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Review

Left–right asymmetry in embryonic development: a comprehensive review

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Abstract

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 recently begun to be understood at the molecular level. The mechanisms which ensure invariant LR asymmetry of the heart, viscera, and brain represent a thread connecting biomolecular chirality to human cognition, along the way involving fundamental aspects of cell biology, biophysics, and evolutionary biology. An understanding of LR asymmetry is important not only for basic science, but also for the biomedicine of a wide range of birth defects and human genetic syndromes. This review summarizes the current knowledge regarding LR patterning in a number of vertebrate and invertebrate species, discusses several poorly understood but important phenomena, and highlights some important open questions about the evolutionary origin and conservation of mechanisms underlying embryonic asymmetry.

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1. Introduction

The geometrical invariance known as symmetry is a prominent aspect of developmental morphology during embryogenesis. Animal body-plans occur in a wide variety of symmetries: spherical (e.g. volvox), radial (e.g. sea anemone), chiral (e.g. snails, ciliates), bilateral (e.g. planaria) and pseudo-bilateral (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). Symmetries are repeatedly broken during development. For example, the radial symmetry of the early chick blastoderm is broken into a bilateral symmetry by the appearance of Köhler's sickle and then the primitive streak. This is further broken into a definitive pseudo-symmetry by

the right-sided looping of the heart tube. A fascinating atlas of morphological asymmetries throughout the animal kingdom is given in Neville (1976).

Developmental noise often results in pseudo-random characteristics and minor stochastic deviations known as fluctuating asymmetry; however, the most interesting phenomenon is invariant (i.e. consistently biased among all normal individuals of a given type) differences between the left and right sides. For reasons of space as well as because these are likely to be secondary to embryonic asymmetries, this review largely neglects behavioral/sensory asymmetries (such as lobster claw morphology which is determined by neurological activity). A huge literature on brain lateralization phenomena in human beings exists as well (Harnad, 1977), but many of these asymmetries are secondary and arise as a result of cultural environmental biasing factors.

The establishment of left–right (LR) asymmetry raises a number of fascinating 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

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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 phenotypically impaired)? 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)? At what developmental stages is asymmetry initiated in vertebrate embryos? How conserved are the molecular mechanisms establishing correct asymmetry in animals with drastically different modes of gastrulation? And, how can the LR axis be consistently oriented with respect to the anterior–posterior (AP) and dorso-ventral (DV) axes in the absence of any macroscopic feature of chemistry or physics which distinguishes left from right? Answers to these questions require a detailed understanding, at the molecular, genetic, and biochemical levels, of the formation of biased asymmetry in embryos.

The LR axis itself follows automatically from the definition of the AP and DV axes, as it is perpendicular to both; however, consistently imposed asymmetry across it is fundamentally different from patterning along the other two axes. Firstly, while the AP and DV axes can be set by exogenous cues such as gravity, or sperm entry point, there is no independent way to pick out the left (or right) direction, since no known macroscopic aspect of nature differentiates left from right. One possible way to use a fundamental force to orient the LR axis relative to the other two axes was suggested by Huxley and deBeer (1963). They proposed that LR asymmetry was oriented during embryonic development by an electric current running down the length of

the notochord, which would generate a magnetic field vector pointing R or L, if measured at the dorsal or ventral sides. Although a correlation between the Earth's geomagnetic field reversals and shell chirality has been observed (Harrison and Funnel, 1964), the nature of a causal relationship (if any) is unknown, and there is no evidence to date of a magnetic field being utilized during LR patterning in any species.

While in most species all normal individuals are asymmetrical in the same direction, animals with complete mirror reversal of internal organs can arise (*situs inversus totalis*) and are otherwise phenotypically unimpaired. Thus, while it is possible to devise plausible evolutionary reasons for why organisms might be asymmetric in the first place (optimal 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 racemic population), than for biasing the LR axis to a given direction. It is possible that tight concordance (which is advantageous) requires specific bias, but no data yet indicate why these two logically distinct processes must be linked.

2. Pre-molecular data

Prior to the first molecular studies of the LR axis, glimpses into mechanisms of asymmetry were provided by a variety of drugs which cause defects in a LR-asymmetric manner or randomize asymmetry (Table 1). These form

Table 1
Drugs which have LR-relevant phenotypes

Drug	Function	Species	Result	Reference
A23187	Ca ⁺⁺ ionophore	Xenopus	Heterotaxia	Toyoizumi et al. (1997)
Cadmium	Heavy metal	Xenopus	Gut malrotations	Sunderman et al. (1991, 1992)
Cadmium	Heavy metal	Rat	Left limb deformities	Barr (1973)
Cadmium	Heavy metal	Mouse	Right limb deformities	Layton and Layton (1979)
Acetazolamide	Carbonic anhydrase inhibitor	Rat	Right limb deformities	Layton and Hallesy (1965)
MNNG	Alkylating agent	Mouse	Left ectodactyly	Inouye and Murakami (1978)
Acetoxymethyl-methylnitrosamine	Alkylating agent	Mouse	Left limb deformities	Bochert et al. (1985)
Xyloside	Proteoglycan synthesis inhibitor	Frog	No cardiac looping	Yost (1990b)
Nitrous oxide	Anesthetic	Rat	Heterotaxia	Fujinaga et al. (1990)
Retinoic acid	Teratogen	Hamster	Heterotaxia	Shenefelt (1972)
Phenylephrine	Adrenergic agonist	Rat	Heterotaxia	Fujinaga and Baden (1991)
Methoxamine	Adrenergic agonist	Rat	Heterotaxia	McCarthy (1990)
Staurosporine	PKC inhibitor	Rat	Heterotaxia	Fujinaga and Baden (1993a)
Lidocaine	Anesthetic	Rat	Heterotaxia	Fujinaga and Baden (1993b)
Nitrofurazone	Antibiotic	Rat	Right-sided hypoplasia	Greenaway et al. (1986)
RGD polypeptides	Block ECM interactions	Frog	Heterotaxia	Yost (1992)

a diverse group of compounds, which includes even such simple substances as cadmium. The developmental pharmacology of these drugs has not yet been explored to characterize endogenous targets involved in LR patterning, with the important exceptions that an adrenergic pathway is known to be involved (Fujinaga and Baden, 1991), and that PKC is known to participate in asymmetry in *Xenopus* (Kramer et al., 2002).

Of particular interest are the effects that present in a unilateral manner; no tractable genetic models of this phenomenon have yet been described. The drugs which cause more extensive limb defects on one side were suggested (Milaire, 1985) to be due to a differential blood supply to the two limbs (due to asymmetry in blood vessels exiting the heart). This is made somewhat unlikely by the fact that cadmium causes opposite-sided defects in rats and mice (Barr, 1973; Layton and Layton, 1979), while cardiac anatomy and relative vessel size of both species are quite similar. Such observations hint that subtle molecular differences may exist between organs which are assumed to be symmetrical (such as left and right limbs, eyes, etc.), establishing a true LR axis rather than a medio-lateral one (although the data do not require graded positional information along this LR axis, but merely L vs. R identity). This counter-intuitive 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 (Bruneau et al., 1999; Hatcher et al., 2000; Newbury-Ecob et al., 1996; Smith et al., 1979), 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 exist in which a large number of tissues, all on one side of the body, resume growth in adulthood (Clericuzio, 1993; Fraumeni et al., 1967; Kloepffel et al., 2002; Leung et al., 2002; Sarkar et al., 1992; Stalens et al., 1993), 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.

