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Is the Early Left-Right Axis like a Plant, a Kidney, or a Neuron? The Integration of Physiological Signals in Embryonic Asymmetry

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Embryonic morphogenesis occurs along three orthogonal axes. While the patterning of the anterior-posterior and dorsal-ventral axes has been increasingly well-characterized, the left-right (LR) axis has only relatively recently begun to be understood at the molecular level. The mechanisms that ensure invariant LR asymmetry of the heart, viscera, and brain involve fundamental aspects of cell biology, biophysics, and evolutionary biology, and are important not only for basic science but also for the biomedicine of a wide range of birth defects and human genetic syndromes. The LR axis links biomolecular chirality to embryonic development and ultimately to behavior and cognition, revealing feedback loops and conserved functional modules occurring as widely as plants and mammals. This review focuses on the unique and fascinating physiological aspects of LR patterning in a number of vertebrate and invertebrate species, discusses several profound mechanistic analogies between biological regulation in diverse systems (specifically proposing a nonciliary parallel between kidney cells and the LR axis based on subcellular regulation of ion transporter targeting), highlights the possible importance of early, highly-conserved intracellular events that are magnified to embryo-wide scales, and lays out the most important open questions about the function, evolutionary origin, and conservation of mechanisms underlying embryonic asymmetry. **Birth Defects Research (Part C) 78:191–223, 2006.** © 2006 Wiley-Liss, Inc.

Key words: embryogenesis; left-right asymmetry; physiology; modeling

INTRODUCTION

The geometrical invariance known as symmetry is a prominent aspect of developmental morphology during embryogenesis. Animal bodyplans occur in a wide variety of symmetries: spherical (e.g., volvox), radial (e.g., sea anemone), chiral (e.g., snails, ciliates), bilateral (e.g., housefly), and pseudobilateral (e.g., man). Vertebrates have a generally bilaterally-symmetrical body plan, but this symmetry is broken by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen, and gut, or the asymmetric development of paired organs (such as brain hemispheres and lungs). A fascinating atlas of such morphological left-right (LR) asymmetries throughout the animal kingdom is given in Neville (1976), and evolutionary surveys have analyzed asymmetries in diverse phyla (Palmer, 1996).

Developmental noise often results in pseudorandom characteristics and minor stochastic deviations known as fluctuating asymmetry (Klingenberg and McIntyre, 1998); however, the most interesting phenomenon is invariant (i.e., consistently biased) differences between the left and right sides. For brevity, as well as because these are likely to be secondary to embryonic asymmetries, this review largely neglects behavioral/sensory asymmetries (Harnad, 1977; Bisazza et al., 1998).

The Unique Fascination of the LR Axis

The establishment of LR asymmetry raises a number of interesting biological questions. Why does asymmetry exist at all? What are the implications of asymmetry for the normal structure and physiology of the heart, gut, and brain? Why are all normal individuals not only asymmetric, but asymmetric to the same direction (i.e., why a consistent bias and not a 50%/50% racemic population, given that individuals with full inversion are not obviously impaired)? While it is possible to devise plausible evolutionary reasons for why organisms might be asymmetric in the first place (op-

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timal packing, fluid dynamics, maximizing surface area of tubes, etc.) (Kilner et al., 2000), there is no obvious reason for why they should all be asymmetric to the same direction. It is, after all, much easier to imagine a developmental mechanism for generating antisymmetry (such as local amplification and long-range inhibition of stochastic biochemical differences resulting in a morphologically biphasic population), than for biasing the LR axis to a given direction. When, during evolution, did handed asymmetry appear, and were there true bilaterally-symmetrical organisms prior to the invention of oriented asymmetry (Cooke, 2004)? Is it connected to chirality in lower forms (such as snail shell coiling and chirality in some plants) or even the asymmetry (lack of quantum parity conservation) in weak nuclear decay (Wu et al., 1957)? At what developmental stages is asymmetry initiated in vertebrate embryos? Are the establishment of bilaterality, imposition of asymmetry, and bias of that asymmetry with respect to the other two axes separate events? How conserved are the molecular mechanisms establishing correct asymmetry in animals with drastically different modes of cleavage and gastrulation? And, how can the LR axis be consistently oriented with respect to the anterior-posterior and dorsal-ventral axes in the absence of any macroscopic feature of the world that distinguishes left from right?

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Answers to these questions require a detailed understanding, at the molecular, genetic, and biochemical levels, of the formation of biased asymmetry in embryos.

Not Just Basic Biology: Asymmetry and Human Medicine

Errors of LR patterning during embryogenesis are relevant to the clinical considerations of several fairly common human birth defects: syndromes such as Kartagener's and Ivemark's (Winer-Muram, 1995), dextrocardia, situs inversus (a complete mirror-image reversal of the sidedness of asymmetrically positioned organs and asymmetric paired organs), heterotaxia (a loss of concordance where each organ makes an independent decision as to its situs), and right or left isomerism (in which the organism is completely symmetrical, for example, polysplenia or asplenia). Heterotaxia and isomerism often result in serious health problems for the patient (Burn, 1991). The LR asymmetry of the heart is intimately connected to its function, and errors in cardiac situs represent a significant source of human heart disease (Kathiriya and Srivastava, 2000). Laterality defects can arise in a single individual (Winer-Muram, 1995; Kosaki and Casey, 1998) but are especially associated with monozygotic twinning (see below).

Interestingly, complete (and rare) situs inversus totalis is not associated with severe difficulties in most patients, although it does appear to be accompanied by an estimated higher incidence of congenital heart disease on the population level (Ramsdell, 2005). This is at first puzzling: if everything is exactly mirror-image, why should there be any problem at all? All connections and structures should be preserved by this reflection. However, it was recently found that in situs inversus patients, where the large-scale asymmetry of the heart is reversed, asymmetric myofiber orientation (small scale structure) in the apex of left ventricle is not, making it opposite to that of the large-scale morphology (Delhaas et al., 2004). This suggests that the gross asymmetry of the organs is separable from the chirality of subcellular components, and also potentially explains why 50-50 racemic populations are not seen: discordance between subcellular asymmetries and large-scale structures in full situs inversus individuals may sufficiently contribute to lower fitness in the reversed individuals (over evolutionary scales) to explain the existence of a consistent and robust biasing mechanism.

LR asymmetries contribute to human physiology involving many organs in addition to the heart. For example, proper propulsion of the digesta appears to depend on digestive tract asymmetry (Arun, 2004). There are also links to more subtle features of human physiology; asymmetric histamine skin responses in L versus R arms are altered in patients with left cerebral epileptic focus (Meador et al., 2004), and in mice, interleukin level difference in L and R cortex corresponds to their paw preference (Shen et al., 2005a, 2005b). Synergy between clinical data and model systems has significantly contributed to the understanding of LR patterning (Bisgrove and Yost, 2001; Zhu et al., 2006), and clinical data have suggested important components of the pathway. One example is PA26 (sestrin), discovered through the analysis of human laterality patients (Peeters et al., 2003) but not yet explored in model systems. Another is the inversion of various organs long observed in the context of human conjoined twins (Torgersen, 1950; Aird, 1959; Cuniff et al., 1988; Burn, 1991; Winer-Muram, 1995). The discovery of the spatial signals propagated by asymmetric gene expression in chick embryos has allowed a partial understanding of laterality defects in human conjoined twins (Kapur et al., 1994), which appear to be induced by crossover of LR morphogen molecules from one twin to the adjacent one (Levin et al., 1996).

The LR Pathway in Three Easy Phases

Conceptually, LR patterning is divided into three phases (Levin and

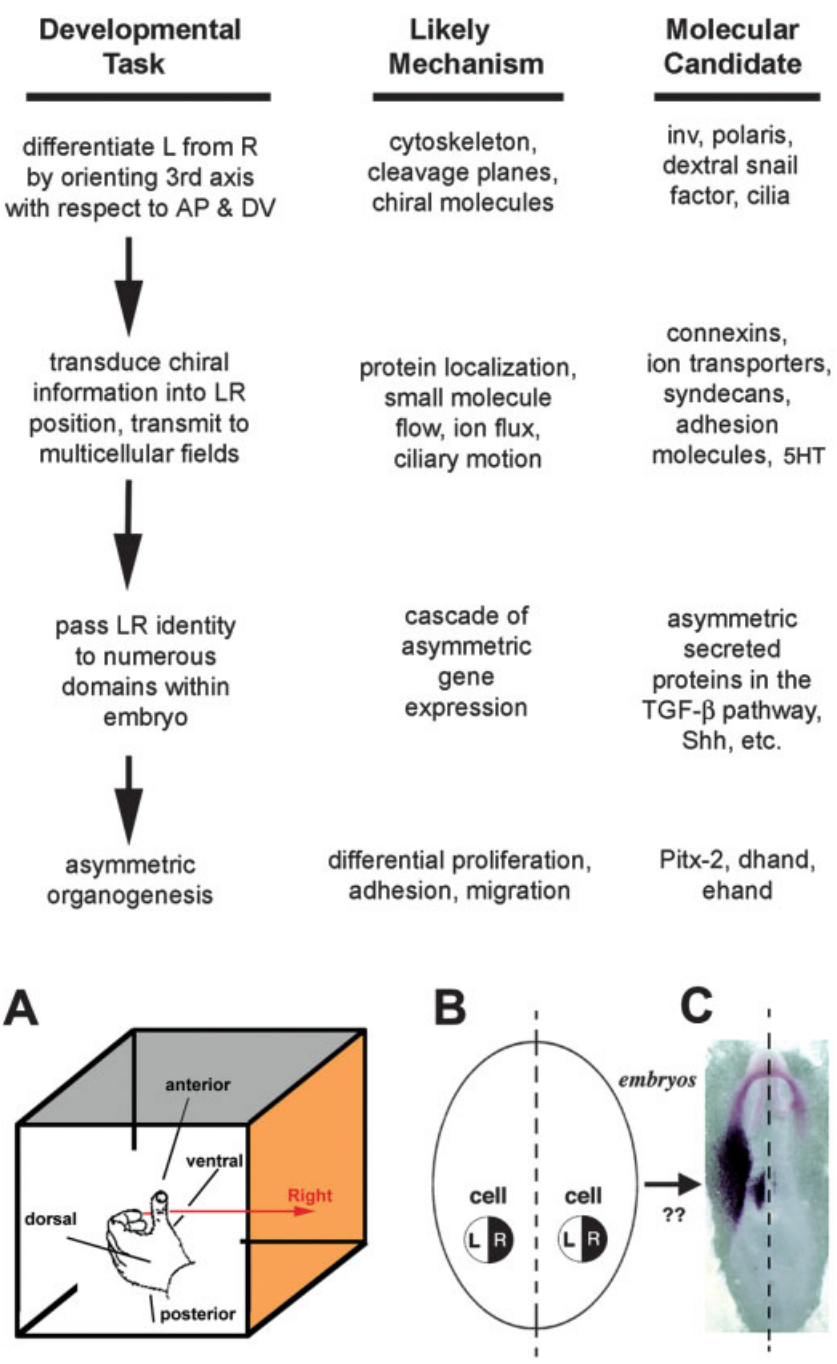

Figure 1. Overview of LR patterning pathway. The LR pathway proceeds from an initial microscopic orientation event (either extracellular, as in the cilia model, or intracellular, as in the cytoskeleton model; **A**), through the imposition of LR positional information on cell fields (**B**), to downstream asymmetric transcriptional cascades (**C**).

Mercola, 1998b); the flow of information is schematized in Figure 1. In the final phase, individual organs utilize cell migration, differential proliferation, cytoskeletal organization, and other mechanisms to achieve asymmetries in their location or morphogenesis (Stalsberg, 1969a, 1969b; Manasek, 1981; Horne-Badovinac et al., 2003;). Consistent with their downstream position, and counter to earlier proposals (Waddington, 1937), a number of studies have shown that the individual organs' lateralities are set, and can be experimentally randomized, independently of each other (Levin et al., 1997; Chin et al., 2000). The topological deformations undergone by asymmetric tissues are more complex than usually assumed (Manner, 2004), and complete understanding is likely to require mathematical or physical models in addition to molecular biology. Biophysical mechanisms used to shape organogenesis include the extracellular matrix (Tsuda et al., 1996; Yue et al., 2004) and actin bundles (Itasaki et al., 1989, 1991; Latacha et al., 2005) in the heart tube, as well as differential rates of elongation in the frog gut tube (Muller et al., 2003). A number of informative recent studies have addressed the mechanics of asymmetric organogenesis (Ramsdell et al., 2005, 2006), including computational models and direct meas-

urements of stiffness in tissues of heart tube (Zamir et al., 2003). Mechanical forces in looping have now been analyzed (Voronov and Taber, 2002; Alford and Taber, 2003; Zamir et al., 2003; Voronov et al., 2004; Zamir and Taber, 2004; Latacha et al., 2005; Nerurkar et al., 2006; Taber, 2006), though myosin does not appear to be involved (Remond et al., 2006). Genetic control over these pathways is mediated proximately by genes such as flectin, the bHLH family members EHAND and DHAND, and the transcription factor Tbx5 (Srivastava, 1995; Tsuda et al., 1996; Sparrow et al., 1998; Bruneau et al., 1999; Angelo et al., 2000; Fernandez-Teran et al., 2000; Hatcher et al., 2000; Takeuchi et al., 2003).

