

Motor protein control of ion flux is an early step in embryonic left–right asymmetry

Michael Levin

Summary

The invariant left–right asymmetry of animal body plans raises fascinating questions in cell, developmental, evolutionary, and neuro-biology. While intermediate mechanisms (e.g., asymmetric gene expression) have been well-characterized, very early steps remain elusive. Recent studies suggested a candidate for the origins of asymmetry: rotary movement of extracellular morphogens by cilia during gastrulation. This model is intellectually satisfying, because it bootstraps asymmetry from the intrinsic biochemical chirality of cilia. However, conceptual and practical problems remain with this hypothesis, and the genetic data is consistent with a different mechanism. Based on wide-ranging data on ion fluxes and motor protein action in a number of species, a model is proposed whereby laterality is generated much earlier, by asymmetric transport of ions, which results in pH/voltage gradients across the midline. These asymmetries are in turn generated by a new candidate for “step 1”: asymmetric localization of electrogenic proteins by cytoplasmic motors. *BioEssays* 25:1002–1010, 2003. © 2003 Wiley Periodicals, Inc.

Introduction: LR asymmetry

Upon the basically bilaterally symmetrical body plan of vertebrates are imposed consistent asymmetries in the morphology and location of organs such as the heart, viscera, and brain. This consistent chirality, in the absence of any macroscopic feature of nature that distinguishes left from right, has high intrinsic interest for those seeking to understand the molecular mechanisms and evolutionary biology of morphogenesis, as well as biomedical relevance to a variety of laterality defects.⁽¹⁾ Recently, significant progress has been made toward uncovering the molecular basis for handed asymmetry.^(2,3) For example, a variety of asymmetrically

expressed genes have been described in several species, and it has been shown that cascades of these signaling factors control the situs of the visceral organs.

However, orienting the LR axis with respect to the other two axes presents a profound conceptual challenge. The dominant model in the field (Fig. 1) is the “chiral molecule” theory.⁽⁴⁾ In this paradigm, 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 orientation 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 that underlies “Step 1” of asymmetry. While candidates abound (since many biological molecules have a chirality), the nature of the LR-relevant chiral molecule and precise knowledge of how early it acts in development have proven elusive.

Cilia: a good theoretical prototype for “step 1”

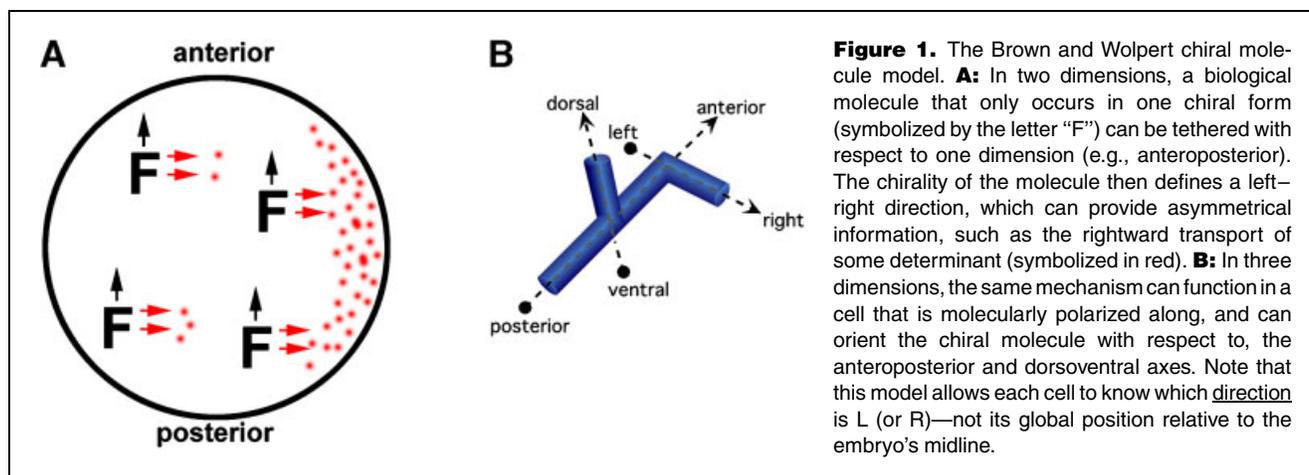
The observation that human Kartagener’s syndrome patients exhibited randomization of visceral *situs* (heterotaxia) and had ultrastructural defects in the dynein component of cilia⁽⁵⁾ was of great interest because it suggested that asymmetry could be bootstrapped from molecular chirality of some ciliary component. This idea was supported by the finding that the murine *iv* mutation, which unbiases laterality,⁽⁶⁾ encodes a dynein called left-right dynein (LRD) that is expressed in cells of the mouse node.⁽⁷⁾ Axonemal dynein is a component of the motor that 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,⁽⁸⁾ 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 extraembryonic space.⁽⁹⁾ Thus, it was proposed that vortical action of cilia may initiate asymmetry by moving an extracellular signaling molecule to one side, where it can induce asymmetric gene expression. This mechanism re-

Cytokine Biology Department, The Forsyth Institute and Department of Craniofacial and Developmental Biology, Harvard School of Dental Medicine, Boston, MA. E-mail: mlevin@forsyth.org

Funding agencies: American Cancer Society (Research Scholar Grant RSG-02-046-01), the American Heart Association (Beginning Grant in Aid #0160263T), a Basil O’Connor fellowship from The March of Dimes (#5-FY01-509), and the Harcourt General Charitable Foundation.

