick embryo, the gastrocoel roof plate in the clawed frog (*Xenopus*), and Kupffer's vesicle in zebrafish.[18),19] The breaking of L-R symmetry is followed first by differential patterning of the lateral plate mesoderm (LPM) on the two sides in a manner dependent on signaling by the secretory protein Nodal and subsequently by asymmetric organogenesis (Fig. 2).

Hereafter, this report will describe L-R symmetry breaking in vertebrates such as mice, frogs, and fish, which is dependent on motile cilia and fluid flow at the LRO (although the description presented is based on studies in mice unless indicated otherwise). Some vertebrates, such as chickens[20] and reptiles,[21] do not rely on such a mechanism for L-R symmetry breaking. Breaking of L-R symmetry in invertebrates will be described briefly in section 7.

**3.1. Two types of cilia at the LRO: motile and immotile.** The node of the mouse embryo is a small cavity located at the midline that exists transiently at embryonic day (E) 7.5–8 (Fig. 3). The ventral surface of the node is covered with a layer of epithelial-like cells, each of which possesses a single cilium. However, there are actually two types of ciliated cells at the node. Cells in the central region of the node, often referred to as pit cells, have motile cilia that rotate in a clockwise (when viewed from the ventral side) direction. About 200 motile cilia thus protrude from the central region of the node into the node cavity[22] (Fig. 3) and rotate at a speed of 600 rpm[12),23] in the mouse embryo. How can motile cilia at the node rotate instead of beating in a planar manner? One reason is that they possess a distinct axonemal structure characterized by a lack of radial spokes.[24] Furthermore, the clockwise direction of rotation may be determined by structural chirality of the axoneme, as suggested by mathematical modeling.[25]

Cells at the periphery of the node, often referred to as crown cells, possess immotile cilia. Such cilia (also known as primary cilia) are solitary protrusions that are found at the surface of nearly all cells, and they are rendered immotile by the lack of dynein arms in their axonemes. Primary cilia function as

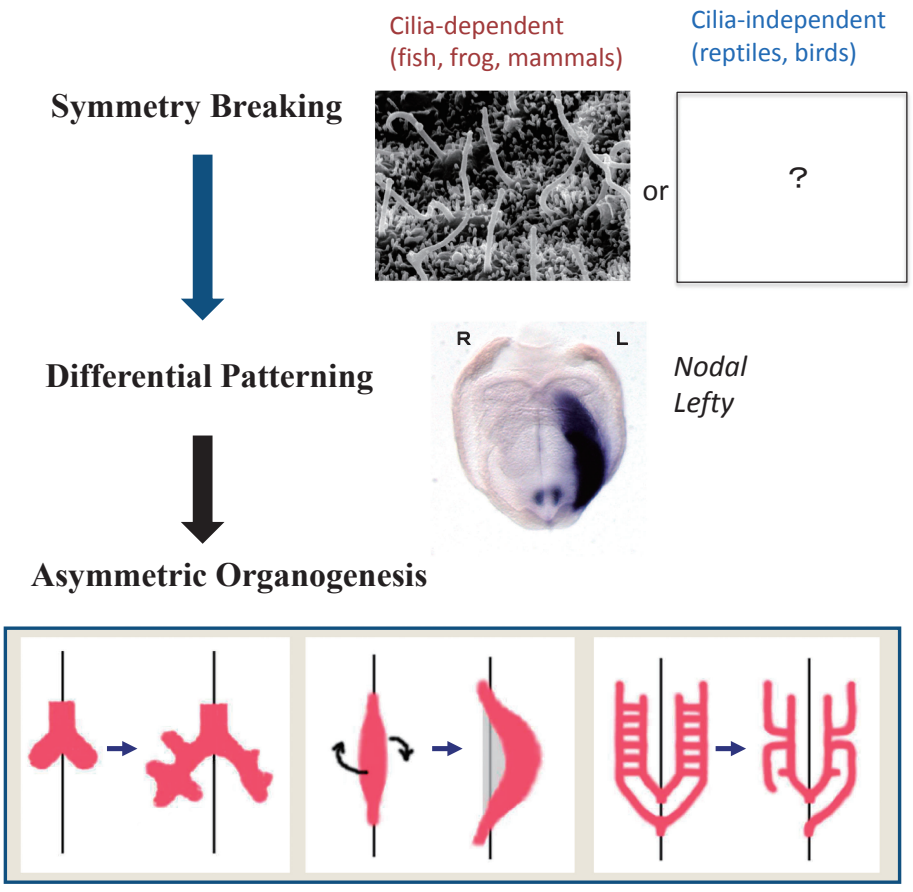


Fig. 2. Three steps in the establishment of L-R asymmetry in vertebrates. The three steps include symmetry breaking, patterning, and organogenesis. In the first step, many vertebrates rely on cilia for symmetry breaking, whereas others deploy a cilia-independent, largely unknown mechanism. In the second step, the LPM on the right and left sides undergoes differential patterning as a result of asymmetric Nodal signaling on the left side. In the final step, asymmetric morphogenesis takes place in visceral organs. Modified with permission from Shiratori and Hamada[155] and Yoshiba and Hamada.[156]

antennae that are able to sense the surrounding environment, and they are able to detect not only molecules such as the signaling proteins Hedgehog and Wnt but also mechanical stimuli.[26] In particular, evidence suggests that primary cilia are essential for Hedgehog signaling during development. In kidney epithelial cells, primary cilia also respond to mechanical force generated by fluid flow by activating the $Ca^{2+}$ channel Pkd2.[27),28] On the other hand, a recent study suggested that most primary cilia, including those at the node of the mouse embryo, do not function as mechanosensors that generate a $Ca^{2+}$ signal.[29] This issue may need further investigation, however, given that intraciliary $Ca^{2+}$ oscillations were detected at the LRO of zebrafish embryos.[30]

How crown cells and pit cells are specified during development remains unknown. Specification of crown cells in the mouse embryo requires Notch signaling.[31] In the zebrafish embryo, anisotropic mechanical strain graded along the medial–lateral axis specifies the identity of cilia at the LRO.[32] Short immotile cilia are thus generated at the medial region of the LRO where mechanical strain is low, whereas long motile cilia are generated at the lateral region where mechanical strain is high. It remains to be seen whether a similar mechanism specifies motile and immotile cilia at the LRO of other vertebrates such as in mice. Nevertheless, the spatial distribution of motile and immotile cilia at the LRO is conserved between zebrafish and mice.

**3.2. Unidirectional fluid flow generated by rotational movement of cilia breaks L-R symmetry.** The rotational movement of the motile cilia at the node generates a leftward laminar flow of extraembryonic fluid present in the node cav-

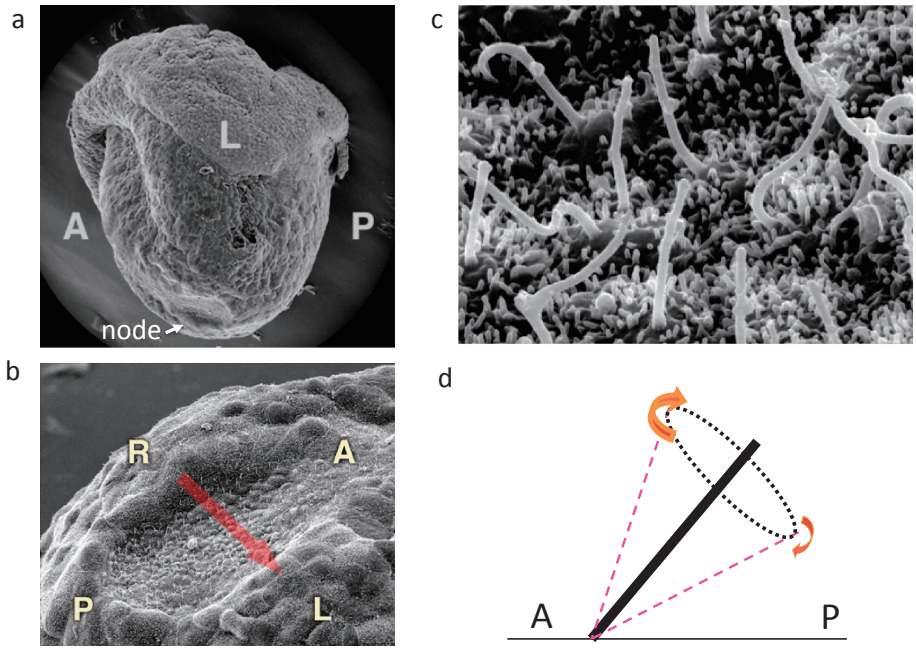


Fig. 3. Cilia at the node of a mouse embryo as revealed by scanning electron microscopy. **a.** Lateral view of a mouse embryo at E7.5. The arrow indicates the location of the node. **b.** Ventral view of the mouse node. The arrow indicates the direction of fluid flow at the node. **c.** Higher magnification of the node showing the presence of motile cilia. **d.** Posterior tilt of motile cilia. Clockwise rotation of posteriorly tilted cilia generates fluid flow (leftward) most efficiently when the cilia are farthest from the surface. A, anterior; L, left; P, posterior; R, right. Modified with permission from Shiratori and Hamada.[155)]

ity[12),33),34)] (Fig. 3), which occurs at a speed of ~15–20 μm/s. This leftward fluid flow at the node, known as nodal flow,[12),33)] is responsible for L-R symmetry breaking. The loss of nodal flow as a result of the lack of motile cilia or the loss of their motility thus results in aberrant L-R patterning of the LPM.[12),35),36)] Furthermore, L-R patterning of the mouse embryo was reversed when the direction of the flow was experimentally reversed,[37)] providing direct evidence that the direction of the fluid flow determines L-R asymmetry. Moreover, humans with laterality defects have been found to harbor mutations in genes that are required for ciliary motility.[38)] There are more than 20 genes (http://www.informatics.jax.org/disease/DOID:0110620), including those for dynein proteins (such as Dnah5 and Dnah11), proteins required for cytoplasmic assembly of dynein complexes, proteins that contribute to the transport of these complexes from the cytoplasm to cilia, and proteins necessary for docking of the complexes to axonemal microtubules.

