CONTROVERSY

The consistent asymmetry of organs such as the heart, viscera, and brain raises profound questions in developmental and cell biology. It is also of high biomedical significance, because several human syndromes affect the right-left axis. Significant progress in our understanding of the molecular genetic basis of right-left patterning has recently been made, but the crucial details of the origin of asymmetry remain unknown. In particular, the roles of cilia and motor proteins have been hotly debated in the field. The dominant model stems from work on rodent embryos, but it is controversial because numerous observations in other species suggest a different mechanism for the initiation of asymmetry. The following review outlines the current state of the field and proposes an alternative to the currently popular cilia model.

— *Olav Alvares*, Editor

THE EMBRYONIC ORIGINS OF LEFT-RIGHT ASYMMETRY

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ABSTRACT: The bilaterally symmetric body plan of vertebrates features several consistent asymmetries in the placement, structure, and function of organs such as the heart, intestine, and brain. Deviations from the normal pattern result in *situs inversus*, isomerisms, or heterotaxia (independent randomization), which have significant clinical implications. The invariance of the left-right (LR) asymmetry of normal morphology, neuronal function, and phenotype of several syndromes raises fascinating and fundamental questions in cell, developmental, evolutionary, and neurobiology. While a pathway of asymmetrically expressed signaling factors has been well-characterized in several model systems, very early steps in the establishment of LR asymmetry remain poorly understood. In particular, the origin of consistently oriented asymmetry is unknown. Recently, a candidate for the origins of asymmetry has been suggested: bulk transport of extracellular morphogens by rotating primary cilia during gastrulation. This model is appealing because it 'bootstraps' morphological asymmetry of the embryo from the intrinsic structural (molecular) chirality of motile cilia. However, conceptual and practical problems remain with this hypothesis. Indeed, the genetic data are also consistent with a different mechanism: cytoplasmic transport roles of motor proteins. This review outlines the progress and remaining questions in the field of left-right asymmetry, and focuses on an alternative model for 'Step 1' of asymmetry. More specifically, based on wide-ranging data on ion fluxes and motor protein function in several species, it is suggested that laterality is driven by pH/voltage gradients across the midline, which are established by chiral movement of motor proteins with respect to the cytoskeleton.

Key words. Left-right asymmetry, developmental biology, embryo, ion transport, motor proteins, cilia.

Introduction: LR Asymmetry

The development of embryos in three-dimensional space requires specification of pattern along three orthogonal axes during morphogenesis. In attempts to understand the generation of biological form, the type of symmetry exhibited by the organism is a key feature that must be explained. The external body-plan of vertebrates possesses bilateral symmetry, and much research has addressed the molecular mechanisms of patterning along the anterior-posterior (AP), dorsoventral (DV), and mediolateral body axes. However, vertebrate embryos (and indeed, many invertebrates) also exhibit a strikingly conserved left-right (LR) asymmetry of the internal organs. Nearly all visceral organs of the thorax and abdomen are LR asymmetrical in their anatomy, placement, and, in some cases, physiology. Directional LR asymmetry is conserved throughout chordate evolution, although the details of anatomical asymmetry can vary among species, such as sidedness of the venous system. Nonetheless, it is striking that all normal individuals of a given species have identical asymmetry (Neville, 1976). In some species, in particular among higher mammals, stereotypic LR asymmetry extends to the brain and nervous system, a situation that has profound implications for the understanding of behavior and cognition (Harnad, 1977; Snyder and Harris, 1997), not only of the individual but perhaps of the species as well (McManus, 2002).

Human Laterality

Abnormalities in the proper development of laterality, caused by chemical agents or heritable genetic lesions, form a class of human birth defects with significant clinical implications (Casey and Hackett, 2000). These defects occur in more than 1 in 8000 live births, and can be classified into several basic types. *Situs inversus* is a complete mirror-image reversal of the organs; this condition is rare, presenting in only about 1 individual *per* 20,000 (Burn, 1991). This is the only class with no major effects on the health of the patient and as such, is likely to be under-detected. In contrast, isomerism (loss of asymmetry), single organ inversions (*e.g.*, dextrocardia), or heterotaxia (a loss of concordance among the individual organs) often has grave consequences.

Human beings also exhibit several less-well-known asymmetries. The basis of human hand preference has been studied for many years, and genetic mechanisms are still controversial