DOI 10.1002/bies.10339

Published online in Wiley InterScience (www.interscience.wiley.com).



quires 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.

Unanswered questions

The idea that vortical cilia motion results in a net asymmetric localization of a morphogen is intellectually satisfying, but it possesses a number of problems. First, there are inconsistencies^(2,10–12) between the predictions of the cilia model in the data on directly measured ciliary flow, the patterns of asymmetric gene expression, and visceral *situs* phenotypes in the various motor protein knockouts and mutations (although a model based on sensory functions of cilia has been proposed, which is more compatible with all of the data, Ref. 10). Second, a number of the relevant knockouts exhibit some degree of midline defects;^(9,13) this raises the possibility that the LR phenotype is secondary to an alteration of dorsoanterior development or barrier function, since it has been known for some time that disruptions of midline barrier or defects in anteroposterior or dorsoventral patterning leads to non-specific destabilization of LR asymmetry.⁽¹⁴⁾ Third, the vortical flow hypothesis does not yet explain how fluid flow created in the narrow gap between the node and Reichert’s membrane consistently results in the proper localization of the putative morphogens despite the movements of the node relative to the membrane (which may be expected to be overwhelming, considering the mobility of pregnant mice). Fourth, 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 this question, it is useful to critically examine aspects of the data and their relationship to other observations that are not often discussed in the literature. Thus, here I consider in detail a set of key questions inherent in this hypothesis, and attempt to

synthesize a number of 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, I would like to draw attention to and explore alternate hypotheses consistent with the data that may otherwise remain unnoticed, and which I believe will greatly repay attention at the molecular level.

Three key (but orthogonal) questions must be asked of the cilia model. (1) 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. 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 extraembryonic medium, mechanical clipping of motile cilia, etc. The beautiful and mechanically intricate experiments of Nonaka et al.⁽¹⁵⁾ 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.⁽¹⁶⁾

(2) 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 address 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 that 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.^(17–19) Indeed, *Ird* is

expressed in prestreak mouse embryos⁽⁷⁾ and expression of a number of 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.⁽²⁾ Most importantly, a number of 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 2–3 (prior to node formation) for correct LR asymmetry⁽²⁰⁾ and, in the frog, very early mechanisms include gap junctional communication (GJC),⁽²¹⁾ ion flux,⁽²²⁾ the LR coordinator,⁽²³⁾ and syndecans.⁽²⁴⁾ Thus, if bias of the mouse LR axis is initiated by cilia, the mechanism is probably a highly divergent from the way in which other species establish asymmetry; this would present its own problems, since then it would have to be explained how animals with such different early mechanisms all converge on the well-conserved left-sided expression of genes such as *Nodal*. The definitive answer to this question awaits LR analysis of conditional mouse mutants where the function of the relevant proteins is abrogated only at late (node) stages, leaving possible early roles untouched. Interestingly, recent evidence suggests that human patients with classical primary ciliary dyskinesia (and the attendant heterotaxia) do not exhibit reversals in the normal prevalence of right-handedness,⁽²⁵⁾ suggesting that at least some aspects of laterality are indeed upstream of mutations affecting ciliary function.

(3) How general is a ciliary mechanism for the orientation of the LR axis? While cilia have been described in a number of species,⁽²⁶⁾ functional LR roles have not been tested in any embryo other than mouse. Asymmetry is initiated by mechanisms not involving cilia (and indeed is present from the first blastomere cleavages) in the chirality of snail embryos⁽²⁷⁾ and *C. elegans*.⁽²⁸⁾ All of the LR-relevant cilia-specific data has come from studies in the mouse (although molecular motor mutations are also associated with asymmetry defects in man, Ref. 29). However, the mouse is an atypical mammal and develops in a cone. 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. Interestingly, unlike the chick, mouse embryos in which the node is ablated do not correctly orient the LR axis during regulative morphogenesis,⁽³⁰⁾ perhaps suggesting an important species difference in events surrounding the formation and spatial patterning of the node.

An alternative model: cytoplasmic transport

Contrasting with the cilia hypothesis, which focuses on hydrodynamics as a motive force for LR signals, we propose another model⁽³¹⁾ that is based on a different aspect of developmental biophysics: voltage and pH gradients driven by ion flux. A number of observations link ion transport and asymmetry; as early as 1956, it was found that an imposed

