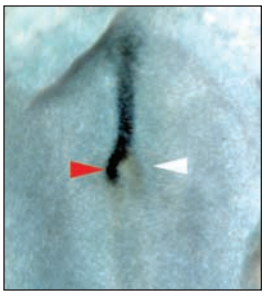

EARLY PATTERNING OF THE LEFT/RIGHT AXIS

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INTRODUCTION

Vertebrates have a 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). 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 asymmetric, but asymmetric in the same direction (i.e., why a consistent bias and not a 50%/50% racemic population)? When, during evolution, did handed asymmetry appear, and were there true bilaterally symmetrical organisms prior to the invention of oriented asymmetry? 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? How can the left/right axis be consistently oriented with respect to the anterior-posterior and dorsoventral axes in the absence of any macroscopic feature of chemistry or physics that distinguishes left from right? None of these questions can be properly addressed until we have a detailed understanding, at the molecular, genetic, and biochemical levels, of the formation of biased asymmetry in embryos.

Whereas 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, although it is possible to come up with plausible evolutionary reasons that organisms might be asymmetric in the first place (optimal packing, fluid dynamics, maximizing surface area of tubes, etc.), there is no obvious reason they should all be asymmetric to the same direction. It is, after all, much easier to imagine a developmental mechanism for generating asymmetry (such as positive feedback and amplification of stochastic biochemical differences) than for biasing it to a given direction.

Although mechanisms underlying anterior–posterior and dorsoventral asymmetry have been studied in detail with the advent of molecular genetics, the mechanistic basis for LR asymmetry was, until recently, completely unknown. However, within the last 10–15 years, significant advances in embryonic asymmetry have been made by a number of groups (Levin and Mercola 1998a; Burdine and Schier 2000; Mercola and Levin 2001; Yost 2001; Mercola 2003). Tables 1–3 summarize the molecular players in the LR pathway and show which ones are conserved among various model systems. Gene products in the LR pathway have been identified in forward and reverse genetics approaches (exemplified by the zebrafish mutants and Sonic Hedgehog, respectively), and almost all have roles in embryonic processes other than LR asymmetry. Although a few of the components have no known homology or function

Table 1. *Asymmetrically expressed genes in embryos that 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	Meno et al. (1996, 1998); Branford et al. (2000); Cheng et al. (2000); Essner et al. (2000)
Activin βB	chick	TGF-β-family signaling molecule	right	Levin et al. (1997)
cAct-RIIa	chick	Activin receptor	right	Levin et al. (1995)
Shh	chick	signaling molecule	left	Levin et al. (1995)
cSnR	chick	zinc finger protein	right	Isaac et al. (1997)
Nodal	chick, mouse, frog	TGF-β-family signaling molecule	left	Levin et al. (1995); Collignon et al. (1996); Lowe et al. (1996); Lohr et al. (1997); Morokuma et al. (2002)
cPTC	chick	Shh receptor	left	Levin (1998b); Pagan-Westphal and Tabin (1998)
Cerberus/Caronte	chick	signaling molecule	left	Yokouchi et al. (1999); 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); Ryan et al. (1998); Morokuma et al. (2002)
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	chick	extracellular matrix molecule	left	Tsuda et al. (1996)
dHAND	chick, mouse, frog	bHLH transcription factor	right	Srivastava (1995); Angelo et al. (2000)
eHAND	chick, mouse, frog	bHLH transcription factor	left	Cserjesi et al. (1995); Srivastava (1995); Biben and Harvey (1997); Sparrow et al. (1998); Angelo et al. (2000)
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-a	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)
cMid-1	chick	microtubule-associated protein	right	Granata and Quaderi (2003)
lsy-6	*C. elegans*	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)

Bold entries indicate genes expressed during gastrulation.

(such as INV), the remainder form a fairly diverse group of molecules including secreted signaling factors, regulators of ion flux, motor proteins, and transcription factors. Some are asymmetrically expressed at the level of mRNA or protein, whereas others appear to have no asymmetry with respect to their localization.