How can unidirectional fluid flow be generated by rotational movement of the cilia? Evidence from modeling[39)] and *in vivo* observations[40),41)] suggests that the clockwise rotation of node cilia generates a leftward flow because the cilia are tilted toward the posterior side.

**3.3. L-R symmetry breaking by translation of preexisting information.** Given that the L-R axis is the last axis to be determined during development, L-R symmetry breaking is thought to be achieved in a manner dependent on preexisting positional cues. Indeed, two preexisting positional cues are represented in the cilia of node pit cells: The A-P and D-V axes are thus represented by the posterior tilt and ventral protrusion of the cilia, respectively (Fig. 4). A third cue is provided by the apparent chirality of the cilia that is attributable to a distinctive arrangement of microtubules and dynein arms and which is thought to allow the cilia to rotate in the clockwise direction. The node cilia thus generate the leftward flow by making use of the preexisting A-P and D-V positional cues and their structural chirality.

How is A-P information translated into the posterior tilt of the node cilia? Node cells are polarized along the A-P axis of the embryo by the planar cell polarity (PCP) pathway (Fig. 4), with PCP core proteins such as Prickle2 and Vangl1 being localized to the anterior side of node cells and another

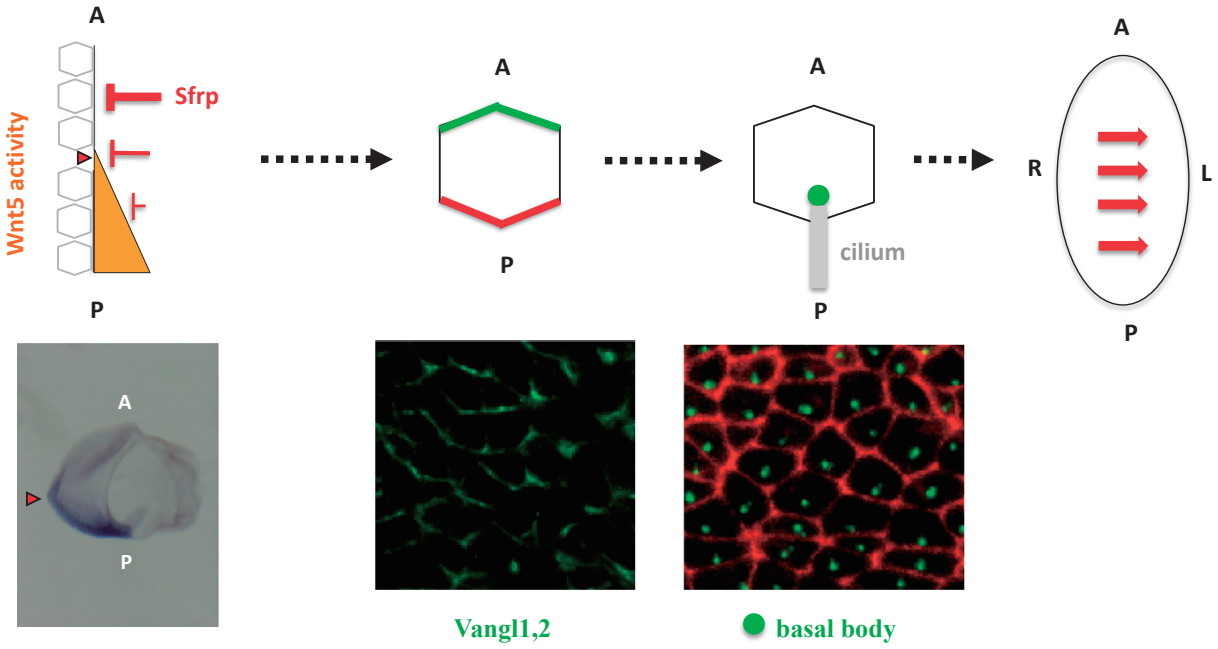


Fig. 4. Polarization of node cells with motile cilia along the A-P axis. Asymmetric expression of Wnt5 and its inhibitor Sfrp along the A-P axis generates a graded distribution of Wnt5a/b activity (orange). This will induce polarized localization of PCP core proteins (such as Vangl1, 2 in green and Dvl in red) in node cells with motile cilia (pit cells) along the A-P axis of the mouse embryo. Finally, the basal body will be localized to the posterior side of pit cells. Modified with permission from Minegishi *et al.*[42]

PCP core protein, Dvl, being localized to the posterior side. This polarized distribution of PCP proteins results in positioning of the basal body (an organelle located at the base of the cilium that serves as the template for ciliogenesis) at the posterior side of each node cell and subsequently confers a posterior tilt on the cilium.

The positional cue that polarizes node cells along the A-P axis is a gradient of Wnt5 activity along this axis[42] (Fig. 4). Two noncanonical Wnt ligands, Wnt5a and Wnt5b, are expressed in the posterior region of the node, whereas Sfrps (secreted Frizzled-related proteins), inhibitors of Wnt signaling, are expressed anterior to the node. The reciprocal expression of Wnt5a/b and their inhibitors may be expected to generate a gradient of Wnt5 activity along the A-P axis, with the activity being highest on the posterior side of the node. Overall, evidence suggests that a gradient of Wnt5 activity polarizes node cells so that PCP core proteins become localized to either anterior or posterior sides of the cells.

The basal body is initially localized at the center of pit cells of the node before its positioning at the posterior side of the cells. It remains unknown how the basal body changes its position within individual node cells. However, actomyosin may provide mechanical force capable of shifting the position of the basal body, given that Rho-associated kinase (Drok) links PCP signaling to the actin cytoskeleton in *Drosophila*[43] and that the polarized distribution of PCP proteins such as Celrs1 and Shroom3 regulates cytoskeletal dynamics and apical actomyosin contractility.[44),45] Of note, the basal body of ependymal ciliated epithelial cells is not correctly positioned and the beating pattern of airway cilia is disrupted, whereas laterality defects are not apparent, in a rat mutant lacking myosin 1d, an unconventional myosin.[46] In contrast, myosin 1d is required for leftward fluid flow and L-R asymmetric gene expression in *Xenopus*.[47] Whether actomyosin contractility contributes to positioning of the basal body of LRO cells such as pit cells in the mouse embryo remains to be demonstrated.

**3.4. Sensing of unidirectional fluid flow by immotile cilia.** The precise action of nodal flow remains unknown, but there are two prevailing hypotheses. The flow may transport small molecules or vesicular particles to the left side,[33),48] where they may then act as an L-R determinant. Alternatively, mechanical stimuli generated by the flow may be sensed by the embryo.[49),50]

Genetic evidence has indicated that it is the immotile cilia at the periphery of the node that sense the fluid flow and that such sensing triggers activation of the $Ca^{2+}$ channel Pkd2.[51] However, it remains unknown what the immotile cilia sense for L-R symmetry breaking—a chemical determinant, mechanical force, or some other signal? A chemical L-R determinant transported by the flow has not been identified in the more than 20 years since the discovery of nodal flow. Mathematical modeling favors either chemical sensing[52] or mechanical sensing.[39] A recent study failed to detect $Ca^{2+}$ influx in primary cilia in response to fluid flow, and it was suggested that immotile cilia at the node are not $Ca^{2+}$-responsive mechanosensors.[29] Further investigation is thus required to clarify how nodal flow is sensed by the embryo.

**3.5. Role of $Ca^{2+}$ in flow sensing.** Several lines of evidence suggest that $Ca^{2+}$ plays a role in the sensing of nodal flow. First, a $Ca^{2+}$ channel composed of Pkd2[53] and Pkd1l1[54)–56] is required for L-R patterning (Fig. 5). Given that the L-R defects of *Pkd2*$^{-/-}$ mutant mice can be rescued by expression of a *Pkd2* transgene specifically in crown cells,[51] Pkd2 in these cells is sufficient for flow sensing. Pkd2, together with Pkd1l1, also likely functions specifically in the cilium of crown cells, given that a mutation of *Pkd2* that disrupts the ciliary localization of the encoded protein gives rise to L-R defects similar to those of *Pkd2*$^{-/-}$ embryos.[51),54] *Pkd2* encodes a $Ca^{2+}$ channel with a short extracellular domain (although, to be more precise, Pkd2 is regarded as a nonselective cation channel and may not be specific for $Ca^{2+}$). The Pkd1l1 protein possesses a much larger extracellular domain at its amino terminus, suggesting that Pkd1l1 may be responsible for sensing the flow signal. Genetic evidence[57] also suggests that Pkd1l1 may inhibit the channel activity of Pkd2 in condition with no flow, and that flow may relieve this inhibition and thereby activate the Pkd2 channel.