DC electric current across the LR axis of the early chick blastoderm specifically induced cardiac reversals.⁽³²⁾ An endogenous asymmetry in the response of calcium channels to Ca^{2+} depletion has been reported in ascidians,⁽³³⁾ and indeed some aspect of Ca^{2+} flux has been implicated in LR asymmetry in amphibian⁽³⁴⁾ and chick⁽³⁵⁾ 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.⁽²²⁾ A directly observable, consistently biased, LR-asymmetric ion flux and membrane voltage gradient across the midline exists in both species, and is dependant 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,^(36,37) thus, I propose 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 (stage 2) in chick—before node cilia exist and before the earliest known asymmetric gene expression.^(22,38) Thus, at least in chick and 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. Analogously 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.⁽³⁹⁾ Approximately half a dozen mRNAs and proteins have now been found to be LR asymmetric at the first few cell cleavages in *Xenopus* (Levin et al., unpublished), and H^+ pumps are known to associate with the cytoskeleton.⁽⁴⁰⁾ We propose that these phenomena reflect potential non-ciliary functions of motors in the LR pathway: asymmetric cargo transport. The possibility of cytoplasmic transport functions of motor proteins that might be relevant to LR patterning have been suggested in several reviews^(31,41–43) and primary papers,^(7,22) because the ciliary and cytoplasmic roles have not yet been experimentally distinguished in any LR context.

This model, specifically centered on cytoplasmic movement of ion transport proteins, is summarized in Fig. 2 (the

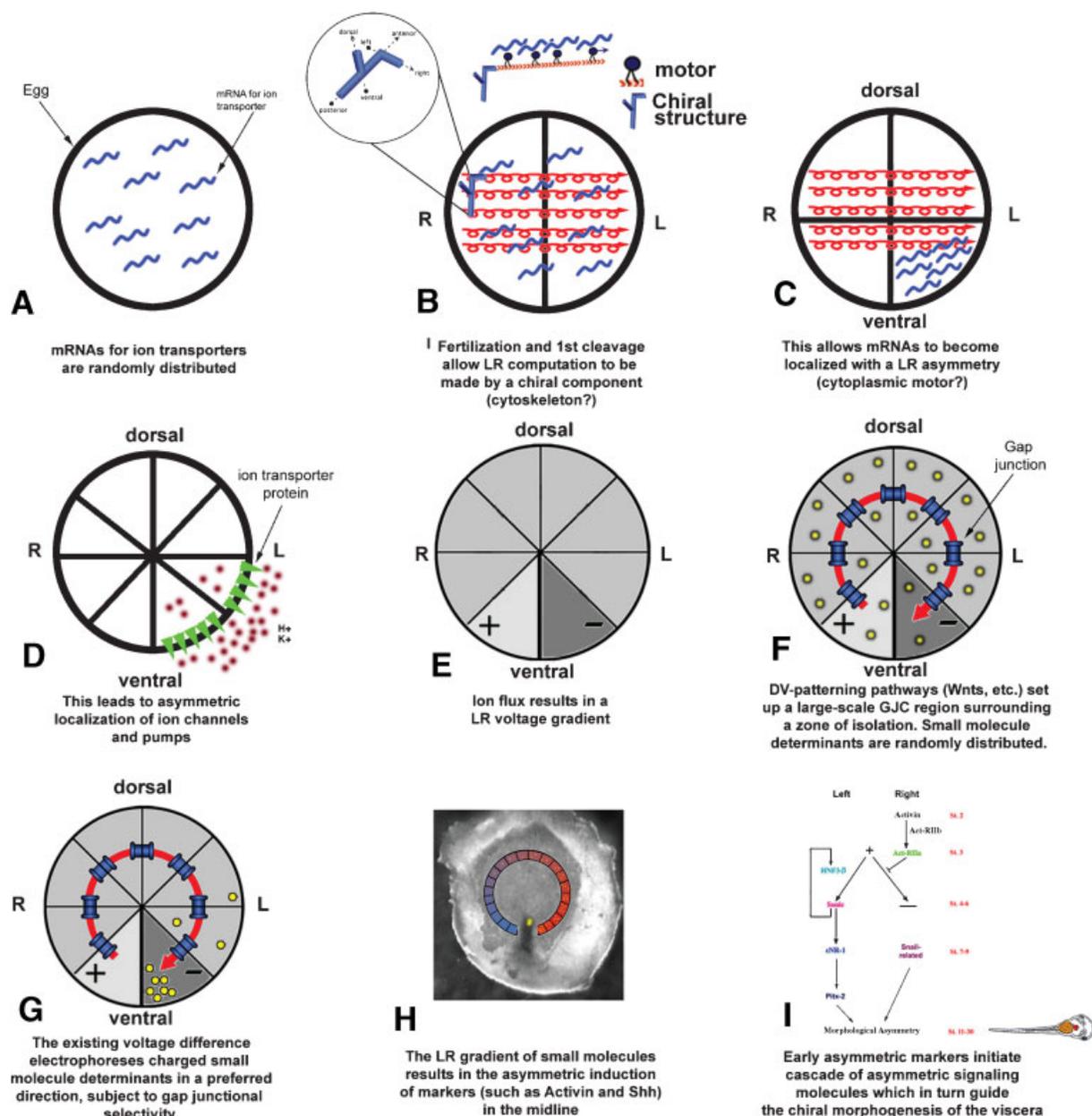


Figure 2. A model of the LR pathway based on cytoplasmic motor protein movement. This highly schematized diagram draws mainly on *Xenopus* embryogenesis and attempts to follow known timing data for each step. **A:** In the unfertilized egg (which is thought to possess radial symmetry about the animal–vegetal axis), maternal mRNAs for key ion transporters are evenly distributed. **B:** Cytoskeletal rearrangements following fertilization set up microfilaments or microtubules, which are oriented along the newly established LR axis. **C:** Motor proteins (such as dynein (*Ird*) and kinesin (*Kif3-b*)) 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.