Conceptually, LR patterning is divided into three phases. In the final phase, individual organs utilize cell migration, differential proliferation, and other mechanisms to achieve asymmetries in their location or morphogenesis. Upstream of these processes lies a pathway of asymmetric genes: genes which are expressed in cell fields only on one side of the embryonic midline, and which propagate signals that dictate sidedness for the organs undergoing asymmetric morphogenesis. These cascades of asymmetric gene expression form the middle phase of LR patterning. However, for whichever asymmetric gene is at the top of the pathway, it is necessary to ask what determines 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 (Brown and Wolpert 1990).

The developmental timing of each phase differs among species, although asymmetric gene expression almost always

Table 2. *Asymmetrically expressed genes that have not been the focus of a LR paper*

Gene	Species	Product/Role	Side	Reference
HNF-3β	chick, mouse	winged-helix transcription factor	left	Levin et al. (1995); Collignon et al. (1996)
cWnt-8C	chick	wnt-family signaling molecule	right	Levin (1998b); Pagan-Westphal and Tabin (1998)
hLAMP-1[a]	chick	extracellular matrix molecule	left	Smith et al. (1997)
JB3[a]	chick	extracellular matrix molecule	right	Wunsch et al. (1994); Smith et al. (1997)
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)

Bold entries indicate genes expressed during gastrulation.
[a]Antibody epitopes.

Table 3. *Genes involved in LR patterning that are not asymmetrically expressed*

Gene	Species	Product/Role	Reference
Iv	mouse	dynein (cytoplasmic transport or ciliary motor)	Lowe et al. (1996); Supp et al. (1997, 1999, 2000)
Inv	mouse	?	Mochizuki et al. (1998); Morgan et al. (1998, 2002)
Vg-1	frog	TGF-β-family signaling molecule	Hyatt et al. (1996); Hyatt and Yost (1998)
Connexins	frog, chick, human	system of gap-junctional cell–cell signaling	Britz-Cunningham et al. (1995); Levin and Mercola (1998b); Levin and Mercola (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. (1999)
Polaris	mouse	?	Murcia et al. (2000)
HFH-4	mouse	transcription factor	Chen et al. (1998); Brody et al. (2000)
Lin-12	*C. elegans*	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. (2003)
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	mouse	TGF-β-family signaling molecule	Rankin et al. (2000)
Lrd	mouse	Dynein	Supp et al. (1997, 1999)
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	mouse, fish	extracellular receptor	Yan et al. (1999)
Furin	mouse	pro-protein convertase	Roebroek et al. (1998); Constam and Robertson (2000)
Brachyury	mouse	transcription factor	King et al. (1998)
Ednrb	mouse	piebald deletion complex	Welsh and O'Brien (2000)
Rotatin	mouse	transmembrane protein	Faisst et al. (2002)
PDI-P5	zebrafish	protein disulfide isomerase	Hoshijima et al. (2002)
Pol-1	mouse	DNA polymerase	Kobayashi et al. (2002)
PA26	human	sestrin-family	Peeters et al. (2003)
Cryptic	mouse, human, zebrafish	EGF-CFC gene	Gaio et al. (1999); Yan et al. (1999); Bamford et al. (2000)

begins at or shortly after gastrulation. The LR axis is probably specified after the anterior–posterior (AP) and dorsoventral (DV) axes, and is determined with respect to them (McCain and McClay 1994; Danos and Yost 1995). The timing of the initiation of LR asymmetry is particularly controversial. In the following text, we review the most important data on mechanisms of asymmetry elucidated in a number of model systems.

FISH

Flatfishes acquire a profound asymmetry in eye location (and scale/skin pigmentation) during metamorphosis from bilaterally symmetric larvae (Matsumoto and Seikai 1992; Okada et al. 2001; Hashimoto et al. 2002). Analysis of mutants in the zebrafish embryo has identified a number of loci which, when altered, cause aberrant LR patterning (Yost 1998), although some of these are likely to represent secondary LR effects of disrupted notochord or AP/DV patterning. In zebrafish, asymmetric markers such as *lefty*, *nodal*, and *pitx2* exhibit well-conserved asymmetric expression during neurulation and somitogenesis (Cheng et al. 2000; Essner et al. 2000; Liang et al. 2000). Unfortunately, almost nothing is known about early, upstream mechanisms in this model system.