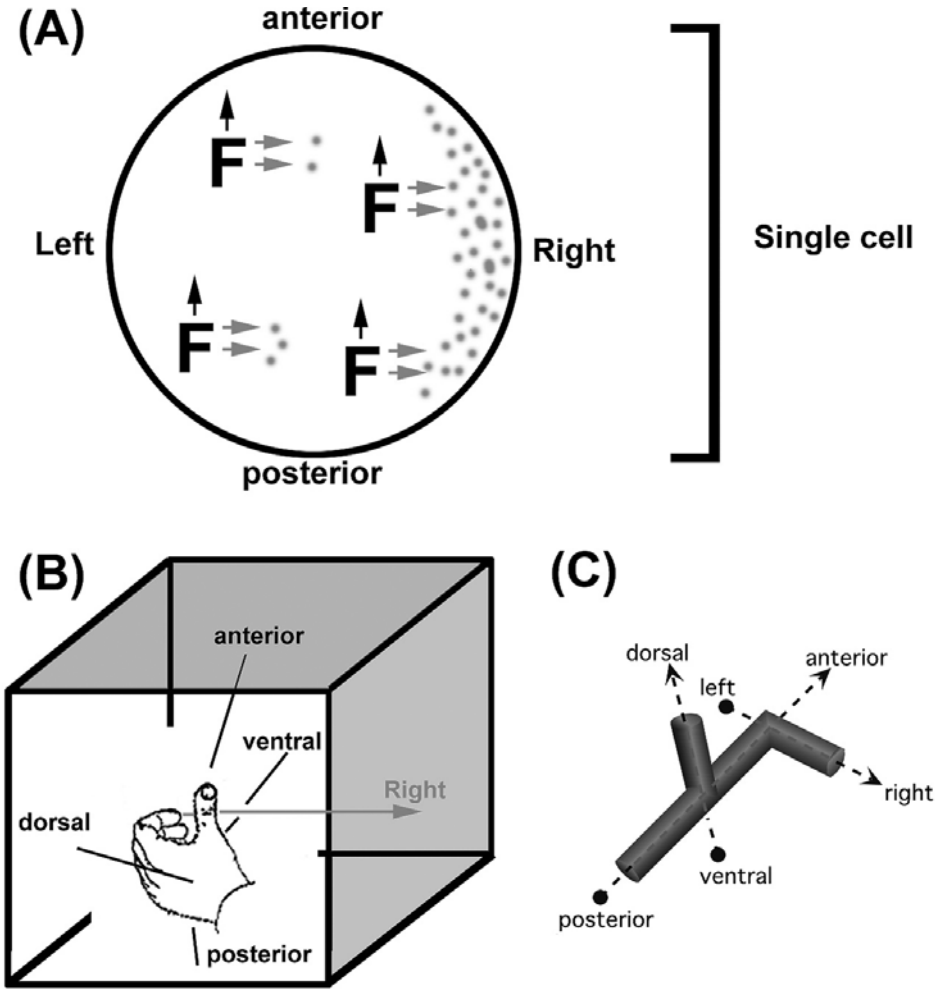

Figure 1. The Brown and Wolpert chiral molecule model. **(A)** In two dimensions, a biological molecule which occurs only as one enantiomer (schematized by the letter 'F') can be tethered with respect to one dimension (*e.g.*, anterior-posterior). The chirality of the molecule then defines a left-right direction, which can provide asymmetrical information, such as the rightward transport of some determinant (schematized in red). **(B,C)** In three dimensions, the same mechanism can function in a cell that is molecularly polarized along, and can orient the chiral molecule with respect to, the anterior-posterior and dorso-ventral axes. This type of mechanism allows each cell to know which direction is Left and which is Right.

(McManus, 1991). Interestingly, many neurological or behavioral asymmetries do not correlate with visceral asymmetry (Tanaka *et al.*, 1999). For example, *situs inversus totalis* individuals still have language lateralization seen in 95% of right-handed normal individuals (Kennedy *et al.*, 1999), and the incidence of left-handedness appears to be the same in *situs inversus* individuals as in the rest of the population (Cockayne, 1938; Torgersen, 1950). Oddly, reversed cerebral asymmetry is associated with breast cancer (Sandson *et al.*, 1992), suggesting that some aspect of laterality mechanisms also participates in growth and pattern control processes.

Immune hypersensitivity is higher on the left side (Dane *et al.*, 2001); directed asymmetries are also found in the fingertip ridge-counts and other dermatoglyphics (Holt, 1968). The left side of the scrotum descends lower than the right; this pattern is correlated with handedness (Chang *et al.*, 1960). In several of the non-visceral asymmetries, there appears to be an interesting relationship between laterality and gender. While the right hand has a higher ridge count in both males and females (Kimura and Carson, 1995), the left foot is larger in women, while the right is larger in males, in dextral subjects; this phenomenon is reversed in non-dextrals (Levy and Levy, 1978). A relationship with asymmetry has especially been described in hermaphrodites (Mittwoch, 2000). For example, in human hermaphrodites with only one cell line, ovaries occur more than twice as often on the left, and testes are more frequent on the right (van Niekerk and Retief, 1981).

Non-conjoined monozygotic twins illustrate another fascinating aspect of human asymmetry. While such twins do not exhibit the visceral laterality defects that characterize conjoined twins, they do manifest a subtle conservation of chirality. "Bookend" or enantiomer twin pairs exhibit opposite directionality of markers such as hand preference, hair whorl direction, tooth patterns, unilateral eye and ear defects, cleft lip, cleft palate, supernumerary teeth, and even tumor locations and un-descended testicles (see discussion in Levin, 1999; Mercola and Levin, 2001). The mirror/bookending phenomenon is not just structural but also pertains to functional parameters such as sleep deviations (Golbin *et al.*, 1993). Most of these cases affect features of the head, adding weight to speculation that laterality may be determined independently in the head and body.

In the field of craniofacial development, non-right-handedness is associated with cleft lip (Wentzlaff *et al.*, 1997), and unilateral lip clefts are more frequent on the left side (Abyholm, 1978; Chenevix-Trench *et al.*, 1992; Holder *et al.*, 1992). The left side of the face has a lower pain threshold (Pauli *et al.*, 1999). Right-handers have a right ear advantage and a larger left craniofacial region (reversed in left-handers), consistent with the speculation that hand preference may be related to craniofacial and consequently aural asymmetries (Dane *et al.*, 2002). The mechanisms linking basic body asymmetry and 'secondary' lateralization phenomena are very poorly understood. In particular, it is not known whether these asymmetries are linked directly with the mechanisms specifying the LR axis, or are secondary to pre-existing asymmetries in nervous system structure or function.

Theoretical Considerations

The establishment of left-right asymmetry is a fascinating problem in embryonic morphogenesis, and glimpses into the molecular mechanisms that determine LR asymmetry have been possible only recently (Burdine and Schier, 2000; Mercola and Levin, 2001). Many key questions still remain. Why are so many organisms asymmetric in a world in which no macroscopic feature distinguishes Left from Right? Even more puzzling, why is LR asymmetry uniform (consistently oriented) throughout a wild-type population? Given that complete *situs*

inversus presents no medical disadvantage, why don't individuals with *situs solitus* co-exist in roughly equal numbers with those with *situs inversus*? After all, it is much easier to imagine mechanisms for generating random asymmetries (by amplifying stochastic differences in cells) than to orient asymmetry consistently with respect to the other two axes. To what extent are the structure and physiology of complex asymmetric organs like the heart shaped directly by the genetics of LR patterning or, indirectly, by other forces such as hemodynamics? When did LR asymmetry first appear during evolution? Is it linked to the chirality inherent in lower forms (such as direction of snail shell coiling)? And finally, is it connected in any way to the puzzle of why living forms appear to utilize only one enantiomer of many chiral biomolecules (Pasteur, 1860)?