early steps have been superimposed on the developmental architecture of *Xenopus* because most of the details on LR-relevant ion flux are available in this species). 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. How might the voltage/pH gradient be transduced to known downstream events? 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.^(20,21) The voltage gradient across the midline might regulate the permeability state of gap junctional paths, or alternatively provide electro-motive force which electrophoresis charged factors through gap junction paths in a consistent direction.

It should also be noted that the systems of cytoplasmic motor transport, ion flux, and large-scale gap junctional paths together provide a way to resolve the question 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.⁽⁴⁴⁾ 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 a number of 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,⁽⁴⁴⁾ so it might be expected that mutations in either kind of gene might result in laterality defects. This of course was observed in the various 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,⁽⁴⁵⁾ and some dyneins classified as axonemal have been found in non-ciliated cells and appear to be associated with cytoplasmic protein localization pathways.⁽⁴⁶⁾ Moreover, motor proteins are extremely pleiotropic and have numerous roles in cell biology;⁽⁴⁷⁾ 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 a number of observations. *LRD* (left–right dynein) possesses no 5th P-loop in the N-terminal region, in contrast to other axonemal-type dyneins.^(48,49) Moreover, both the timing and location of expression suggest non-ciliary roles for *Ird*. Not only is *Ird* expressed in mouse embryos before the formation of the node (day 3.5),⁽⁷⁾ it is present throughout the primitive streak in chick, not just in ciliated node cells.⁽²⁶⁾ It is also expressed in the developing limb where it is thought to participate in cartilage condensation.⁽⁴⁸⁾ Conversely, in mice bearing a targeted *Ird* mutation (which have the predicted laterality defects), sperm motility and tracheal ciliary beating are normal.⁽⁴⁸⁾ Thus, as noted by the authors of the original identification of *Ird* in *inversus viscerum* mice,⁽⁷⁾ these data are quite consistent with the existence of other, non-ciliary functions of *Ird*. The same possibility is suggested in the case of kinesins, since *KIF3B* and *KIF3A* mice exhibit a host of developmental defects not obviously related to ciliary function,^(9,13,50) and these KIF proteins are expressed in non-ciliated cells such as muscle⁽⁵¹⁾ and appear to function in cytoplasmic protein localization.⁽⁵²⁾ The cytoplasmic motor/ion flux model implies that the ciliary phenotypes observed in motor protein knock-out animals are 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 laboratory, who showed that disruption of the cytoskeletal arrays during the first cell cycle in *Xenopus* can randomize LR asymmetry.⁽⁵³⁾ 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 classical “F-molecule” (a chiral molecule tethered with respect to the AP and DV axes);⁽⁴⁾ candidates for the nucleation center which may set up the LR-oriented tracks include the centriole, centrosome⁽⁵⁴⁾ and basal body. Centrosomes are an attractive possibility because they are known to participate in the determination of cellular polarity,⁽⁵⁵⁾ and have been recently implicated in a novel mechanism for asymmetric

inheritance of mRNA during early cleavage,⁽⁵⁶⁾ consistent with our proposal of establishment of electrical polarity of cells by targeted mRNA localization. Interestingly, two gene products known to be involved in LR asymmetry (Polaris and INV) are known to localize to basal bodies⁽⁵⁷⁾ consistent with mechanisms relying on these cellular structures to orient the LR axis.

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. Consistently with this prediction, ion channel and pump localization and function is 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.⁽⁵⁸⁾ The model also suggests that motor protein localization be asymmetric. Indeed, we have observed that Kif3b protein exhibits a strikingly asymmetric localization during the first few cleavages (Levin et al., unpublished observations).

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 chick and *Xenopus*, Ref. 22). Significantly, this appears to be true in mammals as well: a recent analysis of the PCKD (polycystic kidney disease) mouse⁽⁵⁹⁾ found LR defects in knockout animals. Not only is polycystin an ion channel⁽⁶⁰⁾ that can be expressed on the cell membrane,⁽⁶¹⁾ it is regulated by pH and voltage.⁽⁶²⁾ Thus, the PCKD LR 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 by virtue of ion transport and not ciliary action. This possibility is greatly supported by the observations^(63–65) that PCKD cells in both mouse and human 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). Thus, similarly to the situation in chick and frog, recent data in mammals link a LR phenotype to (1) aberrant cell membrane voltage, (2) the P-type cation exchanger (of which the Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase are closely-related members), and (3) polarity of ion pump localization along a major cellular axis. PCKD is expressed in mice from the 2-cell stage⁽⁵⁹⁾ (consistent with the early function of ion channels in frog embryos), and PCKD embryos exhibit incorrect localizations of other electrogenic genes that we have also implicated in LR asymmetry.^(66,67)

