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Linking early determinants and cilia-driven leftward flow in left–right axis specification of *Xenopus laevis*: A theoretical approach

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ABSTRACT

In vertebrates, laterality – the asymmetric placement of the viscera including organs of the gastro-intestinal system, heart and lungs – is under the genetic control of a conserved signaling pathway in the left lateral plate mesoderm (LPM). A key feature of this pathway, shared by embryos of all non-avian vertebrate classes analyzed to date (e.g. fish, amphibia and mammals) is the formation of a transitory midline epithelial structure. Remarkably, the motility of cilia projecting from this epithelium produce a leftward-directed movement of extracellular liquid. This leftward flow precedes any sign of asymmetry in gene expression. Numerous analyses have shown that this leftward flow is not only necessary, but indeed sufficient to direct laterality. Interestingly, however, cilia-independent mechanisms acting much earlier in development in the frog *Xenopus* have been reported during the earliest cleavage stages, a period before any major zygotic gene transcription. The relationship between these two distinct mechanisms is not understood. In this review we present the conserved and critical steps of *Xenopus* LR axis formation. Next, we address the basic question of how an early asymmetric activity might contribute to, feed into, or regulate the conserved cilia-dependent pathway. Finally, we discuss the possibility that Spemann's organizer is itself polarized in the left–right dimension. In attempting to reconcile the sufficiency of the cilia-dependent pathway with potential earlier-acting asymmetries, we offer a general practical experimental checklist for the *Xenopus* community working on the process of left–right determination. This approach indicates areas where work still needs to be done to clarify the relationship between early determinants and cilia-driven leftward flow.

1. Introduction

Most animals display distinct morphological polarizations along the three body axes: anterior–posterior (AP; head–tail), dorsal–ventral (DV; back–belly) and mediolateral (left–right; LR). The phenotypic manifestation of mirror-image asymmetries, or lateralities, along the left–right body axis varies considerably between animal groups. Observed lateralities range from shell chirality in snails to the asymmetric appearance of the pentameric adult rudiment in the echinoderm pluteus larva and to the arrangement of heart and gastro-intestinal tract in vertebrates (Basu and Brueckner, 2008; Duboc and Lepage, 2008; Grande and Patel, 2009). Despite the considerable morphological differences displayed among these organisms, asymmetric expression of a conserved gene cassette, the so-called *Nodal*-cascade, precedes and governs the morphogenetic events resulting in laterality in all cases. The *Nodal* signaling cascade consists of the *TGFβ* growth factor *Nodal* (*Xnr1* or *nodal1* in frog), which directly activates the asymmetric gene transcription of the *TGFβ* feedback inhibitor *Lefty* (*Lefty2* in mouse, in fish and frog also termed *Antivin*) and the homeobox gene *Pitx2*. Whereas *Nodal* transcription is shut down quickly by Lefty activity, *Pitx2* continues to be expressed asymmetrically in the vertebrate organ anlagen during morphogenesis (Schier, 2003; Shen, 2007; Shiratori and Hamada, 2006). The broad phylogenetic conservation of this asymmetrical signaling cassette raises the question of whether the molecular process underlying its initiation is conserved as well (Levin, 2005; Tabin, 2005). Even solely among vertebrate model systems, the extent of conservation of the mechanism by which the *Nodal*-cascade acquires its initial lateral bias remains an unresolved problem.

Two different scenarios can be found in current literature, which differ in mechanism, factors involved, location and most importantly the developmental stage of activity. Basically, an early mechanism referred to herein as the "ion-flux" hypothesis of symmetry breakage (Levin, 2003), acting during early cleavage stages of development, stands in stark contrast to a well-studied, cilia based "leftward flow" or "Nodal flow" model, operating much later in development at neurula stages (Essner et al.,

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2002; Blum et al., 2009). In this review we explore possible relationships and interactions between these signaling mechanisms at a theoretical level using the *Xenopus* model in which evidence for both early and late mechanisms has been reported (Levin, 2003; Schweickert et al., 2007).

2. Cilia-driven leftward flow

The leftward flow hypothesis is based on the motility of monocilia: membrane-bounded, microtubule-containing projections that extend into the extracellular space during neurulation. Motile monocilia are found in homologous embryonic structures in mouse and rabbit (posterior notochord; PNC or ventral node), fish (Kupffer's vesicle; KV) and frog (gastrocoel roof plate; GRP) (Blum et al., 2009, 2007). Monocilia project from the posterior-facing apical surfaces of these epithelial cells and carry out a vigorous, uniformly clockwise circular beat. Because the cilia extend at an angle relative to the apical surface, the rightward portion of the rotation sweeps near the epithelial surface and the leftward portion sweeps through the extracellular medium. The viscous drag along the surface interferes with rightward flow, and a net leftward flow of extracellular fluid above the ciliated epithelium results (Marshall and Nonaka, 2006; Nonaka et al., 2005).