It is difficult to address these questions without a detailed understanding, at the molecular, genetic, and biochemical levels, of the formation of biased asymmetry in embryos. However, orienting the LR axis with respect to the other two axes presents a profound conceptual challenge. The dominant model in the field has been the 'chiral molecule' theory (Brown and Wolpert, 1990). In this paradigm (Fig. 1), LR asymmetry is leveraged from the chemical chirality of a molecule or other subcellular structure. Such an 'F' molecule can potentially nucleate consistently oriented processes in one direction, if tethered in the correct alignment with respect to the other two axes. Much recent work has attempted to pursue LR mechanisms upstream, in the hopes of eventually identifying the chiral structure which underlies 'Step 1' of asymmetry. While many biological molecules are chiral, the identity of the LR-relevant chiral molecule and precise knowledge of how early it acts in development have proven elusive. Another puzzle concerns the scope of LR information (Fig. 2). A chiral molecule is sufficient to indicate direction at the level of a single cell. However, asymmetric gene expression (which has been characterized in many species at gastrulation) requires that cells know upon which side of the midline they are located; understanding how directional information is converted into global position will be a key piece of the puzzle.

Downstream Mechanisms of LR Asymmetry

The asymmetries of organogenesis are beginning to be unraveled by studies of downstream genes such as *Pitx-2* (Piedra *et al.*, 1998). Upstream of these lies a fairly well-characterized pathway of asymmetrically expressed genes which function in cascades of induction and repression lateral to the midline (Burdine and Schier, 2000). The discovery of these cascades has also shed light on the cause of *situs* defects in conjoined twins (Levin *et al.*, 1996). However, the asymmetric gene pathway begs the question: What determines the sidedness of the first gene which is asymmetrically expressed? Earlier mechanisms include syndecans, adhesion junctions, gap junctions, and ion flux (Levin and Mercola, 1998; Brizuela *et al.*, 2001; Kramer and Yost, 2002; Levin *et al.*, 2002). The question of how these mechanisms feed into the asymmetric gene cascade is currently an exciting and open area of research; however, arguably the most exciting and controversial question concerns the origin of asymmetry: What is the first embryonic event which distinguishes Left from Right and aligns the LR axis?

Cilia: a Good Theoretical Candidate for 'Step 1'

The observation that human Kartagener's syndrome patients exhibited randomization of visceral *situs* (heterotaxia) and had ultrastructural defects in the dynein component of cilia

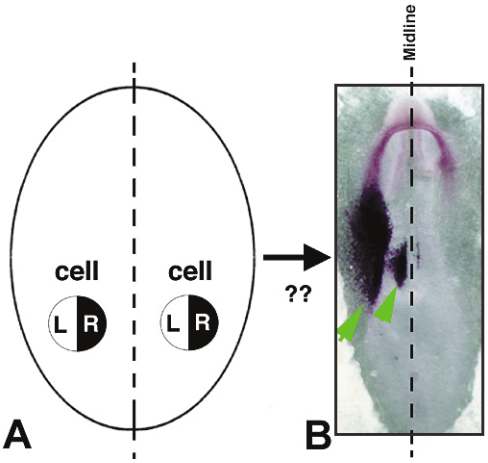

Figure 2. Direction *vs.* position on the Left-Right axis. **(A)** A tethered chiral structure can give information as to which direction is Right or Left. However, gastrulating embryos exhibit paired cell fields on either side of the midline, only one of which expresses certain genes. **(B)** For example, *Nodal*, a TGF-β family member, is expressed in a small and large domain (green arrows) on only one side of the midline. This requires that cells know their position with respect to the midline; this information is different from direction, and requires coordination with respect to the whole embryo (in contrast to the localized directional information generated by a chiral molecule).

(Afzelius, 1976) was of great interest because it suggested that asymmetry could be 'bootstrapped' from molecular chirality of some ciliary component. This idea was supported by the finding that the murine *iv* mutation, which unbiases laterality (Lowe *et al.*, 1996), encodes a dynein called left-right dynein (LRD) that is expressed in cells of the mouse node (Supp *et al.*, 1997). Axonemal dynein is a component of the motor which drives ciliary motion; the chirality of this motion is intrinsic to the protein components. Genetic deletions of KIF3-A or KIF3-B, two microtubule-dependent kinesin motor proteins, resulted in randomization of the *situs* of the viscera, and this finding is also often interpreted as evidence for a primary role for cilia in LR determination. Most importantly, following the first observation of cilia in the murine node (Sulik *et al.*, 1994), elegant experiments have revealed a clockwise rotation of monocilia extending ventral to the node that produces a localized net right-to-left flow of fluorescent beads placed in the extra-embryonic space (Nonaka *et al.*, 1998). Thus, it was proposed that vortical action of cilia initiates asymmetry by moving an extracellular signaling molecule to one side, where it can induce asymmetric gene expression. This mechanism requires vectorial bulk transit of medium directly across the field of beating cilia at a wide angle to the embryo's long axis; it has been suggested that the wedge-shape of the node might convert rotational motion to net lateral motion. More recently, it has been proposed that motile, LRD-containing monocilia generate nodal flow, and that non-motile polycystin-2-containing cilia sense nodal flow, initiating a calcium signal at the left border of the node (McGrath *et al.*, 2003).