There exist a number of specific ways to establish and manipulate endogenous voltage gradients, which are predicted to have LR roles. Electrical polarity between cell groups is often regulated by tight junction proteins, which control extracellular current paths; tight junctions also regulate the movement of cell membrane proteins (including ion pumps) between the apical and basolateral locales.⁽⁶⁸⁾ Our model predicts that disruption of tight junction proteins may result in

LR defects by virtue of short-circuiting key voltage gradients in the early embryo either directly or by changing the localization of electrogenic proteins. This has indeed been observed in *Xenopus*,⁽⁶⁹⁾ and a role for cell adhesion molecules in asymmetry has also been demonstrated in chick.⁽⁷⁰⁾

Aligning electrical polarity with morphological polarity of cells and tissues depends on correct insertion of electrogenic proteins in cell membranes and their anchoring to the proper location. A number of ion transporters' localizations are controlled by ankyrin proteins.^(71,72) Most interestingly, mutation of *inversin*, which produces a protein with highly conserved ankyrin repeats, results in almost full LR reversal in mice.⁽⁷³⁾ INV localization in cleavage-stage embryos has not been described, and the sequence of the *inv* protein currently provides no clues as to function aside from the ankyrin repeats. Our model proposes a ready explanation: if *inv* is important for the correct localization of key ion flux proteins, it can easily be imagined that a mutation leading *inv* to be localized or oriented in an opposite configuration will result in *situs inversus totalis*. Significantly, *inv* mice exhibit a polycystic kidney phenotype with its attendant reversals in electric polarity and ion pump localization,^(74,75) and *inversin* has been observed to be localized to the cell membrane in some mammalian cell types.⁽⁷⁵⁾ This model of *inv* function is applicable to *Xenopus* as well. Gain-of-function experiments indicate that overexpression of *inv* on the right side of the *Xenopus* embryo randomizes the LR axis, while overexpression on the left side does not.⁽⁷⁶⁾ This observation makes sense in light of the model: since the right-sided H⁺/K⁺-ATPase localization is necessary for correct asymmetry in *Xenopus*,⁽²²⁾ introducing an excess of *inv* protein in the early right blastomeres can randomize the LR axis by interfering with the stoichiometry of the complex that targets ion flux proteins to appropriate locales.

A priori, it might be argued that mis-localization of ion flux proteins would have disastrous effects on general cell health. However, in our screen of large numbers of electrogenic targets in frog development,⁽²²⁾ we observed that inhibition of a surprising range of such proteins produced no generalized teratogenic defects. The ion flux system, similarly to gap junctional communication,⁽²²⁾ appears to function mainly in LR signaling during early development. This phenomenon probably reflects LR-specific roles of a few individual channels and pumps which, by virtue of tight spatial and temporal restriction or tight 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 that are distinct from the cilia model, allowing us to distinguish between the two models (in contrast to much of the previous discussion which highlighted the fact that all current data except for timing is consistent with both models). One such concern is the spatial origin of LR

information. The cilia model 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, Refs. 20,21) predicts that the midline cells receive LR information from lateral tissue (see Fig. 2). In the chick, current data strongly indicates that, indeed, Hensen's node is instructed with respect to the LR axis by adjacent lateral cell groups;^(21,77,78) moreover, we recently showed 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,⁽²⁰⁾ arguing against an intrinsic mechanism for the node.

Conclusion and future prospects

The simple ciliary motion model has a number of problems, but these can probably be taken into account by more sophisticated schemes based on sensory cilia.⁽¹⁰⁾ The data make 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. On the one hand, 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 towards the alternative hypothesis. If cilia is 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,⁽⁷⁹⁾ or by ion flux-regulated ciliary beat.⁽⁸⁰⁾

In summary, most of the genetic data is 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 a number of questions of its own. For example, how do the events schematized in Fig. 2 translate to the very different developmental architectures of the rabbit, mouse or zebrafish? Especially in 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, data indicate that in the wild-type case, embryonic axes may indeed be set up very early.^(81,82)

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, bookending phenomena (opposite sidedness of hair whorls,

tooth and eye defects, etc.) 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 (discussed in Refs. 2,83). The strict midline demarcation of pigmentation in CHLD syndrome arising from X-inactivation⁽⁸⁴⁾ and differences in key proteins between the two blastomeres of human embryos after the first cell division⁽⁸⁵⁾ 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 a number of animal species including man are likewise consistent with the first cell cleavage separating the L and R sides.^(86,87) Our model proposes that mechanisms of laterality function at the first blastomere cleavages, exhibiting timing which is conserved among *C. elegans*, coiled molluscs and a number of vertebrates.

We predict that increasing evidence for endogenous voltage and pH LR gradients will be found in other species such as rabbit. Indeed, circumferential expression of connexins (paralleling the GJC system in chick and frog) has been found in early streak rabbit embryos (C. Viebahn, personal communication and Ref. 88). Importantly, since the relevant ion fluxes can be generated by the action of any of a large number of electrogenic genes, the molecular identity of the proteins generating ion flow may differ among species. A Ca^{2+} channel and the Na^+/K^+ -ATPase are particularly implicated in mice given the polycystic kidney data, in contrast with K^+ and the H^+/K^+ -ATPase in chick and 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. We are currently pursuing these issues and examining the roles of ion flux in other embryonic model systems (mouse, rabbit, 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.