Upstream of these processes lies a pathway of asymmetric genes that are expressed in cell fields only on one side of the embryo's midline. By inducing or repressing transcription of downstream asymmetric targets, they propagate signals among subpopulations of cells (such as node and lateral plate mesoderm) that eventually dictate sidedness for the organs undergoing asymmetric morphogenesis (Lowe et al., 1996; Levin et al., 1997; Lohr et al., 1998; Piedra et al., 1998). These cascades of asymmetric gene expression form the middle phase of LR patterning (Levin, 1998; Whitman and Mercola, 2001; Mercola, 2003), and include gene families such as *Hedgehogs*, bone morphogenetic proteins (*BMP*s), and *Pitx* (see Levin (2005) for an exhaustive list of asymmetric genes and their respective lateralities).

However, for whichever asymmetric gene is at the top of the pathway, it is necessary to ask what determined its asymmetry. Thus, in the first phase of LR patterning, an as-yet unknown mechanism orients the LR axis with respect to the other two axes. While theoretical candidate mechanisms have been proposed (Brown and Wolpert, 1990), no mechanism has been conclusively shown to initiate asymmetry de novo. The developmental timing of each phase differs among species, though asymmetric gene expression almost always begins at or shortly after gastrulation. The LR axis is probably specified after the anterior-posterior (AP) and dorsal-ventral (DV) axes, and is likely to be determined with respect to them (McCain and McClay, 1994; Danos and Yost, 1995). The timing of the initiation of LR asymmetry in the various species is particularly controversial, but the mechanisms underlying different aspects of LR patterning in various species are beginning to be uncovered in significant detail.

a unique and fascinating aspect of LR patterning: the physiological components, which reveal surprising aspects of evolutionary conservation, feedback loops, and biophysical mechanisms.

A number of excellent recent reviews have summarized the progress in various areas of asymmetry (Burdine and Schier, 2000; Hamada et al., 2002; Hobert et al., 2002; McGrath and Brueckner, 2003; Palmer, 2004; Ramsdell, 2005; Tabin, 2005; Vallortigara and Rogers, 2005; Raya and Belmonte, 2006). In this review, moving among model species as needed, I focus on what I believe to be a unique and fascinating aspect of LR patterning: the physiological components, which reveal surprising aspects of evolutionary conservation, feedback loops, and biophysical mechanisms.

PHYSIOLOGICAL COMPONENTS OF THE LR PATHWAY

Gap Junctional Communication

The fairly dense pathway of LR cascade members in chick embryos (Levin et al., 1995; St Amand et al., 1998; Garcia-Castro et al., 2000; Ohuchi et al., 2000; Wang et al., 2004) suggested an immediate question: what mechanisms are upstream of the very first asymmetrically-expressed gene? Contrary to the paradigm of genetically separate L and R compartments that applies after mid-gastrulation, it was observed that events occurring on the far R side were required for establishment of L identity on the L side at the beginning of streak initiation (Levin and Mercola, 1999). Thus, gap junctional communication (GJC) was examined as a candidate for a mechanism that would enable cells to communicate across large distances along the LR axis and assign LR identities to cell fields.

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Gap junctions are channels connecting adjacent cells that allow the direct transfer of small molecule signals. The cell biology of gap junctions has been described extensively (Goodenough et al., 1996; Falk, 2000), and gap junctional flow is involved in a number of important patterning events in embryonic development and tumor progression (Guthrie and Gilula, 1989; Lo, 1996; Levin, 2001). Based on a report that several unrelated patients with visceroatrial heterotaxia contain potential mutations within Connexin43 (Britz-Cunningham et al., 1995), and data from frog embryos that indicated asymmetric patterns of GJC (Guthrie, 1984; Guthrie et al., 1988) in early blastomeres, Levin and Mercola (1998a) tested the hypothesis that gap junctional paths were a mecha-

nism by which LR information was communicated across large cell fields. *Xenopus* embryos at early cleavage stages were shown to contain a junctional path across the dorsal blastomeres, and a zone of junctional isolation on the ventral midline (confirming with a double-dye system in previous observation using a single small-molecule probe (Guthrie, 1984; Olson et al., 1991; Brizuela et al., 2001); but see Landesman et al. (2000)). Injection of mRNA encoding a dominant negative connexin protein into dorsal blastomeres or wild-type connexins into ventral blastomeres both resulted in heterotaxia and randomization of *XNR-1* expression in the absence of other developmental defects (Levin and Mercola, 1998a).

These results indicate that an endogenous path of GJC between dorsal and lateral blastomeres, as well as the isolation across the ventral midline, is necessary for normal LR asymmetry in *Xenopus*. Pharmacological blocker experiments suggested that the gap-junctional system begins to function in LR asymmetry during cleavage stages. These data led to the hypothesis that a circumferential path of GJC, around a zone of isolation, could be the mechanism that bridges asymmetry at the level of a cell (step 1) to the embryo-wide cascades of asymmetric gene expression (step 2). It was proposed (Levin and Nascone, 1997; Levin and Mercola, 1998a) that small molecule determinants are initially randomly distributed, but traverse the circumferential GJC path net unidirectionally, accumulate on one side of the midline, and then induce asymmetric gene expression in conventional ways.

Similarly to the results in *Xenopus*, it was discovered that differential GJC is required upstream of asymmetric *Shh* expression in the chick node and one Connexin, *Cx43*, was implicated by treatment with specific antisense oligonucleotides or blocking antibodies (Levin and Mercola, 1999). Interestingly, *Cx43* mRNA is broadly expressed in the epiblast of streak stage embryos, but not in the streak itself. Thus, GJC required for LR asymmetry may propagate signals throughout the epiblast but not across an insulating zone at the streak. In support of this model, surgical incisions made along various radii emanating from the developing node abolish node asymmetry. While a topological transformation is required to map the GJC system onto the different embryonic architectures of the chick and *Xenopus*, the basic schematic is the same in both systems: correct laterality determination upstream of asymmetric gene expression depends on an uninterrupted contiguous region of GJC around a small zone of junctional isolation.

Ion Flows: Asymmetrical Bioelectricity

One of the key aspects of the GJC model is that the junctional flow must be net unidirectional, in order to derive a LR asymmetry from the existing dorsoventral difference in GJC (although no individual small molecule needs to make it all the way around the path). In some contexts, chemically-rectifying or one-way junctions have been observed (Flagg-Newton and Loewenstein, 1980; Robinson et al., 1993; Xin and Bloomfield, 1997; Zahs and Newman, 1997; Zahs, 1998), and it is tempting to visualize unidirectional paths of heterotypic gap junctions arranged appropriately to provide a ratchet mechanism for accumulation of LR morphogen on one side of the midline. However, because thermodynamics forbids the generation of a gradient without an expenditure of energy, GJC models require an energetic process to drive the chiral distribution of the small molecules through the circumferential path. Hypothesizing that a voltage difference might provide an electromotive force that can be used to electrophorese charged molecules in preferred directions through GJC paths, Levin et al. (2002) tested the hypothesis that ion fluxes (needed to generate the standing voltage gradients) were an obligatory aspect of early LR patterning in *Xenopus.*

A pharmacological screen of hundreds of various types of ion channels, pumps, and cotransporters (Levin et al., 2002) specifically implicated four target genes involved in H^+ and K^+ flux (Adams and Levin, 2003; Chen and Levin, 2004). Two of these, the H^+/K^+-ATPase and V-H^+-ATPase, are already known to function during early cleavage stages (Levin et al., 2002 Adams et al., 2006); the K^+ channel components have not yet been extensively characterized. Maternal H^+/K^+-ATPase mRNA and protein subunits of both pumps are asymmetrically localized during the first two cell divisions, demonstrating that asymmetry in *Xenopus* is generated by about 2 hr postfertilization. Analysis of the situs of asymmetric genes (*xNR-1*, *xLefty*, and *xPitx-2*) following pharmacological and genetic inhibition of these pumps (as well as gain-of-function experiments) showed that, consistently with the early asymmetrical localization, the ion flux mechanism is upstream of asymmetric gene expression. Equalization of membrane voltage and cytoplasmic pH by methods independent of V-ATPase and H^+/K^+-ATPase also randomize asymmetry, demonstrating that it is indeed the ion transport that is crucial for laterality, not some other cryptic function of these protein complexes, which has been reported for some other channels and pumps (Paul et al., 2003; Baumgartner et al., 2004). Voltage-sensitive dyes and self-referencing ion-selective probe measurements during early cleavage stages in vivo revealed the predicted consistent asymmetries in H^+ efflux from L versus R ventral cells and the resultant membrane voltage differences across the midline.

Because the GJC system is conserved to both chick and *Xenopus*, Levin et al. (2002) and Adams et al. (2006) tested whether embryonic laterality was dependent upon ion flux in the chick and zebrafish. An asymmetry in current flows in the chick streak was already observed (but not discussed with respect to LR patterning) in Jaffe and Stern (1979). Analysis of the chick embryo using an in vivo reporter of membrane voltage indicated that cells on the left side of the primitive streak were consistently depolarized with respect to those on the contralateral side. This indicates that the chick embryo has assigned L and R identi-

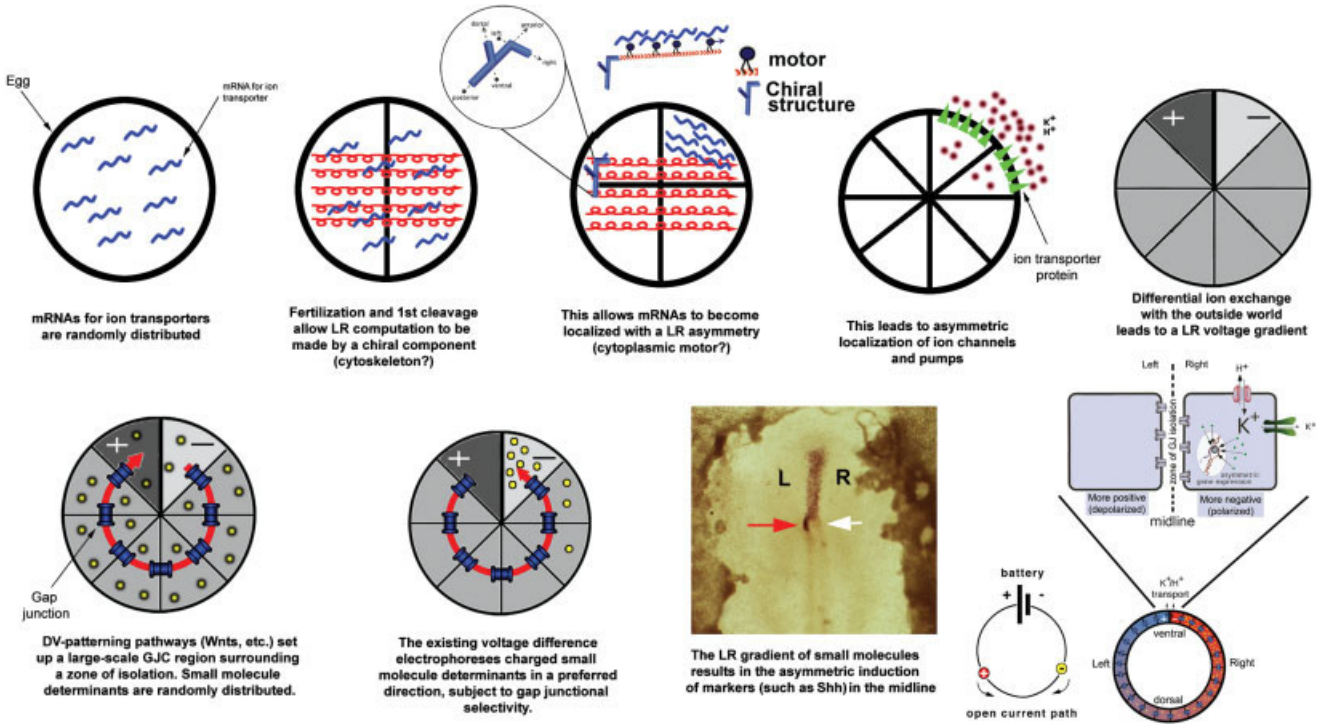

Figure 2. Integrative model of early physiological events based on *Xenopus* system (time-sequence moving left to right). Initially randomly-distributed maternal mRNAs and proteins encoding H^+ and K^+ transporters become asymmetrically distributed due to the action of motor proteins (cued in turn by an asymmetric cytoskeleton deriving chirality from a basal body or other oriented ''F-molecule''). The function of asymmetrically-distributed ion channels and pumps results in a LR gradient in membrane voltage. When the gap junction system provides an open circuit around the zone of isolation (battery), small molecule determinants (e.g., serotonin) become asymmetrically localized, inducing downstream gene expression (such as *Shh*).

ties by stage 3-prior to the earliest known asymmetric gene expression. Specific inhibition of the H^+/K^+-ATPase prior to gastrulation equalized the depolarization of cells across the midline, and randomized the asymmetric expression of *Shh*, *cWnt-8C*, and other markers (including *Cerberus*-a marker of head asymmetry). Subsequent work in another laboratory revealed that in the chick, the asymmetric function of the H^+/K^+-ATPase functions through downstream Ca^{++} and Notch signals (Raya et al., 2004). Although no direct measurements of early voltage or pH have yet been reported for zebrafish, functional data indicate that both the H^+/K^+-ATPase and the V-ATPase are utilized at early stages (before Kupffer's vesicle formation) of zebrafish development to direct the normal asymmetry of gene expression and organ situs (Kawakami et al., 2005; Adams et al., 2006).