Second, L-R asymmetric $Ca^{2+}$ signaling has been detected at the node of mouse embryos,[50),58] because they have L-R asymmetric $Ca^{2+}$ oscillations at the

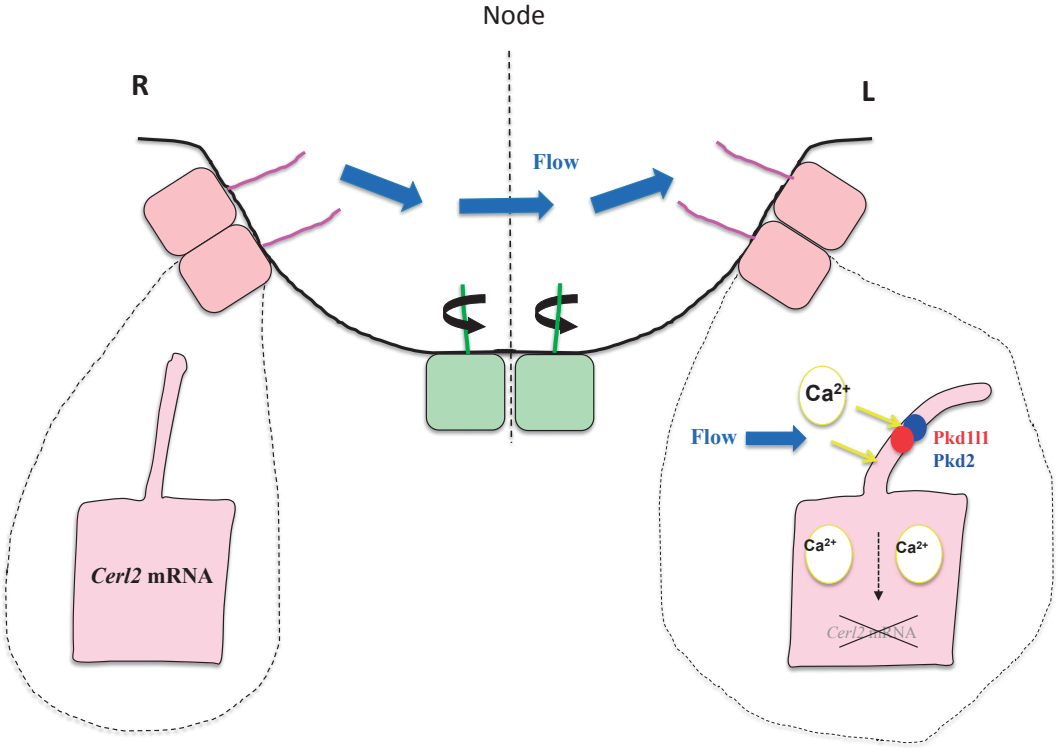


Fig. 5. Immotile cilia at the periphery of the node sense nodal flow. Ciliated cells located in the central region of the node of the mouse embryo (green) possess motile cilia that rotate and generate nodal flow, whereas those located peripherally (pink) possess immotile cilia that sense the flow. Sensing of the flow requires a Pkd2-Pkd1l1 complex with $Ca^{2+}$ channel activity that is localized to the cilium. The flow-mediated signal, the identity of which remains unknown, triggers the degradation of *Cerl2* mRNA preferentially on the left side.

LRO of the zebrafish embryo.[30] Third, several $Ca^{2+}$ blockers have been found to disrupt asymmetric gene expression in crown cells.[51] Thus, $GdCl_3$ (an inhibitor of stretch-sensitive transient receptor potential [TRP] channels), 2-aminoethyl diphenylborinate (an inhibitor of the inositol 1,4,5-trisphosphate [$IP_3$] receptor), and thapsigargin (an inhibitor of $Ca^{2+}$-dependent ATPase activity in the endoplasmic reticulum) impair L-R asymmetric expression of a flow-responsive transgene.[51),59] Finally, inhibition of fibroblast growth factor (FGF) signaling, which is essential for normal L-R asymmetry, was found to attenuate the $Ca^{2+}$ signal on the left side of the ventral node of the mouse embryo without disturbing the leftward fluid flow.[48]

These observations suggest a possible route for $Ca^{2+}$ within node cells. First, $Ca^{2+}$ can enter crown cells via a stretch-sensitive TRP channel (such as Pkd2) located in the membrane of immotile cilia. This intraciliary $Ca^{2+}$ would then reach the cell body, act at the $IP_3$ receptor directly or indirectly, and induce the release of more $Ca^{2+}$ from the endoplasmic reticulum into the cytosol.

**3.6. *Cerl2/Dand5* mRNA as a target of flow-mediated signaling.** What are the molecular events that take place in crown cells when they receive the flow-mediated signal? *Cerl2* (also known as *Dand5*) mRNA is the target of this signal.[60),61] The Cerl2 protein inhibits the activity of the transforming growth factor $\beta$ (TGF$\beta$)-related protein Nodal, likely by directly interacting with it. The activity of Nodal is greatly increased by its formation of a heterodimer with another TGF$\beta$-related protein, Gdf1 (growth differentiation factor 1),[62] and Cerl2 may inhibit formation of the Nodal-Gdf1 heterodimer. The level of *Cerl2* mRNA is R > L asymmetric (higher on the right side than on the left side) among crown cells, and *Cerl2* mutant mice manifest randomization of L-R decision-making.[63]

The abundance of *Cerl2* mRNA is initially symmetric (R = L) at the early headfold stage of mouse embryogenesis, but it becomes R > L asymmetric as the velocity of nodal flow increases, with the amount on the left side being down-regulated.[59),61] The L-R asymmetry of *Cerl2* expression is determined not at the level of transcription but rather posttranscriptionally,[64] in particular, by the decay of *Cerl2* mRNA in a manner dependent on its 3′ untranslated region. The degradation of *Cerl2* mRNA preferentially on the left is triggered by the leftward fluid flow (Fig. 5) and is further enhanced by the operation of complex Wnt-Cerl2 interlinked feedback loops, in which Wnt3 increases *Wnt3* expression and promotes *Cerl2* mRNA decay whereas Cerl2 promotes the degradation of Wnt3. Both mathematical modeling and experimental data[64] suggest that these feedback loops constitute a bistable switch that is capable of amplifying in a noise-resistant manner a small L-R bias conferred by nodal flow.[61]

The precise mechanism of *Cerl2* mRNA decay in response to nodal flow remains unknown. However, it may be relevant that Bicc1, a putative RNA-binding protein specifically expressed at the node of mouse embryos, is essential for L-R asymmetry.[65] Furthermore, Bicc1 interacts with ANKS3 and ANKS6,[66),67] and *ANKS3* mutations have been identified in patients with laterality defects.[68] Finally, Bicc1 also binds to *Cerl2* mRNA in zebrafish and thereby inhibits its translation.[69] It remains to be determined whether a Bicc1-Anks3-Anks6 complex plays a role in the degradation of *Cerl2* mRNA and how $Ca^{2+}$ might be involved in this process.

**3.7. The mechanism of L-R symmetry breaking is not conserved among vertebrates: cilia- and flow-independent symmetry breaking in chick and reptile embryos.** The chick embryo deploys a mechanism of L-R symmetry breaking that is independent of motile cilia and fluid flow.[20] Motile cilia are absent at Hensen's node of the chick embryo. Furthermore, the avian *talpid2* mutant, in which the gene for C2CD3, a protein essential for ciliogenesis, is disrupted, manifests a ciliopathy phenotype, including polydactyly and facial clefting. However, it does not show laterality defects,[70] indicating that cilia are not required for L-R symmetry breaking in the chick. Instead, asymmetric cell rearrangement, in particular a leftward movement of cells around Hensen's node, is responsible for L-R symmetry breaking in this species. This rearrangement results in the relative displacement of cells expressing Sonic hedgehog (Shh) and those expressing FGF8, and it thereby gives rise to asymmetric expression domains. It has also recently been shown that reptile embryos develop L-R asymmetry without motile cilia and fluid flow at the LRO.[21] It will thus be of interest to learn the origin of L-R asymmetry in reptiles and birds.

Although it is generally believed that the mechanism of L-R symmetry breaking is conserved among mammals, this conclusion requires further evidence. LRO morphology varies substantially among mammals,[71] and it has been suggested[20] that the LRO of the pig embryo does not have sufficient space for motile cilia to generate fluid flow.

## 4. Signaling from the LRO to the lateral plate

**4.1. Asymmetric signals at the node.** Three signaling molecules expressed in crown cells play a key role in setting up molecular L-R asymmetry at the node in mouse embryos (Fig. 6). First, *Nodal* is expressed bilaterally in node crown cells in a manner dependent on a crown cell-specific enhancer (NDE), the activity of which requires binding sequences for Rbpjk, a downstream mediator of Notch signaling, suggesting that such expression is induced by Notch.[31] The expression of *Nodal* in crown cells is followed by that in the left LPM, with the former being essential for the latter, given that specific ablation of NDE results in the loss of *Nodal* expression in the LPM.

Second, *Gdf1* is also bilaterally expressed in node crown cells. Furthermore, as mentioned above, the activity of Nodal complexed with Gdf1 is much greater (~100-fold) than that of the Nodal homodimer. In a *Gdf1* mutant mouse, Nodal activity in or near crown cells is greatly attenuated[59] and the expression of *Nodal* in the LPM is absent.[72] Collectively, these observations suggest that Gdf1 itself is not active at physiological concentrations but that it instead acts as a co-ligand for Nodal at the node.