Note added in proof

The elegant work by McGrath et al. (Ref. 89) which was published after this paper went to press directly supports two of the predictions made above: that asymmetric ion flux will be detected in mammals, and that it is likely to depend on the function of PCKD as an ion channel. Such asymmetries may well exist during even earlier stages in mice.

Acknowledgments

I would like to thank Mark Mercola, Cliff Tabin, Masahiko Fujinaga, Taisaku Nogi, Takahiro Fukumoto, Vladimir Gelfand, Richard Vallee, and Hiroshi Hamada for numerous helpful discussions on this topic, and Martin Blum, Christoph Viebahn,

and Dany Adams for their suggestions on the manuscript. I am grateful to Christoph Viebahn, Martin Blum, and Chris McManus for sharing unpublished data.

References

- Kosaki K, Casey B. Genetics of human left-right axis malformations. *Seminars in Cell Dev Biol* 1998;9:89–99.
- Mercola M, Levin M. Left–right asymmetry determination in vertebrates. *Annual Rev Cell Dev Biol* 2001;17:779–805.
- Yost HJ. Establishment of left–right asymmetry. *Int Rev Cytol* 2001;203:357–381.
- Brown N, Wolpert L. The development of handedness in left/right asymmetry. *Development* 1990;109:1–9.
- Afzelius B. A human syndrome caused by immotile cilia. *Science* 1976;193:317–319.
- Lowe LA, et al. Conserved left–right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* 1996;381:158–161.
- Supp DM, Witte DP, Potter SS, Brueckner M. Mutation of an axonemal dynein affects left–right asymmetry in *inversus viscerum* mice. *Nature* 1997;389:963–966.
- Sulik K, et al. Morphogenesis of the murine node and notochordal plate. *Dev Dyn* 1994;201:260–278.
- Nonaka S, et al. Randomization of left–right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* 1998;95:829–837.
- Tabin CJ, Vogal KJ. A two-cilia model for vertebrate left–right axis specification. *Genes Dev* 2003;17:1–6.
- Wagner M, Yost H. Left–Right development: the roles of nodal cilia. *Current Biology* 2000;10:R149–R151.
- Burdine R, Schier A. Conserved and divergent mechanisms in left–right axis formation. *Genes Dev* 2000;14:763–776.
- Takeda S, Yonekawa Y, Tanaka Y, Nonaka YOS, Hirokawa N. Left–right asymmetry and kinesin superfamily protein KIF3A. *J Cell Biol* 1999;145:825–836.
- Danos MC, Yost HJ. Linkage of cardiac left–right asymmetry and dorsal-anterior development in *Xenopus*. *Development Supplement* 1995;121:1467–1474.
- Nonaka S, Shiratori H, Saijoh Y, Hamada H. Determination of left right patterning of the mouse embryo by artificial nodal flow. *Nature* 2002;418:96–99.
- Fujinaga M, Baden JM. Evidence for an adrenergic mechanism in the control of body asymmetry. *Dev Biol* 1991;143:203–205.
- Levin M, et al. Left/Right patterning signals and the independent regulation of different aspects of *situs* in the chick embryo. *Dev Biol* 1997;189:57–67.
- Levin M, Johnson R, Stern C, Kuehn M, Tabin C. A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell* 1995;82:803–814.
- Stern C, et al. Activin and its receptors during gastrulation and the later phases of mesoderm development in the chick embryo. *Developmental Biology* 1995;172:192–205.
- Levin M, Mercola M. Gap junction-mediated transfer of left–right patterning signals in the early chick blastoderm is upstream of *Shh* asymmetry in the node. *Development* 1999;126:4703–4714.
- Levin M, Mercola M. Gap junctions are involved in the early generation of left right asymmetry. *Dev Biol* 1998;203:90–105.
- Levin M, Thorlin T, Robinson K, Nogi T, Mercola M. Asymmetries in H⁺/K⁺-ATPase and cell membrane potentials comprise a very early step in left–right patterning. *Cell* 2002;111:77–89.
- Hyatt B, Yost H. The left–right coordinator: the role of *Vg1* in organizing left–right axis. *Cell* 1998;93:37–46.
- Kramer KL, Barnette JE, Yost HJ. PKC γ regulates syndecan-2 inside-out signaling during *xenopus* left–right development. *Cell* 2002;111:981–990.
- McManus IC, Martin N, Stubbings G, Chung EMK, Mitchison HM. Handedness and situs inversus in primary ciliary dyskinesia. in press.