FROGS

Embryos of the frog *Xenopus laevis* are analogous to the fish and chick with respect to a number of asymmetrically expressed left-sided genes (e.g., *Nodal*, *Lefty*, and *Pitx-2*) that function after neurulation (Levin 2004b). Although the mechanisms that process LR information during gastrulation in amphibia are largely unknown, the *Xenopus* embryo has allowed discovery of a number of mechanisms that underlie asymmetry at the earliest stages known in any species. Experiments in *Xenopus* were the first to suggest 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 axis, exhibited laterality defects, suggesting that the LR axis may be dependent on the transient microtubule array during the first cell cycle. The microtubule-associated motor proteins kinesin and dynein have been linked with LR asymmetry in mammals (see below). The appearance of LR asymmetry between fertilization and the first cell division is also consistent with the recent work on ion fluxes and the appearance of asymmetric mRNA and 14-3-3 protein localization during early cleavage (Levin et al. 2002; Bunney et al. 2003).

Syndecans

Localized perturbation of a small patch of extracellular matrix (ECM) by microsurgery, as well as global perturbation of the ECM by microinjection of Arg-Gly-Asp peptides or heparinase into the blastocoel, resulted in randomization of LR asymmetry. This work provided the first molecular entry point into LR asymmetry and suggested that the ECM participated in transfer of LR information in development. Inhibition of proteoglycan synthesis with the drug p-nitrophenyl-β-D-xylopyranoside prevents heart looping in *Xenopus* (Yost 1990). The sensitivity window was between stages 12 and 15—just after gastrulation.

On the basis of 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 the 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, suggesting that these two molecules function together during LR patterning at gastrulation.

Vg1 and the "Coordinator"

Another key finding in *Xenopus* was the discovery of an experimental perturbation that 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 can be interpreted as signifying that Vg1 normally acts in descendants of the L3 blastomere, which contribute to the left lateral plate mesoderm, 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, as it cannot be mimicked by Activin. Although 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. Confirmation of the role of endogenous Vg1 in this process remains uncertain

pending characterization of processed, endogenous Vg1 in early *Xenopus* embryos (and especially, asymmetries therein) (see Chapter 35).

Gap Junctional Communication

Gap junctions are channels connecting adjacent cells which allow the direct transfer of small molecule signals. The cell biology of gap junctions has been described in several excellent recent reviews (Falk 2000), and gap junctional flow is involved in a number of important patterning events in embryonic development and tumor progression (Lo 1996; Levin 2001). Briefly, the most frequently studied gap junction channel is formed by the assembly and docking of hexamers of proteins from the connexin family (one hexamer in each of two adjacent cell membranes). Functional gap junctional communication (GJC) is dependent on the existence of compatible hemichannels on the cells' surfaces, the permeability of the hemichannels to the substance, and the open status of the gap junction.

On the basis of a report that several unrelated patients with viscero-atrial heterotaxia contain potential mutations within *Connexin43* (Britz-Cunningham et al. 1995), and data from frog embryos that indicated asymmetric patterns of GJC in early blastomeres (Guthrie 1984; Guthrie et al. 1988), Levin and Mercola (1998b) tested the hypothesis that gap junctional paths are a mechanism by which LR information is 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 previous observation using a 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 *Nodal* expression in the absence of other developmental defects (Levin and Mercola 1998b).

These results indicated 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 and is upstream of asymmetric XNR-1 and heart tube looping. These data have 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 1998b) 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 in conventional ways. Similar data were later obtained in the chick embryo (see below). The identities of the putative low-molecular-weight determinants remain unknown.

Ion Flux

One key aspect of the GJC model is that the net junctional flow must be unidirectional in order to derive a LR asymmetry from the existing DV difference in GJC. Hypothesizing that a voltage difference might provide an electromotive force which can be used to electrophorese charged molecules in preferred directions through GJC paths, Levin et al. (2002) tested the model that ion fluxes (needed to generate the standing voltage gradients) might 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. 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 2 hours postfertilization. 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 constructs 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 during 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.