Unanswered Questions

The idea that vortical ciliary motion results in a net asymmetric localization of a morphogen is intellectually very satisfying but possesses several problems. First, there are inconsistencies (Burdine and Schier, 2000; Wagner and Yost, 2000; Mercola and Levin, 2001; Tabin and Vogan, 2003) between the predictions of

the cilia model and the data on directly measured ciliary flow, the patterns of asymmetric gene expression, and visceral *situs* phenotypes in the various motor protein knock-outs and mutations (although a model based on sensory functions of cilia has been proposed, which is more compatible with all of the data [Tabin and Vogan, 2003]). Second, several of the relevant knockouts exhibit some degree of midline defects (Nonaka *et al.*, 1998; Takeda *et al.*, 1999), raising the possibility that the LR phenotype is secondary to an alteration of dorso-anterior development or barrier function (disruptions of midline barrier or defects in anterior-posterior or dorso-ventral patterning lead to non-specific destabilization of LR asymmetry [Danos and Yost, 1995]). Third, biased motion of molecules of physiological size (not latex beads) has not been observed.

The cilia model is currently a very popular candidate for the first step in LR asymmetry. Because of the importance of understanding the origin of consistent asymmetry, it is useful to examine critically various aspects of the data and their relationship to other observations that are not often discussed in the literature. Thus, this review considers in detail a set of key questions inherent in this hypothesis, and attempts to synthesize several recent findings into a different model for the initiation of LR asymmetry. The goal is not to focus on problems with the cilia model or to rule out cilia as part of LR patterning; some aspect of the cilia model is almost surely right (at least in mice). Instead, attention is drawn to alternate hypotheses consistent with the data that may otherwise remain unnoticed; exploration of these will greatly repay attention at the molecular level.

Three key (but independent) questions must be asked of the cilia model. First: Are cilia themselves causally involved in LR patterning? The published mouse gene knockouts and mutants (as well as natural human mutations) do not distinguish between cytoplasmic and ciliary roles of cytoskeletal motors—both are impaired in the resulting embryo, leaving the possibility that interference with the cytoplasmic functions is what actually causes laterality defects. Indeed, some human patients with immotile cilia have normal LR asymmetry (Rooklin *et al.*, 1980). The crucial experiment would be direct functional interference with ciliary motion in the absence of a genetic deletion of motor protein function. This might be accomplished by changing the viscosity of the extra-embryonic medium, mechanical clipping of motile cilia, etc. The mechanically intricate experiments of Nonaka *et al.* (2002) indeed demonstrate that exogenous flow can randomize asymmetry. However, technical challenges (such as a requirement for nutrient flow to the embryo) have so far prevented testing a true "no flow" condition or "viscous medium" test. Such a negative control is particularly crucial, given the established finding that control culture of early mouse embryos randomizes LR asymmetry in and of itself (Fujinaga and Baden, 1991).

The next aspect of the cilia model to consider is timing: Can ciliary motion be the first step of asymmetry? While this is *a priori* quite plausible, none of the published data addresses this issue. At best, the mutants and embryo culture studies show that some aspect of ciliary action is important for LR, but they do not indicate that this is the first step which initiates asymmetry. Moreover, it is hard to reconcile the hypothesis of nodal cilia as 'Step 1' with studies in the chick that show asymmetric gene expression prior to node formation (Levin *et al.*, 1995, 1997; Stern *et al.*, 1995). Indeed, *lrd* is expressed in pre-streak mouse embryos (Supp *et al.*, 1997), and expression of several kinesin and dynein genes can be detected at the base of the chick primitive streak, which has previously been suggested as the locus of the primary DV/AP/LR computation event (Mercola and Levin, 2001). Most importantly, several mechanisms appear to be required for LR patterning at pre-cilia stages. In the chick, a system of gap-junctional communication is required at stage 2^+ (early primitive streak stage, prior to node formation) for correct LR asymmetry (Levin and Mercola, 1999), and in the frog, very early mechanisms include gap junctional communication (GJC) (Levin and Mercola, 1998), ion flux (Levin *et al.*, 2002), the LR coordinator (Hyatt and Yost, 1998), syndecans (Kramer *et al.*, 2002), and 14-3-3 proteins, which show an asymmetry at the first cleavage (Bunney *et al.*, 2003). Thus, if bias of the mouse LR axis is initiated by cilia, it is probably a highly divergent mechanism from the way in which other species establish asymmetry. The definitive answer to this question awaits analysis of laterality in targeted or conditional mouse mutants where the function of the relevant proteins is abrogated only at late (mature node) stages, leaving early roles untouched. Interestingly, recent evidence suggests that human patients with classic primary ciliary dyskinesia (and the attendant heterotaxia) do not exhibit reversals in the normal prevalence of right-handedness (McManus, in press), suggesting that at least some aspects of laterality in humans are indeed upstream of mutations affecting ciliary function.

Last, how general is a ciliary mechanism for the orientation of the LR axis? While cilia are present in several species (Essner *et al.*, 2002), functional LR roles have not been reported in any embryo other than the mouse. Asymmetry is initiated by mechanisms not involving cilia (and indeed is present from the first blastomere cleavages) in the chirality of snail embryos (Freeman and Lundelius, 1982) and *C. elegans* (Wood, 1991). Although molecular motor mutations are also associated with asymmetry defects in man (Olbrich *et al.*, 2002), all of the LR-relevant cilia-specific data have come from studies in the cultured mouse embryo. However, the mouse is an atypical mammal and develops in a cylinder. Most mammals—specifically including rabbits and primates but excluding rodents—develop as flat blastodiscs like the chick. Thus, it is unknown whether ciliary motion (which has been observed directly in mouse embryos) is relevant to other species.