The third important signaling molecule expressed in crown cells is Cerl2 (Dand5), a member of the Cerberus/Dan family of proteins that inhibit Nodal signaling.[63] As mentioned above, the level of *Cerl2* mRNA in crown cells is symmetric before the development of nodal flow, but becomes R > L asymmetric in response to the flow. This asymmetric (R > L) expression of *Cerl2* ensures that Nodal activity in crown cells is higher on the left side

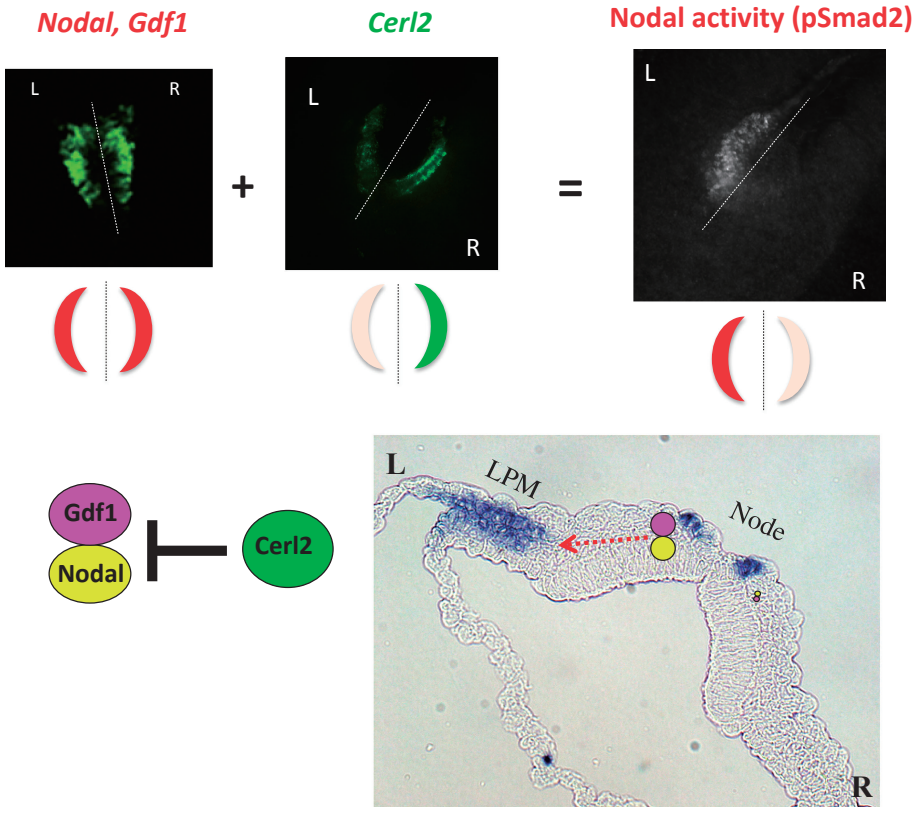


Fig. 6. Generation of molecular asymmetries at the node. Whereas *Nodal* mRNA and *Gdf1* mRNA are present at similar levels on both sides of the node of a mouse embryo, *Cerl2* mRNA shows an asymmetric (R > L) distribution (top panels). Nodal and Gdf1 form a heterodimer that constitutes an active form of Nodal. Given that Cerl2 is an inhibitor of Nodal (bottom left panel), the level of Nodal activity, which is reflected by the abundance of phosphorylated Smad2/3 (pSmad2), shows a R ≪ L pattern (top panels). The Nodal-Gdf1 heterodimer produced by and secreted from perinodal crown cells is thought to be transported to the LPM on the left side via an intraembryonic route (red dotted arrow in the bottom right panel). On reaching the LPM, the Nodal-Gdf1 heterodimer is thought to activate expression of *Nodal* (indicated by purple staining in the bottom right panel), which is responsive to Nodal signaling. Modified with permission from Shiratori and Hamada,[155] Yoshiba and Hamada,[156] and Shiratori and Hamada.[157]

(Fig. 6). The Cerl2-generated asymmetry (R < L) of Nodal activity at the node closely correlates with the asymmetry of *Nodal* expression in the LPM.[59] It thus appears that Cerl2 selectively inhibits Nodal-Gdf1 heterodimer activity in crown cells on the right side, which then leads to induction of *Nodal* expression in the left LPM. This mechanism is conserved at least in zebrafish,[73] *Xenopus*, and mouse.

**4.2. Transfer of the Nodal signal from the node to the LPM.** Nodal produced in crown cells is required for subsequent *Nodal* expression in the left LPM.[74),75] Evidence suggests that active Nodal protein (presumably, the Nodal-Gdf1 heterodimer) produced in crown cells on the left side is transported to the left LPM, where it directly activates *Nodal* expression. First, a transcriptional enhancer (ASE) required for *Nodal* expression in the LPM is responsive to Nodal. Second, Cryptic, a co-receptor of Nodal essential for left-sided *Nodal* expression in the lateral plate, is required only in the LPM for correct L-R patterning.[76]

Available data also suggest that active Nodal protein (Nodal-Gdf1 heterodimer) synthesized in crown cells is transported from the node to the LPM via an intraembryonic route (Fig. 6). First, *Nodal* expression in the LPM remained unaffected when mouse embryos were incubated with recombinant Nodal in culture medium. Second, Nodal interacts with sulfated glycosaminoglycans that are localized specifically to the basement membrane between the node and lateral plate. Furthermore, inhibition of sulfated glycosaminoglycan synthesis abolished *Nodal* expression in the LPM.[76]

## 5. Differential patterning of the lateral plate on both sides

**5.1. Role of the TGF$\beta$-related factors Nodal and Lefty.** Two TGF$\beta$-related proteins, Nodal and Lefty, are expressed asymmetrically in the LPM (Fig. 7) and play a major role in its patterning.[7)–9),77] Genetic and biochemical studies have established that Nodal and Lefty have opposite functions. Nodal functions as a left-side determinant,[74] whereas Lefty is an antagonist of Nodal that restricts Nodal activity to the left side.[78] Mutant mice lacking *Nodal* expression in the LPM thus show right isomerism of bilateral organs such as the lung, whereas mutant mice that express *Nodal* on both sides of the LPM manifest left isomerism. On the other hand, the absence of Lefty results in bilateral *Nodal* expression in the LPM and subsequent left isomerism.[78),79] Vertebrates possess two *Lefty* genes, *Lefty1* and *Lefty2*, both of which are expressed in the left LPM and at the midline.[80] Given that Lefty1 and Lefty2 have indistinguishable activities and show similar expression patterns, they will be referred to collectively as Lefty in this review.

Nodal activates intracellular signaling through interaction with type I (ALK4 and ALK7) and type II (ActRIIa and ActRIIb) TGF$\beta$ receptors[81] (Fig. 8). As described above, Nodal forms a heterodimer with Gdf1 (Vg1 in frog), with Gdf1 serving as a co-ligand that increases Nodal activity.[62),82] Unlike other TGF$\beta$ family members, however, Nodal requires an EGF-CFC (epidermal growth factor–Cripto-FRL1-Cryptic) family protein (such as Cryptic or Cripto) as a co-receptor. Smad2 and Smad3, together with Smad4, are intracellular components of the Nodal signaling pathway. FoxH1 is the major transcription factor that interacts with Smad2/3 and transduces the Nodal signal. Lefty inhibits Nodal signaling by interacting in a competitive manner with the EGF-CFC co-receptor[83] and the type II TGF$\beta$ receptor chain.[84]

Nodal signaling is precisely regulated both positively and negatively at several levels by various molecules during development (Fig. 8). Drap1 is a transcriptional repressor that interacts with FoxH1 and inhibits its DNA binding activity.[85] Ectodermin negatively controls Nodal signaling by inhibiting Smad4 activity.[86] Mice lacking Drap1 or Ectodermin thus manifest increased Nodal signaling. Nodal signaling is also subject to translational repression,[87] at least three different mechanisms have been identified in the zebrafish embryo: (1) repression of *Nodal* mRNA translation by Ybx1, (2) repression of *Cr1* (*Cripto*) mRNA translation, and (3) repression of *Nodal* mRNA translation by the microRNA miR-430.[88] Nodal signaling is essential not only for L-R patterning but also for A-P patterning as well as mesoderm induction and patterning.[89] These various negative regulators of Nodal signaling have been shown to be essential for mesoderm formation, but it is unknown whether they play a role in L-R patterning.

**5.2. Transcriptional regulation of *Nodal* and *Lefty* and formation of a self-enhancement lateral inhibition system by the encoded proteins.** L-R asymmetric expression of *Nodal* and *Lefty* is transient, with a duration of only several hours in the mouse embryo, from the two- to six-somite stage. This transient expression is established by positive and negative regulatory loops connecting these genes (Fig. 8). Asymmetric expression of *Nodal* and *Lefty*