CHICK

The first morphological asymmetry in the chick embryo is a subtle tilt of Hensen's node toward the end of gastrulation (Kölliker 1879; Hertwig 1902; Dathe et al. 2002). The first obvious sign of asymmetry is the looping of the heart tube, which has been shown to be determined during gastrulation

in transplantation experiments (Hoyle et al. 1992). The chick was the first system in which asymmetric gene expression was demonstrated, and this organism provides the most detailed picture of left/right mechanisms functioning during gastrulation.

Asymmetric Genes

Characterizing the expression of 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 et al. 1995, 1997; Levin 1998a). *Sonic Hedgehog* (*Shh*) encodes a signaling molecule that is also involved in patterning of the limb and the neural tube (Capdevila and Johnson 2000) and is expressed symmetrically within the ectoderm of Hensen's node (the chick organizer; see Chapters 15 and 29) before stage 4, at which time it becomes restricted to the left side of the node (Fig. 1A). This is followed at stage 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 (Fig. 1B). 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* (Levin et al. 1995). Although right-sided asymmetric expression of *Activin βB* has been reported in the early chick streak (Levin et al. 1997; Levin 1998a), Act-RIIa is now thought more likely to be a receptor for Nodal-related ligands than Activin (see Chapter 35); thus, the details of these interactions remain to be elucidated, and it is still unknown whether cAct-RIIa itself plays a causal role in LR patterning.

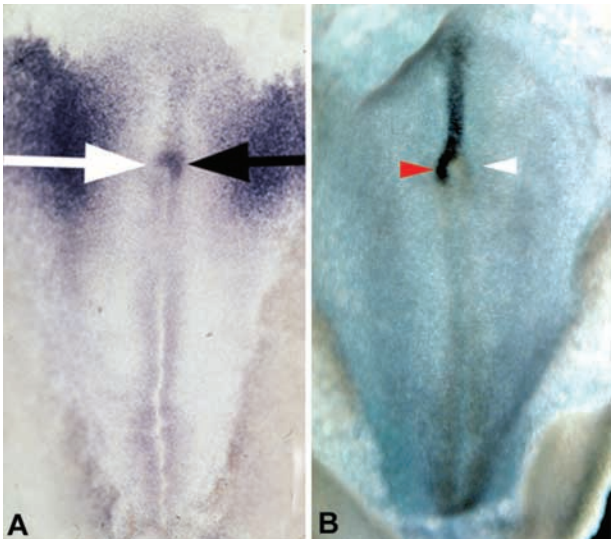

Figure 1. Asymmetric gene expression in chick embryos. During gastrulation, a number of genes are asymmetrically expressed. Two of the best characterized are *Activin Receptor 2a* (*cAct-RIIa*) on the right side of Hensen's node (*A*), and *Sonic Hedgehog* (*Shh*) on the left (*B*). (Reprinted, with permission, from Levin et al. 1995 [© Elsevier].)

Many more asymmetric genes have been identified in chick embryos (Levin 1998b); these factors participate in cascades of induction and repression of asymmetric gene pathways taking place on the left and right of the midline (see Tables 1 and 2). The signaling molecules functioning during gastrulation dictate heart and gut situs as well as embryonic turning through control of the expression of the highly conserved left-sided *Nodal*.

Gap Junctional Communication

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? Interestingly, 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, GJC was examined in the chick embryo 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.

Similar 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). 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. Although 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 appears to depend on an uninterrupted contiguous region of GJC around a small zone of junctional isolation.

An essential feature of the GJC model in both *Xenopus* and chick is circumferential GJC around a zone of junctional insulation (the streak in chick and the ventral midline in *Xenopus*). Although consistent with the idea that the epiblast influences node asymmetry, this set of findings 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). 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.

