Vertebrates display left-right (L-R) asymmetric organ positioning and morphologies, which are established during embryonic development. These asymmetries are conserved among individuals and species. How, when and where do embryos first break the symmetry? Why is it broken in a consistent direction? How is the asymmetry transmitted to and coordinated within the whole embryo? Which of these elements are conserved between different organisms? These questions have been the focus of intense research during the last decade, and much has been learned. Nonetheless, our understanding of how tissue and organ L-R differences are established during embryogenesis is scarce. A systems biology approach may enable us to better understand the dynamics of gene networks, epigenetics, cilia, fluids, and charged molecules as well as other processes involved in the generation of the vertebrate L-R axis.

INTRODUCTION

The external vertebrate body plan exhibits bilateral symmetry. However, internally, both the morphology and position of organs are left–right (L–R) asymmetric (Figure 1). These asymmetries are conserved among species and consistently preserved among individuals within a species. Thus, humans have their heart on the left side, as do mice, chickens, zebrafish, and frogs, to name a few examples. Accurate L–R asymmetric positioning and morphogenesis of organs is essential for proper body functioning, and alterations of the asymmetric body plan in humans result in severe medical conditions.1 Laterality defects at the genetic and morphological level can involve complete inversion of the asymmetry, randomization of the asymmetry (within individuals or within the different organs of a single individual), or bilaterality, and can be classified in different types accordingly.2 These phenotypes emphasize three main aspects of L–R axis determination: the consistent orientation of the asymmetry, the symmetry-breaking event, and the transmission and coordination of asymmetric information between different parts of the body at different times.

The L–R axis is determined during embryonic development, after the two other primary body axes [anteroposterior (A-P) and dorsoventral (D-V)] have been established. In the last decade, pioneering studies showed that L–R axis determination is under genetic control.3 Subsequently, genetic cascades and epigenetic factors controlling L–R axis determination have been unveiled (see Refs. 4,5,6–12 for reviews). Some molecular elements directing L–R asymmetry have been found to be conserved among species (e.g., the expression of transcription growth-β factor Nodal (Figure 1) and the paired-like homeodomain transcription factor Pitx2 on the left lateral plate mesoderm (LPM)), while others remain specific to each species, with it remaining still unknown whether L–R axis determination follows an evolutionary conserved design with characteristic species-specific details, or it involves a key divergent step.13–16 Research in L–R axis determination has been devoted to finding asymmetric cues, such as asymmetric gene expression patterns (e.g., Nodal, Pitx2, Shh to name a few), asymmetric activities and molecular concentrations (e.g., H⁺/K⁺-ATPase activity, Notch activity, intracellular and extracellular calcium), and asymmetric transports (e.g., nodal fluid flow, ionic transport through gap junctions). Altogether, these decades of research have shown that L–R axis determination involves molecular, cellular, and tissue dynamics with complex biological, chemical, and physical processes.

Systems biology aims at deciphering a rationale of the system dynamics and commonly involves its quantification. This kind of approach is starting in
the context of L–R axis determination. Thus, we find examples where multidisciplinary approaches, which involve theoretical and computational analyses and engineered systems jointly with challenging experiments utilizing molecular biology, have been used recently to tackle different questions of L–R axis determination. Herein, we review some aspects of L–R axis determination in which this kind of multidisciplinary approaches have been essential to foster our knowledge and emphasize open issues on this broad topic that lie within the field of systems biology.

**DIRECTIONAL SYMMETRY BREAKING**

The robust specification of the left side with respect to the two remaining body axes poses a challenge on how L–R axis is determined. When first defining left and right body sides, two different fates must appear and cells must decide whether to be a left-sided cell or a right-sided cell. Cells must decide this in the proper place and direction relative to the two other body axes: if the A-P axis or the D-V axis is inverted, the L–R axis is inverted as well. This kind of asymmetry is termed fixed or directional asymmetry. To account for the directionality of L–R axis establishment, Brown and Wolpert proposed a theoretical model based on a chiral molecule. According to the scenario envisaged by Brown and Wolpert, this chiral molecule indicates the left and right directions and thus settles down the bias for directional L–R asymmetry at the molecular level. Then, transport mechanisms coupled to the molecular asymmetry defined by the chiral molecule can spread the asymmetry first to the whole cell and to the tissue level afterwards.