Alongside the ion transporters that produce asymmetric ion flows (Levin, 2003a) and the gap junctions that determine the distribution of isopotential and iso-pH cell fields in embryos (Levin, 2001), another passive component shapes current paths (leaks between cells) and thus controls resulting voltage gradients: tight junctions. Consistently with the role of bioelectric signals in asymmetry, protein components of tight junctions have been implicated in LR patterning (Brizuela et al., 2001; Simard et al., 2006).

Serotonin: A Small Molecule With a New Role

Another main line of inquiry raised by the implication of gap junction paths in asymmetry was, what is the molecular nature of the small-molecule LR signals that are exchanged between cells on the L and R sides? The ideal candidate would be smaller than the size cut-off of gap junctions ($< \sim$1 kDa) and would be charged (to enable regulation of movement via ion pump-dependent voltage gradients) (Levin and Nascone, 1997; Levin, 2003c). Moreover, it should be water-soluble: signaling molecules such as retinoic acid (which has been implicated in later LR patterning steps (Chazaud et al., 1999; Adam et al., 2003; Kawakami et al., 2005; Tanaka et al., 2005; Vermot and Pourquie, 2005; Sirbu and Duester, 2006)) do not need gap junctions to move between cells. The neurotransmitter serotonin (5HT) fits these criteria, has been demonstrated to go through gap junctions in some contexts (Wolszon et al., 1994), has been suggested to have nonneural roles in early embryogenesis (Buznikov and Shmukler, 1981; Buznikov et al., 2001), and offers the benefits of a well-developed pharmacological tool set (Gaster and King, 1997). Serotonin is thus an ideal candidate for an early LR signal (Levin et al., 2006).

Fukumoto et al. (2005a, 2005b) took advantage of the large number

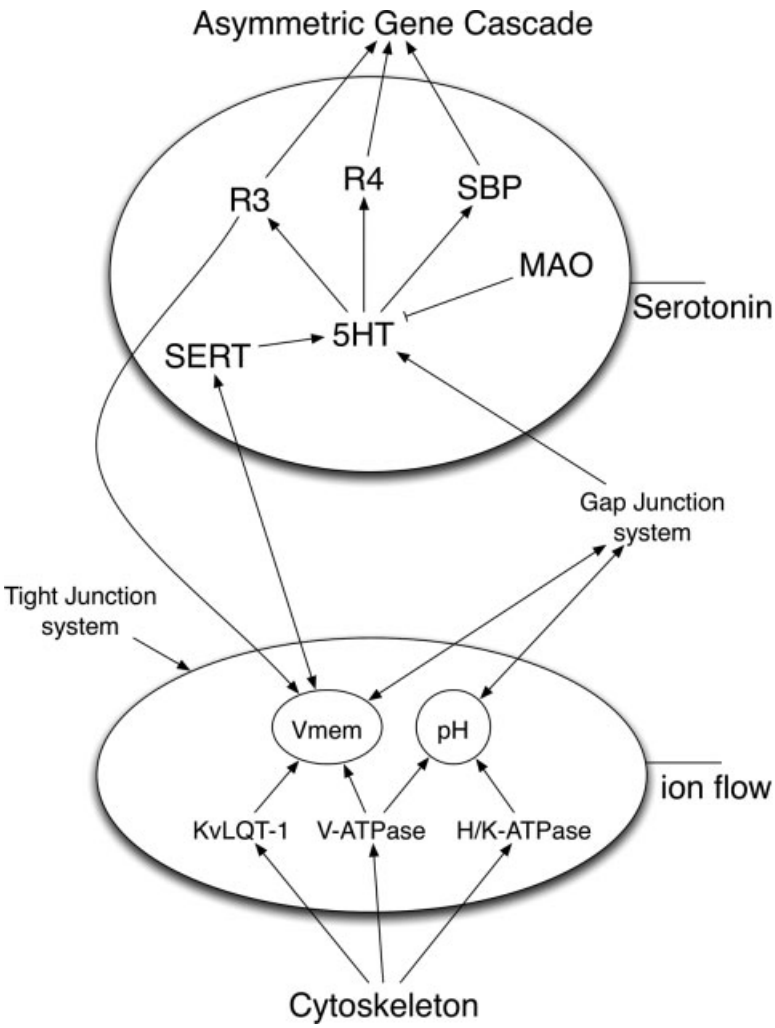

Figure 3. Feedback loops involving early physiological systems. The physiological module consists of several components: tight junctions and gap junctions that control current paths and thus voltage gradients, cytoskeletal elements that control localization of ion transporters, the ion flow module consisting of pH and membrane voltage (regulated by several transporters), and the serotonergic module (consisting of 5HT itself, receptors, serotonin binding protein, MAO degradation enzyme, and the SERT plasma membrane transporter). Arrows indicate functional relationships. Some connections are bidirectional; for example, gap junction paths dictate membrane voltage in cells but are themselves gated by transjunctional potential.

of well-characterized reagents available to test and characterize serotonin's role in LR asymmetry in chick and frog embryos. Using analysis of endogenous localization of 5HT and related proteins, and functional experiments designed to probe the properties of the 5HT pathway, serotonin signaling was shown to be upstream of early asymmetric gene expression in both chick and frog, and revealed new developmental aspects of this versatile signaling molecule. An inverse drug screen and subsequent molecular loss- and gain-of function specifically implicated receptor subtypes R3 and R4, the cell-membrane serotonin transporter SERT, and the degradation enzyme monoamine oxidase (MAO). Moreover, evidence indicated the importance of a novel (as yet uncharacterized) intracellular 5HT receptor in the right ventral cells. Manipulation of any of these components resulted in randomization of asymmetric gene expression and organ situs. Interestingly, manipulation of R3 activity led mostly to complete situs inversus, a very rare phenotype in the LR field (Hyatt and Yost, 1998).

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Analysis of endogenous localization of serotonin revealed a striking shift from homogenous distribution to a gradient, ultimately culminating in coalescence of serotonin in a single blastomere-precisely as predicted by the GJC path model (schematized in Fig. 2). This serotonin gradient was dependent on open gap junctions and on the function of the previously-implicated ion pumps. MAO appears to function by destroying the maternal serotonin by about the 64-cell stage, presumably after the relevant signaling has taken place. In the chick, MAO and SERT proved to be asymmetrically localized in Hensen's node, adding to the available markers of asymmetry (Levin, 2005). Serotonin itself was localized throughout the streak, consistent with older radiographic (Emanuelsson et al., 1988) and fluo-

rescence immunocytochemical data (Wallace et al., 1982). The asymmetric catalytic activity of MAO is likely to provide differential 5HT levels on the R versus L sides of Hensen's node, but this has not been demonstrated quantitatively.

A number of important questions remain. First, the chick data do not directly support a GJC morphogen model for 5HT function, because asymmetric localization of 5HT itself has not been demonstrated in this species, and no evidence has been presented to support the circumferential gradient of serotonin in chick that was observed in *Xenopus* (Fukumoto et al., 2005b). Moreover, the available chick data concern macroscopic (embryo-wide localization). Subcellular investigation of 5HT movement as well as sensitive comparisons between the two sides of Hensen's node will be required to understand the role of SERT-mediated transport and 5HT signaling in detail. Moreover, in the frog, direct movement of 5HT in vivo has not been demonstrated. Unfortunately, given the small size of serotonin, current technology does not permit labeling it in such a way as to allow in vivo analysis of its movement: all available tags that are compatible with detection in living cells alter its molecular weight by an order of magnitude, likely significantly altering its interaction with endogenous binding partners, as well as the rate and extent of its movement. New techniques will need to be developed so that serotonin movement can be tracked in living embryos.

You Haven't Understood It Until You Can Model It on a Computer

The ultimate goal of the field is to synthesize the molecular data back into a coherent model of the integrated system.

(Mimura and Nishiura, 1979). The complexity of many physiological pathways requires a computer simulation because the effects of perturbations can be difficult or impossible to predict intuitively. Figure 3 reveals the functional relationships between the elements of the early mechanisms; for example, it is known that embryonic pH gradients regulate GJC paths (Turin and Warner, 1980), and some of the components of the serotonin pathway both contribute to and are modulated by membrane voltage. Also, it should be noted that cilia can contain ion channels and serotonin receptors (Brailov et al., 2000; Shin et al., 2005), and readers are invited to produce models involving the known components. Of necessity, this effort must be quantitative, and a true understanding of asymmetry will be demonstrated by a quantitative model that is predictive with respect to symmetry outcomes under a range of stochastic and targeted perturbations in system size, physiological parameter level, gene expression, and geometric (surgical) rearrangement (Cooke, 1972a, 1972b, 1972c, 1973, 1979; Cooke and Webber, 1985a, 1985b; Meinhardt, 2001, 2004; Oviedo et al., 2003; Ravasz and Barabasi, 2003). Robustness in particular is a key trait that must be explained (Eldar et al., 2002, 2003, 2004; England and Cardy, 2005; Houchmandzadeh et al., 2005). For example, *Xenopus* embryos reliably orient the LR axis under a pH range of 6–11 and under the influence of numerous drugs, with a base heterotaxia rate of 1% (Levin et al., 2002; Adams et al., 2006).

The ultimate goal of the field is to synthesize the molecular data back into a coherent model of the integrated system.

One of the key questions is how asymmetries in epigenetic signals become stabilized in asymmetric gene cascades. A recent elegant study that used a variety of gain- and loss-of-function approaches, together with real-time in vivo imaging of Ca^{++} content, produced a mathematical model of gene activation by ion fluxes transduced by *Notch* signaling (Raya et al., 2004). This important contribution used data from the chick system to analyze a complex and robust genetic network that locally activates asymmetric Notch signaling in Hensen's node. This in turn is driven by a transient extracellular calcium spike, itself dependent on asymmetric H^+/K^+-ATPase activity. Although it has not yet been explored, given the serotonin data it is tempting to hypothesize that the Ca^{++} flux may be due to asymmetric 5HT-R3 activity.

Asymmetric signaling by Ca^{++} is also suggested to function in the mouse node (McGrath et al., 2003), leading to the hypotheses of sensory cilia transducing physiological signals to downstream cascades (McGrath and Brueckner, 2003; Tabin and Vogan, 2003) similar to the way ion flow is upstream of the direction of ciliary beating in ciliates (Machemer and Eckert, 1973). Modeling has also been applied to the biomechanics of cilia movement in mammalian asymmetry. While the role of cilia is controversial (see below), modeling of ciliary hydrodynamics (Cartwright et al., 2004) have illuminated necessary properties, such as tilting (Nonaka et al., 2005) and a significant degree of robustness (Brokaw, 2005). It should also be noted that like the model described below, ciliary movement is a potential class of mechanisms that can transduce a biochemical (subcellular) chirality into an embryo-wide asymmetry.

The Embryo is an Electrophoresis Chamber: One Model Synthesizing the Available Data

The considerable amount of quantitative data now available on early LR-relevant physiology in *Xenopus* can be synthesized into a unified model (Fig. 2) explaining the dependence of early asymmetric gene expression (and subsequent organ situs) on ion flows, serotonergic sig-

naling, and gap junctional paths (Levin, 2004; Levin et al., 2006). This hypothesis suggests that net asymmetric serotonin movement occurs through gap junctions, is driven by electrophoretic forces generated by ion pump-dependent membrane voltage differences across the ventral midline, and ultimately induces asymmetric downstream genes (Levin, 2004). This class of models has been previously proposed for a number of morphogen systems (Rose, 1966; Novak and Bentrup, 1973; Lange and Steele, 1978; Larter and Ortoleva, 1981) and is compatible with many other small molecule signals. Inositol polyphosphates are also likely candidates, as they have been implicated in asymmetry (Albrieux and Villaz, 2000; Sarmah et al., 2005), as well as being downstream of a voltage sensor (Murata et al., 2005).