3. Left–right asymmetry meets molecular biology

While mechanisms underlying antero-posterior and DV asymmetry have been studied in detail with the advent of molecular genetics, the mechanistic basis for LR asymmetry was obscure until the last 10–15 years (Burdine and Schier, 2000; Levin and Mercola, 1998a; Mercola, 2003; Mercola and Levin, 2001; Yost, 2001). Tables 2–4 summarize the endogenous developmental molecules players in the LR

pathway, and show available conservation data among various model systems. These gene products have been identified in forward- and reverse-genetics approaches (exemplified by the zebrafish mutants and genes like *Sonic hedgehog*, respectively), and almost all have roles in embryonic processes other than LR asymmetry. Some are asymmetrically expressed at the level of mRNA or protein, while others exhibit no asymmetry. A number of mechanisms have been identified with molecular markers but the details of their function remain poorly understood. One example is the necessity for apoptosis in the midline of the chick embryo for normal LR asymmetry (Kelly et al., 2002); FGF8 and Brachyury are markers for midline cells fated to die, but it is not yet known in detail what endogenous mechanisms underlie the specification of the apoptotic cells, nor how their death provides instructive signals to downstream LR pathways.

Conceptually, LR patterning is divided into three phases (Levin and Mercola, 1998a); the flow of information is schematized in Fig. 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 (Horne-Badovinac et al., 2003; Manasek, 1981; Stalsberg, 1969a,b). Consistent with their downstream position, and counter to earlier proposals (Waddington, 1937), a number of recent studies have shown that the individual organs' literalities are set, and can be experimentally randomized, independently (Chin et al., 2000a; Levin et al., 1997b). 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) in the chick heart tube, and differential rates of elongation in the frog gut tube (Muller et al., 2003). Genetic control over these pathways is mediated proximately (if not directly) by genes such as *flectin*, the bHLH family members *EHAND* and *DHAND*, and the transcription factor *Tbx5* (Angelo et al., 2000; Bruneau et al., 1999; Fernandez-Teran et al., 2000; Hatcher et al., 2000; Sparrow et al., 1998; Srivastava, 1995; Takeuchi et al., 2003; Tsuda et al., 1996). The mechanisms underlying embryonic turning remain poorly understood (Constam and Robertson, 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.

Upstream of these processes lies a pathway of asymmetric genes which 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 sub-populations of cells (such as node and lateral plate mesoderm), which eventually dictate sidedness for the organs undergoing asymmetric morphogenesis. These cascades of asymmetric gene expression form the middle phase of LR

Table 2

Asymmetrically expressed genes in embryos which have been the focus of a paper on LR asymmetry

Gene	Species	Product/role	Side	Reference
Lefty	Mouse, chick, frog	TGF- β -family signaling molecule	Left	Branford et al. (2000), Cheng et al. (2000b), Essner et al. (2000), Meno et al. (1996, 1998)
Lrd	Mouse	Dynein	Either	Supp et al. (1997, 1999)
Activin- β B	Chick	TGF- β -family signaling molecule	Right	Levin et al. (1997a)
cAct-RIIa	Chick	Activin receptor	Right	Levin et al. (1995)
Shh	Chick	Signaling molecule	Left	Levin (1995)
CSnR	Chick	Zinc finger protein	Right	Isaac et al. (1997)
Nodal	Chick, mouse, frog	TGF- β -family signaling molecule	Left	Collignon et al. (1996), Levin et al. (1995), Lohr et al. (1997a), Lowe et al. (1996a), Morokuma et al. (2002)
CPTC	Chick	Shh Receptor	Left	Levin (1998b), Pagan-Westphal and Tabin (1998)
Cerberus	Chick	Signaling molecule	Left	Zhu et al. (1999)
BMP-4	Zebrafish, chick	BMP family signaling molecule	Left	Chen et al. (1997), Monsoro-Burq and LeDouarin (2000)
Pitx-2	Chick, frog, mouse	Transcription factor	Left	Logan et al. (1998), Morokuma et al. (2002), Ryan et al. (1998)
NKX3.2	Chick, mouse	Transcription factor	Left in chick, right in mice	Schneider et al. (1999)
Follistatin	Chick	Signaling molecule	Right	Levin (1998a)
FGF-8	Chick	Growth factor	Right	Boettger et al. (1999)
Flectin ^a	Chick	Extra-cellular matrix molecule	Left	Tsuda et al. (1996)
DHAND	Chick, mouse, frog	bHLH transcription factor	Right	Angelo et al. (2000), Srivastava (1995)
EHAND	Chick, mouse, frog	bHLH transcription factor	Left	Angelo et al. (2000), Biben and Harvey (1997), Cserjesi et al. (1995), Sparrow et al. (1998), Srivastava (1995)
Caronte	Chick	Cerberus/DAN family member	Left	Yokouchi et al. (1999)
N-Cadherin	Chick	Adhesion molecule	Right node, left groove	Garcia-Castro et al. (2000)
Cx43	Chick	Gap junction protein	Right	Levin and Mercola (1999)
Islet-1	Chick	LIM homeobox gene	Left	Yuan and Schoenwolf (2000)
H ⁺ /K ⁺ -ATPase	Frog, chick	H ⁺ and K ⁺ ion pump	Right	Levin et al. (2002)
PKI- α	Chick	PKA inhibitor	Right	Kawakami and Nakanishi (2001), Rodriguez-Esteban et al. (2001)
NCX-1	Chick, mouse	Sodium-calcium exchanger	Right	Linask et al. (2001)
HoxC-8	Frog	Transcription factor	Left	Thickett and Morgan (2002)
Xin	Mouse	?	Right	Wang et al. (1999)
Southpaw	Zebrafish	TGF- β family	Left	Long et al. (2003)
Mid1	Chick	Microtubule-associated protein	Right	Granata and Quaderi (2003)
Isy-6	<i>C. elegans</i>	Micro RNA repressor	Left	Johnston and Hobert (2003)
Dll1	Chick	Delta-like signaling molecule	Left	Raya et al. (2004)
14-3-3E	Frog	14-3-3 family	Right	Bunney et al. (2003)
Kif5C	Chick	Kinesin motor	Right	Dathe et al. (2004)
Cyclops	Zebrafish	Nodal-related	Left	Concha et al. (2000)
Leftover	Zebrafish	?	Left	Concha et al. (2000), Gamse et al. (2003)
Pcl-2	Chick	Transcription repressor	Right	Wang et al. (2004)
HB-EGF	Mouse	Growth factor	Left	Golding et al. (2004a,b)

^a Antibody epitopes.

patterning. 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 must orient

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.

Table 3
Asymmetrically expressed genes which have not been the focus of a LR paper

Gene	Species	Product/role	Side	Reference
HNF3- β	Chick, mouse	Winged-helix transcription factor	Left	Collignon et al. (1996), Levin et al. (1995)
CWnt-8C	Chick	wnt-family signaling molecule	Right	Levin (1998c), Pagan-Westphal and Tabin (1998)
hLAMP-1 ^a	Chick	Extra-cellular matrix molecule	Left	Smith et al. (1997)
JB3 ^a	Chick	Extra-cellular matrix molecule	Right	Smith et al. (1997), Wunsch et al. (1994)
HGF	Chick	Kringle signaling molecule	Left	Streit et al. (1995)
Hrlim	Ascidian	LIM-family signaling molecule	Right	Wada et al. (1995)
Rtk2	Zebrafish	Eph receptor	Right	Schilling et al. (1999)
Fli-1	Zebrafish	Transcription factor	Left	Schilling et al. (1999)
DM-GRASP	Zebrafish	Adhesion protein		Schilling et al. (1999)
Xbap	Frog	Transcription factor	Left	Newman et al. (1997)
Hest1	Zebrafish	ASIC ion channel	Left	Concha et al. (2003)

^a Antibody epitopes.

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 AP and DV axes, and is determined with respect to them (Danos and Yost, 1995a; McCain and McClay, 1994). 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.