Recently, there has been much evidence that highlights cilia, which involve a chiral structure of motor proteins, as a potential F-molecule-like candidate that can drive L–R axis determination. An initial correlation between cilia and L–R axis specification came from a congenital disease in humans termed Kartagener’s syndrome. The human population with this syndrome has immotile cilia in the sperm and trachea and has a high incidence of L–R reversed organs. A similar condition can be found in mutant mice *inversus viscerum* (iv), eliciting a thorough study of ciliary dynamics and L–R asymmetry in these mammals (see Ref. 23 for a review). The first asymmetric event observed in mouse development occurs around embryonic days 7.5–8 and corresponds to a leftward fluid flow in a cavity termed the node. The node is located along the midline at the anterior end of the primitive streak and has a few hundred monocilited cells. The cilia in these cells, with a 9 + 0 architecture, rotate clockwise when viewed from the ventral side and create a flow of extraembryonic fluid crossing the node from right to left. The absence of fluid flow when cilia are lacking or are immotile suggested that the motion
FIGURE 2 | Nodal ciliary motion and leftward fluid flow. Cells (grey circles) are monociliated. Left–right asymmetry is broken at the level of each single cell and is transferred to a field of cells through the fluid flow (green arrow). (a) Motion in wildtype mouse embryos. The motion is clockwise (red arrow) when viewed ventrally and tilted to the posterior (dashed line). As a guide to the eye, the simplified trajectory of the tip of cilia is depicted by a dotted line. Cilia perform a two-phase nonplanar beating motion with a power stroke (blue) and a recovery stroke (orange). The shape and bending of cilia along its motion (blue and orange lines) are depicted in four cells. In two additional cells, the extent of the two phases of motion is schematically drawn in the cone defined by the motion of the tip of cilia. A leftward fluid flow arises (green arrow). (b) Motion in inv mutants. A minority of cilia perform an inverted beating motion, tilted to the anterior (red dashed line), which elicits swirls and slowing down of the leftward flow (green arrow).

of cilia drives directional flow. In addition, the flow itself was shown to direct L–R asymmetry, since reversing it in wildtype embryos elicits L–R inversion, while introducing a leftward fast fluid flow in inv mutants restores proper L–R asymmetry. Therefore, these data provided a scenario in which cilia induce a leftward fluid flow that directs the asymmetry (Figure 2(a)).

But still there was an open issue: could the clockwise motion of cilia per se elicit a leftward fluid flow? A whole set of techniques have been put forward to solve this hydrodynamic problem. The small spatial and velocity scales involved in the node sets the fluid dynamics in a regime where our intuition fails. In this regime (called low Reynolds number), viscosity dominates over inertia. A first novel theoretical and computational approach based on steady fluid dynamics at low Reynolds numbers predicted a key element: the motion of cilia must be around an axis tilted to the posterior to elicit a leftward flow. Subsequently, high-speed recording of the projection of the motion of the tip of cilia on the surface of the node provided evidence that the projected trajectory has an elliptic shape as could be expected from a motion around an axis tilted to the posterior. Indeed, by assuming such a motion, the angle of tilting could be inferred and a posterior tilting angle of 30°–45° was found. Scanning electron microscopy images of cilia in wildtype mice and inv mutants confirmed a posterior tilting of cilia, while an engineered mechanical system supported experimentally that posteriorly tilted clockwise rotation can induce a leftward flow. Taking into account the two-phase beating motion 9 + 2 cilia and flagella perform, a theoretical and computational study that characterized, for the first time, the spatiotemporal dynamics of cilia and of beads embedded in the fluid flow proposed that the motion of cilia corresponds indeed to an asymmetric two-phase nonplanar beating which ensures the asymmetric flow. According to the nonplanar beating motion proposal, cilia move faster towards the left when moving far from the cell surface (effective or power stroke), and bend and move more slowly when passing rightwards close to the cell surface (recovery stroke). The propulsion of the flow is favored during the power stroke, setting the asymmetry of the flow. Physical arguments on the force required to bend cilia point out that both internal (e.g., cilia motors) and external (e.g., viscous resistance created by the cell surface) factors are necessary to dictate the directionality of the power and recovery strokes. The two-phase beating motion of cilia was supported by high-speed temporal imaging of nodal ciliary motion. In addition, this kind of motion clarified the origin of the features exhibited by the flow in inversion of embryonic turning (inv) mutants. In this mutant, the flow is leftward, albeit slower and more swirlily. The presence of a minority of cilia (20%) with reversed beating motion (i.e., rightward power stroke and leftward recovery stroke) in a node containing mainly immobile and wildtype cilia elicits a slower meandering motion of beads embedded in the flow, which overall move from right to left, as observed through video microscopy and confirmed computationally (Figure 2(b)).