An Alternative Model: Cytoplasmic Transport

In contrast to the cilia hypothesis, which focuses on hydrodynamics as a motive force for LR signals, another model has been proposed (Levin, 1997; Levin and Nascone, 1997) which is based on a different aspect of developmental biophysics: voltage and pH gradients driven by ion flux. Numerous observations link ion transport and asymmetry. Over 45 years ago, it was found that an imposed DC electric current across the LR axis of the early chick blastoderm specifically induced cardiac reversals (Sedar, 1956). An endogenous asymmetry in the responses of calcium channels to Ca^{++} depletion has been reported in ascidians (Albrieux and Villaz, 2000), and indeed some aspect of Ca^{++} flux has been implicated in LR asymmetry in amphibian (Toyoizumi *et al.*, 1997), mouse (McGrath *et al.*, 2003), and chick (Linask *et al.*, 2001) embryos.

More recently, it was shown that H^+ and K^+ ion flux functions upstream of the asymmetric expression of the LR gene cascade in directing embryonic *situs* in both chick and frog embryos (Levin *et al.*, 2002). A directly observable, consistently biased, LR-asymmetric ion flux and membrane voltage gradient across the midline exist in both species, and are dependent on the activity of an ion exchanger (the H^+/K^+-ATPase, and a K^+ channel). Steady-state voltage gradients in non-neuronal

cells are known to control gene expression and other aspects of cell behavior (Robinson and Messerli, 1996; Levin, 2003a); thus, it is proposed that asymmetry is driven, at very early stages, by differences in ion flux across the embryonic midline.

This phase of the model is by itself neither a candidate for 'Step 1' of asymmetry (since some upstream factor must still consistently dictate which side will be negative with respect to the other side) nor mutually exclusive with the cilia hypothesis. However, two aspects of this bioelectric phenomenon set it up as an integral part of an alternative scheme for the initiation of asymmetry. First, the asymmetry in ion fluxes and mRNA localization for the LR-relevant ion exchanger exists during the first few cell divisions in *Xenopus* and during early streak formation (st. 2) in the chick—before node cilia exist and before the earliest known asymmetric gene expression (Levin, 1998; Levin *et al.*, 2002). Thus, at least in the chick and the frog, ciliary action at the node (or its equivalent in *Xenopus*) cannot be the initiating step of asymmetry: Embryos of those two species know their Left from their Right well before the appearance of such cilia!

Second, the LR asymmetry in mRNA localization in *Xenopus* suggests an immediate upstream mechanism distinct from but related to cilia. Analogous to the animal-vegetal asymmetries in mRNA localization in the frog oocyte and many other cell types, the LR asymmetries in mRNA localization may plausibly be driven by cytoplasmic motor proteins such as dynein and kinesin (Tekotte and Davis, 2002). Approximately half a dozen mRNAs and proteins have now been found to be LR-asymmetric at the first few cell cleavages in *Xenopus* (*e.g.*, Bunney *et al.*, 2003). These phenomena may reflect potential non-ciliary functions of motors in the LR pathway: asymmetric cargo transport. The possibility of cytoplasmic transport functions of motor proteins which might be relevant to LR patterning has been suggested in several reviews (Levin and Nascone, 1997; Tamura *et al.*, 1999; Hobert *et al.*, 2002; Robinson and Messerli, 2003) and primary papers (Supp *et al.*, 1997; Levin *et al.*, 2002), because the ciliary and cytoplasmic roles have not yet been experimentally distinguished.

The present model, specifically centered on cytoplasmic movement of ion transport proteins, is summarized in Fig. 3 (the early steps have been superimposed on the developmental architecture of *Xenopus* because, in the chick, it is not even known how the anterior-posterior direction is specified). Briefly, cytoplasmic motor movement results in an asymmetric distribution of specific ion pump mRNA and protein cargo in a key group of early cells. The presence of electrogenic proteins on the cell surface on one side of the midline allows those cells to carry out an ion exchange with the extracellular space which is not replicated on the contralateral side. This ion flux results in differential membrane voltage and pH among cells on either side of the midline. For example, strong H^+ pumping on one side will cause a loss of positive charges and will result in those cells being more negative (depolarized) than their counterparts on the opposite side. These changes in pH and voltage eventually result in differential gene expression downstream, feeding into the known LR-asymmetric gene cascade. There are many ways in which membrane voltage can be transduced to downstream intracellular signaling events, but it is not yet clear how this occurs in the context of LR patterning. One possibility is that the voltage difference across the midline is important in regulating the exchange of small signaling molecules between the L and R sides of the embryo that takes place through gap junctions (Levin and Mercola, 1998, 1999). The voltage gradient across the midline might regulate the permeability state of gap junctional paths or, alternatively, provide electro-motive force which electrophoreses charged factors through gap junction paths in a consistent direction.

It should also be noted that, together, the systems of cytoplasmic motor transport, ion flux, and large-scale gap junctional paths provide a way to resolve the question (Fig. 2) of how LR orientation information on the level of a single cell (given by an oriented F-molecule) is converted into global information on LR position relative to the midline of the whole embryo (which is necessary for the specification of asymmetric gene expression in cell fields). The initial steps of this model are analogous to similar mechanisms by which other species, such as *Drosophila*, achieve polarized axes via mRNA localization (Januschke *et al.*, 2002). By setting up localized ion gradients across the midline which can control the movement of LR determinants through embryo-wide gap junctional paths, motor proteins can initiate the cascade by which oriented intracellular movement is transduced into large fields of gene expression.

Alternative Interpretations

This model makes several predictions and offers different ways to look at available data that are commonly taken to be evidence for a ciliary role. First, it predicts that mutations or deletions of motor proteins in mice would result in LR defects, due to a disruption of a cytoplasmic shuttling of important cargo by dynein or kinesin motors. In particular, mRNA localization is now known to be dependent on both kinesin and dynein (Januschke *et al.*, 2002), so it might be expected that mutations in either family might result in laterality defects. This was observed in the mouse knockouts described above.