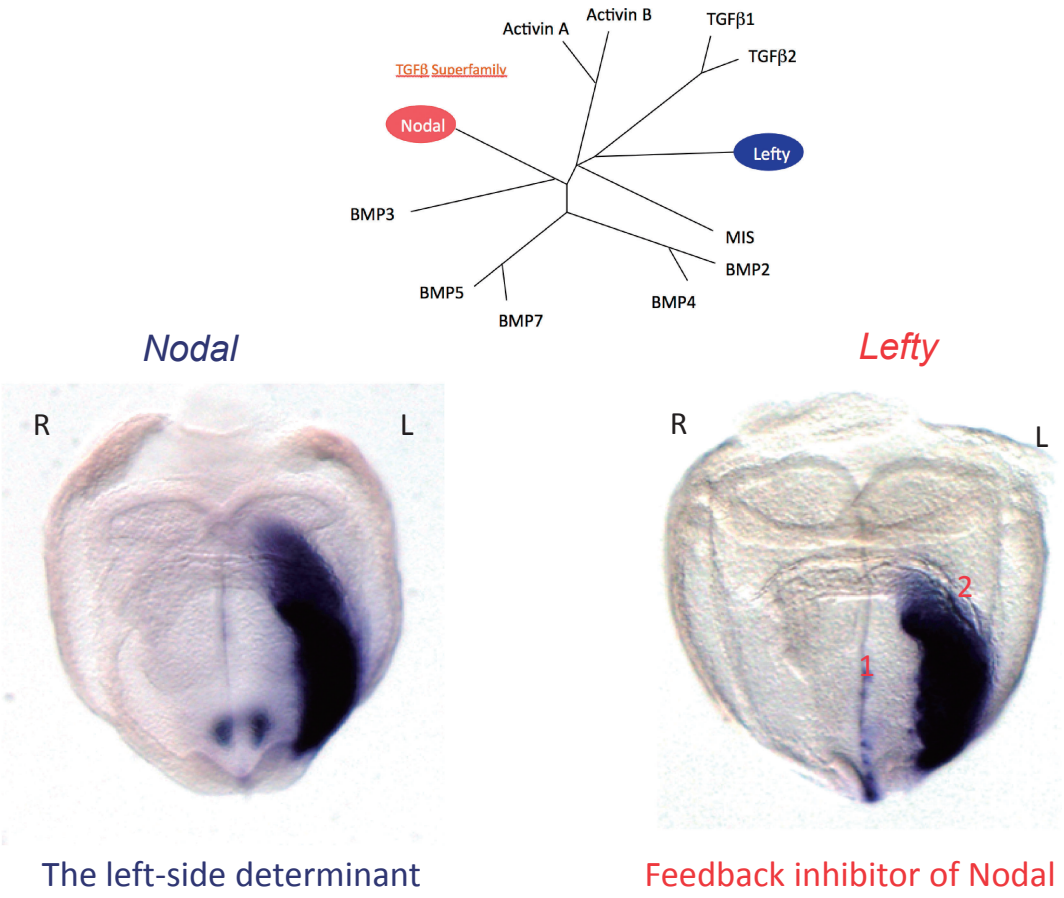


Fig. 7. L-R asymmetric expression of *Nodal* and *Lefty* in the mouse embryo. Cluster analysis (top) shows the relations among members of the TGF$\beta$ superfamily of proteins. *In situ* hybridization (bottom) reveals the L-R asymmetric expression of *Nodal* (which encodes a left-side determinant) and *Lefty* (which encodes a feedback inhibitor of Nodal) in the E8.0 mouse embryo. Note that *Nodal* and *Lefty* are expressed in the LPM on the left side, whereas *Lefty* is also expressed at the midline (left side of the floor plate). There are actually two *Lefty* genes, with *Lefty2* being preferentially expressed in the LPM and *Lefty1* at the midline. Modified with permission from Shiratori and Hamada.[157]

in the LPM relies on the Nodal-responsive, FoxH1-dependent enhancer ASE.[90] *Nodal* expression is thus positively regulated by Nodal itself and negatively regulated by the feedback inhibitor Lefty. Interaction of Nodal protein with target cells triggers the transcription of both *Nodal* and *Lefty*. The Nodal thereby produced amplifies expression of *Nodal* and *Lefty*, whereas the Lefty produced eventually terminates the expression of both genes. The Lefty-mediated negative loop is therefore expected to restrict the duration and area of Nodal signaling in a highly precise manner, accounting for the transient nature of asymmetric *Nodal* and *Lefty* expression. The activities of Nodal and Lefty proteins and this transcriptional regulatory relation between the corresponding genes suggest that the two proteins may constitute a self-enhancement lateral inhibition system,[91] a type of Turing reaction-diffusion system.[92] Such a system comprising an activator and an inhibitor requires in principle that the inhibitor diffuses faster than the activator, and this is indeed the case for Nodal-Lefty at least in the zebrafish embryo.[93),94] Computational simulations of gradient formation indicate that diffusivity, extracellular interactions, and selective ligand destruction collectively shape the Nodal morphogen gradient.[94] The Nodal-Lefty network may contribute to various patterning events during development including scaling.[95]

Asymmetric gene expression is also subject to epigenetic regulation. Depletion of DNA methyltransferase enzymes (Dnmt1 or Dnmt3bb.1) in zebrafish embryos thus gives rise to hypomethylation of the *Lefty2* enhancer, resulting in up-regulation of

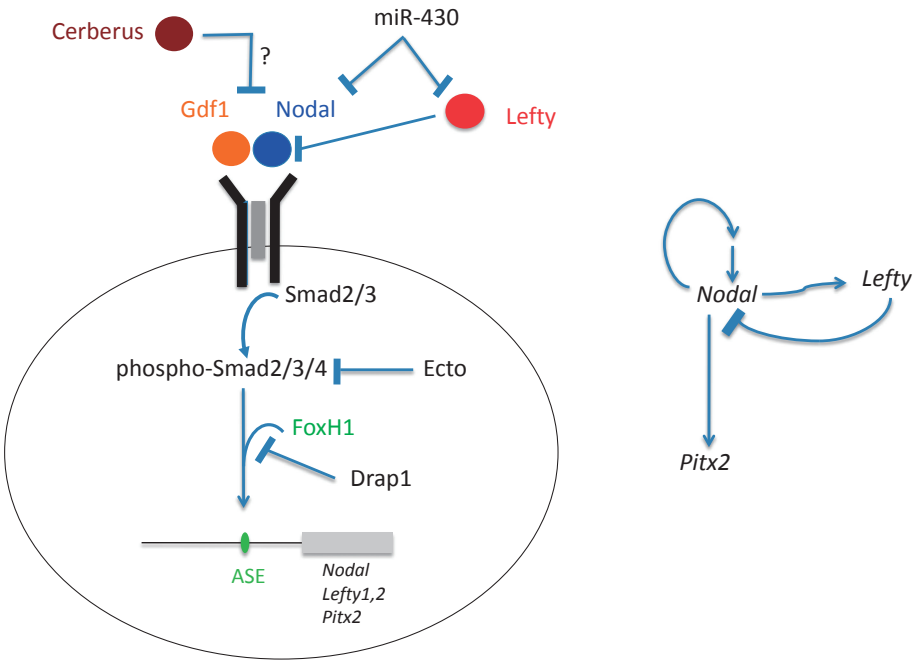


Fig. 8. Control of Nodal signaling by various regulators in the developing vertebrate embryo. The Nodal signaling pathway in vertebrate embryos (Nodal → receptor → Smad2/3/4 → FoxH1 → target genes) is regulated in a spatiotemporal manner at multiple levels by various inhibitors including Lefty, Cerberus (Cerl2), Ectodermin (Ecto), Drap1, and miR-430 (left panel). Nodal and Lefty also participate in positive and negative regulatory loops (right panel).

*Lefty2* expression, attenuation of Nodal signaling, down-regulation of *Nodal* expression in the LPM, and disruption of organ laterality.[96] *Lefty2* expression in the postimplantation mouse embryos is also regulated by the DNA demethylase Tet1.[97]

## 6. Asymmetric organogenesis

**6.1. Asymmetric organs.** Various visceral organs begin to develop anatomic asymmetries after asymmetric *Nodal* expression in the LPM has disappeared. Theoretically, a symmetric structure can become asymmetric in at least three ways (Fig. 9). First, a primordial organ that is present in just one copy in an embryo could initially be located at the midline but subsequently undergo a directional morphological change such as looping that would result in positioning of the organ on one side. Examples of such morphogenesis by directional looping include the heart and gut. Second, a primordial organ could be initially formed as a bilaterally symmetric structure, but the two sides might subsequently undergo differential growth or branching. Examples of this type of morphogenesis include the lung, which has one lobe on the left and four lobes on the right in the adult mouse (two lobes on the left and three lobes on the right in human).

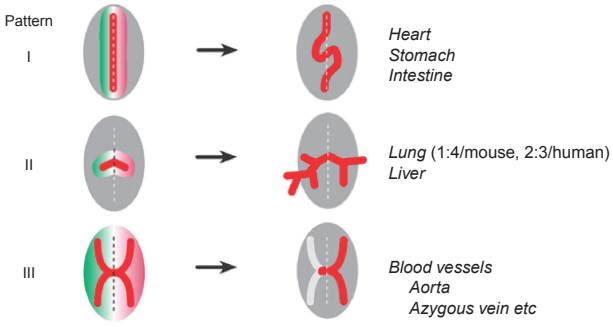


Fig. 9. Generation of morphological asymmetries. Three different patterns for generation of morphological asymmetries rely on (I) directional looping, (II) differential branching, or (III) one-sided regression. Examples of anatomic structures generated by each mechanism are shown. Modified with permission from Shiratori and Hamada[155] and courtesy of Yukio Saijoh (University of Utah).

The third type is unilateral regression, as exemplified during remodeling of the vascular system. The embryonic vascular system is thus initially bilaterally symmetric, but some parts subsequently undergo regression specifically on one side.

**6.2. Genetic basis of the asymmetric morphogenesis: role of Nodal signaling and the transcription factor Pitx2.** The main intracellular

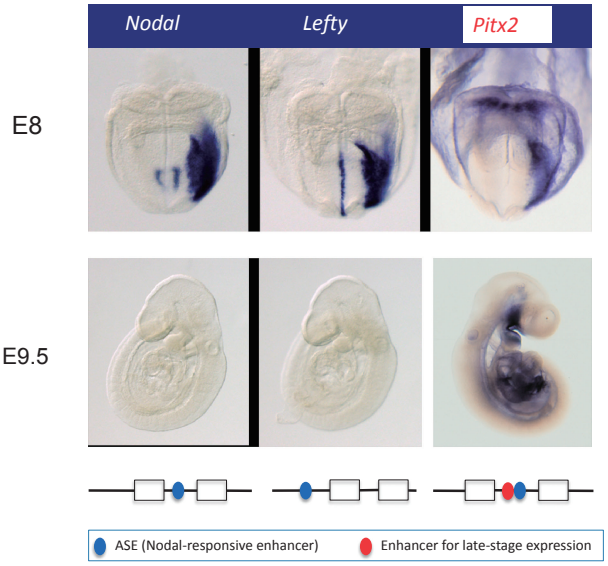


Fig. 10. L-R asymmetric expression of *Nodal*, *Lefty*, and *Pitx2* in the mouse embryo. *In situ* hybridization reveals that, whereas L-R asymmetric expression of *Nodal* and *Lefty* is transient (detectable around E8), L-R asymmetric expression of *Pitx2* persists much longer. Asymmetric expression of *Nodal* and *Lefty* is controlled by a Nodal-responsive enhancer (ASE), which renders their asymmetric expression transient. *Pitx2*, however, contains both ASE and an additional enhancer that maintains its expression at later stages. Modified with permission from Shiratori and Hamada.[157]

mediator of asymmetric organogenesis is the transcription factor Pitx2,[98)–101)] the left-sided expression of which is induced by Nodal.[102)] Like *Nodal* and *Lefty*, *Pitx2* is expressed asymmetrically in the LPM, but its left-sided expression persists until much later stages of development (Fig. 10). Mice deficient in Pitx2 (to be more precise, Pitx2c, the isoform that is asymmetrically expressed) manifest laterality defects in most visceral organs.[103)] Pitx2c is responsible for the generation of left-side morphology. Thus, in its absence, bilateral organs such as the lung exhibit right isomerism.