- Essner J, et al. Conserved function for embryonic nodal cilia. *Nature* 2002;418:37–38.
- Freeman G, Lundelius JW. The developmental genetics of dextrality and sinistrality in the gastropod *Lymnaea peregra*. *Wilhelm Roux Arch Dev Biol* 1982;191:69–83.
- Wood W. Evidence from reversal of handedness in *C. elegans* embryos for early cell interactions determining cell fates. *Nature* 1991;349:536–538.
- Olbrich H, et al. Mutations in *DNAH5* cause primary ciliary dyskinesia and randomization of left–right asymmetry. *Nature Genetics* 2002;30:143–144.
- Davidson BP, Kinder SJ, Steiner K, Schoenwolf GC, Tam PP. Impact of node ablation on the morphogenesis of the body axis and the lateral asymmetry of the mouse embryo during early organogenesis. *Dev Biol* 1999;211:11–26.
- Levin M, Nascone N. Two molecular models of initial left–right asymmetry generation. *Medical Hypotheses* 1997;49:429–435.
- Sedar JD. The influence of direct current fields upon the developmental pattern of the chick embryo. *J Exp Zool* 1956;133:47–71.
- Albrieux M, Villaz M. Bilateral asymmetry of the inositol triphosphate-mediated calcium signaling in two-cell ascidian embryos. *Biology of the Cell* 2000;92:277–284.
- Toyoizumi R, Kobayashi T, Kikukawa A, Oba J, Takeuchi S. Adrenergic neurotransmitters and calcium ionophore-induced situs inversus viscerum in *Xenopus laevis* embryos. *Development Growth Differentiation* 1997;39:505–514.
- Linask K, Han M, Artman M, Ludwig C. Sodium-calcium exchanger (NCX-1) and calcium modulation: NCX protein expression patterns and regulation of early heart development. *Dev Dynamics* 2001;221:249–264.
- Levin M. Bioelectromagnetic patterning fields: roles in embryonic development regeneration and neoplasm. *Bioelectromagnetics* 2003;24:295–315.
- Robinson K, Messerli M. In: McCaig C, editor. *Nerve Growth and Guidance*. Portland: Portland Press 1996.
- Levin M. Left–Right asymmetry and the chick embryo. *Seminars Cell Dev Biol* 1998;9:67–76.
- Tekotte H, Davis I. Intracellular mRNA localization: motors move messages. *Trends Genet* 2002;18:636–642.
- Vitavska O, Wieczorek H, Merzendorfer H. A novel role for subunit C in mediating binding of the H⁺-V-ATPase to the actin cytoskeleton. *J Biol Chem* 2003.
- Tamura K, Yonei-Tamura S, Belmonte JC. Molecular basis of left–right asymmetry. *Development Growth Differentiation* 1999;41:645–656.
- Hobert O, Johnston RJ, Jr, Chang S. Left–right asymmetry in the nervous system: the *Caenorhabditis elegans* model. *Nature Reviews Neuroscience* 2002;3:629–640.
- Robinson KR, Messerli MA. Left/right up/down: the role of endogenous electrical fields as directional signals in development repair and invasion. *Bioessays* 2003;25:1–8.
- Januschke J, et al. Polar transport in the *Drosophila* oocyte requires Dynein and Kinesin I cooperation. *Curr Biol* 2002;12:1971–1981.
- Pazour GJ, Dickert BL, Witman GB. The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. *J Cell Biol* 1999;144:473–481.
- Vaisberg E, Grissom P, McIntosh J. Mammalian cells express three distinct dynein heavy chains that are localized to different cytoplasmic organelles. *J Cell Biol* 1996;133:831–841.
- Vale RD. Millennial musings on molecular motors. *Trends Cell Biol* 1999;9:M38–M42.
- Supp DM, et al. Targeted deletion of the ATP binding domain of left–right dynein confirms its role in specifying development of left–right asymmetries. *Development Supplement* 1999;126:5495–5504.
- Kandl KA, Forney JD, Asai DJ. The dynein genes of *Paramecium tetraurelia*: the structure and expression of the ciliary beta and cytoplasmic heavy chains. *Mol Biol Cell* 1995;6:1549–1562.
- Marszalek J, Ruiz-Lozano P, Roberts E, Chien K, Goldstein L. Situs inversus and embryonic ciliary morphogenesis defects in mouse mutants lacking the KIF3A subunit of kinesin-II. *Proc Nat Acad Sci USA* 1999;96:5043–5048.
- Ginkel LM, Wordeman L. Expression and partial characterization of kinesin-related proteins in differentiating and adult skeletal muscle. *Molec Biol Cell* 2000;11:4143–4158.