4. Invertebrates

While the genetics of symmetry determination have been addressed in plants (Endress, 1999, 2001; Theissen,

Table 4
Genes involved in LR patterning which are not asymmetrically expressed

Gene	Species	Product/role	Reference
Iv	Mouse	Dynein (cytoplasmic transport or ciliary motor)	Lowe et al. (1996b), Supp et al. (1997, 1999, 2000)
Inv	Mouse	?	Mochizuki et al. (1998), Morgan et al. (1998, 2002)
Connexins	Frog, chick, man	System of gap-junctional cell-cell signaling	Britz-Cunningham et al. (1995), Levin and Mercola (1998c, 1999)
No turning	Mouse	Midline patterning	Melloy et al. (1998)
SIL	Mouse	Midline patterning	Izraeli et al. (1999)
KIF-3	Mouse	Component of ciliary motor	Nonaka et al. (1998), Takeda et al. (1999a)
Polaris	Mouse	?	Murcia et al. (2000)
HFH-4	Mouse	Transcription factor	Brody et al. (2000), Chen et al. (1998)
Lin-12	<i>C. elegans</i>	Notch signaling molecule	Hermann et al. (2000)
Delta-1	Mouse	Notch signaling molecule	Przemeck et al. (2003)
Notch	Mouse, zebrafish	Notch signaling molecule	Krebs et al. (2003), Raya et al. (2003a)
Smo	Mouse	Membrane protein involved in hedgehog signaling	Zhang et al. (2001)
Ihh	Mouse	Member of hedgehog signaling proteins	Zhang et al. (2001)
GDF-1/Vg-1	Mouse, frog	TGF- β -family signaling molecule	Hyatt et al. (1996), Hyatt and Yost (1998), Rankin et al. (2000), Wall et al. (2000)
DNAH5	Human	Dynein	Ibanez-Tallon et al. (2002), Olbrich et al. (2002)
PCKD-2	Mouse	Polycystin-2 ion channel	Pennekamp et al. (2002)
ZIC3	Human, mouse, frog	Zinc-finger protein	Gebbia et al. (1997), Kitaguchi et al. (2000), Purandare et al. (2002)
EGF-CFC	Mice, Zebrafish	Extracellular receptor	Yan et al. (1999a)
Furin	Mice	Proprotein convertase	Constam and Robertson (2000), Roebroek et al. (1998)
Brachyury	Mice	Transcription factor	King et al. (1998)
Ednrb	Mice	Piebald deletion complex	Welsh and O'Brien (2000)
Rotatin	Mice	Transmembrane protein	Faisst et al. (2002)
PDI-P5	Zebrafish	Protein disulfide isomerase	Hoshijima et al. (2002)
Pol- λ	Mouse	DNA polymerase	Kobayashi et al. (2002)
PA26	Human	Sestrin-family	Peeters et al. (2003)
Cryptic	Mouse, human, zebrafish	EGF-CFC gene	Bamford et al. (2000), Gaio et al. (1999b), Yan et al. (1999b)
Charon	Zebrafish	Cerberus-family protein	Hashimoto et al. (2004)

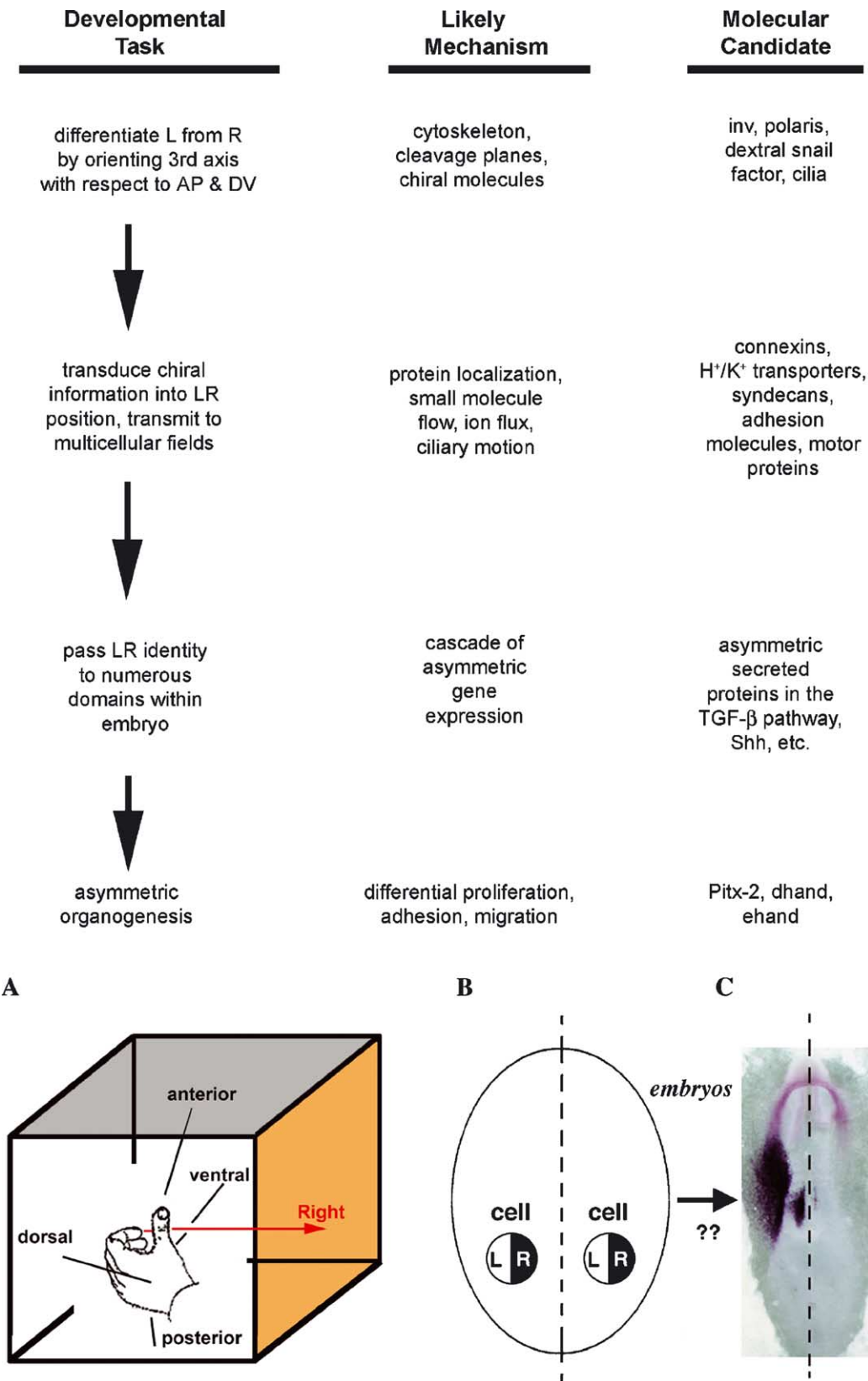


Fig. 1. The progressive flow of LR information is shown as a function of time, from its orientation with respect to the LR axis (A), to its conversion to positional information with respect to the midline and its imposition upon large-scale cell fields (B,C). Candidate mechanisms for each step are shown.

2000), and chiral forms exist among the protozoa (Frankel, 1991; Nelsen et al., 1989), true LR asymmetry appears to be mainly a feature of the animal kingdom. The organs possessing asymmetries, as well as the direction of their asymmetry, are evolutionarily well conserved. The heart is asymmetrically located in the mollusks (McMurrich, 1894); the *situs* of the stomach and the liver (Romer, 1962) is the same among fish, reptiles, birds, and mammals. In sea urchins, the asymmetric position of the adult rudiment in the larva has been studied (Aihara and Amemiya, 2000, 2001; McCain and McClay, 1994).