Whereas the development of most L-R asymmetric organs requires Pitx2, some asymmetric morphogenic events are independent of this protein. One such example is heart looping in mice, which is under the control of left-sided Nodal signaling but not of Pitx2. In mice specifically lacking Pitx2c, heart looping and the stomach thus remain normal even though other asymmetric organs show laterality defects.[103),104)] Similarly, asymmetric looping of the heart and gut remains normal in a zebrafish *Pitx2* mutant (most likely a null mutant).[105)] The direction of heart looping is also largely normal in a zebrafish mutant lacking *southpaw* (*Nodal* in zebrafish), suggesting that heart looping is independent of Nodal signaling in this organism.[106)] In support of this notion, a recent study suggests that heart looping in zebrafish and chick is controlled by a bone morphogenetic protein (BMP)–Prrx1a axis on the right side.[107)] The Nodal-Pitx2 pathway in the left LPM and the BMP-Prrx1a pathway in the right LPM seem to operate in parallel and to engage in mutual repression. Whereas *Prrx1* or *Prrx1/Prrx2* mutant mice show normal heart looping,[108),109)] the role of Prrx1 in chicks may be filled by Snail1 in mice.[107)] The precise role of right-side-specific signaling in asymmetric organogenesis awaits further clarification.

**6.3. Heart and outflow tract.** Heart formation is under the control of correct L-R patterning,[110)] which determines the position of the atria relative to nearby organs (such as the stomach and spleen), the orientation of ventricular looping, and the position of the great vessels. In fact, screening of a large number of chemically mutagenized mice for congenital heart disease (CHD) identified 61 genes that can give rise to this condition.[111)] Mutations in 30 of these 61 genes gave rise to CHD with laterality defects, and 23 of these 30 genes belong to the ciliome family of genes that are required for the motility of rotating cilia or for the sensing of fluid flow by immotile cilia at the mouse node. Similarly, among 28 CHD genes identified in humans, 11 are cilia related.[112),113)] The L-R symmetry-breaking event mediated by cilia thus has profound effects on heart formation.

Heart development begins with the specification of bilateral precardiogenic mesoderm, which subsequently converges at the midline to generate a linear heart tube. The heart is symmetric until this stage, but as the heart tube elongates along the A-P axis it starts to bulge on its ventral side and to bend rightward. The developing heart thus changes its shape from a simple linear tube to a structure with a rightward helical loop. This process, known as heart looping, is crucial for the subsequent formation of the atrial and ventricular chambers. In addition to heart looping, other events that take place during heart formation also depend on L-R asymmetry including (1) spiral septum formation, defects in which result in transposition of the great arteries and double outlet right ventricle, and (2) formation of the pulmonary vein by angiogenic sprouting from the left atrium.

How does the linear heart tube undergo rightward (dextral) looping? Three theoretical mechanisms have been postulated: differential growth within the heart tube, oriented growth within the

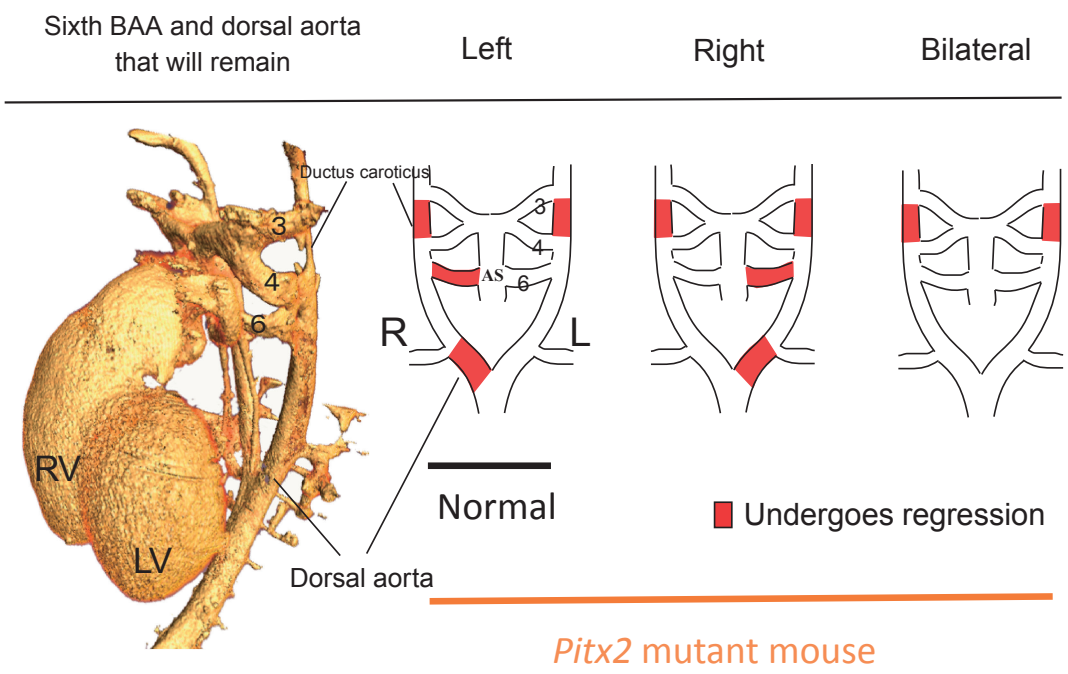


Fig. 11. L-R asymmetric remodeling of the sixth branchial arch artery in the mouse embryo. In a normal embryo, the right side of the sixth branchial arch artery (BAA) and the right side of the dorsal aorta regress as development proceeds, eventually resulting in arching of the aorta toward the left side. In *Pitx2* mutant mice; however, this pattern of remodeling is impaired. AS, aortic sac; RV and LV, right and left ventricle.

heart tube, and buckling.[110] The heart tube elongates as a result of ingression of cardiac progenitor cells from the surrounding tissues at both ends of the tube, which are known as the venous and arterial poles. Observation of the developing mouse heart at this stage by high-resolution microscopy has identified two types of asymmetry at these poles.[114] First, the arterial pole manifests rotational asymmetry and is also positioned asymmetrically across the midline. This rotational asymmetry present before heart looping is apparent not only in the mouse but also in other animals such as chick and zebrafish.[115),116] Second, the venous pole exhibits asymmetric ingression and proliferation of cardiac progenitor cells, with more cells ingressing through the right half of the venous pole than through the left half.[114] A computational model suggested that biomechanical forces generated by the asymmetric rotation and asymmetric cell ingression can drive rightward heart looping.[114] Indeed, cells that surround and will undergo ingression into the heart show mechanical stress, and this epithelial tension contributes to elongation and looping of the developing heart.[117] However, the molecular and cellular mechanisms underlying the asymmetric rotation and asymmetric cell ingression remain unknown.

The developing heart and outflow tract also undergo asymmetric remodeling. For example, the aortic arch, which is connected to the left ventricle and arches toward the left side, is derived from the dorsal aorta and branchial arch arteries (BAAs) at embryonic stages. The dorsal aorta and BAAs are initially formed symmetrically but subsequently undergo asymmetric remodeling. The left sixth BAA thus persists and gives rise to the aortic arch, whereas the right sixth BAA regresses (Fig. 11). A dynamic morphological change in the outflow tract of the heart, which is under the control of *Pitx2*, results in provision of an asymmetric blood supply to the left sixth BAA.[118] This uneven distribution of blood flow results in differential signaling by both the platelet-derived growth factor receptor and vascular endothelial growth factor receptor 2. The consequent stabilization of the left sixth BAA and regression of the right sixth BAA underpin left-sided formation of the aortic arch. Hemodynamics generated by a Pitx2-induced morphological change in the outflow tract are thus responsible for the asymmetric remodeling of the great arteries.