What about the fact that the relevant mammalian motor proteins are thought to be axonemal, not cytoplasmic? While sequence analysis is often used to assign dynein and kinesin genes into cytoplasmic or ciliary groups, this classification is not conclusive in the absence of functional data ruling out the alternative role. Sequence may be misleading: Cytoplasmic dynein DHC1b is required for flagellar assembly (Pazour *et al.*, 1999), and some dyneins classified as axonemal have been found in non-ciliated cells and appear to be associated with cytoplasmic protein localization pathways (Vaisberg *et al.*, 1996). Moreover, motor proteins are extremely pleiotropic and have numerous roles in the cell (Vale, 1999); thus, it would not be too surprising if flagellar or ciliary functions were impaired in mutants as a side-effect and did not play a causal role in LR asymmetry.

This possibility is suggested by several observations. *LRD* (left-right dynein) possesses no 5th P-loop in the N-terminal region, in contrast to other axonemal-type dyneins (Kandl *et al.*, 1995; Supp *et al.*, 1999). Moreover, both the timing and location of expression suggest non-ciliary roles for LRD. Not only is *lrd* expressed in mouse embryos before the formation of the node (day 3.5) (Supp *et al.*, 1997), but also it is present throughout the primitive streak in the chick, not just in ciliated node cells (Essner *et al.*, 2002). It is also expressed in the developing limb, where it is thought to participate in cartilage condensation (Supp *et al.*, 1999). Conversely, in mice bearing a targeted *lrd* mutation (with the predicted laterality defects), sperm motility and tracheal ciliary beating are normal (Supp *et al.*, 1999). Thus, as noted by the authors of the original identification of *lrd* in *inversus viscerum* (*inv*) mice (Supp *et al.*, 1997), these data are quite consistent with the existence of other, non-ciliary functions of LRD. The same possibility is suggested in the case of kinesins, since KIF3B and KIF3A mice