Several kinds of molluscs undergo spiral cleavage and secrete an exoskeleton shaped like a conical spiral. In 3D 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 which utilize each type of coiling. Murray and Clarke (1966) found that the direction of coiling of *Plionoma suturalis* is maternally inherited and sinistrality is dominant to dextrality. Freeman and Lundelius (1982), studying a different species, found that dextrality is dominant; the dextral gene apparently functions via an as yet unidentified cytoplasmic component.

Amphioxus, considered to be the ancestor of vertebrates, exhibits many LR asymmetries (Jefferies et al., 1996); one of the most striking occurs in somitogenesis (Minguillon and Garcia-Fernandez, 2002). Some of the genes known to be asymmetrically expressed in vertebrates are beginning to be cloned in amphioxus, and information on the conservation of lateralized expression should become available soon (Araki et al., 1996; Boorman and Shimeld, 2002a,b; Minguillon and Garcia-Fernandez, 2002; Shimeld, 1999; Terazawa and Satoh, 1995, 1997). The Ascidian *Nodal*- and *Pitx*-related genes exhibit the well-conserved left-sided expression (Morokuma et al., 2002).

In contrast to its pivotal contributions to other aspects of developmental biology, *Drosophila* has not played a key role in uncovering mechanisms of LR asymmetry. For example, selection for LR asymmetries in *Drosophila*, in hopes of generating a genetically tractable mutant, has not been successful (Tuinstra et al., 1990). However, it is now known from mutant analysis that *Drosophila* possesses genes which govern the helical torsion of the body (Martin-Blanco and Garcia-Bellido, 1996) and the rotation of the embryonic gut proventriculus (Adam et al., 2003; Hayashi and Murakami, 2001; Ligoxygakis et al., 2001). Both of these asymmetries are instances of chirality, which appears to dominate in the invertebrates (ciliates, mollusks, etc.). However, recent morphometric analysis has revealed a subtle but real directed asymmetry in wing size (Klingenberg et al., 1998), suggesting that novel mechanisms orienting the LR axis in fruit flies remain to be discovered.

In *Caenorhabditis elegans*, the embryonic cell lineage is asymmetrical: although the animal exhibits 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 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 left/right 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. Interestingly, a gene related to the *C. elegans* cell polarity determination pathway (*Par* family) has now been shown to be involved in the LR pathway in *Xenopus* (Bunney et al., 2003), suggesting possible conservation of basic vertebrate and invertebrate asymmetry-generating molecules.

The relationship and evolutionary origin of asymmetry-generating mechanisms in vertebrates and invertebrates is currently a very controversial issue (Boorman and Shimeld, 2002a; Jefferies, 1991). In both amphioxus and ascidians, expression of the homeobox transcription factor *Pitx2* is left-sided, suggesting that LR asymmetry in all chordates is regulated by a conserved developmental pathway which evolved before the separation of the lineages leading to living chordates. Other molecules such as *Notch* are involved in asymmetry in both vertebrates and invertebrates, but the molecular details of their action are distinct (Delattre and Felix, 2001; Hermann et al., 2000; Krebs et al., 2003; Przemek et al., 2003; Raya et al., 2003a,b, 2004; Vincent, 2003).

5. Fish

Flatfishes acquire a profound asymmetry in eye location (and scale/skin pigmentation) during metamorphosis from bilaterally symmetric larvae (Hashimoto et al., 2002; Matsumoto and Seikai, 1992; Okada et al., 2001a,b). Analysis of mutants in the zebrafish embryo has identified a number of genes involved in LR patterning (Yost, 1998), though some of these are likely to represent secondary LR effects of disrupted notochord or AP/DV patterning (see discussion of midline barrier below). In zebrafish, asymmetric markers such as *Lefty*, *Nodal*, and *Pitx2* exhibit well-conserved asymmetric expression during neurulation and somitogenesis (Cheng et al., 2000b; Essner et al., 2000; Liang et al., 2000).

Very little is known about early LR patterning steps in the zebrafish and it remains to be determined whether mechanisms implicated upstream of asymmetric gene expression (discussed below) function in fish, and whether the initial steps of asymmetry occur prior to or after the start of gastrulation. Rhythmic Ca^{++} waves have been described during fish gastrulation (Gilland et al., 1999), consistent with the calcium fluxes discovered (McGrath et al., 2003; Raya et al., 2004) in mice and chicks (see below), although the connection to downstream LR markers is not understood. However, the zebrafish has enabled some unique insight into neurological asymmetry.

The dorsal diencephalon of zebrafish embryos is LR asymmetric. The pineal complex consists of the pineal organ anlage and a left-sided parapineal. The neighboring brain nuclei (L and R dorsal habenulae) show consistent differences in size, density of neuropil, and gene expression. Zebrafish mutants have demonstrated a correlation between LR position of parapineal and the laterality of habenular nuclei; these asymmetries are downstream of Nodal signaling, illustrating the link between brain and visceral asymmetry. Selective ablation of the parapineal organ results in the loss of habenular asymmetry (Gamse et al., 2003; Halpern et al., 2003). One of the recent asymmetric genes expressed in zebrafish habenulae is *hest1* (Concha et al., 2003), which is a pH-sensitive ion channel. In light of the involvement of H^+ flux in early LR asymmetry in frog and chick embryos (see below), it will be interesting to find out whether H^+ flux is also involved in much later generation of brain asymmetry. Importantly, it has been shown that one of the downstream endpoints of the early brain asymmetry is behavioral. Most species of fish examined preferentially utilize the right eye when attacking rivals (Bisazza and de Santi, 2003; Miklosi and Andrew, 1999).

6. Amphibia

Experiments in *Xenopus* first suggested that the LR axis might be established extremely early, and to be intimately linked with DV axis formation (Yost, 1991). The DV axis is initiated by sperm entry during fertilization, followed by a cytoplasmic rotation during the first cell cycle, driven by a microtubule array at the vegetal cortex (Gerhart et al., 1989). Work from the Yost lab showed that embryos in which the microtubule array was blocked, but which were tilted manually to rescue the DV and AP axes exhibited laterality defects, suggesting that the LR axis may be dependent on the transient microtubule array during the first cell cycle. The appearance of LR asymmetry between fertilization and the first cell division is consistent with the recent work showing asymmetric mRNA and protein localization during early the first few cleavages (Bunney et al., 2003; Levin et al., 2002). While the mechanisms which process LR information during

gastrulation in amphibia are largely unknown, the *Xenopus* embryo has allowed discovery of a number of mechanisms which underlie asymmetry at the earliest stages known in any species.

The *Xenopus* system was the first to allow ‘molecular’ studies of LR asymmetry in embryogenesis (Yost, 1990a, 1992). Localized perturbation of a small patch of extracellular matrix by microsurgery, as well as global perturbation of the extracellular matrix by microinjection of Arg-Gly-Asp peptides or heparinase into the blastocoel, resulted in randomization of LR asymmetry. This work provided the first molecular entrypoint into LR asymmetry, and suggested that the extracellular matrix participated in transfer of LR information in development. Inhibition of proteoglycan synthesis with the drug β -xyloside prevents heart looping in *Xenopus* (Yost, 1990b). The effect occurred well before heart looping, was cell-autonomous, and was proposed to involve migratory cells of the cardiac primordia.