Leftward flow was first identified in mouse by Nonaka et al. (2002) and was subsequently observed in rabbit, fish and frog by other workers (Schweickert et al., 2007; Essner et al., 2005; Kramer-Zucker et al., 2005; Okada et al., 2005). Importantly, leftward flow precedes asymmetric gene expression in the LPM, suggesting that it plays a fundamental signaling role. Numerous reports of knockouts, mutants and morphants affecting ciliogenesis, cilia motility or polarization in PNC, KV and GRP reveal the close connection between fluid flow and organ placement. In addition humans suffering from syndromes based on ciliary dysfunctions (primary ciliary dyskinesia) often show alterations in the lateral asymmetry of inner organs. Taken together, the experimental and genetic evidence demonstrate the necessity of leftward flow for the development of the LR axis (Basu and Brueckner, 2008; Afzelius, 1976; Fliegauf et al., 2007).

A substantial body of literature describing nongenetic manipulations in fish, mouse and frog supports the causal connection between leftward flow and LR axis specification. For example, KV ablation experiments in medaka and zebrafish embryos resulted in lack of asymmetric gene expression and randomization of organ placement (Essner et al., 2005; Hojo et al., 2007). Corresponding dissection experiments have been performed in frog and mouse neurulae as well (Davidson et al., 1999; Ohi and Wright, 2007), reaching the same conclusion that LR patterning requires leftward ciliary flow. Laser ablation or surgical removal of the precursor cells of KV or GRP (dorsal forerunner cells in fish; superficial mesoderm (SM) in frog at gastrula stages) altered laterality specifically without impacting on the AP or DV axis (Essner et al., 2005; Blum et al., 2009). In *Xenopus*, leftward flow can be interfered without seriously damaging or removing cells. For example, at neurula stages, embryos were injected with methylcellulose (MC) into the gastrocoel nearby the ciliated GRP epithelium. The high viscosity of MC efficiently prevents leftward liquid flow. MC treatment prior to and at flow stages alters organ laterality and asymmetric gene expression of tadpoles. MC manipulations at later, post-flow stages has no effect on the LR axis, indicating the nontoxic nature of this treatment (Schweickert et al., 2007).

Finally, the application of an artificial flow on mouse embryos provides another substantial piece of evidence that flow is indeed part of the LR pathway. Hamada and co-workers administered a strong rightward flow on wild-type embryos, thereby overriding the endogenous leftward flow. This artificially reversed stimulus induced the *Nodal*-cascade on the right but not on the left side of treated embryos. Moreover the same technique could be used to direct laterality in mutants lacking ciliary motility and leftward flow. Homozygous *iv* (*inversus viscerum*) embryos show randomized *Nodal* expression (left, right, bilateral and absent) due to a mutation in a motor protein (left–right dynein) necessary for cilia movement. Leftward artificial flow rescued to wild-type left and rightward flow reversed *Nodal* expression in *iv* mutants (Nonaka et al., 2002).

These experiments and the growing literature on the role of ciliary function in left–right determination have established that an ancient, conserved mechanism specifies the left–right axis and asymmetric organ laterality in vertebrates. While this mechanism is, by the bulk of experimental evidence and criteria, regarded as both necessary and sufficient for left–right determination, it does not specifically indicate whether other earlier pathways might be considered "necessary" as well. In the case of frog development, this possibility is exemplified by the ion-flux hypothesis, which posits that very early cleavage-stage localization of maternally deposited factors plays a patterning role in LR axis specification (Levin, 2003). In addition, factors reported to be active in the ion-flux model have been implicated in laterality in a wide range of species including sea urchin, ciona, fish and chick (Fukumoto et al., 2005; Hibino et al., 2006; Shimeld and Levin, 2006; Adams et al., 2006), suggesting a conserved requirement for LR development.

In this review, we attempt to reconcile these disparate models by considering – at a theoretical level – how early determinants might contribute, whether morphogenetically or in some signaling capacity, to the patterning consequences of leftward flow, i.e. specification of the LR axis. We hope that this approach will provide an instructive framework upon which ideas bridging these two models can be tested experimentally

3. LR pathway in *Xenopus laevis*

The critical and conserved steps in LR development, which are considered both necessary and sufficient for the left–right axis, are illustrated in the following section by five sequential processes in *Xenopus*. Relevant work in fish and mouse is cited to underscore conservation.

3.1. Step 1: superficial mesoderm specification and Foxj1 expression.

The superficial mesoderm (SM), the outer cell layer of Spemann's organizer, is specified at early gastrula stages (Fig. 1A and A′, (Shook et al., 2004)). Homologous tissues have been identified in mouse and fish as well (Shook et al., 2004). Cell labeling experiments in *Xenopus* have shown that after involution, SM cells are fated to form the gastrocoel roof plate (GRP) during neurula stages (Fig. 1B; Shook et al., 2004) and to express the forkhead-family transcription factor *Foxj1* (Pohl and Knochel, 2004; Stubbs et al., 2008). *Foxj1* is considered a master control gene for ciliogenesis in the GRP, as its overexpression is sufficient to induce the whole ciliogenesis program. Further, loss of *Foxj1* function prevents cilia formation and consequently results in laterality defects (frog (Stubbs et al., 2008); fish (Stubbs et al., 2008; Yu et al., 2008); mouse (Zhang et al., 2004)). How *Foxj1* expression in the SM itself is regulated is not yet fully understood.

3.2. Step 2: GRP morphogenesis.

During early neurula stages the GRP cilia continuously grow (Fig. 1C, (Schweickert et al., 2007)). The motility of GRP cilia is provided by microtubule-dependent motor proteins: the