In this scheme, the early embryo is viewed as an open circuit (GJC zone) around a region of differential voltage potential (zone of junctional isolation). In the frog, the circuit is the set of animal pole cells during early cleavages. In the chick, this occurs through the blastoderm around the primitive steak. Serotonin accumulates on one side of the midline and thus induces different genes on the R side through a cytoplasmic receptor. In *Xenopus*, the initial asymmetry of the battery

subcellular asymmetries can be transduced into asymmetric gene expression through physiological mechanisms

(source of electrophoretic force through gap junctions) is established by the asymmetric localization of channel and pump subunits to the L or R side. Thus, the earliest known step of asymmetry in this model is a bias in asymmetric localization of ion transporters driven by asymmetric subcellular motor protein transport, which was suggested previously in the context of LR asymmetry (Levin and Nascone, 1997) and other systems (Brawley and Robinson, 1985; Goida et al., 1992; Bregestowsky et al., 1993; Brezhestovsky et al., 1993). Indeed, it is known that the motor protein KIF3A, and the ion channal Polycystin-2, both of which are important for normal LR asymmetry in mouse embryos, are now known to interact directly, as is predicted by this model (Li et al., 2006). This scheme shows how subcellular asymmetries can be transduced into asymmetric gene expression through physiological mechanisms and suggests that the origin is to be sought inside early blastomeres within the first hour after fertilization (see discussion below on cytoskeletal subcellular chirality), but it should be noted that the originating event (cytoskeletal chirality) has not yet been experimentally demonstrated.

Roles for electrophoretic movement of morphogens (Cooper, 1984; Cooper et al., 1989; Fear and Stuchly, 1998a, 1998b, 1998c) have been observed in the context of follicle-egg systems (Woodruff and Telfer, 1980; Telfer et al., 1981; Bohrmann and Gutzeit, 1987; Woodruff et al., 1988; Woodruff and Cole, 1997; Adler and Woodruff, 2000), self-electrophoresis in *Fucus* symmetry-breaking (Jaffe et al., 1974; Jaffe and Nuccitelli, 1977), and regeneration in both vertebrate and invertebrate systems (Rose, 1966, 1970; Smith, 1967; Lange and Steele, 1978). Theoretical analysis indicates that gap junctional coupling increases the sensitivity of cells to electric fields produced in their milieu (Cooper, 1984; Cooper et al., 1989). However, having proposed a role for this mechanism in chick and frog, it was important to answer the question: are physiological-strength endogenous electric fields actually sufficient to produce a meaningful gradient in serotonin in the time provided, given the known properties of embryonic cells and the geometry of the early frog embryo?

One key feature of the serotonin dataset is that all of the important parameters can be quantitatively estimated. This model was tested by construction of a computer simulation of electrophoresis of serotonin in a GJC-coupled system with the geometry of an early *Xenopus* embryo (Esser et al., 2006). The simulation revealed that electrophoresis under the force generated by realistic ion pump-generated gradients can indeed result in a significant gradient of serotonin in the two hours in which the serotonin gradient is known to form. Moreover, it allowed investigation of the dependence of the kinetics of this gradient on relevant parameters such as GJC density, voltage gradient, and morphogen size/charge. Since it is possible to individually manipulate all of the relevant parameters in this system, the specific quantitative predictions of this model must now be tested in experimental work.

An essential feature of this class of models is circumferential GJC around a zone of junctional insulation (the primitive streak in chick, the ventral midline in *Xenopus*). While consistent with the idea that the epiblast influences node asymmetry, this set of findings in chick also indicates that the information does not originate from a single source, but that contiguity of the blastodisc on both sides of the midline is necessary (Levin and Mercola, 1999). The GJC model predicts that the midline cells receive LR information from lateral tissue during gastrulation. In the chick, current data strongly indicate that indeed Hensen's node is instructed with respect to the LR axis by adjacent lateral cell groups (Psychoyos and Stern, 1996; Pagan-Westphal and Tabin, 1998; Yuan and Schoenwolf, 1998; Levin and Mercola, 1999), rather than generating LR information intrinsically (which would be required by the cilia-based models). Another essential feature that must be investigated is selectivity, since clearly it cannot be the case that every free charged small molecule in the embryo is driven into a LR gradient. Gap junctions are known to be selective for permeability to differ-

ent small molecule signals (Weber et al., 2004; Ek-Vitorin and Burt, 2005; Ek-Vitorin et al., 2006), and the basis for endogenous selectivity, and perhaps even chemical rectification (Robinson et al., 1993; Zhang et al., 2003), must be addressed experimentally.

MAJOR PUZZLES: THE FUTURE OF ASYMMETRY AND SOME HYPOTHESES

Everyone in the Field Scores this Phenotype, But What Exactly Is ''Randomization''?

One of the key remaining questions is the molecular meaning of ''randomization,'' which is the phenotype most often observed when organ situs or asymmetric gene expression is scored after experimental perturbation. Upon the initial discovery of the LR pathway, it was observed that embryos with double-sided *Nodal* expression or lack of *Nodal* expression (produced by Shh or Activin implants, respectively), show a randomization of visceral situs (Levin et al., 1995, 1997)-not a symmetrical heart and gut, but heterotaxia. This was interpreted as suggesting that this pathway of genes imparts LR information to the organs but does not control their morphogenesis per se, leading the organs to independently and randomly choose their situs when presented with identical molecular signals from the L and R sides. However, it is now known that global equalization of signaling in a number of LR pathways also induce randomization of asymmetric genes such as *Shh* and *Nodal*. A mechanistic model for this process would have to explain not simply consistent induction (or repression) of genes such as *Shh* by GJC or cell depolarization, but a process by which cells in both sides of the node can be driven to randomly express *Shh* or not. Moreover, existing in situ hybridization data require that the cells making this decision be synchronized. For example, *Shh* expression on a given side is always homogenous-it is not that single cells make this decision (resulting in a speckled pattern on both sides of the node), but that L and R sides of the node each makes its decision as a group. One candidate for such a bistable mechanism would be a short-range activation/long-range inhibition system such as that which establishes cell polarity via the Notch-Delta pathway (Hermann et al., 2000; Delattre and Felix, 2001; Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003, 2004; Vincent, 2003). It is possible that such a mechanism works in the node to integrate a number of epigenetic biasing factors into stable domains of downstream gene expression. Future work is necessary to understand how this works in the node and streak; recent mathematical models are beginning to tackle this issue (Meinhardt and Gierer, 2000; Rasskin-Gutman and Izpisua-Belmonte, 2004).

A mechanistic model for this process would have to explain not simply consistent induction (or repression) of genes such as *Shh* by GJC or cell depolarization, but a process by which cells in both sides of the node can be driven to randomly express *Shh* or not.

Some sort of feedback amplification system in cell fields on each side is likely, to turn a morphogen concentration into a sharp yes/no for (for example) *Shh* expression. If this mechanism's threshold is similar to the initial ubiquitous concentration of morphogen (before the electrophoresis acts), then loss-of-function of the gap junction or the ion pump systems will cause the morphogen to stay near this threshold. Then, each side would be equally likely to drive themselves to a yes or no for *Shh* expression, giving the four possible different states (left, right, bilateral, or no expression). Even more puzzling for simple gene cascade models is the observation that in several vertebrate and invertebrate systems, symmetric expression of an upstream normally asymmetric gene does not lead to uniformly bilateral or missing expression of downstream genes (in the case of positive and negative regulation, respectively) but rather results in a randomization of downstream gene expression (Morokuma et al., 2002), or does not affect downstream LR pathway targets at all (Kelly et al., 2002)!

Regardless of the mechanism, it is clear that heterotaxia can present significant problems for the embryo (Casey, 1998; Ramsdell et al., 2006; Zhu et al., 2006). Thus, a candidate mechanism explaining randomization may also answer the long-standing puzzle of why populations are biased and not split 50-50 among situs inversus and situs solitus-perhaps the amplification and synchronization mechanisms do not allow such fluctuating asymmetry to occur without a significant background of heterotaxia. Indeed, this is observed in mouse mutants such as *inv*, which are not purely reversed (Morgan et al., 1998, 2002;Eley et al., 2004). This in turn suggests a search for models that do not separate clearly between the symmetry breaking phase and the orientation of the LR axis with respect to the other two axes.

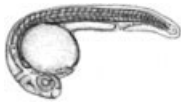
Evolutionary Conservation 1: What Is Known More Broadly Through Phyla?

How many different asymmetry mechanisms have been invented throughout evolutionary history? Table 1 summarizes the physiological mechanisms and their functional implication in various model systems. Consistent LR asymmetry has been described in plants (Luo et al., 1999; Andalo et al., 2000; Waites and Hudson, 2001; Galego and Almeida, 2002; Rao et al.,

TABLE 1. Conservation of Physiological Mechanisms among Phyla (in rough Evolutionary Order)

Embryonic time: →

Rough Evolutionary time (complexity) ↓

Model system	Cytoskeleton/ motor proteins	Ion flux	GJC	5HT	Cilia
Plants 	✓				
Ciliates 	✓				
C. elegans 	✓				
Snails 	✓				
Drosophila 	✓				
Sea urchin 		✓			
Ciona 		✓			
Zebrafish 		✓			✓
Xenopus 	✓	✓	✓	✓	
Chick 		✓	✓	✓	
Rabbit 			✓		*
Mouse 					✓
Human 					✓

✓ = functionally implicated; * = described but not yet functionally tested.

2002; Rolland-Lagan et al., 2003; Corley et al., 2005; Costa et al., 2005; Korn, 2006), and chiral forms exist among the protozoa (Nelsen et al., 1989; Frankel, 1991). The heart is asymmetrically located in the mollusks (McMurrich, 1894) and in sea urchins, the asymmetric position of the adult rudiment in the larva has been studied (McCain and McClay, 1994; Aihara and Amemiya, 2000, 2001; Kitazawa et al., 2004; Duboc et al., 2005).

Several kinds of mollusks undergo spiral cleavage and secrete an exoskeleton shaped like a conical spiral (Crampton, 1894; Sturtevant, 1923; Meshcheryakov and Beloussov, 1975). In three-dimensional space, such spirals can have two possible variants: a left-handed and a right-handed helix (which are otherwise identical). Each particular species of snail has invariant (consistent) chirality, but there are species that utilize each type of coiling. Murray and Clarke (1966) found that the direction of coiling of *P. suturalis* is maternally inherited and sinistrality is dominant to dextrality. Freeman and Lundelius (1982), studying a different species, found that dextrality is dominant; interestingly, the dextral gene apparently functions via a cytoplasmic component since it is possible to rescue the sinistral phenotype by direct transfer of cytoplasm from the dextral variant of the snail into the sinistral variety. The biochemical nature of this activity has not yet been identified, and although molecular work is ongoing (Hosoiri et al., 2003; Harada et al., 2004; Shibazaki et al., 2004; Wandelt and Nagy, 2004), it is not yet known whether the mechanisms of chiral blastomere cleavage are related to those regulating asymmetric motor protein movement and cytoskeletal components in vertebrates (Yost, 1991; Qiu et al., 2005).

In *C. elegans*, the embryonic cell lineage is asymmetrical: although the animal is generally bilaterally symmetrical with only a few LR asymmetries, many of its contralaterally analogous cells arise via different lineages on the two sides of the embryo (Wood and Kershaw, 1991). Larvae and adults also exhibit invariant LR asymmetries in the nervous system and gut. It is now known that induction at the 12-cell stage by the mesomere (MS) blastomere is necessary to establish the differences between left and right pairs of blastomeres in the anterior part of the embryo (Hutter and Schnabel, 1995). The microRNA lsy-6 controls the neuronal LR asymmetry of chemosensory receptor expression (Johnston and Hobert, 2003). Two lateral blast cells P(11/12)L and P(11/12)R are symmetric at hatching but migrate subsequently in opposite AP directions during the first larval stage and adopt different fates; this is downstream of the Notch pathway (Delattre and Felix, 2001; Hermann et al., 2000), as are the consistent cell movements leading to a twist of the intestinal primordium.

Amphioxus exhibits many LR asymmetries (Jefferies et al., 1996); one of the most striking is the asymmetry of somitogenesis (Minguillon and Garcia-Fernandez, 2002), which has recently been shown to be linked to the LR axis in vertebrates through retinoic acid signaling (Saude et al., 2005; Vermot and Pourquie, 2005; Sirbu and Duester, 2006). Downstream asymmetric gene cassettes appear to be conserved (Terazawa and Satoh, 1995, 1997; Araki et al., 1996; Shimeld, 1999; Boorman and Shimeld, 2002a, 2002b; Minguillon and Garcia-Fernandez, 2002; Morokuma et al., 2002).