Ion Flux

Because the GJC system has been shown to be conserved to both chick and *Xenopus*, Levin et al. tested whether embryonic laterality was dependent on ion flux in the chick as well (Levin et al. 2002). Analysis of the chick embryo using an in vivo membrane voltage reporter dye (Fig. 2A) indicated the existence of a consistently biased depolarization of cells on one side of the early primitive streak (*prior* to the formation of Hensen's node). This indicates that the chick embryo has assigned L and R identities by stage 3^{-}—prior to the earliest known asymmetric gene. Similar to the data in *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, whereas 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 observed in the chick at the level of mRNA. This echoes a theme that highlights an important difference between species. Although both chick and *Xenopus* appear to use GJC and ion flux to pattern the LR axis, there are differences in how this mechanism is regulated. The dorsoventral difference in GJC in frog embryos takes place posttranslationally (by gating control of existing gap junctions). In contrast, the chick embryo seems to establish the zone of isolation at the level of mRNA (by not transcribing *Cx43* mRNA in the primitive streak). Similarly, whereas 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).

The most interesting 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. What are the *first* asymmetrically expressed genes on the left and right sides in the chick embryo? Some of the details of this process have recently been provided by a study which showed 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). Does asymmetric gene expression begin prior to gastrulation? It has previously been suggested (Levin and Mercola 1998a) 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, the molecular mechanism of this process cannot be elucidated until we have a good understanding of how (and whether) individual cells in the chick blastoderm have an anterior–posterior polarity.

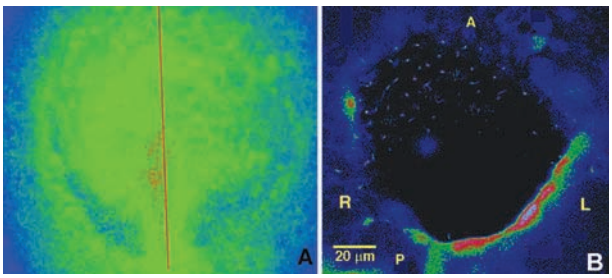

Figure 2. Asymmetric ion flux during gastrulation. A voltage-sensitive fluorescent dye allowed in vivo detection of an endogenous asymmetry in the steady-state membrane voltage levels of cells on the left and right sides of the early primitive streak (*A, red line* indicates midline of streak). The left side of the streak is depolarized with respect to the right. (*B*) In the mature node of the mouse embryo, an asymmetric Ca^{++} signal is detected. (*A*, Reprinted, with permission, from Levin et al. 2002; *B*, reprinted, with permission, from McGrath and Brueckner 2003 [© Elsevier].)

MAMMALS

Errors of LR patterning during embryogenesis are relevant to the clinical considerations of several fairly common human birth defects: syndromes including 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). Laterality defects can arise in a single individual (Winer-Muram 1995; Kosaki and Casey 1998), but are especially associated with monozygotic twinning (see below).

One crucial question in 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 without affecting normal development. This has been suggested to signify that the patterning of axes in mammalian embryos takes place later than in 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). Although the extent of LR patterning (if any) during early cell divisions in mammals remains unknown, recent findings in mammalian embryos have shed light on processes that may generate or transmit LR information.

Cilia

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 (Singh et al. 1991; Schreiner et al. 1993; Lowe et al. 1996), encodes a dynein called Left-Right Dynein (LRD) that is expressed in cells of the mouse node (Supp et al. 1997). Axonemal dynein is a component of the motor driving ciliary motion; the chirality of this motion is intrinsic to the protein components. Genetic deletions of KIF3-A or KIF3-B, two microtubule-dependent kinesin motor proteins, resulted in randomization of the situs of the viscera, and this finding is also often interpreted as evidence for a primary role for cilia in LR determination (Vogan and Tabin 1999). Most importantly, following the first observation of cilia in the murine node (Sulik et al. 1994), elegant experiments have revealed a clockwise rotation of monocilia extending ventral to the node that produces a localized net right-to-left fluid flow of fluorescent beads placed in the extraembryonic space (Nonaka et al. 1998; Marszalek et al. 1999; Okada et al. 1999; Takeda et al. 1999). 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 (Nonaka et al. 1998; 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 certain LR mutants (Tabin and Vogan 2003). In addition to kinesin and dynein, a number of other proteins have also been linked to asymmetry that has 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 (Pennekamp et al. 2002).