**6.4. Gut.** The developing gut consists of the foregut, midgut, and hindgut, from anterior to posterior. The foregut will give rise to the anterior

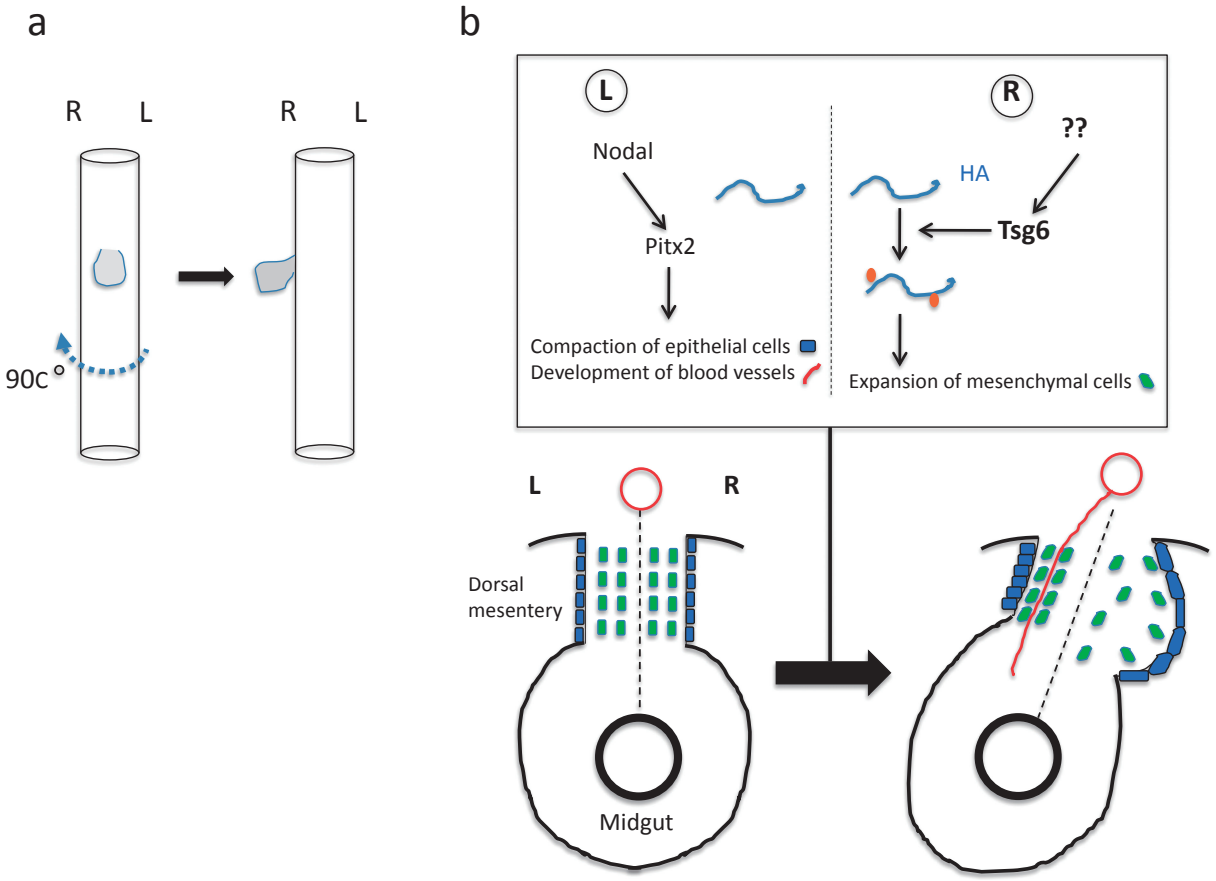


Fig. 12. Rotation and looping of the developing gut. **a.** Clockwise rotation of the foregut results in translocation of the liver primordium (gray) to the right side of the body cavity. **b.** Cellular changes that initiate L-R asymmetry in the midgut tube of the chick embryo.[120),123)] Asymmetries arise in the dorsal mesentery between Hamburger–Hamilton (HH) stages 20 and 22. Left side-specific Nodal-Pitx2 expression drives compaction of mesenchymal cells (green rectangles) within the left dorsal mesentery and promotes retention of a columnar morphology of epithelial cells (blue rectangles) on the left side of the dorsal mesentery. In the right dorsal mesentery, hyaluronan (HA) undergoes modification by Tsg6, which catalyzes the covalent attachment of a heavy chain (orange) of inter-$\alpha$-trypsin inhibitor. Signals that induce *Tsg6* expression in the right half of the dorsal mesentery remain unknown. The modified form of hyaluronan is more stable and accumulates in the right dorsal mesentery, resulting in expansion of mesenchymal cells and exclusion of blood vessels (red line) in the right dorsal mesentery. Together, these cellular asymmetries drive leftward tilting of the gut tube. Left (L) and right (R) sides of the dorsal mesentery are indicated together with the midline (broken line). Modified with permission from Hamada.[158)]

portions of the digestive tract including the esophagus, stomach, and duodenum, as well as to adjacent organs such as the pancreas and liver. The liver is an asymmetric organ in that it is located asymmetrically in the body cavity. A 90-degree clockwise rotation (looking down from the rostral side) of the foregut results in translocation of the liver primordium to the right side (Fig. 12a). The liver also manifests morphological asymmetries in its size and shape. In humans, for example, the right side of the liver is five to six times as large as is the left side, and it shows a distinct morphology and more complex lobation pattern. Similar morphological asymmetries are present in the liver of other vertebrates, although the precise morphologies differ among species. During formation of the liver in *Xenopus* embryos, endoderm cells on the right side become columnar and apically constricted, whereas those on the left side become rounder and rearrange into a compact structure.[119)] The development of such cellular asymmetries is regulated by Pitx2c and is responsible for the generation of future morphological asymmetries of the adult liver. Of interest, the cellular response to Pitx2c in the developing liver is opposite to that apparent in the embryonic midgut, in which endoderm cells on the left side of the dorsal mesentery

remain columnar and those on the right side become compact.

The midgut is the future small intestine. Given that the gut is a long tubular organ that far exceeds the length of the body, it must be packaged to fit within the limited abdominal space. This feat is accomplished by a process known as gut looping, which takes place early in development. At the early embryonic stage, the gut is a simple tube composed of endodermal epithelium with mesenchyme recruited from the LPM. The mesenchyme portion becomes the dorsal mesentery, which will connect the dorsal edge of the gut to the body wall along its entire length. As the gut elongates rapidly along the A-P axis, the midgut undergoes a 270-degree anticlockwise rotation. The symmetry-breaking event that initiates this rotation and will result in midgut looping is a leftward tilt of the dorsal mesentery (Fig. 12b).[120),121)] It has been suggested that two types of cellular changes in and around the dorsal mesentery are responsible for this leftward tilt.[121),122)] First, mesenchymal cells of the right half of the dorsal mesentery become dispersed, whereas those of the left half become compacted. Second, epithelial endoderm cells on the right side of the dorsal mesentery expand and flatten, whereas those on the left side retain a narrow columnar shape. Together, these cellular asymmetries drive the leftward tilting of the midgut. These cellular changes are under the control of Pitx2, which is expressed in the left half of the dorsal mesentery. The genes for N-cadherin and Daam2, which are targets of Pitx2, are also expressed on the left side of the dorsal mesentery, and their expression may confer adhesive and condensed features to cells on the left side. These observations suggest that left side-specific events governed by Nodal-Pitx2 determine the direction of midgut looping. However, a right side-specific pathway may also exist. Covalent modification of the extracellular matrix component hyaluronan by Tsg6 takes place on the right side of chick and mouse embryos, and this modification is essential for midgut looping.[123)] *Tsg6* is expressed in the dorsal mesentery on the right side of chick embryos, and *Tsg6* mutant mice manifest aberrant rotation of the midgut.[123)] The precise relation between the Nodal-Pitx2 pathway and the Tsg6-hyaluronan pathway remains to be clarified.

Additional loops form as the gut elongates, bends, and twists. Association of the gut and dorsal mesentery is required for formation of the mature loops in the gut.[124)] The looping morphology can thus be maintained after dissection of the intestine together with the dorsal mesentery away from all other embryonic tissues. In contrast, separation of the intestine from the dorsal mesentery results in uncoiling of the gut and further coiling of the dorsal mesentery. These observations suggest that the gut tube is under compression whereas the dorsal mesentery is under tension. Such isotropic forces arise from differential growth between the faster-growing gut and the slower-growing dorsal mesentery.[124)]

**6.5. Brain.** Most insight into the development of brain asymmetry has come from studies of zebrafish, which shows a pronounced anatomic asymmetry in the epithalamus.[125)] The epithalamus consists of bilateral habenular nuclei and an unpaired pineal complex (Fig. 13). The habenular nuclei form a conserved limbic conduction system linking the telencephalon (forebrain) to the mesencephalon (midbrain). The pineal is situated at the midline

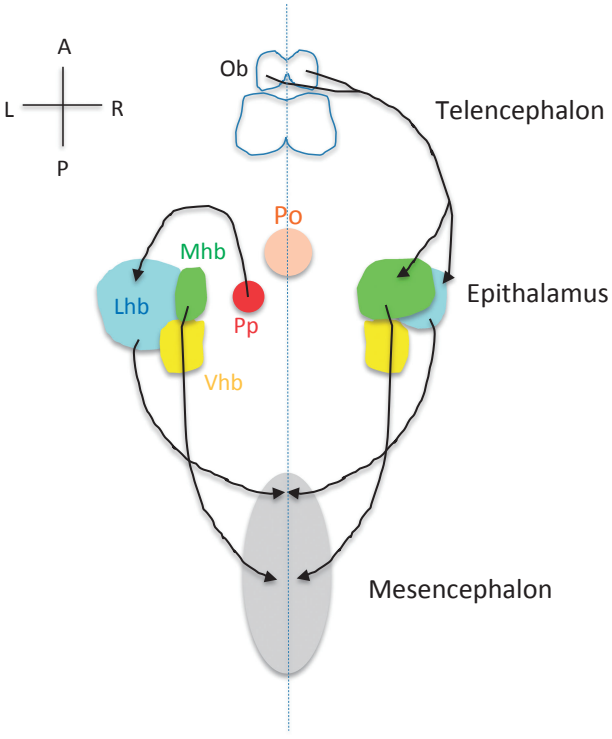


Fig. 13. The epithalamic system and L-R asymmetric neural projection in zebrafish. The habenula of zebrafish is divided into a dorsal component and a ventral component (Vhb), with the dorsal habenula being constituted by the lateral (Lhb) and medial (Mhb) subnuclei of unequal size. Further components of the zebrafish epithalamus are the photosensitive pineal (Po) and the asymmetrically organized parapineal organ (Pp). The Lhb and Mhb project to the mesencephalon. The olfactory bulb (Ob) projects to the Lhb and Mhb on the right side. Bilaterally symmetric neural projections are not shown.