Based on the proposal that heparan sulfate proteoglycans (HSPGs) or the ECM on the basal surface of the ectoderm transmits LR information to mesodermal primordia during gastrulation (Yost, 1992), Teel and Yost examined the roles of the Syndecan family; syndecan-1 and -2 are maternally expressed HSPGs specifically located in the animal cap ectoderm (Teel and Yost, 1996). Using dominant-negative and loss-of-function approaches, it was shown that syndecan-2 is involved in LR asymmetry (Kramer and Yost, 2002) in *Xenopus*. A cytoplasmic domain of Syndecan-2 is phosphorylated in cells on the right but not left half of the frog embryo during gastrulation. Moreover, they showed that attachment of multiple heparan sulfate glycosaminoglycans on syndecan-2 and functional interaction of these sites with the cytoplasmic domain are an obligate part of LR patterning during gastrulation, immediately prior to the migration of mesoderm across ectoderm. Kramer and Yost also presented biochemical data on the direct interaction of Syndecan-2 with Vg1 (a candidate for the LR coordinator (Hyatt and Yost, 1998)), suggesting that these two molecules function together during LR patterning at gastrulation.

Another key finding in *Xenopus* was the discovery of an experimental perturbation which can produce almost full *situs inversus*; this is especially interesting since almost every other reported manipulation results in heterotaxia—an independent randomization of *situs* and not full reversal (or loss of asymmetry). The active form of Vg1, a TGF β family member, can almost completely invert the LR axis when misexpressed on the right side (R3 blastomere) of a *Xenopus* embryo (Hyatt et al., 1996; Hyatt and Yost, 1998). This suggests that Vg1 normally acts in descendants of the L3 blastomere, which contribute to the left LPM, and the model suggests signaling through ALK2 and mutual antagonism with BMP on the right side of the embryo (Ramsdell and Yost, 1999). Axial inversion is specific to the activated Vg1 and cannot be mimicked by Activin. While these data are

consistent with an early LR pattern in the pre-gastrula stage *Xenopus* embryo, the precise timing remains uncertain since the persistence of the injected mRNA to later stages raises the possibility that the injected Vg1 persists in the embryo and mimics a later signal. Endogenous Vg1 protein in early *Xenopus* embryos (and especially, asymmetries therein) have yet to be characterized. Along-side the above-mentioned secreted signaling molecules, *Xenopus* embryos have also allowed the discovery of LR mechanisms which depend on a different channel for cellular communication: gap junctions.

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 in several excellent recent reviews (Falk, 2000; Goodenough et al., 1996), and gap junctional flow is involved in a number of important patterning events in embryonic development and tumor progression (Guthrie and Gilula, 1989; Levin, 2001; Lo, 1996). Based on a (now controversial) report that several unrelated patients with viscerio-atrial heterotaxia contain potential mutations within Connexin43 (Britz-Cunningham et al., 1995), and data which indicated asymmetric patterns of GJC (Guthrie, 1984; Guthrie et al., 1988) in early frog blastomeres, Levin and Mercola tested the hypothesis that gap junctional paths were a mechanism by which LR information was communicated across large cell fields (Levin and Mercola, 1998c). *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 previous observation using a single small-molecule probe (Brizuela et al., 2001; Guthrie, 1984; Olson et al., 1991, 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, 1998c).

These results indicate that an endogenous path of GJC between dorsal and lateral blastomeres, as well as the isolation across the ventral midline, are 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, and is upstream of asymmetric *XNR-1* and heart tube looping. It was proposed (Levin and Mercola, 1998c; Levin and Nascone, 1997) that small molecule determinants are initially randomly distributed, but traverse the circumferential GJC path unidirectionally, accumulating on one side of the midline, and then induce asymmetric gene expression. Similar data were later obtained in the chick embryo (see below). The identities of the putative low molecular weight determinants remain unknown (although pre-nervous neurotransmitters are now a plausible candidate (Fukumoto et al., 2003)).

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 signaling molecule needs to traverse the entire path). In some contexts, chemically rectifying or 1-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 (net unidirectional) distribution of the small molecules through the circumferential path. A model in which a voltage difference provides an electrophoretic force pushing charged molecules in preferred directions through GJC paths suggested the hypothesis that ion fluxes may be an obligatory aspect of early LR patterning in *Xenopus*.

A pharmacological screen of hundreds of various types of ion channels, pumps, and co-transporters (Levin et al., 2002) specifically implicated four target genes involved in H^+ and K^+ flux (Adams and Levin, 2003; Chen and Levin, 2004). One of these, the H^+/K^+ -ATPase, functions during early cleavage stages. Moreover, maternal H^+/K^+ -ATPase mRNA is asymmetrically localized during the first two cell divisions, demonstrating that asymmetry is generated by two hours post-fertilization. Examination of the *situs* of asymmetric genes (*xNR-1*, *xLefty*, and *xPitx-2*) following early exposure to blockers of the H^+/K^+ -ATPase revealed that, consistently with the early asymmetrical expression, the ion flux mechanism is upstream of the asymmetric expression of those genes. Gain-of-function experiments using H^+/K^+ -ATPase and K^+ channel overexpression also demonstrated that equalizing H^+ and K^+ flux on either side of the midline randomizes the LR axis.

Taken together, these data demonstrate that the *Xenopus* embryo assigns L and R identities to cells at the first few cleavages. This conclusion is also confirmed by the finding of asymmetric 14-3-3E protein localization, which is crucial for normal LR asymmetry (Bunney et al., 2003). However, a key series of experiments demonstrated that under some circumstances, ectopic organizers induced much later are still able to impose correct LR identity on nearby tissue (Nascone and Mercola, 1997). Thus, the *Xenopus* embryo is likely to contain an endogenous very early mechanism for aligning the LR axis, but also the capacity for regulatory patterning of the LR axis at later stages.

7. Chick

The first morphological asymmetry in the chick embryo is the tilt of Hensen's node toward the end of gastrulation (Dathe et al., 2002; Hertwig, 1902; Kölliker, 1879). Transplantation experiments in the chick had shown that heart sidedness is determined during gastrulation

(Hoyle et al., 1992), and soon thereafter the chick became the first system in which asymmetric gene expression was demonstrated. Characterizing a number of known genes during early chick embryogenesis, Levin et al. found that several had consistently asymmetric expression patterns during gastrulation and at the beginning of neurulation (Levin, 1998b; Levin et al., 1995, 1997b). Sonic hedgehog (*Shh*), a signaling molecule which is also involved in several other patterning contexts, is expressed symmetrically within the ectoderm of Hensen's node (the chick organizer [Streit et al., 1994]) before st. 4, at which time it becomes restricted to the left side of the node. This is followed at st. 7 by the left-sided expression of *Nodal* (a TGF- β family member, originally called *cNR-1* in the chick). *Nodal* is first expressed in a small domain of endoderm cells directly adjacent to the ectoderm cells expressing *Shh*, and then in a large domain in the lateral plate mesoderm.

The juxtaposition of the proximal domain of *Nodal* to the cells expressing *Shh* suggested an inductive interaction, and indeed, implanting cells expressing *Shh* on the right side of Hensen's node is sufficient to induce an ectopic domain of *Nodal* expression on the right side. The Activin-inducible gene *Activin receptor IIa* (*cAct-RIIa*) becomes expressed on the right side of Hensen's node at the same time that *Shh* becomes restricted to the left. This suggested the right-sided presence of an Activin-like repressor upstream of *Shh*; it was then shown that a local source of Activin protein implanted on the left side is able to induce *cAct-RIIa* there, and to repress the expression of left-sided *Shh*. It is still unknown whether *cAct-RIIa* itself plays a causal role in LR patterning, but an endogenous right-sided expression of *Activin- β B* has been reported (Levin, 1998d; Levin et al., 1997a). Many more asymmetric genes have been identified in chick embryos (Tables 2 and 3); these factors participate in cascades of induction and repression of asymmetric gene pathways taking place on the left and right of the midline, ultimately dictating heart and gut *situs* as well as embryonic turning.