52. Le Bot N, Antony C, White J, Karsenti E, Vernos I. Role of xklp3 a subunit of the *Xenopus* kinesin II heterotrimeric complex in membrane transport between the endoplasmic reticulum and the Golgi apparatus. *J Cell Biol* 1998;143:1559–1573.
53. Yost HJ. Development of the left–right axis in amphibians. *Ciba Foundation Symposium* 1990;162:165–176.
54. Wood WB. Left–right asymmetry in animal development. *Annu Rev Cell Dev Biol* 1997;13:53–82.
55. Bornens M. Centrosome composition and microtubule anchoring mechanisms. *Curr Opin Cell Biol* 2002;14:25–34.
56. Lambert JD, Nagy LM. Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages. *Nature* 2002;420:682–686.
57. Taulman PD, Haycraft CJ, Balkovetz DF, Yoder BK. Polaris a protein involved in left–right axis patterning localizes to basal bodies and cilia. *Mol Biol Cell* 2001;12:589–599.
58. Hamm-Alvarez SF, Sheetz MP. Microtubule-dependent vesicle transport: modulation of channel and transporter activity in liver and kidney. *Physiol Rev* 1998;78:1109–1129.
59. Pennekamp P, et al. The ion channel polycystin-2 is required for left–right axis determination in mice. *Curr Biol* 2002;12:938–943.
60. Koulen P, et al. Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol* 2002;4:191–197.
61. Hanaoka K, et al. Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature* 2000;408:990–994.
62. Gonzalez-Perrett S, et al. Voltage dependence and pH regulation of human polycystin-2-mediated cation channel activity. *J Biol Chem* 2002;277:24959–24966.
63. Wilson PD, et al. Apical plasma membrane mispolarization of NaK-ATPase in polycystic kidney disease epithelia is associated with aberrant expression of the beta2 isoform. *Am J Pathol* 2000;156:253–268.
64. Barisoni L, et al. Analysis of the role of membrane polarity in polycystic kidney disease of transgenic SBM mice. *Am J Pathol* 1995;147:1728–1735.
65. Ogborn MR, Sareen S, Tomobe K, Takahashi H, Crocker JF. Renal tubule Na,K-ATPase polarity in different animal models of polycystic kidney disease. *J Histochem Cytochem* 1995;43:785–790.
66. Gillespie GA, Somlo S, Germino GG, Weinstat-Saslow D, Reeders ST. CpG island in the region of an autosomal dominant polycystic kidney disease locus defines the 5' end of a gene encoding a putative proton channel. *Proc Natl Acad Sci USA* 1991;88:4289–4293.
67. Haragsim L, Rankin C, Bastani B. H-ATPase distribution in the cpk- and ppcy-mouse models of polycystic kidney disease (PKD). *J Am Soc Nephrol* 1995;6:375.
68. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Prog Biophys Mol Biol* 2003;81:1–44.
69. Brizuela B, Wessely O, DeRobertis E. Overexpression of the *Xenopus* tight-junction protein Claudin causes randomization of the left–right body axis. *Dev Biol* 2001;230:217–229.
70. Garcia-Castro MI, Vielmetter E, Bronner-Fraser M. N-Cadherin a cell adhesion molecule involved in establishment of embryonic left–right asymmetry. *Science* 2000;288:1047–1051.
71. Kretschmer T, et al. Ankyrin G and voltage gated sodium channels colocalize in human neuroma—key proteins of membrane remodeling after axonal injury. *Neurosci Lett* 2002;323:151–155.
72. Srinivasan Y, Elmer L, Davis J, Bennett V, Angelides K. Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature* 1988;333:177–180.
73. Mochizuki T, et al. Cloning of *inv* a gene that controls left/right asymmetry and kidney development. *Nature* 1998;395:177–181.
74. Mochizuki T, Tsuchiya K, Yokoyama T. Molecular cloning of a gene for inversion of embryo turning (*inv*) with cystic kidney. *Nephrology Dialysis Transplantation* 2002;17:68–70.
75. Nurnberger J, Bacallao RL, Phillips CL. Inversin forms a complex with catenins and N-cadherin in polarized epithelial cells. *Mol Biol Cell* 2002;13:3096–3106.
76. Yasuhiko Y, et al. Calmodulin binds to *inv* protein: implication for the regulation of *inv* function. *Development Growth Differentiation* 2001;43:671–681.
77. Pagan-Westphal S, Tabin C. The transfer of left–right positional information during chick embryogenesis. *Cell* 1998;93:25–35.
78. Psychoyos D, Stern C. Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo. *Development* 1996;122:3263–3273.
79. Pazour GJ, Witman GB. The vertebrate primary cilium is a sensory organelle. *Curr Opin Cell Biol* 2003;15:105–110.
80. Tamm SL, Terasaki M. Visualization of calcium transients controlling orientation of ciliary beat. *J Cell Biol* 1994;125:1127–1135.
81. Gardner RL. Specification of embryonic axes begins before cleavage in normal mouse development. *Development Supplement* 2001;128:839–847.
82. Piotrowska K, Zernicka-Goetz M. Role for sperm in spatial patterning of the early mouse embryo. *Nature* 2001;409:517–521.
83. Levin M. Twinning and embryonic left–right asymmetry. *Laterality* 1999;4:197–208.
84. Happle R, Mittag H, Kuster W. The CHILD nevus: a distinct skin disorder. *Dermatology* 1995;191:210–216.
85. Antczak M, Van Blerkom J. Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod* 1997;3:1067–1086.
86. Farmer A. A bilateral gynandromorph of *Nephrops norvegicus*. *Marine Biology* 1972;15:344–349.
87. Mittwoch U. Genetics of mammalian sex determination: some unloved exceptions. *J Exp Zool* 2001;290:484–489.
88. Liptau H, Viebahn C. Expression patterns of gap junctional proteins connexin 32 and 43 suggest new communication compartments in the gastrulating rabbit embryo. *Differentiation* 1999;65:209–219.
89. McGrath J, Somlo S, Makova S, Tian X, Brueckner M. Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell* 2003;114:61–73.