Drosophila, which has provided inroads to so many other developmental questions, has not played an early role in uncovering mechanisms of LR asymmetry but has recently provided some interesting data. While selection for LR asymmetries in *Drosophila* has not been successful (Tuinstra et al., 1990), it is now known from mutant analysis that *Drosophila* possesses genes that govern the helical torsion of the body (Martin-Blanco and Garcia-Bellido, 1996) and the rotation of the embryonic gut proventriculus (Hayashi and Murakami, 2001; Ligoxygakis et al., 2001; Adam et al., 2003). Both of these asymmetries are instances of chirality, which appears to dominate in other invertebrates such as ciliates and mollusks (chirality is mathematically equivalent to asymmetry but does not require a linear midline). However, recent morphometric analysis has revealed a subtle but real directed asymmetry in wing size (Klingenberg et al., 1998), suggesting that mechanisms orienting the LR axis in fruit flies remain to be discovered. In another genus of flies, a large-scale (not subtle, requiring statistical analysis) asymmetry in wings has been reported (Runyon and Hurley, 2004). The fly system has the potential to shed light on the linkage of the LR axis with the AP axes (since inversion of AP polarity does not alter LR polarity of proventriculus (Hayashi et al., 2005); though see Ligoxygakis et al. (2001)), as well as on the potential role of motor proteins in invertebrate asymmetry (Hozumi et al., 2006; Speder et al., 2006) that may parallel the known roles of motor proteins in rodent embryos (Supp et al., 1997; Marszalek et al., 1999; Takeda et al., 1999).

Evolutionary Conservation 2: Which of the Known Mechanisms Are Conserved?

A number of mechanisms appear to be conserved. For example, chick Syndecan-2 is asymmetrically expressed (Fukumoto and Levin, 2005), and syndecans are known to be crucial for embryonic asymmetry in *Xenopus* (Kramer et al., 2002; Kramer and Yost, 2002). Even more strikingly, intracellular microtubules are important for asymmetry in plants (Hashimoto, 2002; Thitamadee et al., 2002; Abe et al., 2004), similar to their likely role in vertebrate asymmetry (Yost, 1991; Qiu et al., 2005). While the details of ion flux usage in the LR pathway of various species differ, H^+ and K^+ flux roles are conserved to sea urchin and *Ciona* (Duboc et al., 2005; Hibino et al., 2006; Shimeld and Levin, 2006). In the zebrafish, the H^+/K^+-ATPase (Kawakami et al., 2005; Adams et al., 2006) and Na^+/K^+-ATPase (Ellertsdottir et al., 2006) are also known to be important for normal asymmetry, but no asymmetric localization has yet been reported. Rhythmic Ca^{++} waves have

been described during fish gastrulation (Gilland et al., 1999), consistent with the calcium fluxes discovered in mice and chick (McGrath et al., 2003; Raya et al., 2004), although the connection to downstream LR markers is not yet characterized.

The GJC, ion flux, and serotonin pathways have been extensively compared in chick and frog embryos (Levin, 2005) and, while all of the basic components appear to be utilized by both systems despite their radically-different gastrulation architecture, details differ in interesting ways. For example, while the H^+/K^+-ATPase and V-ATPase ion pumps are expressed, as predicted by the GJC model (which requires the motive force "battery" to be located in the zone of isolation) in the primitive streak during early gastrulation of the chick, no asymmetry in pump localization has been reported in the chick at the level of mRNA or protein. Thus, while asymmetric ion flux is provided by asymmetric localization of mRNA in early frog embryos, it appears to be established in the chick embryo by a posttranslational mechanism (such as gating of electrogenic activity of mature pump complexes). Likewise, the difference in GJC in frog embryos takes place posttranslationally, by gating control of existing gap junctions (Levin and Mercola, 1998a). In contrast, the chick embryo establishes the zone of isolation at the level of mRNA, by not transcribing Cx43 mRNA in the primitive streak (Levin and Mercola, 1999). With respect to serotonin, both species use 5HT as part of an early LR signaling step; both rely on MAO, R3, R4, and SERT (Fukumoto et al., 2005a, 2005b). However, frog embryos operate with a maternal pool of serotonin that is meant to be degraded by the blastula stage, while chick embryos utilize zygotically-synthesized serotonin during gastrulation. The asymmetric serotonin gradient in *Xenopus* is generated by progressive relocalization of maternal 5HT through paths, while it appears to be generated in chick by differential degradation on the L versus R sides.

The conservation of subcellular and physiological mechanisms throughout vertebrates and even invertebrates is considerable; the situation with mammalian embryos is more complex. For example, no mouse mutants in gap junction genes have as yet reported a true LR phenotype; thus, knockins of dominant negative constructs will be required to determine whether GJC plays a role in LR asymmetry of rodents (since many different connexin genes exist in embryos and are thus potentially able to exhibit compensation during single gene deletion experiments). Likewise, although the ion channel polycystin is known to be important in mice (Pennekamp et al., 2002; McGrath et al., 2003) as well as zebrafish (Bisgrove et al., 2005), existing knockouts for many members of the serotonin pathway and various channels and pumps deleted as part of neurobiology efforts have not resulted in any reported asymmetry phenotypes. Thus, it is possible that the relevant physiological components remain to be identified, since experiments with early mouse embryos are much more difficult than in other model systems and many of the elements have not been directly examined. Especially in the case of ion transport, considerable molecular divergence may occur since any number of channels or pumps can produce the same physiological effect and thus potentially substitute for one another through evolutionary change. Another possibility is that while the downstream effectors such as the Nodal-Lefty-Pitx cascade are conserved throughout phyla, early components diverge significantly.

Important insight into the evolutionary conservation of GJC mechanisms is expected from analysis of GJC in rabbits; the rabbit embryo exhibits circumferential patterns of connexin expression (Liptau and Viebahn, 1999), and GJC has been functionally implicated in rabbit asymmetry (Muders et al., 2006). Thus functional analysis of GJC in a mammal with the flat architecture of the chick is likely to shed significant light on the evolutionary conservation and origin of the GJC system as it participates in LR patterning. Data on downstream components suggests that asymmetry mechanisms track more closely to geometric architecture than taxonomical relationships (Fischer et al., 2002).

What Happens When There Are Many Small Cells Instead of a Few Large Ones?

Consideration of early mechanisms in chick or rabbit raises an important issue about what occurs in organisms that do not have the benefit of large cell cleavages. What happens when early asymmetry steps occur in a cell field of thousands of small cells as opposed to a few large blastomeres whose cleavage planes are oriented with respect to the final embryonic body-plan? (as in *Xenopus*)? The alignment of axes in mammalian cleavage is somewhat controversial (Gardner, 1996, 1997; Zernicka-Goetz et al., 1996; Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002), but it is known that the human 3′ untranslated region (UTR) for *squint* mRNA is able to drive asymmetric localization in cleaving zebrafish embryos (Gore et al., 2005), so future work must determine the extent to which cleavage-based localization mechanisms are also utilized in mammals. One of the most exciting new developments has been the discovery that this cytoplasmic asymmetry may in fact be a rotational (East-West) chirality in *Xenopus* (Danilchik et al., 2006); this is conceptually very

What happens when early asymmetry steps occur in a cell field of thousands of small cells as opposed to a few large blastomeres whose cleavage planes are oriented with respect to the final embryonic body-plan?

attractive because the sperm entry point (which defines the DV axis) can be anywhere on the circumference of the egg, making a rotational model more likely. Rotational chirality provides information on LR direction (not absolute position), and is a good candidate for asymmetry generation in small cells because it is an ideal match for a subcellular "F-molecule" function. It is likely that subcellular events related to the cytoskeleton (perhaps organizing centers and basal bodies) are where the origin of asymmetry is to be found, and this theme is discussed in the context of polarized cells below.

The next phase of asymmetry is also very important (Fig. 1B and C), and cell size and number are crucial to formulating models of how it might happen. Because no macroscopic force distinguishes R from L, a powerful paradigm has been proposed to leverage large-scale asymmetry from the chirality of subcellular components (Brown and Wolpert, 1990; Brown et al., 1991). In this class of models, some molecule or organelle with a fixed chirality is oriented with respect to the AP and DV axes, and its chiral nature is thus able to nucleate asymmetric processes such as transport of ion channel and pump proteins to the proper side of the early embryo (Levin and Mercola, 1998b). Thus, the first developmental event that distinguishes L from R would take place on a subcellular scale. However, a mechanism must then exist to transduce subcellular signals to cell fields (Brown and Wolpert, 1990; Levin and Nascone, 1997; Levin and Mercola, 1998b). Asymmetric gene expression in embryos requires that fairly large fields of cells already know on which side of the midline they are located (such as the expression of the left-sided gene *Nodal*). In contrast, proposed mechanisms of step 1 of asymmetry (such as the F-molecule model) rely on subcellular mechanisms for determining which direction is L and which is R. Thus, one of the key questions concerns how orientation information can be turned into information on a cell's location, relative to the midline, within the context of the whole embryo. This information flow must take place between cells; ciliary motion driving extracellular movement of signaling molecules (see below) and embryo-wide gap junction paths (through which unidirectional transport is guided by events oriented within single cells at the zone of isolation) are natural candidates for such a signal exchange.

Does asymmetric gene expression exist prior to gastrulation? It has been suggested (Levin and Mercola, 1998b) that the computation which aligns the LR axis with the DV and AP axes in the chick takes place at the initiation of gastrulation, at the base of the primitive streak (which reliably progresses from the periphery to the center of the blastoderm). However, no detailed model of this process in the chick has been proposed, and may have to wait for a good understanding of how (and whether) individual cells in the chick blastoderm determine their AP polarity (Wei and Mikawa, 2000). Existing data suggest that the simple early cleavage and ion transporter partitioning model cannot be the whole story. First, it cannot be mapped directly onto the chick or rabbit body-plan. Second, even in *Xenopus*, ectopic organizers can be induced at later stages (when there are approximately 1000 cells) that correctly orient their own LR asymmetry (Nascone and Mercola, 1997), showing that even frog embryos do not have to rely on large early blastomeres. The model shown in Figure 2 is a beginning, toward a framework that can be used to think about the components known to be important for asymmetry, but it clearly must be modified in important ways to be more widely applicable.

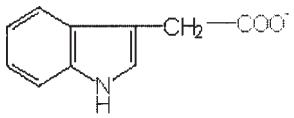
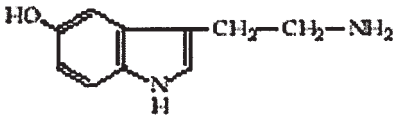
Cilia

An important physiological mechanism that has been implicated in LR asymmetry is ciliary motion. This subject has been extensively covered in recent reviews (Hirokawa et al., 2006), and we have presented the minority view (Levin, 2003c, 2004) that while cilia are likely to be an important aspect of mouse asymmetry, it is not at all clear that they initiate asymmetry in mammals, or that they are causally involved in organisms other than the mouse and fish. Ciliary data have given rise to two main physiological hypotheses: a sensory model (McGrath and Brueckner, 2003; McGrath et al., 2003; Tabin and Vogan, 2003) and one in which cilia are wafting morphogens such as *Sonic hedgehog* (Okada et al., 2005; Tanaka et al., 2005; Hirokawa et al., 2006).

while cilia are likely to be an important aspect of mouse asymmetry, it is not at all clear that they initiate asymmetry in mammals, or that they are causally involved in organisms other than the mouse and fish.

While the results of a true "no flow" condition (such as in viscous medium) have not yet been demonstrated, and culture of rodent embryos randomizes situs in and of itself (Fujinaga et al., 1990; Fujinaga and Baden, 1991), in rodent embryos, cilia at the node are likely to play a functional role in the LR pathway, although it is not known whether they generate LR information de novo or function in transmission of as yet unknown upstream LR signals. Consistent with the theoretically pleasing hypothesis that cilia initiate LR orientation, no earlier LR mechanisms have been described in rodents. However, many types of cilia can reverse the direction of their beat (Bone, 1958), and it is not clear whether the biochemical structure of cilia uniquely determines their function (allowing asymmetry to be generated from molecular chirality).