The discovery that *Nodal* can positively regulate its own gene expression highlights the enduring problem of explaining how *Nodal* signaling is constrained spatially. Earlier studies, predating the elucidation of promoter elements in the *Nodal* gene, suggested the existence of a barrier at the dorsal midline of embryos to prevent signaling on one side of the embryo from interfering with cascades on the other. It has long been known, for instance, that twins conjoined at the level of the trunk often exhibit laterality disturbances and that the defects reside primarily in the right sibling. Spontaneous chick conjoined twins or experimentally induced *Xenopus* conjoined twins develop with a similar defect (Levin et al., 1996; Nascone and Mercola, 1997). Molecular genetic analyses of these embryos, however, pointed out that the defects observed in the right sibling are not due to an intrinsic error in the orientation of the LR axis, but results from interference from signals that probably originate from the right flank of the left twin.

A barrier model was proposed initially (Danos and Yost, 1995b, 1996a; King et al., 1998; Lohr et al., 1997b; Yost, 1998) to explain why interference does not occur during normal development and also to account for the severity of laterality defects seen in embryos with midline anomalies or patterning deficits created by either genetic or microsurgical means.

Possible candidates for the barrier include the primitive pit and notochord. Consistent with this, removal of the notochord destabilizes LR asymmetry (Danos and Yost, 1996b). Likewise, the *flh* ('floating head') and *ntl* ('no tail' or Brachyury) mutants have notochord defects that are often accompanied by liver and cardiac inversions (Chin et al., 2000b; Concha et al., 2000; Halpern et al., 1993; Korzh et al., 2001; Talbot et al., 1995). Molecular level insight into relatively late aspects of this important problem was provided by the discovery of Lefty homologues. Lefty proteins are divergent TGF β superfamily ligands (Heymer et al., 1997a; Meno et al., 1996) that antagonize *Nodal* signaling in embryological assays (Bisgrove et al., 1999; Cheng et al., 2000a; Meno et al., 1999); reviewed in Schier and Shen (2000). *Lefty* genes, like *Nodal*, are implicated in LR signaling because their expression is altered by mutations that affect organ *situs* (Dufort et al., 1998; Gaio et al., 1999a; Heymer et al., 1997b; Izraeli et al., 1999; King et al., 1998; Meno et al., 1996, 1998; Meyers and Martin, 1999; Nonaka et al., 1998; Tsukui et al., 1999a; Yan et al., 1999a). Conversely, mouse embryos lacking *lefty1* have bilateral expression of normally left-sided markers, including *Nodal* (Meno et al., 1998). Given their ability to antagonize *Nodal*, *Lefty1* in the midline may provide a barrier to prevent the unwanted propagation of *Nodal* signals to the right flank of the embryo while *Lefty2* acts as a feedback inhibitor within the left lateral plate mesoderm.

The fairly dense pathway of LR cascade members in chick embryos suggests an immediate question: what mechanism is upstream of the very first asymmetrically expressed gene? Contrary to the paradigm of genetically separate L and R compartments which begins during mid-gastrulation, it was observed that events occurring on the far R side were required for establishment of L identity on the left side at the beginning of streak initiation (Levin and Mercola, 1999). Thus, gap junctional communication was examined in the chick embryo, as a candidate for a mechanism which would enable cells to communicate across large distances along the LR axis and assign LR identities to cell fields. Similarly to the results in *Xenopus*, it was discovered that differential GJC is required upstream of asymmetric *Shh* expression in the node and one Connexin, *Cx43*, was implicated by treatment with specific antisense oligonucleotides or blocking antibodies (Levin and Mercola, 1999). *Cx43* 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 of this system is the same in both systems: correct laterality determination upstream of asymmetric gene expression depends on an uninterrupted region of GJC around a zone of isolation.

The fact that contiguity of the blastodisc on both sides of the midline is necessary (Levin and Mercola, 1999) suggest a model whereby instructive signals traverse lateral tissue in the plane of the GJC path, which in turn predicts that the midline cells receive LR information from lateral tissue during gastrulation. In the chick, current data strongly indicates that indeed Hensen's node is instructed with respect to the LR axis by adjacent lateral cell groups (Levin and Mercola, 1999; Pagan-Westphal and Tabin, 1998; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998), rather than generating LR information intrinsically (which would be required by the cilia-based models described below). Important open areas of research include identification of upstream signals that orient GJC in embryos, characterization of the determinants that traverse gap junctions and downstream target genes that they regulate, and the targets that are immediately downstream of GJC flow.

Because the GJC system has been shown to be conserved to both chick and *Xenopus*, a role for ion flux was tested in the chick as well (Levin et al., 2002). 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 identities by st. 3—prior to the earliest known asymmetric gene. Similarly to *Xenopus*, 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). Interestingly, while the H^+/K^+ -ATPase is 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, no asymmetry in pump localization has been reported in the chick at the level of mRNA. This echoes a theme which highlights an important difference between species. While both chicks and *Xenopus* appear to use GJC and ion flux to pattern the LR axis, the regulation of the mechanisms differs. The difference in GJC in frog embryos takes place post-translationally (by gating control of existing gap junctions). In contrast, the chick embryo establishes the zone of isolation at the level of mRNA (by not transcribing *Cx43* mRNA in the primitive streak). Similarly, 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 post-translational mechanism

(such as gating of electrogenic activity of mature pump complexes).

While the details of ion flux in the LR pathway of other species are not yet clarified, a H^+ and K^+ flux appears to be conserved in sea urchin (Yuichiro Ishii et al., 2003) and *Ciona* (Shimeld, 2003). Important future data are likely to come from pursuing the asymmetric gene cascade upstream, and determining how it interfaces with the GJC and ion flux systems. Some of the details of this process have recently been provided by an elegant study which used a variety of gain- and loss-of-function approaches, together with real-time *in vivo* imaging of Ca^{++} content, to show that an H^+/K^+ -ATPase-dependent extracellular calcium accumulation on the left side of Hensen's node is sensed by a *Notch* pathway mechanism (Raya et al., 2004). The epistasis between the earlier *Shh* asymmetry and the Ca^{++} -dependent *Notch* signaling is not yet clear. Raya et al. developed a mathematical model that predicts the robust *Notch* expression, and is a prototype for the kind of approach which will surely be necessary to understand and control the transduction of epigenetic factors such as ion flux into domains of stable gene expression. Does asymmetric gene expression exist prior to gastrulation? It has previously been suggested (Levin and Mercola, 1998b) that the computation which aligns the LR axis with the DV and AP axes 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; such a model may have to wait for an understanding of how (and whether) individual cells in the chick blastoderm determine their AP polarity (Wei and Mikawa, 2000).

8. Mammals

Errors of LR patterning during embryogenesis are relevant to the clinical considerations of several fairly common human birth defects: syndromes 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 (where the organism is completely symmetrical, for example, polysplenia or asplenia); these alterations of normal asymmetry are recapitulated in a number of animal models (Bisgrove and Yost, 2001). Of these, only the complete (and rare) *situs inversus totalis* is not associated with physiological difficulties. The rest, especially heterotaxia, 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 (Kathiriyi and Srivastava, 2000). Laterality defects

can arise in a single individual (Kosaki and Casey, 1998; Winer-Muram, 1995) but are especially associated with monozygotic twinning. One interesting and poorly understood phenomenon is the relationship with sex determination: in human hermaphrodites, ovaries develop on the left, testes on the right (Mittwoch, 2000).