Altogether, these results suggest that, in mice, symmetry is broken first at the molecular and at the...
A CONSERVED EVENT?

Importantly, the cilia and fluid flow mechanism is able to break bilateral symmetry in a consistent way, without requiring any L–R preexisting asymmetry. Moreover, support to this mechanism in zebrafish and *Xenopus laevis* embryos has been provided as well.\(^{42-45}\) In addition, it could potentially play a role in rabbits and medaka embryos where a leftward flow and posteriorly tilted clockwise ciliary motion have been observed, but no evidence for their relation to L–R axis determination has been provided yet.\(^{34}\) Thus, it may be the conserved symmetry-breaking event developmental biologists have been searching for. Interestingly, the actual quantitative details of cilia and fluid flow motion differ among species (Table 1), which can be evaluated through mathematical and computational modeling.\(^{32}\) For instance, ciliated organs with more sparsely distributed or stiffer cilia are expected to exhibit slower leftward fluid flows.\(^{32}\) Significantly, those elements relevant for settling down a leftward fluid flow are conserved: low Reynolds numbers and the directional features of cilia motion and position (Table 1). However, *inv* mutants, which have a leftward fluid flow but reverse L–R asymmetry, exemplifies the notion that the L–R axis is not just defined by the direction of flow and that additional information such as the flow speed may play a role as well. Accordingly, further features may be conserved, such as the Peclet number that takes into account the speed of the flow and evaluates which is the dominating transport mechanism.\(^{30}\)

Several data suggest additional complexities. The leftward fluid flow is the first known asymmetry in mice. In contrast, earlier L–R asymmetries exist in fish and amphibians before the action of cilia and the leftward fluid flow. In zebrafish embryos the earliest element known to control L–R axis determination is the action of the H\(^+\)/K\(^+\) ATPase.\(^{43}\) Interestingly, this activity does not alter cilia or fluid flow dynamics, which emerge at later stages.\(^{43}\) Therefore, L–R axis determination seems to use parallel processes to transfer L–R asymmetric information, at least in fish. In *Xenopus*, asymmetries in microfilament-dependent organization have been suggested during the first cell cycle,\(^{46}\) while H\(^+\)/K\(^+\)-ATPase (H-V) mRNA is asymmetrically localized along the three spatial axes during the first cleavage stages.\(^{37-49}\) These asymmetries are observed at stages much earlier than the emergence of the leftward fluid flow.\(^{45}\) It remains to be elucidated whether these early asymmetries in *Xenopus* embryos alter cilia and the nodal flow dynamics. Moreover, in rabbits, the fibroblast growth factor fgf8 has been shown to control L–R asymmetry at earlier stages than the onset of asymmetric fluid flow.\(^{50}\) Finally, while nodal cilia have been reported in chick embryos,\(^{51}\) it remains to be elucidated whether they are motile, induce a leftward fluid flow, and control L–R asymmetry.