TABLE 2. Parallels between the Serotonin and Auxin Systems

Component	Plant	Vertebrate
K^+ channel	(Bandurski et al., 1990; Fuchs et al., 2003; Philippar et al., 1999; Philippar et al., 2004; Vicente-Agullo et al., 2004)	(Adams and Levin, 2004; Chen and Levin, 2004; Levin, 2003b)
H^+ pump	H^+-ATPase (Coenen et al., 2002; Hager, 2003; Palmgren, 1998; Rober-Kleber et al., 2003)	H^+-ATPase, H^+, K^+-ATPase (Adams et al., 2006; Hibino et al., 2006; Levin et al., 2002b)
Small molecule signal	auxin 	serotonin 
Influx cell membrane transporter	AUX1 (Reinhardt et al., 2003; Yang et al., 2006)	SERT (Fukumoto et al., 2005a)
Soluble receptor	TIR1, ABP57 (Dharmasiri et al., 2005; Kim et al., 1998; Kim et al., 2001; Woodward and Bartel, 2005a; Woodward and Bartel, 2005b)	SBP (Del Rio, 1995; Fukumoto et al., 2005b; Jimenez Del Rio, 1993; Tamir et al., 1994)
Long-range gradient	10-fold (Edlund et al., 1995; Uggla et al., 1996)	5-fold (estimated) (Esser et al., 2006; Fukumoto et al., 2005b)
Electrophoretic mechanism proposed	(Bandurski et al., 1990; Goldsworthy and Rathore, 1985; Rathore and Goldsworthy, 1985; Schrank, 1951; Webster and Schrank, 1953)	(Esser et al., 2006; Fukumoto et al., 2005a; Fukumoto et al., 2005b)

Because rodent embryos in which molecular motors have been mutated are also likely to have impaired cytoplasmic function of motor transport (which may be very important for asymmetry (Qiu et al., 2005)), it has not been possible to cleanly separate the ciliary functions of the LR-relevant motors from their cytoplasmic transport roles in mammalian embryos. Most ciliary proteins have subcellular roles, such as motor protein transport, cell polarity determination, and transcription control (Wang et al., 2006); it is also possible that ciliary motion itself has physical (traction) effects on cytoplasmic components through the ciliary rootlet (Yang et al., 2002, 2005; Yang and Li, 2005). Ciliary protein deletion mutants tend to give laterality defects, but are also often implicate other roles, like the OFD1 knockout that also has altered Hoxa and Hoxd expression in the limb (Ferrante et al., 2006). Interestingly, OFD1 is a centrosomal/basal body protein, and is an ideal candidate for intracellular F-molecule orientation-based models. In contrast to extracellular ciliary movement (Romio et al., 2004). The resolution to this question will come through a more sophisticated mutant: if a mouse can be produced in which loss-of-function of LR dynein or other ciliary components can be restricted to the node or made to begin only at day 7, and if this mutant has a LR phenotype, it will be convincingly demonstrated that it is indeed the nodal ciliary roles of the relevant proteins that are important for mouse asymmetry. Regardless of the outcome in rodent embryos, human primary ciliary dyskinesia patients still have normal cerebral lateralization, demonstrating that cilia are not a unique symmetry-breaking event in humans (McManus et al., 2004).

The rodent embryo is somewhat unusual in its large scale and node architecture, compared to more typical mammals such as rabbit and human. Consistent with the possibility that the functional involvement of cilia in asymmetry is not specific to rodents, recent studies in Zebrafish demonstrated that knockdown of the *ntl* gene specifically in the dorsal forerunner cells (ciliated cells in Kupffer's vesicle) results in randomization of situs (Amack and Yost, 2004; Bisgrove et al., 2005; Essner et al., 2005; Kramer-Zucker et al., 2005). These data argue for a more widely-conserved role of ciliary components. Since an Na^+/K^+-ATPase mutation in fish resulted in laterality defects but normal cilia (Ellertsdottir et al., 2006), it is possible that the fish uses parallel pathways. Taken together, the data in vertebrates and invertebrates suggest the possibility that subcellular motor protein- and

cytoskeleton-driven localization LR pathways and ion flows were evolutionarily older and were the way that evolution first oriented asymmetry (now having been implicated in plants, snails, *C. elegans*, *Drosophila*, sea urchins, *ciona*, fish, frog, and chick). At some point, ciliary movement became involved in the middle of the pathway (perhaps to strengthen or transmit earlier asymmetries), and rodent embryos may indeed have dispensed with the earlier mechanisms once the ciliary components were fully in place. Consistent with this imaginary timeline (Table 1), organisms with fully-fledged LR asymmetry, such as sea urchins and *Drosophila*, do not seem to have anything resembling a node or an internal ciliated pocket (Amemiya, 1971, 1986, 1989; Martinez-Campos et al., 2004). The cilia is likely to have entered the evolutionary stream around the time of fish, since recent data in zebrafish implicate cilia (Amack and Yost, 2004; Bisgrove et al., 2005; Essner et al., 2005; Kramer-Zucker et al., 2005), though a direct extracellular viscosity test has not been done in this system.

The question of timing and possible ciliary roles in asymmetry has been addressed in another mammal. Rabbit embryos (like most mammals including humans) develop as a flat blastodisc similar to chick embryos, and their study has contributed significant advances which complement and contrast the mouse system (Viebahn et al., 1995; Viebahn, 2001; Fischer et al., 2002). In rabbit, *Cer1* expression is asymmetric at stage 3 (Idkowiak et al., 2004), prior to ciliary movement, although it is not clear whether this is consistently biased. Mice may not recapitulate all phenomena important for LR patterning (see discussion of midline determination below). This becomes of particular importance when considering the mechanistic implications of laterality phenotypes observed in human monozygotic twins.

How Do Physiological Events Control Gene Expression?

One of the challenges facing this field is to understand precisely how asymmetric gene expression is controlled by upstream physiological events. In *Xenopus*, the first known asymmetric gene is *Nodal*, which is expressed long after neurulation begins. Thus, a number of interesting mechanisms must yet remain to be identified functioning between early serotonin signaling and *Nodal* regulation. One possibility is syndecan signaling, which functions in frog embryos during the correct timeframe (Kramer et al., 2002; Kramer and Yost, 2002). Future experimental work must uncover the receptors for serotonin and other GJC-permeable LR morphogens, and perhaps array approaches can identify proximal immediate-early response genes to Ca^{++}, H^{+}, and K^{+} asymmetries. Screens for pH- and $[K^{+}]$-sensitive promoter elements may reveal genes sensitive to intracellular ionic conditions. Likewise, the identification and characterization of a morphogen that depends on ciliary movement remains a high priority.

The Minds of Plants: Parallels Between Serotonin and Auxin Asymmetry

The patterning of the LR axis has some fascinating similarities to mechanisms of other patterning systems. One of these concerns the parallels between serotonin and the plant hormone auxin (Table 2), in the determination of morphogenesis and LR asymmetry in plants, which is now being addressed molecularly (Endress, 1999, 2001; Theissen, 2000). Auxin, a plant hormone that bears a striking structural similarity to the neurotransmitter serotonin, is a positional signal in a number of plant patterning events (Sabatini et al., 1999; Vroemen et al., 1999; Baluska et al., 2003; Friml, 2003; Barlow, 2005) and is involved in establishment of bilateral symmetry (Lee and Evans, 1985; Liu et al., 1993; Zgurski et al., 2005) and even LR asymmetry (Pekker et al., 2005) in plant systems. Indeed, many of the same players that have been implicated in LR asymmetry (K^{+} channels, plasma membrane H^{+} flux, cell membrane transporters and gradients of serotonin/auxin, regulatory roles of pH, electrophoretic movement, etc.) are now known to be crucial components of auxin signaling (Arend et al., 2002; Coenen et al., 2002; Pasternak et al., 2002; Hager, 2003; Rober-Kleber et al., 2003; Wind et al., 2004).

In addition to the similarity of the molecular components involved, a functional loop mechanism appears to be conserved as well. The ability of 5HT to localize to a specific blastomere depends on gap junctional communication and H^{+}, K^{+}-ATPase function, which (in concert with a potassium channel) may provide an electromotive force for moving charged small molecules (such as serotonin) between cells (Levin, 2004). Conversely, a number of ion transporters are controlled by 5HT, most notably R3 and SERT (Maricq et al., 1991; Quick, 2003). As this field matures, it will be necessary to develop quantitative models of the bidirectional relationship between 5HT movement in an electric field and the regulation of ion transporters by 5HT. The activation of asymmetric ion flux by serotonin and the unidirectional movement of 5HT due to an electrophoretic force may thus be a positive-feedback loop that could magnify small asymmetries on the cellular level into asymmetry on the scale of cell

activation of asymmetric ion flux by serotonin and the unidirectional movement of 5HT due to an electrophoretic force may thus be a positive-feedback loop that could magnify small asymmetries on the cellular level into asymmetry on the scale of cell fields;

fields; such feedback loops are likely to be absolutely central to establishment of asymmetry.

The movement may be intracellular or intercellular in various patterning systems. Indeed, plant root tips set up a sink-driven gradient of auxin (Friml et al., 2002); the serotonin gradient in frog embryos is likewise dependent on the degradation machinery to function after the relocalization steps have taken place. A model has been proposed (Paponov et al., 2005) in which auxin flow, starting by diffusion, induces formation of the polar transport system. This in turn promotes auxin transport, leading to canalization of auxin flow along a narrow column of cells and ultimately controlling differentiation. A similar amplification loop between 5HT and K^+ flow through R3 is precisely the kind of mechanism that has been proposed to account for the function of 5HT movement during LR patterning (Fukumoto et al., 2005b). The involvement of electrophoretic mechanisms relevant to serotonin-like molecules in plant and animal patterning (Goldsworthy and Rathore, 1985; Fischer et al., 1997; Palmgren, 1998; Rober-Kleber et al., 2003) suggest that fascinating aspects of evolutionary conservation of such patterning mechanisms await investigation. Such wide conservation of mechanism between the common ancestor of animals and vascular plants requires that protists had access to serotonin-like molecules. Though we do not have access to the protist basal to both plants and animals, we know that protists such as plasmodium and tetrahymena have both serotonin and its receptors (Brizzi and Blum, 1970; Janakidevi et al., 1966; Pan et al., 1994).

The LR Axis Is Like a Kidney: Insights from Epithelia and Other Polarized Cells

The mechanisms characterized in the LR pathway revealed parallels to other systems in addition to the plant connection described above. Subcellular polarity and cytoskeleton are the common theme. For example, in *Caenorhabditis elegans*, a 14-3-3 protein (PAR-5) is required for cellular asymmetry in the early embryo (Morton et al., 2002). PAR-5 likewise functions in axial patterning in *Drosophila* (Benton et al., 2002). Interestingly, it was shown that the PAR homolog 14-3-3E protein is LR-asymmetrically localized during the first two cleavages in frog embryos and functions in the LR pathway upstream of *Nodal*; moreover, this signaling can be perturbed by fusicoccin-a fungal compound previously thought to only interact with plant cells (Bunney et al., 2003). The involvement of 14-3-3 proteins in cellular asymmetry in early cleavages of both *C. elegans* and *Xenopus* is further evidence of a deep and fundamental underlying conservation of mechanisms by which asymmetry and polarity, whether on the cellular level, or on the scale of the organism, is established. The finding that elements of fusicoccin/14-3-3 signaling are conserved from fungi-plant interaction to animal embryogenesis presents a new perspective from which to investigate novel aspects of large-scale morphogenetic control in vertebrates and highlights the possible connection between fundamental subcellular polarity machinery (cytoskeleton and motor proteins) and morphological asymmetry.

Another example of this polarity is seen in the development of the nervous system. Neuroblasts are able to polarize without external cues, but such cues are needed to orient their polarization to a consistent angle (Pearson and Doe, 2003; Rolls and Doe, 2003). The LR axis may also involve a similar distinction between generating asymmetry and establishing its orientation with respect to the other two axes. Ca^{++}-dependent polarization in pollen tubes illustrates another unique aspect of the LR axis: excess and ubiquitous Ca^{++} stops directional growth, even though increased Ca^{++} in the tip dictates the polarity of extension (Rathore et al., 1991; Messerli and Robinson, 1997, 2003; Messerli et al., 1999, 2000; Robinson and Messerli, 2002). Similarly, in the LR field, the global up- or downregulation of almost every component (serotonin, ion flow, GJC, and *Shh* in vertebrates, myosin in *Drosophila*) both result in randomization! This spatial distribution is paramount for these physiological mechanisms and is not like many pathways, in which loss-of-function and gain-of-function have opposite effects. The necessity for high GJC/5HT/membrane voltage in one area and simultaneously low GJC/5HT/membrane voltage elsewhere, precludes simple "one knockout-one phenotype" models that are common in other aspects of morphogenesis. On the other hand, recent efforts to mathematically model morphogen gradients in *Drosophila* have also revealed that such robust patterning mechanisms must keep the system between uniformly low or uniformly high morphogen levels across the embryo (Gregor et al., 2005; Goentoro et al., 2006)-as in the case of LR asymmetry, driving the system in either direction (through a very high or very low Thiele modulus) destabilizes the axial-patterning event.

Another interesting parallel of the LR axis exists in the way neurons regulate their membrane voltage. Normal neurons build up a voltage potential using the Na^+/K^+-ATPase in combination with a K^+ channel to provide the negative membrane voltage as the excess K^+ escapes (Hille, 2001). The early *Xenopus* embryo utilizes exactly the same system, involving an H^+/K^+-ATPase exchanger in combination with a K^+ channel to set up its LR voltage differences (Levin et al., 2002; Levin, 2003b; Chen and Levin, 2004), although there is an additional hyperpolarizing pump involved in *Xenopus*-the V-ATPase (Adams et al., 2006). Gastric cells appear to do the same (Fujita et al., 2002), using the H^+/K^+-ATPase pump and the same KCNQ1 channel that is implicated in frog asymmetry.

In addition to the control of ion current paths by subcellular localization, the reverse relationship exists as well: ion transport is important for polarity on the level of single cells (Patel and Barber, 2005). This is yet another feedback loop that must be incorporated into the complex models of early embryonic physiology; discoveries from the kidney and gut epithelial fields

will continue to provide insights for the LR field that will contribute to the development of quantitative models encompassing all of the relevant physiological and genetic events involved (Martin and Harvey, 1994; Pribyl et al., 2003; Fischbarg and Diecke, 2005). The future development of such models and the resulting understanding of robustness, dynamic cycles, and feedback are essential to perfect functional techniques for manipulating these pathways rationally in biomedical settings.