One of the crucial questions regarding mammalian embryos concerns when LR information is first generated. Mouse embryos have been shown to be able to reconstitute normal morphology after significant experimental manipulation—early blastomeres can be removed or added apparently without affecting normal development. This has been suggested to signify that the patterning of axes in mammalian embryos takes place later than other species such as *Xenopus*. However, a number of recent studies have suggested that the polar body may indicate the future axis of bilateral symmetry in fertilized mouse eggs (Gardner, 2001; Johnson, 2001), perhaps indicating the existence of early axial orientation mechanisms that could be masked by later regulation in the event of experimental perturbation.

The observation that human Kartagener's syndrome patients exhibited randomization of visceral *situs* (heterotaxia) and had ultrastructural defects in the dynein component of cilia (Afzelius, 1976, 1985) was of great interest because it suggested that asymmetry could be bootstrapped from molecular chirality of some ciliary component. This idea was supported by the finding that the murine *iv* mutation, which unbiases laterality (Lowe et al., 1996b; Schreiner et al., 1993; Singh et al., 1991), encodes a dynein called left–right dynein (LRD) that is expressed in cells of the mouse node (Supp et al., 1997). Axonemal dynein is a component of the motor which drives ciliary motion; the chirality of this motion is intrinsic to the protein components. Genetic deletions of KIF3-A or KIF3-B (Marszalek et al., 1999; Nonaka et al., 1998), two microtubule-dependent kinesin motor proteins, resulted in defects in nodal cilia and randomization of the *situs* of the viscera, and this finding provides evidence for a role for cilia in LR determination (Vogan and Tabin, 1999). Most importantly, following the first observation of cilia in the murine node (Sulik et al., 1994), elegant and technologically challenging experiments have revealed a clockwise rotation of monocilia extending ventral to the node that produces a localized net right to left fluid flow of fluorescent beads placed in the extra-embryonic space (Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999b). Thus, it was proposed that vortical action of cilia (coupled with the wedge-shape of the node) may initiate asymmetry by moving an extracellular signaling molecule to one side, where it can induce asymmetric gene expression (Vogan and Tabin, 1999). A more sophisticated version of this model, invoking two kinds of cilia (motile and sensory) was later proposed, to account for discrepancies between data from observations of ciliary beating in cultured mouse embryos, and the molecular and morphological phenotype observed in the LR mutants (McGrath and Brueckner, 2003;

Tabin and Vogan, 2003). In addition to Kinesin and Dynein, a number of other proteins have also been linked to asymmetry which have been interpreted to result from impaired ciliary function. These include Inversin (Morgan et al., 1998, 2002; Otto et al., 2003; Watanabe et al., 2003), Polaris (Murcia et al., 2000; Taulman et al., 2001), and Polycystin-2 (Pennekamp et al., 2002).

The strongest version of this model (McGrath et al., 2003) hypothesizes that LR asymmetry is initiated by the motion of the cilia in the mature node (toward the end of gastrulation). A recent set of intricate experiments demonstrated that artificial flow around the cultured node of rodent embryos is able to affect asymmetry. While the results of a 'no flow' condition (such as in viscous medium) have not been demonstrated, and culture of rodent embryos randomizes *situs* in and of itself (Fujinaga and Baden, 1991; Fujinaga et al., 1990), these data demonstrate that 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 upstream LR signals. Consistent with the theoretically pleasing hypothesis that cilia initiate LR orientation, no earlier LR mechanisms have yet been described in rodents. However, many types of cilia can reverse the direction of their beat (Bone, 1958), and it is not yet clear whether the biochemical structure of cilia uniquely determines their function (allowing asymmetry to be generated from molecular chirality). Because rodent embryos in which molecular motors have been mutated are also likely to have impaired cytoplasmic function of motor transport, it has not yet been possible to separate the ciliary functions of the LR-relevant motors from cytoplasmic transport roles.

The rodent embryo is quite unusual in its large-scale and node architecture, compared to most mammals. However, consistent with the possibility that the relationship between cilia and asymmetry is not unique to rodents, a recent study in Zebrafish demonstrated that knock-down of the *ntl* gene specifically in the dorsal fore-runner cells (ciliated cells in Kupffer's vesicle) results in randomization of *situs* (Amack and Yost, 2004). While these data do not address the role of the cilia *per se*, they do suggest that the role of *ntl* in asymmetry is not confined to midline barrier function, and indicate that the dorsal fore-runner cells are a crucial component of the LR pathway in zebrafish. While a function for motor proteins in LR patterning is fairly certain, the mechanisms by which they control laterality and whether cilia *per se* contribute to the initiation or transmission of asymmetry in each species remains controversial (Hamada et al., 2002; Levin, 2003b, 2004a,b).

The earliest known endogenous LR mechanisms (GJC, H^+/K^+ flux, Vg1 coordinator) have not been found to contribute to mammalian asymmetry. However, ion flux has been implicated in mouse embryos. A genetic deletion experiment has suggested that the ion channel Polycystin is required for normal asymmetry in the mouse (Pennekamp

et al., 2002). More directly, it has recently been shown that asymmetric calcium signaling appears at the left margin of the node at the time of nodal flow (McGrath et al., 2003); this cytoplasmic Ca^{++} gradient may be analogous to the extracellular Ca^{++} flux recently demonstrated in the chick (Raya et al., 2004). Whether additional ion flows play a role in rodents and other mammals, and whether Ca^{++} flow is important for LR patterning prior to mature node stages, remains uncertain.

Rabbit embryos (like most mammals including human embryos) develop as a flat blastodisc similar to the chick, and have contributed significant advances which complement and contrast mouse data (Fischer et al., 2002; Viebahn, 2001; Viebahn et al., 1995). Indeed, mice may not recapitulate all phenomena important for LR patterning. For example, the striking strict one-sided midline pigment lateralization observed in human children affected by CHILD (Happle, 2002; Happle et al., 1995; Konig et al., 2000) syndrome is not seen in the corresponding mouse mutant (Liu et al., 1999; Phillips et al., 1973), suggesting that important aspects of early gene function and demarcation of the LR midline may differ between rodents and other mammals. This becomes of particular importance when considering the mechanistic implications of laterality phenotypes observed in human monozygotic twins (below). Moreover, while mice do exhibit the strong linkage between sidedness of hermaphroditic organs observed in human cases (Biddle et al., 1994; Eicher and Washburn, 1983; Ward et al., 1987), the consistent laterality of testes' vs. ovaries' placement is opposite of that observed in humans (Krob et al., 1994; van Niekerk and Retief, 1981). Tractable mouse models of these fascinating phenomena may not always be possible, and may not provide data directly applicable to human development.

9. Twinning and asymmetry

Inversions of various organs have been observed in the context of human conjoined twins (Aird, 1959; Cuniff et al., 1988; Torgersen, 1950). It is also known that conjoined twins of armadillo (Newman, 1916), fish (Morrill, 1919), frog (Spemann and Falkenberg, 1919), and man (Burn, 1991; Winer-Muram, 1995), often exhibit alterations of *situs* in one of the twins. The discovery of the spatial signals propagated by asymmetric gene expression in chick embryos have allowed a partial understanding of laterality defects in human conjoined twins (Kapur et al., 1994). Analysis of spontaneous twins of chick embryos in various orientations suggests that laterality defects in one twin may be induced by cross-over of LR morphogen molecules from the adjacent twin (Levin et al., 1996). More detail is provided in Supplement 1.

Non-conjoined monozygotic twins, while not exhibiting the kinds of visceral laterality defects that occur in conjoined twins, do 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 (Beere et al., 1990; Carton and Rees, 1987; Cidis et al., 1997; Gedda et al., 1981; Lauterbach, 1925; Mensing, 1983; Morini et al., 2002; Morison et al., 1994; Newman et al., 1937; Okamoto et al., 2001; Potter and Nance, 1976; Rife, 1933, 1940, 1980; Satoh et al., 1995; Schneider, 1985; Sommer et al., 1999, 2002; Sperber et al., 1994; Townsend and Richards, 1990; West, 1985; Yager, 1984). The mirror/bookending phenomenon also pertains to functional parameters such as sleep deviations, and hearing and cerebral functional localization (Golbin et al., 1993; Sommer et al., 1999, 2002; Springer and Searleman, 1978a,b).