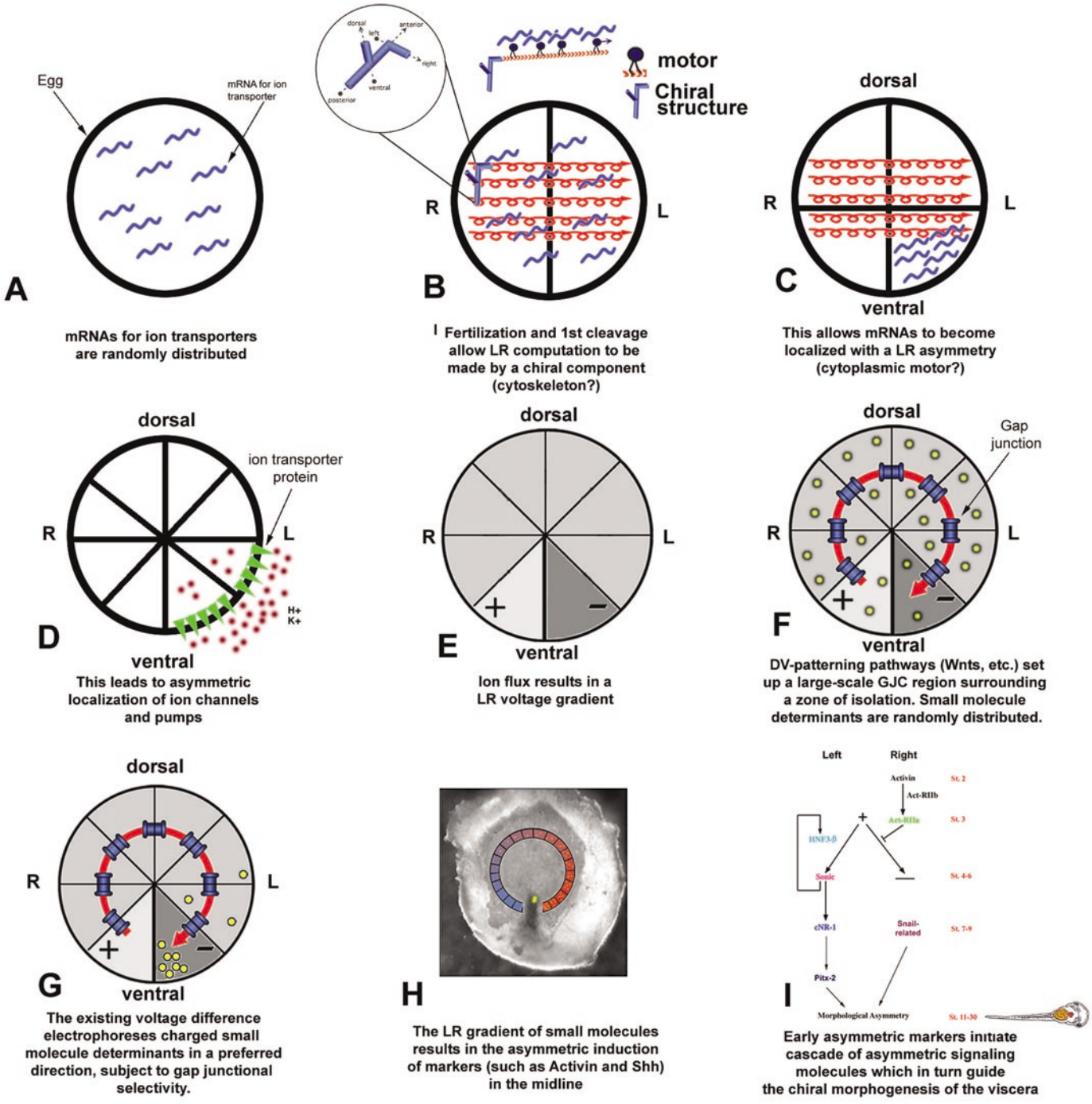

Figure 3. A model of the LR pathway based on cytoplasmic motor protein movement. This highly schematized diagram draws mainly on *Xenopus* embryogenesis and attempts to follow known timing data for each step. **(A)** In the unfertilized egg (which is thought to possess radial symmetry about the animal-vegetal axis), maternal mRNAs for key ion transporters are evenly distributed. **(B)** Cytoskeletal re-arrangements following fertilization set up microfilaments or microtubules which are oriented along the newly established LR axis. **(C)** Motor proteins (such as dynein [LRD] and kinesin [KIF-3B]) translocate along these tracks and result in an asymmetric localization of certain mRNAs. **(D)** These mRNAs are translated, the resulting proteins perhaps targeted to correct regions and held in place by ankyrin proteins such as *inv*, and thus initiate ion flux. **(E)** The differential ion flux results in LR-asymmetric gradients of pH and voltage. In particular, cells across the ventral midline possess significantly different membrane potential levels. **(F)** The system of gap-junctional communication is set up, featuring junctional isolation across the ventral midline and a path of GJC circumferentially around it. **(G)** The voltage gradient between the L and R sides imposes a unidirectional net movement of as-yet-uncharacterized small signaling molecules. This results in accumulation on one side of the midline from an initially random (homogenous) distribution. **(H)** The accumulation of these small molecule morphogens on one side induces gene expression in conventional ways. **(I)** This initiates the known cascade of asymmetrically expressed signaling factors which form the middle of the LR pathway which dictates the *situs* of asymmetric organs.

exhibit a host of developmental defects not obviously related to ciliary function (Nonaka *et al.*, 1998; Marszalek *et al.*, 1999; Takeda *et al.*, 1999), and these KIF proteins are expressed in non-ciliated cells such as muscle cells (Ginkel and Wordeman, 2000) and

appear to function in cytoplasmic protein localization (Le Bot *et al.*, 1998). The cytoplasmic motor/ion flux model implies that the ciliary phenotypes observed in motor protein knock-out animals may be secondary and mask the true cause of the laterality defect.

Another strong prediction of this model is that the early cytoskeleton is crucial to LR asymmetry. This was presciently demonstrated by the Yost lab, who showed that disruption of the cytoskeletal arrays during the first cell cycle in *Xenopus* can randomize LR asymmetry (Yost, 1990). More specifically, this model requires that some aspect of the cytoskeleton be oriented with respect to the LR axis. Such oriented cytoskeletal tracks are ideally suited for nucleation by the classic 'F-molecule'. Plausible candidates for the nucleation center which may set up the LR-oriented tracks include the centriole, centrosome (Wood, 1997), and basal body. Both inversin and BBS8 proteins localize to centrosomes and basal bodies, and null mutants for these genes have strong laterality phenotypes (Ansley *et al.*, 2003; Watanabe *et al.*, 2003).

This model predicts that modulation of cytoskeleton and motor protein function will affect ion channel and pump activity, and ultimately alter the electrical polarity of cells. Consistent with this prediction, ion channel and pump localization and function are known to be dependent on microtubule-based motor protein movement, and, in some cases, direct interaction between dynein and ion channel proteins has been observed (Hamm-Alvarez and Sheetz, 1998). The model also suggests that motor protein localization be asymmetric. Indeed, KIF-3B protein exhibits a strikingly asymmetric cytoplasmic localization during the first few cleavages (Levin, 2003b).

The model also predicts that genetic modulation of ion flux (through deletion or mutation of electrogenic genes) will result in LR defects (as has already been shown in the chick and *Xenopus*; Levin *et al.*, 2002). Significantly, this appears to be true in mammals as well. Polycystin is an ion channel expressed on the cell membrane (Hanaoka *et al.*, 2000; Koulen *et al.*, 2002) as well as on primary cilia in the kidney (Pazour *et al.*, 2002), and it is regulated by pH and voltage (Gonzalez-Perrett *et al.*, 2002). Recent analysis of the PCKD (polycystic kidney disease) mouse (Pennekamp *et al.*, 2002) found LR defects in polycystin knock-out animals. Thus, the PCKD laterality phenotype suggests that ion flux is a necessary component of LR patterning in mammals and that the primary role of polycystin in LR asymmetry may be early ion transport and not ciliary action. This possibility is greatly supported by the observations that PCKD cells in both mice and humans exhibit altered electrical polarity (standing long-term cell membrane voltage levels) due to mislocalization of the Na^+/K^+-ATPase protein to the opposite pole of the cell (along the apical-basal axis) (Barisoni *et al.*, 1995; Ogborn *et al.*, 1995; Wilson *et al.*, 2000). Thus, similar to the situation in the chick and the frog, recent data in mammals link a LR phenotype to (1) aberrant cell membrane voltage, (2) the P-type cation exchanger (a family which includes Na^+/K^+-ATPase and H^+/K^+-ATPase), and (3) polarity of ion pump localization along a major cellular axis. While polycystin has been localized to primary cilia in the mouse node (McGrath *et al.*, 2003), consistent with the ciliary model, polycystin is expressed in mice from the two-cell stage (Pennekamp *et al.*, 2002) (consistent with the early function of ion channels in frog embryos), and PCKD embryos exhibit incorrect localizations of other electrogenic genes which are also implicated in LR asymmetry (Gillespie *et al.*, 1991; Haragsim *et al.*, 1995).

A priori, it might be argued that mis-localization of ion flux proteins would have disastrous effects on general cell health. However, in a screen of large numbers of electrogenic targets in frog development, inhibition of a surprising range of such proteins produced no generalized teratogenic defects (Levin *et al.*, 2002). The ion flux system appears to function mainly in LR signaling during early development (the same was discovered to be true of gap junctional communication in early frog and chick embryos; Levin and Mercola, 1998). This phenomenon probably reflects LR-specific roles of a few individual channels and pumps which, by virtue of tight spatial and temporal restriction or strict functional regulation, do not severely affect general housekeeping functions of cellular electrophysiology in most embryonic tissues throughout development.