The controversy of whether cilia and fluid flow are the first symmetry-breaking events highlights further possibilities.\(^{6,7,11,52-56}\) A model for a conserved mechanism of L–R asymmetry (the ‘intracellular model’) has been proposed.\(^{53,57}\) According to this model, L–R asymmetry arises at very early stages inside cells driven by an F-like molecule, which is proposed to be a cytoskeletal element.\(^{53}\) The F-molecule elicits a directional transport of key molecules (maternal mRNAs and ion-transporter proteins) within a cell, which subsequently can revert on the whole body through ion fluxes. Notably, despite the lack of identification of the F-molecule, motor proteins, ion fluxes, and gap junction communication have been shown to be relevant for L–R axis determination in a wide variety of species, ranging from ciliates and plants, to mammals.\(^{53}\)

TRANSFER OF THE ASYMMETRY

A conserved feature among vertebrate embryos is the left-sided expression of the gene *Nodal* in the LPM, which is key for the proper establishment of asymmetric morphologies such as the looping of the heart and gut (Figure 1).\(^{5,58,59}\) *Nodal* asymmetric expression in the LPM requires *Nodal* expression around the node at earlier stages.\(^{60-64}\) Actually, in all vertebrate embryos examined so far, the cascade of L–R asymmetric information goes at some point...
TABLE 1  Cilia and Flow Dynamics in Different Model Organisms

<table>
<thead>
<tr>
<th></th>
<th>WT Mouse</th>
<th>iv Mutant</th>
<th>inv/inv Mutant</th>
<th>Rabbit</th>
<th>Zebrafish</th>
<th>Medaka</th>
<th>Xenopus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cilia length (µm)</td>
<td>~5</td>
<td>~5</td>
<td>~9</td>
<td>~3</td>
<td>~5</td>
<td>~3</td>
<td>~5</td>
</tr>
<tr>
<td>Cilia rotation speed (Hz)</td>
<td>11 ± 3</td>
<td>0</td>
<td>~100 (50%)</td>
<td>7 ± 2</td>
<td>~26</td>
<td>43 ± 3</td>
<td>~20, 25</td>
</tr>
<tr>
<td>Cilia rotation direction</td>
<td>CW</td>
<td>—</td>
<td>CW CCW (5%)</td>
<td>CW</td>
<td>CCW (Dv)</td>
<td>CW</td>
<td>CW</td>
</tr>
<tr>
<td>Cilia tilting</td>
<td>P (40°)</td>
<td>—</td>
<td>A (20%)</td>
<td>P(45°)</td>
<td>P (40°)</td>
<td>P (40°)</td>
<td>P (60%)</td>
</tr>
<tr>
<td>Cilia protrusion</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>D</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>Ciliated organ</td>
<td>Node</td>
<td>Node</td>
<td>Node</td>
<td>NP</td>
<td>KV</td>
<td>KV</td>
<td>GP</td>
</tr>
<tr>
<td>and dimensions</td>
<td>100 µm</td>
<td>100 µm</td>
<td>100 µm</td>
<td>150 µm</td>
<td>600 µm</td>
<td>150 µm</td>
<td>240 µm</td>
</tr>
<tr>
<td>Main direction of flow</td>
<td>L</td>
<td>None</td>
<td>L</td>
<td>L</td>
<td>L, CCW</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>Speed fluid flow (µm/s)</td>
<td>4–19</td>
<td>0</td>
<td>Less than wildtype</td>
<td>1</td>
<td>—</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>References</td>
<td>34</td>
<td>26</td>
<td>26 and 34</td>
<td>34</td>
<td>42, 43 and 44</td>
<td>34</td>
<td>45</td>
</tr>
</tbody>
</table>