However, perhaps the most important analogy is between kidney function and the LR axis. A number of mouse knockout studies have identified targets whose abrogation causes both a kidney and a LR defect (Mochizuki et al., 1998, 2002; Murcia et al., 2000; Haycraft et al., 2001; Otto et al., 2003; Kramer-Zucker et al., 2005). This is most often interpreted with respect to a sensory cilium being necessary for kidney function and asymmetry (Pazour and Witman, 2003; Pazour, 2004; Barr, 2005; Pan et al., 2005). In contrast, I propose that the salient commonality between the kidney and the LR axis is their tight linkage of cell polarization to ion transport localization. Kidney cells (and epithelial cells in general) are highly polarized, and exert strong control over ion fluxes by using cytoskeletal and motor protein transport elements to regulate the intracellular membrane transport of ion channels and pumps, specifically including the H^+ and K^+ transporters known to be important in asymmetry (Nelson et al., 1991; Brown et al., 1992; Nelson, 1992; Suzuki et al., 1995; Al-Awqati et al., 1999; Fujita et al., 2002; Yao et al., 2002; Beyenbach, 2001). Other components implicated in LR asymmetry, such as claudins are also involved in kidney function (Yu et al., 2003; Abuazza et al., 2006; Balkovetz, 2006; Gonzalez-Mariscal et al., 2006; Lee et al., 2006), as they shape the transepithelial voltage gradients resulting from the targeted ion flows. Thus, it may be that the reason kidney defects and asymmetry randomization often appear together is that mutations in components of subcellular targeting (motor proteins, cytoskeletal components, etc.) result in abnormal function in both kidney epithelia and early asymmetry-breaking events.

the salient commonality between the kidney and the LR axis is their tight linkage of cell polarization to ion transport localization.

The intestinal epithelium, in a very wide range of organisms, is another highly-polarized tissue that expresses and utilizes key ion transporters implicated in LR asymmetry (Morley et al., 1992; Kraut et al., 1995; Numata et al., 1995; Cheng et al., 1996; Huang et al., 2006; Jespersen et al., 2005; Jons et al., 2006).

I argue that the origin of asymmetry takes place intracellularly, is conserved from invertebrates (is related to invertebrate chirality), and is intimately connected to motor proteins and the cytoskeletal tracks that guide their localization. This is clear in snails (Freeman and Lundelius, 1982; Shibazaki et al., 2004) and plants (Thitamadee et al., 2002), and the data discussed above implicate it in vertebrates as well (Qiu et al., 2005). Alongside the kinesins and dyneins that have been implicated in rodent asymmetry (Supp et al., 1997; Marszalek et al., 1999; Takeda et al., 1999), recent data in *Drosophila* have also implicated the myosin motor (Hozumi et al., 2006; Speder et al., 2006). Surprisingly, loss of the symmetrically-expressed myosin I results in mirror-image flies and not randomization, like the *inversin* deletion in mice (Morgan et al., 1998). As in vertebrates, in which LR components are present in kidney and digestive system epithelia, oriented actin filaments and myosin I are observed in the polarized cells of the fly gut (Hozumi et al., 2006; Speder et al., 2006). Moreover, β-catenin binds both inversin (Nurnberger et al., 2002; Eley et al., 2004; Simons and Walz, 2006) and the fly myosin I (Speder et al., 2006), suggesting deep conservation of the symmetry biasing mechanism and its linkage to pathways that pattern polarity along the AP and DV axes. Cytoskeletal elements direct the large-scale polarity of other axes from the DV axis in *Xenopus* (Elinson and Rowning, 1988; Gerhart et al., 1989) to the AP axis in planaria (Nentwig, 1978).

Brain Asymmetry

While nervous system lateralization is spread throughout phylogeny (Andrew, 2000) because of a number of ecological advantages (Rogers et al., 2004; Vallortigara and Rogers, 2005), there exist a number of fascinating human behavioral and brain asymmetry phenomena that have bearing on the question of exactly when different aspects of the LR axis become fixed during embryogenesis. While some animals (e.g., mice) often show paw preference, the consistent preference among all individuals only approaches high levels in man (approximately 90% for right-handedness), and its genetic basis is still controversial (McManus and Bryden, 1992; McManus, 1995).

Interestingly, brain asymmetry (Toga and Thompson, 2003) does not correlate with visceral asymmetry (Kennedy et al., 1999; Tanaka et al., 1999). For example, situs inversus totalis individuals still have language lateralization seen in 95% of right-handed normal situs individuals (Kennedy et al., 1999). The incidence of left-handedness is exactly the same in situs inversus individuals as in the rest of the population (Cockayne, 1938; Torgersen, 1950). This suggests that mechanisms establishing the laterality of the brain are, at some early point in the LR pathway, different from those that determine the sidedness of visceral organs. Moreover, 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 (McManus et al., 2004), suggesting that at least some aspects of laterality in humans are indeed upstream of, or exist in parallel to, mutations affecting ciliary function. Bizarrely, the asymmetry of wristwatch-wearing behavior does indeed correlate with situs inversus (McManus et al., 2004). Recent work in zebrafish (Concha et al., 2003; Gamse et al., 2003; Halpern et al., 2003; Barth et al., 2005; Lin and Burdine, 2005; McManus, 2005) has shed light on the regulation of brain asymmetry by known pathways, but it is unclear whether the fish or another available model system will provide a tractable entry point to the study of the fascinating brain asymmetry in man as distinct from the cardiac-visceral pathways.

Two Small Black Clouds in the Human Data: Cryptic Asymmetry, Subtle Chirality, and Sex

Analogous to the seemingly small, incongruous observations in thermodynamics that turned out to revolutionize the tidy picture of classical physics at the beginning of the last century, a number of clinical and experimental data have been obtained that do not fit neatly into the existing paradigms and have the potential to reveal untapped areas of the asymmetry field. These relate to subtle and cryptic asymmetry, in contrast to the more obvious morphological and behavioral differences.

Nonconjoined monozygotic twins, not only exhibiting a higher-than-normal incidence in laterality defects (Kuehl and Loffredo, 2002), also manifest many subtler kinds of mirror-image asymmetry ("bookend" or enantiomer twin pairs). Pairs of such twins have been noted to present mirror asymmetries in hand preference, hair-whorl direction, tooth patterns, unilateral eye and ear defects, cleft lip, cleft palate, supernumerary teeth, and even tumor locations and undescended testicles (Lauterbach, 1925; Rife, 1933, 1940, 1980; Newman et al., 1937; Potter and Nance, 1976; Gedda et al., 1981; Mensing, 1983; Yager, 1984; Schneider, 1985; West, 1985; Carton and Rees, 1987; Beere et al., 1990; Townsend and Richards, 1990; Morison et al., 1994; Sperber et al., 1994; Satoh et al., 1995; Cidis et al., 1997; Sommer et al., 1999, 2002; Okamoto et al., 2001; Morini et al., 2002). The mirror/bookending phenomenon is not just structural but also pertains to functional parameters such as sleep deviations, hearing, and cerebral functional localization (Springer and Searleman, 1978a, 1978b; Golbin et al., 1993; Sommer et al., 1999, 2002). Though monozygotic twins affected by genetic lesions often show opposite sidedness of limb abnormalities (Richieri-Costa and Opitz, 1986; Opitz and Utkus, 2001), almost all bookending phenomena in healthy twins involve features of the head. Hair whorls originate from the same tissue layer as the nervous system, and are linked to handedness and language dominance (Klar, 2003; Weber et al., 2006), although the proximate mechanisms determining hair-whorl sidedness is not well understood. Thus, consistently with the discordance between brain and body situs discussed above, there may be two separate organizers for the head and body (Meinhardt, 2002), which use different mechanisms of determining laterality (Harland and Gerhart, 1997).

The bookending phenomenon may also speak to the timing of the earliest steps of asymmetry in mammals. Most healthy, nonconjoined twins presumably result from separation of cleavage, morula, or early blastocyst stage embryos (James, 1983). Thus, some chiral information may be present in the very early mammalian embryo, later manifesting as hair whorls, etc., if the cells are separated at an early stage. In contrast, the asymmetry of the major body organs seems to be unspecified (or plastic enough to be respecified) at those stages, and is developed correctly for both monozygotic twins. This may be related to the fact that heterotaxic reversals in hair whorls and tooth patterns would not be expected to be disadvantageous, while discordant situs for internal organs clearly is subject to negative evolutionary pressure. An alternative model is that some as yet unknown pathological mechanism is responsible for both the process of twinning itself and the destabilization of the LR axis (Boklage, 1981, 1987). In support of this view, it has been found that increased incidence of left-handedness in twins is not dependent on zygosity or time of splitting (McManus and Bryden, 1992; Derom et al., 1996). The molecular basis of these phenomena is not understood, although analysis of laterality in twins produced by splitting of embryos during in vitro fertilization procedures may eventually provide important clues. Interestingly, monozygotic twins are often discordant for the imprinting of KCNQ1 (Weksberg et al., 2002), a potassium channel which has been implicated in LR asymmetry in frog embryos (Levin, 2003b).

Increased understanding of epigenetic factors in laterality will come from analysis of monozygotic twins (Steinmetz et al., 1995), but may also be gained by examination of the experimentally-tractable artificial twins of bovine embryos that later exhibit hair whorls (Lanier et al., 2001; Meola et al., 2004; Evans et al., 2005). Why might early splitting have consequences for embryonic chirality? Mirror image cytoskeleton patterns and cell migration tracks have been observed following normal cell division in culture (Albrecht-Buehler, 1977a, 1977b, 1977c, 1978), once again underscoring the importance of cytoskeleton and subcellular structures for large-scale asymmetry and cellular behavior.

Another fascinating phenomenon that has only begun to be explored is that of cryptic asymmetry. This refers to consistent asymmetries that exist in morphologically symmetrical structures and are sometimes only revealed following experimental perturbations (Cohen, 2001). One example is the consistently asymmetric defects in planarian eye regeneration only observed following H^+/K^+-ATPase inhibition (Nogi et al., 2005) and unilateral limb defects produced in rodents by some compounds (Barr,