Finally, it is crucial to explore predictions made by this proposed mechanism which are distinct from the cilia model, which would lead to specific tests of each hypothesis (in contrast to much of the previous discussion, which highlighted the fact that all current data except for timing are consistent with both models). One such concerns the spatial origin of LR information. The cilia model (which views cilia as a chiral molecule which initiates LR asymmetry) strongly predicts that the LR orientation of the node is intrinsic to node cells—that it is generated within the node by the action of ciliated cells. The ion flux model (especially in the context of the gap junction system; Levin and Mercola, 1998, 1999) predicts that the midline cells receive LR information from lateral tissue. In the chick, analysis of current data strongly indicates that, indeed, Hensen's node is 'instructed' with respect to the LR axis by adjacent lateral cell groups (Levin and Mercola, 1998; Pagan-Westphal and Tabin, 1998; Psychoyos and Stern, 1996). Moreover, it was recently shown that the correct LR-sidedness of asymmetric genes in the node is dependent on GJC-mediated communication between quite distal tissues on the L and R sides (Levin and Mercola, 1999), arguing against an intrinsic mechanism for the node. However, these data are compatible with ciliary flow having a downstream role in the LR pathway, such as, for example, in maintaining or amplifying asymmetry generated by earlier mechanisms.

Conclusion and Future Prospects

The simple ciliary motion model has several problems, but these can probably be taken into account by more sophisticated schemes based on sensory cilia (Tabin and Vogan, 2003). Analysis of the data makes it highly unlikely that cilia are the originating event in birds or amphibia, but the mouse may be different. It is uncertain whether cilia are causal in the mouse, and settling this question will require a definitive test that could distinguish between ciliary motion *per se* and motor protein activity. If it can be demonstrated that correct LR patterning does not occur when ciliary beating is disrupted at target points other than motor proteins or ion fluxes, the ciliary flow hypothesis will be strongly supported for the mouse system. On the other hand, data indicating that disrupting cytoplasmic motor transport alters LR asymmetry in the absence of effects on cilia would count toward the alternative hypothesis. If cilia are an instructive factor in LR asymmetry in mammals, they are probably downstream of ion flux events, perhaps linked by mechanisms such as a sensory function for cilia which may transduce voltage information to cells (Pazour and Witman, 2003), or by ion-flux-regulated ciliary beat (Tamm and Terasaki, 1994). A downstream sensory role is quite plausible in the chick as well.

In summary, most of the genetic data are equally compatible with an ion flux model and a ciliary model. Cytoplasmic

transport of ion channel/pump mRNA or protein is an especially strong candidate in light of the developmental timing and links to voltage and polarity in PCKD and *inv* animals. As any model of a complex and poorly understood event, this proposal raises numerous questions of its own. For example, how do the events schematized in Fig. 3 translate to the very different developmental architectures of the rabbit, mouse, or zebrafish? Especially in the mouse, it is thought that embryonic axes are quite plastic until later stages, making it unlikely that chirality can be specified at cleavage stages; however, analysis of the data indicates that, in unmanipulated (wild-type) embryos, embryonic axes may indeed be set up very early (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001). In the chick, understanding of how the LR axis is aligned with the DV and AP axes (which probably occurs at the base of the primitive streak) will require increased insight into the molecular mechanisms determining the direction in which the primitive streak grows.

Of course, the ability to regulate the LR axis in later stages does not rule out very early mechanisms for primary LR orientation. In human monozygotic non-conjoined twins, the sidedness of unilateral eye, tooth, and other defects, as well as the chirality of hair whorls, is often opposite in the two individuals (discussed in Levin, 1999; Mercola and Levin, 2001). Such "bookending" phenomena suggest that some aspects of chirality are established at very early stages, certainly long prior to the appearance of a mature streak and ciliated node. The strict midline demarcation of pigmentation in CHILD syndrome (congenital hemidysplasia with ichthyosiform nevus) arising from X-inactivation (Happle *et al.*, 1995) and differences in LEPTIN and STAT3 protein localization between the two blastomeres of human embryos after the first cell division (Antczak and Van Blerkom, 1997) also suggest that very early cell cleavages may separate the embryo into L and R sides with established identities. Bilateral gynandromorphs (arising from single blastomere sex chromosome loss) in several animal species, including man, are likewise consistent with the first cell cleavage separating the L and R sides (Farmer, 1972; Mittwoch, 2001). The current model proposes that mechanisms of laterality function at the first blastomere cleavages, and exhibits timing that is conserved among *C. elegans*, coiled molluscs, and several vertebrates. Analysis of recent data showing a 14-3-3 protein involved in the very early LR determination process in *Xenopus* suggests that mechanisms determining cellular and organismic polarity may be similar, and are conserved among *C. elegans*, *Drosophila*, and vertebrates (Bunney *et al.*, 2003).

Evidence for endogenous voltage and pH LR gradients should be sought in other mammals such as the rabbit, as well as in primitive chordates and invertebrates. Indeed, circumferential expression of connexins (paralleling the GJC system in the chick and the frog) has been found in early-streak rabbit embryos (Liptau and Viebahn, 1999). Importantly, since the relevant ion fluxes can be generated by the action of any of a great many electrogenic genes, the molecular identity of the proteins generating ion flow may differ among species. A Ca^{++} channel and the Na^{+}/K^{+}-ATPase are particularly implicated in mice, given the polycystic kidney data and a recent demonstration of asymmetric Ca^{++} signaling in gastrulating mouse embryos (McGrath *et al.*, 2003), in contrast to K+ and the H^{+}/K^{+}-ATPase utilized in the chick and the frog. It will also be crucial to characterize the orientation of the various cytoskeletal elements at early stages of embryogenesis, as well as to compare bioelectric parameters in wild-type mouse embryos with those of the various motor protein and *inv* mutants. Several labs are currently pursuing these issues and examining the roles of ion flux in other embryonic model systems (mouse, rabbit, monkey, zebrafish, and various invertebrates). The resolution of these questions will require the interdisciplinary approaches of genetics, biophysics, and electrophysiology, and will likely have fascinating and important implications for many areas of cell and developmental biology, as well as for insight into a plethora of clinical endpoints.

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