Slow and fast phases of cilia motion has been studied only for wildtype (WT) and iv mutant mouse embryos. Direction of rotation from ventral view, if not stated otherwise (Dv). NP, KV, GP stands for notochordal plate, Kupffer’s vesicle, gastrocoel roof plate, respectively. CW and CCW indicate clockwise and counterclockwise motion. L, R, A, P, D, V indicate left, right, anterior, posterior, dorsal, and ventral, respectively. Percentages refer to the fraction of cilia within a node.

through the node, located along the midline. During the last decade, the molecular cascades that process and transfer the L–R asymmetric information from the node to the organs have been a focus of thorough study. For recent reviews on the molecular cascades controlling L–R axis determination, see Refs. 7,8,10,23,65,66. Herein we focus on specific cases for which theoretical modeling has been used to evaluate which dynamics is taking place.

The pattern of Nodal expression around the node differs between species. In chick embryos, Nodal is asymmetrically expressed around the node at stage Hamburger Hamilton 6 and requires Notch signaling.62,63 The formulation and simulation of a network of the main genes and proteins involved, jointly with its experimental validation and testing, helped deciphering how and which asymmetries of earlier stages were transferred to the asymmetric perinodal expression of Nodal.63 Asymmetries in the activity of the H+/K⁺-ATPase at stage 449 elicits a transient higher left-sided extracellular Ca²⁺ level.63 This epigenetic asymmetry is translated into a genetic asymmetric pattern of expression by modifying the binding affinity of receptor Notch with its ligands, as suggested computationally and corroborated experimentally. According to the numerical analysis, such change in binding affinities enables a switch-like response of left-sided Nodal expression at the border between ligand domains if Notch signaling is properly enhanced63 (Figure 3).

The periodic waves of expression, from the posterior end to anterior regions of the embryo, of the glycosyltransferase Lunatic fringe, a modulator of the Notch pathway, enhances the Notch pathway increasingly over time63 and mainly on the left side where receptor-ligand binding affinities are higher because of extracellular Ca²⁺. Thus, after a period of time, the Notch pathway on the left side reaches high enough activity to induce Nodal expression. Therefore, a time-dependent coupling with the A-P triggers left-sided Nodal expression.63 Interestingly, time-dependent specification of left and right cell fates mediated by Notch has been recently proposed to be acting as well during the establishment of subnuclear asymmetry between habenular nuclei in zebrafish.67

Mouse embryos exhibit bilateral expression of Nodal around the node, which depends as well on the Notch pathway and which is essential for left-sided Nodal expression in the LPM.61 Recently, by means of mathematical modeling supported by novel experimental data, asymmetric expression of Nodal in the LPM of mouse embryos has been proposed to be driven by a reaction–diffusion mechanism that transiently amplifies a slight asymmetry, which comes from the leftward fluid flow along the node.69 The nodal flow is assumed to drive a small initial asymmetry of Nodal expression. The reaction-diffusion system then amplifies it through Nodal self-enhancement and long-range inhibition.
mediated by Lefty, another protein of the transforming growth-β family which is activated by Nodal and has longer range effects, suggesting it may diffuse faster. Note that the dynamics of local activation and long-range inhibition can ensure asymmetry (Figure 3), as initially proposed from theoretical grounds, since both left and right sides compete for expression by interacting through diffusible molecules. Therefore, reaction–diffusion dynamics can elicit Nodal expression either on the left or on the right side of the LPM, as found experimentally in manipulated embryos, and the proper bias is thought to be settled down by the nodal flow.

An open issue is how the leftward fluid flow is translated into asymmetric information. One proposal is that the fluid flow transports a morphogen which becomes asymmetrically distributed. The plausibility of this transport mechanism was first confirmed by means of a theoretical analysis of the nodal hydrodynamics which estimated the Peclet number and numerically tested that the direct transport driven by the flow is more relevant than diffusive transport as well as pointed out the relevance of protein half-lives. Experimental validation using exogenous proteins has been confirmed afterwards. In addition, vesicular nodal parcels have been found to be transported by the fluid flow. The plausibility of this transport has been theoretically confirmed as well; albeit physical arguments on the force exerted by the flow and the cilia challenge the proposal that these vesicles are broken down through collisions with the node or cilia as it has been proposed from the motion observed in video microscopy recording. Another scenario for translation of the fluid flow asymmetric information proposes that the flow is mechanosensed by cilia. According to this model, some cilia in the node sense the pressure and stresses of the fluid flow and respond accordingly. Results