and functions as a photosensitive clock that secretes melatonin. The parapineal is located left of the midline and projects exclusively to the dorsal habenula on the left side.[126] As a result, the left habenula and right habenula receive asymmetric inputs, with such asymmetric neural circuits being responsible for certain behaviors. For example, the response to fear depends on the projection from the left habenula to the interpeduncular nucleus of the midbrain.[127] Epithalamic asymmetries are established by collective migration of parapineal cells to the left side of the brain, which is controlled by several signaling pathways including asymmetric Nodal signaling and bilateral FGF signaling.[128] *Nodal* is thus expressed on the left side of the epithalamus, whereas *Fgf8* is expressed bilaterally in the habenular nuclei. In an *Fgf8* mutant, the parapineal fails to migrate and remains at the midline. If *Nodal* expression is lost or becomes symmetric, the parapineal migrates in a random manner, either to the left or right side, suggesting that asymmetric Nodal signaling determines the direction of parapineal migration.[129] FGF signaling is activated in a few cells located at the leading edge of the parapineal during its migration, and asymmetric Nodal signaling contributes to a leftward bias of FGF pathway activation.[130]

Anatomic and functional asymmetries also exist between the left and right cortical hemispheres of the human brain.[131),132] Various cognitive functions are thus lateralized, with the most pronounced asymmetries being apparent in the language system. Other asymmetrically organized cognitive systems include visuospatial processing, auditory processing, and behaviors such as hand or foot preference. However, the neuroanatomic asymmetries underlying these functional differences remain largely unknown. Although genes that are asymmetrically expressed in the human cortex have been identified,[133)–136] the biological relevance of such asymmetry remains to be seen. Linkage analysis has also identified candidate genes for the control of cerebral asymmetry such as *PCSK6*[137] and *LRRTM1*.[138] Specific alleles of such genes were found to be linked to handedness in limited family groups, but the associations failed to achieve statistical significance in genome-wide analysis of samples from the general population.

A molecular asymmetry detected in the mouse brain is the distribution of the NMDA subtype of glutamate receptor in the hippocampus.[139),140] The synaptic distribution of the NMDA receptor subunit GluR$\varepsilon$2 (NR2B) in the adult hippocampus is thus asymmetric between the left and right hemispheres as well as between the apical and basal dendrites of individual neurons. L-R asymmetry in hippocampal circuits may be required for spatial learning and memory,[141] and it is disrupted in the *iv*/*iv* mutant mouse, which lacks motile cilia and thus does not develop nodal flow at the LRO,[142] suggesting that such asymmetry depends on the action of motile cilia. It remains unclear, however, where and when hippocampal asymmetry is generated during development. Although it may originate from ciliary function at the node, it may alternatively be dependent on motile cilia present in the neural tube. The origin of hippocampal asymmetry is an important issue for future studies.

**6.6. Other visceral organs.** In most birds, unlike other animals, adult females have only one functional gonad (ovary) and a single oviduct, both of which are located on the left side. A pair of gonads is initially formed in a female embryo, but subsequently only the left ovary remains while the right ovary degenerates. Such asymmetric gonadal development is regulated by Pitx2, which is expressed in the left gonad.[143),144] The absence of Pitx2 in the right gonad allows expression of retinaldehyde dehydrogenase 2 (RALDH2), which catalyzes the synthesis of retinoic acid, on the right side. Retinoic acid suppresses expression of the nuclear receptor Ad4BP (also known as Sf-1) and estrogen receptor $\alpha$ on the right side, resulting in degeneration of the right gonad.

## 7. L-R asymmetry in invertebrates

There is considerable diversity in mechanisms for the development of L-R asymmetry among animals (Fig. 14). Like vertebrates, chordates such as amphioxus[145),146] and ascidians[147] deploy cilia and the Nodal-Pitx2 signaling pathway. Sea urchins also rely on motile cilia and the Nodal-Pitx2 pathway,[148] but, unlike vertebrates, Nodal specifies the future right side, whereas BMP acts on the left side.[149),150] Snails[17),151),152] and *Drosophila*[153),154] do not depend on cilia but instead rely on cytoskeletal proteins for the generation of cellular chirality, which serves as the symmetry-breaking event. *Drosophila* even lacks the Nodal-Pitx2 pathway, which is conserved in all other species examined.

## 8. Conclusions

There has been rapid progress in our understanding of L-R asymmetry in animals over the last 20 years or so, since the first discovery of L-R

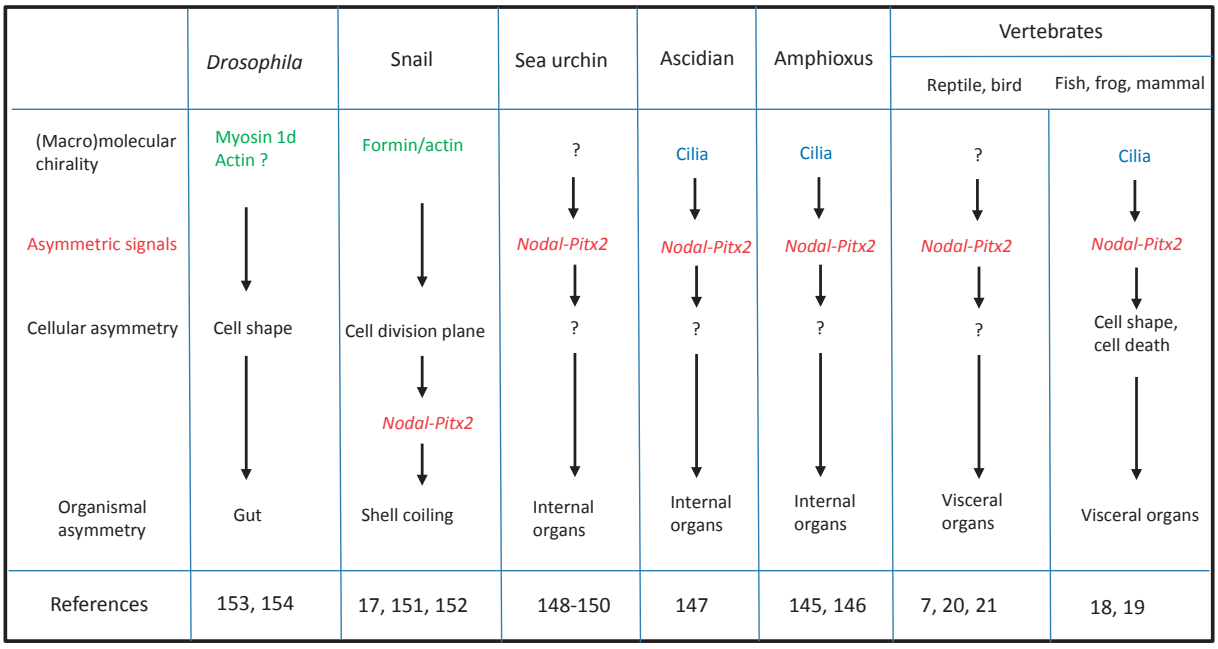


Fig. 14. Mechanisms of L-R asymmetry establishment in various animals. Note that some steps (such as the Nodal-Pitx2 pathway) are conserved, whereas others are divergent.

asymmetrically expressed genes. Systematic screening for L-R mutants in various model animals and genetic studies of human patients with laterality defects have identified a large number of genes that are required for establishment of L-R asymmetry. We also now largely understand the key role of cilia in symmetry breaking in many animals, the role and regulation of asymmetric Nodal signaling, and the cellular basis of asymmetric morphogenesis for some visceral organs. However, despite this recent progress, many challenging questions remain to be answered. For example, how does fluid flow achieve its effects, and how does an embryo sense the flow? What is the origin of L-R asymmetry during embryogenesis? If directional fluid flow breaks L-R symmetry, as in mice, what determines the direction of ciliary rotation? Can it be ascribed to structural chirality of dynein arms in the axoneme? How does brain asymmetry develop in humans? Finally, the evolutionary conservation and diversity of mechanisms for the establishment of L-R asymmetry among animals are still largely unexplored.

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## Profile

Hiroshi Hamada was born in Kagawa prefecture, Japan, in 1950 and graduated from Okayama University School of Medicine in 1975. After 5 years as a post-doc at the NIH, he became an Assistant Professor at Memorial University in Newfoundland, Canada, and started working on the regulation of embryonal carcinoma cell differentiation. This moved his interests toward developmental biology. He returned to Japan as an Associate Professor in The University of Tokyo, and subsequently a Professor at the Institute for Molecular & Cellular Biology, The University of Tokyo/Graduate School of Frontier Biosciences, Osaka University for 20 years. After retirement from Osaka University, he moved to RIKEN Center for Developmental Biology as the Director in 2015. He is currently Team Leader of RIKEN Center for Biosystems Dynamics Research. His laboratory used the mouse system to study embryonic patterning and organogenesis. Prompted by the finding of Lefty, a left–right asymmetrically expressed TGF$\beta$ member, his group investigated how body axes, left–right and anterior–posterior axes, are established during development. His current interests are the mechanism of left–right symmetry breaking and its diversity among various animals. He was elected as an Associate Member of EMBO and received the Keio Medical Science Prize in 2014.