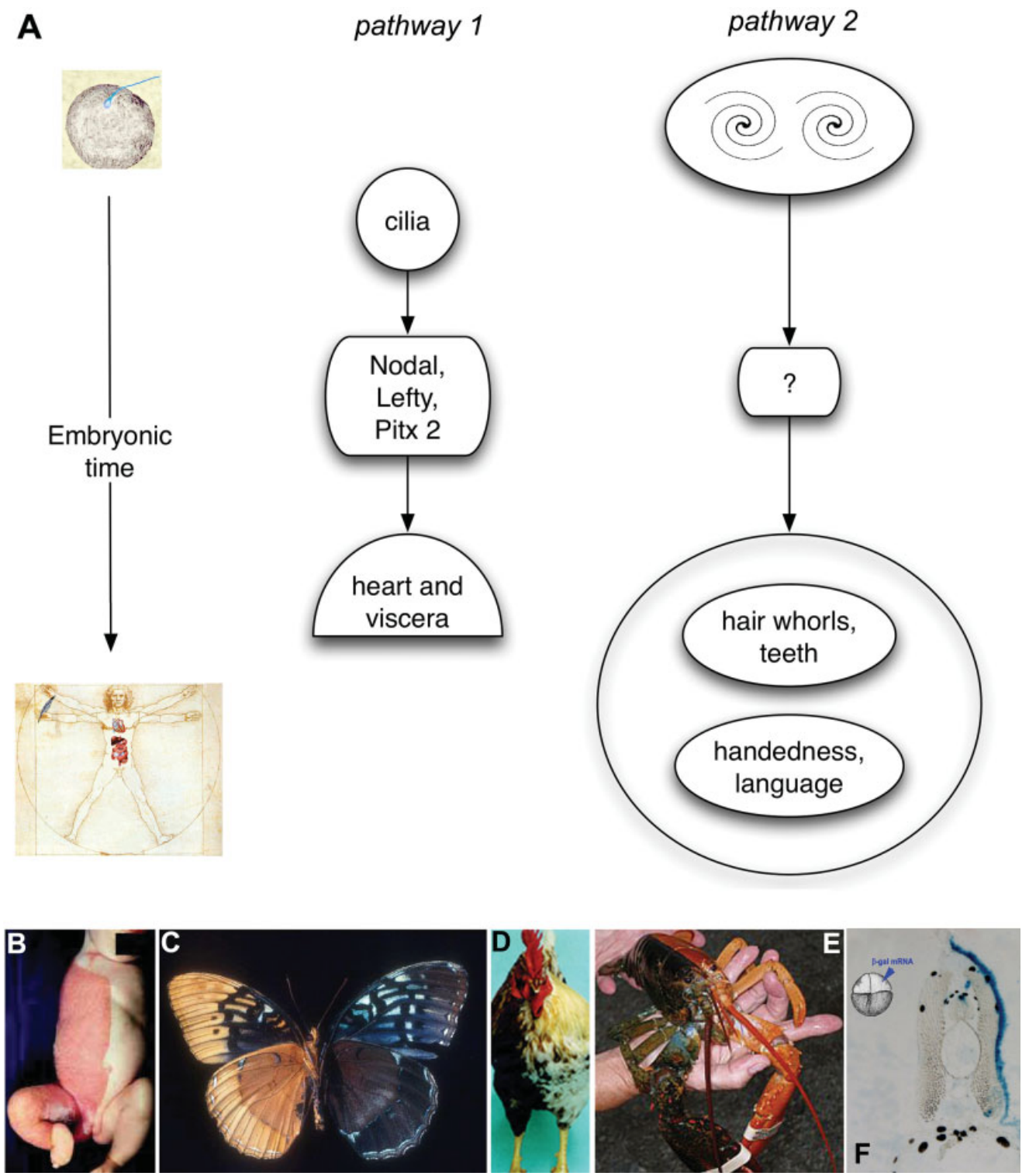


Figure 4. Two systems of asymmetry in human embryos and midline determination. **A:** The biomedical data suggest that human embryos have two different pathways of asymmetry, which may or may not both be downstream of a common originating mechanism. The first pathway could be cilia-related, and feeds into the known asymmetric gene cascade upstream of the situs of the heart and viscera. The other pathway is likely to be active at early cleavage stages, controls unknown downstream mechanisms, and is ultimately revealed by ectodermal structures: cryptic asymmetry in hair whorls and tooth patterns, and behavioral/anatomical asymmetries in the brain. Midline determination: asymmetric cutaneous pigmentation pattern with a sharp midline demarcation in the X-linked CHILD syndrome (**B**) suggests early establishment of midline in human embryos. Similar patterns are observed in gynandromorphs in butterflies (**C**), chickens (**D**), and lobsters (**E**). Alignment of early cleavages with the midline of the embryo is revealed in model species such as *Xenopus* when a marker mRNA such as β-galactosidase is injected into a blastomere at first or second cleavage and results in unilateral distribution one week later (**F**).

1973; Layton and Layton, 1979; Milaire, 1985). Other examples include the consistently chiral rotation observed in *Xenopus* following pharmacological disruption of the cytoskeleton (Danilchik et al., 2006) and asymmetries in craniofacial structures in zebrafish mutants (Albertson and Yelick, 2005). All of these cases demonstrate asymmetries that are not observed in wild-type individuals but are exposed by experimental manipulation. Other related data in vertebrates has revealed asymmetries in normal somites (Saude et al., 2005) and limbs (Sienknecht, 2006), structures thought to be morphologically symmetrical.

Such observations hint that subtle molecular differences may exist between organs that are assumed to be symmetrical (such as left and right limbs, eyes, etc.), establishing a true LR axis rather than a mediolateral one (although the data do not require graded positional information along this LR axis, but merely L vs. R identity). This counterintuitive and as yet unexplored idea is also suggested by several human syndromes. Holt-Oram syndrome (Tbx5-related) presents upper limb malformations which are much more common on the left-side (Smith et al., 1979; Newbury-Ecob et al., 1996; Bruneau et al., 1999; Hatcher et al., 2000), while fibular a/hypoplasia affects the right side more often (Lewin and Opitz, 1986). Indeed, a variety of human syndromes affecting paired organs have a significant bias for one side (Paulozzi and Lary, 1999). Moreover, genetic conditions (such as hemihypertrophy syndrome) exist in which a large number of tissues, all on one side of the body, resume growth in adulthood (Fraumeni et al., 1967; Clericuzio, 1993; Sarkar et al., 1992; Stalens et al., 1993; Kloeppel et al., 2002; Leung et al., 2002), suggesting that a wide range of tissues within morphologically symmetrical organs may not only possess positional information with respect to the LR axis but may maintain this identity for decades after completion of embryogenesis. Molecular insight into this phenomenon is likely to open important advances in developmental patterning; taken together with the bookending phenomena observed in monozygotic twins, these data suggest the presence of two distinct LR pathways in human embryos.

subtle molecular differences may exist between organs that are assumed to be symmetrical

Most of the work in the LR field has naturally addressed the mechanisms controlling the situs of morphologically asymmetric organs. However, the human and rodent data discussed above (unilateral drug effects and global hemihypertrophy syndromes) indicate that seemingly identical paired structures may in fact harbor subtle molecular differences conferring positional information along the LR axis, and that this information may persist well into adulthood. Recent studies have indicated that rodent embryo somites exhibit a striking asymmetric expression of genes such as HB-EGF and MLC3F (Golding et al., 2004a, 2004b); differential gene expression in precursors of paired organs and skeletal elements could potentially provide a heretofore unsuspected mechanism for assigning L and R identity to seemingly identical structures. Human fetuses at 10 weeks of gestation are 85% right-handed (very close to the final ~90% figure); this is not likely to be under brain control but is probably of muscular or spinal determination, suggesting asymmetries in those tissues (Hepper et al., 1998). Future work must characterize novel molecular differences between paired structures and address the functional significance of this asymmetric gene expression. Identification of LR signals in locales other than overtly asymmetric organs, and an understanding of the temporal extent of LR information after completion of embryogenesis, is sure to have important implications for biomedicine and basic developmental biology.

The human data on asymmetry suggests that there are two different pathways operating (Fig. 4A). The primary control of visceral and cardiac asymmetry takes place through activity of the well-understood asymmetric cascade genes (Casey, 1998; Kosaki et al., 1999; Bamford et al., 2000) and can be associated with ciliary dysfunction (Afzelius, 1976, 1999). Interestingly, there appears to be another, largely mysterious, pathway revealed by clinical data (Cohen, 2001). As discussed above, chirality of hair whorls, hand use preference, and brain hemisphere language localization appear to be controlled by a separate pathway not downstream of the mechanisms disrupted in situs inversus and heterotaxia patients. The conservation of asymmetry of unilateral defects in monozygotic twins suggests that mirror-image LR information was already present at the time of splitting: while we do not know the intermediate steps mediating control of the final phenotype, the original symmetry-breaking event must take place quite early (and certainly prior to the late gastrulation stage at which cilia act to transmit or initiate the asymmetry cascade). An important open question is whether these two pathways are completely parallel or simply diverge early from a common starting point.

To define true LR asymmetry, the body-plan first must establish a midplane of symmetry. While mice seem to have indeterminate early development, or at least can respecify the LR axis when disturbed (Brown et al., 1990; Brown and Wolpert, 1990), striking unilateral pigmentation patterns (Fig. 4B) can occur in human patients with X-linked diseases such as CHILD syndrome (Happle et al., 1995; Happle, 2002). The required LR-asymmetric X-inactivation in such patients suggests that the midline decision is made quite early in human embryos. Consistent with the previous proposal that rodent embryos are derived and atypical with respect to LR mechanisms, the mouse models of CHILD syndrome recapitulate all

of the important features except unilateral pigmentation (Konig et al., 2000).

In orthoptera, unilateral gynandromorphs are thought to result when one of the X chromosomes in an XX zygote is eliminated at the first cleavage division (Barranco et al., 1995). While the rodents do not appear to set midline early enough for X-linked mechanisms to produce large-scale asymmetries, the human cases revealed by genetic pigmentation syndromes resemble examples of gynandromorphs found throughout phyla (Fig. 4C-E), including butterflies, ants, crabs, and chickens, can present midline asymmetries of sex and pigmentation (Farmer, 1972; Dang and Peterson, 1979; Sivaradjam and Bierne, 1981; Homsher and Yunker, 1981; Mey, 1982; Taber and Francke, 1986; Taylor, 1986; Micheli, 1991; Stevens and Munk, 1991; Barranco et al., 1995; Moriyasu et al., 1998; Zou and Fingerman, 2000; Sagi et al., 1996, 2002). All of these argue for a conserved early demarcation of the midline, and indeed in the case of crustaceans, it is actually known that this takes place, as in *Xenopus*, at early cleavage stages (Extavour, 2005). The human clinical data also suggest a striking conservation of mechanisms establishing midline, at least as concerns ectodermal derivatives.

Even if the midline is established early, as is suggested by the gynandromorph cases, is asymmetry or its biasing linked to this process? It is likely; in human hermaphrodites, ovaries tend to develop on the left, while testes appear on the right (Mittwoch, 2000). This time, rodents are not the odd man out, although while mice do exhibit the strong linkage between sidedness of hermaphroditic organs observed in human cases (Eicher and Washburn, 1983; Ward et al., 1987; Biddle et al., 1994), the consistent laterality of placement of testes versus ovaries is opposite that observed in humans (van Niekerk and Retief, 1981; Krob et al., 1994). Sex determination provides a largely-untapped entry-point into understanding and gauging the conservation of mechanisms establishing midline and its coordination with the LR and other axes.

Could One Properly Shake Hands with An Alien?

Would the chirality of organisms with a fundamentally different biochemistry be the same as is observed on Earth? The issue of original chirality (i.e., why living organisms contain only L-amino acids and D-sugars) is bound up fundamentally with the origin of life. In vitro synthesis almost invariably results in equal mixtures of enantiomer pairs of compounds, while biosynthetic processes were able to clearly separate such racemic mixtures (Pasteur, 1860). Several theories for this have been proposed. Perhaps, whatever type of isomer happened to have formed first biased the rest of evolution toward that type by competition (Frank, 1953). The chirality of the first one could have been determined by chance, or by exogenous factors such as light polarization (Noyes and Bonner, 1975) or even the geomagnetic field (GMF). Interestingly, the GMF seems to have a relationship with LR chirality (Anderson, 1988): the geological fossil record shows a correlation between flipping of the GMF polarity and reversals of the chirality of several types of mollusks such as *Globorotalia menardi* (Harrison and Funnel, 1964; Dubrov, 1978). Thus, the determination of chirality may be one of the several roles the GMF probably plays in embryogenesis (Cole and Graf, 1974; Shibib et al., 1987; Asashima et al., 1991; Leal et al., 1992; Sandoze et al., 1995).

Alternatively, there may be a fundamental reason for why biological forms prefer one type of molecule over its enantiomer. For example, when racemic mixtures of the amino acids alanine, tryptophan, and tyrosine in alkaline solution are subjected to decomposition by radioactive decay of strontium-90, the D-isomers are destroyed more quickly than the L-isomer (Garay, 1968). There are also arguments based on weak neutral currents which show that L-amino acids will predominate in biochemical reactions in a period of on the order of 15,000 years (Mason and Tranter, 1984; Kondepudi, 1987). Thus, radioactive decay could plausibly have biased enantiomer choice in the prebiotic environment. Likewise, the energy of the right-handed α-helix of poly-L-alanine is a few tenths of a kilocalories per mole per residue lower than that of the left-handed helix, implying that over some length, right-handed forms will be more stable (Morgan, 1977). Both asymmetries are presumably consequences of the non-conservation of parity in subatomic weak nuclear interactions (Wu et al., 1957). Testing such models represents a formidable challenge, but the identification and evolutionary characterization of the biochemical mechanism that initiates asymmetry will ultimately reveal whether a direct link exists between the symmetry breaking events at the origin of the universe and the handedness of biological forms on Earth today.

CONCLUSION

The consistent macroscopic chirality of embryonic structures is a crucial part of developmental biology. This subject touches on issues ranging from evolutionary mechanisms of body-plan dynamics to the subtleties of parity conservation in quantum mechanics. While a number of important advances have been made in several model systems, the most interesting questions remain open, and the field is in need of resolution with respect to conceptual unity (or proving the impossibility thereof). I argue that the LR axis is patterned by mechanisms that are fundamentally conserved throughout phyla and used in establishing cell polarity in a number of tissues. It is likely that future experiments addressing the subcellular roles of motor proteins, small molecule movement (through gap junctions and driven by cilia in the extracellular space), and the mechanisms that generate organ shape from laterality signals, will open up new areas of cell, developmental, and evolutionary biology. Indeed, the increasing efforts to mathematically model and synthesize data from genetics, physiology, and biophysics to truly understand the amplification and feedback loops will give rise to a satisfying understanding of laterality in the animal kingdom.

ACKNOWLEDGMENTS

Many thanks to the members of the Levin laboratory and the LR asymmetry, physiology, and neuroscience communities for useful discussions, and to Dany S. Adams for her detailed comments on a draft of this review. Permissions and attributions for Fig. 4: The image of a *Papilio glaucas* in panel C was obtained with permission from James Adams. The image of the bilateral gynandromorphy chicken in panel D is used by permission of Michael Clinton. The image of a patient with CHILD syndrome and harlequin pigmentation (panel B) is taken with permission of John Wiley and Sons from the American Journal of Medical Genetics, 2000, 90, p. 340. The image of the bilateral gynandromorphy lobster (panel E) was used by permission of the Bangor Daily News, photo by Abigail Curtis.

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