FIGURE 3 | Transfer of body left–right asymmetric information. We show schematically two types of mechanisms (switch dynamics) that transfer a signal (in blue) into two cell types (the left cell type, in blue, and the right cell type, in green). The first column shows the switch dynamics. Cells are initially equivalent (grey), but under the influence of a signal decide to become a left cell or a right cell. The spatial pattern of the signal (depicted in rectangles in the top row) controls where each cell fate is chosen. Subsequent columns show the outcome for each dynamic under the signal pattern indicated at the top row. (middle row) This switch dynamics amplifies the signal or converts it into another type of asymmetry. Therefore, the cell pattern correlates with the pattern of the signal. An example of this mechanism is the conversion of asymmetric extracellular Ca\textsuperscript{2+} into asymmetric Nodal expression around the node in chick embryos. (bottom row) This switch dynamics amplifies the signal and ensures asymmetry through communication (red line) between the states of cells at each side across the midline. Accordingly, for symmetric patterns of the signal, a random outcome is found (50% show inverted phenotype). An example of this mechanism is reaction–diffusion dynamics involving short-range activation and long-range inhibition. In the figure, the dotted line stands for the midline.
on mutants kif3a and iv, which exhibit distinct patterns of expression of Nodal in the LPM (bilateral and absence or random asymmetric expression, respectively) while none of them has a fluid flow (iv has immotile cilia and kif3a cannot assemble cilia), were some of the evidences fostering this model. The finding of two population of cilia within the mouse node, one of motile cilia and one of immotile sensing cilia, supported this model. However, a hydrodynamic study has challenged it, since the pressure and stresses of the flow are expected to be symmetrical on both sides of the node. Notice that both scenarios take into account the direction of the flow as well as other features such as its speed. In order to clarify these issues, it may be worth it to investigate further how Nodal becomes consistently expressed in the right LPM in inv embryos which show a slow leftward and swirly fluid flow.

CONCLUSION

L–R axis determination involves many different aspects ranging from complex nonlinear molecular and cell dynamics, symmetry breaking and pattern formation processes, and hydrodynamics, to name a few. Systems biology approaches characterize and quantify all the relevant different aspects to provide an integrated understanding. Herein, we have reviewed specific examples in which mathematical and computational modeling of these aspects has been essential to evaluate and propose frameworks as well as to raise predictions to understand how the embryo first decides which side is left and which side is right and how it transmits this information to different spatial regions. We have seen as well that these approaches can help pinpointing which elements are essential for the conservation of a mechanism.

Many issues in vertebrate L–R axis determination are still unanswered which pose challenges to systems biology. This is the case of unveiling the mechanisms driving L–R asymmetric morphogenesis. At present, the coupling of early asymmetric molecular events with L–R asymmetric cellular behaviors is starting to be deciphered (see Refs 74,75,76 as examples where multidisciplinary approaches have been used to unveil the forces acting during left-right asymmetric morphogenesis). Relating molecular asymmetries to asymmetric morphogenesis will provide a systems-level understanding of L–R axis determination and will need novel combined computational and experimental efforts. Another issue is the structure of the flow of L–R axis information along the embryo. Is there really a hierarchical structure of information flow (and hence, a first symmetry breaking that controls all subsequent processes)? Is information processed in parallel, eliciting nonlinear cascades? Can L–R axis establishment be decoupled into independent functional modules (e.g., a module to generate random asymmetry and a module for consistent bias (chiral molecule) as proposed in Brown and Wolpert)? Novel multidisciplinary systems biology approaches that can identify and evaluate the plausibility and features of different networks of L–R information flows are necessary to address these questions.

NOTES

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