Interestingly, though monozygotic twins affected by genetic lesions often show opposite sidedness of limb abnormalities (Opitz and Utkus, 2001; Richieri-Costa and Opitz, 1986), almost all bookending phenomena in healthy twins involve features of the head. 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, non-conjoined 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 re-specified) 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 (Derom et al., 1996; McManus and Bryden, 1992). 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, 2003a).

10. Laterality and brain asymmetry

Nervous system lateralization is spread throughout evolution (Andrew, 2000), 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). The genetic basis of handedness in man is still highly controversial (McManus, 1995; McManus and Bryden, 1992). Left-handedness runs in families weakly—almost half of all left-handers have no known left-handed relatives (McManus, 1995). Two right-handed parents have left-handed children in about 5–10% of the cases, while two left-handed parents have left-handed children in about 25–30% of cases (McManus and Bryden, 1992). Monozygotic twins often differ in their handedness.

Interestingly, it has been found that brain asymmetry 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 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 which determine the sidedness of visceral organs. Moreover, human patients with 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 mutations affecting ciliary function.

11. Conservation of mechanisms

While a number of mechanisms (e.g. apoptosis, syndecans) have only been implicated in one species (chick and frog respectively), others (e.g. Vg-1, GJC, ion flux, Notch) appear to be more widely conserved. Indeed, a relationship with the LR axis of Ca^{++} flux for example appears to be conserved in invertebrates, chicks, and mice (Albrieux and Villaz, 2000; McGrath et al., 2003; Raya et al., 2004). Important differences exist, however, in the details of a number of pathways. For example, in chick, *Shh* is a left-sided determinant while *Fgf-8* is a right-sided signal (Boettger et al., 1999; Levin et al., 1995), while in mouse *Fgf-8* appears to determine the left side identity while *Shh* is necessary to confine left-sided proteins to the left (Meyers and Martin, 1999). Interestingly, the details of these pathways appear to be correlated with the geometry of embryogenesis rather than taxonomy. In rabbit (a mammal which might be expected to utilize *Fgf-8* as a left determinant as does the mouse), *Fgf-8* is a right side molecule, suggesting that the flat blastodisc, a feature shared

by rabbit and chick embryos, may be the important parameter (Fischer et al., 2002). A similar inversion between chick and mouse is observed with respect to the gene *NKX3.2* (Schneider et al., 1999).

One crucial open question in the field concerns the conservation of the early members of the asymmetric gene cascade. The earliest conserved asymmetric gene known is *Nodal*, which is left-sided at somite stages in all vertebrates in which it has been examined. Downstream *Lefty* and *Pitx-2* genes appear to be well conserved also. However, neither *Shh* nor any of the other early genes known to be asymmetric during chick gastrulation (*cAct-RIIa*, *cHNF3-β*, *Follistatin*, *cWnt-8C*, etc.) have been reported to asymmetric in other species despite in situ hybridization searches by a number of labs (Ekker et al., 1995; Stolow and Shi, 1995), although *Shh* is left-sided in ducks and quails (Levin, 1996). Interestingly, misexpression of Hedgehog proteins in frog embryos is known to randomize asymmetry (Sampath et al., 1997), raising the possibility that the asymmetric Hedgehog signal exists in amphibia but perhaps utilizes an as yet uncharacterized family member. The situation with respect to the early asymmetric genes is the same in mouse, where genetic deletions have suggested roles for some of the same molecules (Oh and Li, 1997; Tsukui et al., 1999b). It is possible that the asymmetry in Hedgehog signaling exists at a level other than mRNA (protein processing, translation, etc.) or is anatomically so subtle as to have been missed. While no asymmetric expression upstream of *Nodal* has been reported in mice, two mouse pathways (the first conserved to chicks, the second to *Xenopus*) play a role upstream of *Nodal*: the *Notch* pathway (Krebs et al., 2003; Raya et al., 2003b) and *Vg-1* (Rankin et al., 2000). Two more areas which are of relevance to questions of evolutionary conservation are retinoic acid signaling and induction of *Nodal* genes by *Hedgehog* signals in amphibia; details can be found in Supplement 2.

12. Open questions

An interesting aspect of *Nodal* expression in *Xenopus* concerns the degree of bias of asymmetry. In both chick and mouse, the left-sided expression of *Nodal* is observed in all normal embryos (Collignon et al., 1996; Levin et al., 1995; Lowe et al., 1996b). In contrast, left-sided expression of xNR-1 in the frog is quite variable, being undetectable in up to 25% of the embryos, even from groups whose other members go on to form late-stage tadpoles with 100% normal LR asymmetry (Levin and Mercola, 1998c; Lohr et al., 1997b). Nevertheless, *Nodal* is a common left-sided mid-pathway asymmetric gene in all model systems to date; understanding the evolutionary mechanisms which resulted in this stable point, together with divergence of upstream mechanisms (if indeed they differ), is a key challenge.

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,b); 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 (such as limbs). 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 persistence of LR information after embryogenesis, are sure to have important implications for biomedicine and basic developmental biology.

Because no macroscopic force distinguishes right from left, a powerful paradigm has been proposed to leverage large-scale asymmetry from the chirality of sub-cellular components (Brown et al., 1991; Brown and Wolpert, 1990). In this class of models, some molecule or organelle with a fixed chirality is oriented with respect to the antero-posterior and DV axes, and its chiral nature is thus able to nucleate asymmetric processes such as transport (Levin and Mercola, 1998a). Thus, the first developmental event which distinguishes left from right would take place on a subcellular scale. However, a mechanism must then exist to transduce subcellular signals to cell fields (Levin and Mercola, 1998a; Levin and Nascone, 1997). 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 Left and which is Right. 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 flow of signaling molecules and cell–cell communication via gap junctions are both natural candidates for such a signal exchange. Roles for epigenetic factors such as membrane voltage, and Ca^{++} signaling are also likely.

One of the key remaining questions is the molecular meaning of 'randomization'. 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, 1997b)—not a loss of asymmetry in the 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 including GJC, H,K-ATPase, and apoptosis, also induce *randomization* of asymmetric genes such as *Shh*. A mechanistic model for this process would have to explain not simply consistent induction (or repression) of genes such as *Shh* by gap junctional communication or cell depolarization, but a mechanism by which cells in both sides of the node can be driven to randomly express *Shh* or not. Even more puzzling for simple gene cascade models is the observation that in several vertebrate and invertebrate systems, symmetrization of an upstream 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). 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. Interestingly, a role for this pathway has recently been implicated downstream of the H,K-ATPase in the chick (Raya et al., 2004). Thus, 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); potential candidate molecules may include motor proteins from the kinesin family gene which could underlie intracellular transport in node signaling events (Dathe et al., 2004).

13. Conclusion

The consistent macroscopic chirality of embryonic structures is a crucial part of developmental biology. This question touches on issues ranging from evolutionary mechanisms of body-plan dynamics to the subtleties of parity conservation in quantum mechanics (see Supplement 3 which discusses the possible relationship of macroscopic LR asymmetry with chemical chirality). While a number of important advances have been made in several model systems, the most interesting questions remain open. It is likely that future experiments addressing the role of motor proteins, small molecule movement, and the mechanisms which generate organ shape from laterality signals, will open new areas of cell, developmental, and evolutionary biology.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.mod.2004.08.006](https://doi.org/10.1016/j.mod.2004.08.006)

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