The strongest version of this model (McGrath and Brueckner 2003) hypothesizes that LR asymmetry is initiated by the motion of the cilia in the mature node (toward the end of gastrulation). Consistent with this idea, no upstream LR mechanisms have yet been described in rodents although the rodent embryo is unusual in its architecture, compared to more typical mammalian embryos (such as rabbit and human). Despite the existence of cilia in many organisms (Essner et al. 2002), no functional data implicate cilia in establishment of asymmetry in any organism other than rodents. Because 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 roles. Thus, whereas a function for motor proteins in LR patterning is fairly certain, the mechanisms by which they control laterality and the role of cilia in asymmetry remain controversial (Levin 2003, 2004a).

The earliest known endogenous LR mechanisms (Syndecans, GJC, H^+/K^+ flux, Vg1 coordinator) have not been found in mammals. No mouse mutants in gap junction genes have as yet reported a true LR phenotype. Since many different Connexin genes exist in mouse embryos, there is the potential for compensation during single-gene-deletion experiments, so knockins of dominant-negative constructs will be required to determine whether GJC plays a role in LR asymmetry of rodents. Significant 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 functional analysis of GJC in a mammal with a more prototypical flat gastrulation architecture is likely to shed significant light on the evolutionary conservation and origin of the GJC system as it participates in LR patterning.

However, ion flux has been implicated in mouse asymmetry. A genetic deletion experiment 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 (Fig. 2B) appears at the left margin of the node at the time of nodal flow (McGrath et al. 2003); this cytoplasmic Ca^{++} gradient may be related to the extracellular Ca^{++} flux recently demonstrated in the chick at gastrulation (Raya et al. 2004). Although it is still unknown whether flows of ions other than calcium play a role in rodents and other mammals, and whether Ca^{++} flow is important for LR patterning prior to mature node stages, future studies of the conservation of ion flow mechanisms among embryos with very different gastrulation modes (frog, chick, rabbit, rodents) are likely to teach us much about asymmetry and basic development.

Conjoined Twins

It is a long-known but puzzling fact that conjoined twins of armadillo (Newman 1916), fish (Morrill 1919), frog (Spemann and Falkenberg 1919), and man (Aird 1959; Burn 1991; Winer-Muram 1995) often exhibit alterations of situs in one of the twins. It has been proposed that an explanation for the laterality defects might be found in consideration of interactions between signaling molecules in two adjacent primitive streaks. Analysis of spontaneous twins of chick embryos (Levin et al. 1996) by in situ hybridization with probes to asymmetric signaling factors such as *Shh* and *Nodal* have given rise to two models that are predictive with respect to which classes of conjoined twins should exhibit laterality defects, and which twin should be affected. For example, parallel streaks during early gastrulation could result in the right-sided Activin of the left embryo inhibiting the expression of *Shh* in the left side of the right embryo. This would result in a normal left embryo, but the right embryo would have no expression of *Shh* in the node, leading to lack of *Nodal* expression and, ultimately, randomized morphological situs. These models have yet to be tested directly in mammalian embryos.

OPEN QUESTIONS AND EVOLUTIONARY PARADIGMS

Because no macroscopic force distinguishes right from left, 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 anterior–posterior and dorsoventral axes, and its chiral nature is thus able to nucleate asymmetric processes such as transport (Levin and Mercola 1998a). Thus, the first developmental event that 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 Nascone 1997; Levin and Mercola 1998a). Asymmetric gene expression in embryos requires that fairly large fields of cells know on which side of the midline they are located (e.g., the cells on the right side of the chick node express *Activin*, but those on the left side do not). 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 key question concerns how orientation 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, and cell–cell communication via gap junctions is a natural candidate for such a signal exchange (Levin and Nascone 1997). The extracellular matrix, membrane voltage, and Ca^{++} signaling are also likely to play a role in this process.

One crucial open question in the field concerns the conservation of the early members of the asymmetric gene cascade. The earliest asymmetric gene known in *Xenopus* is *Nodal*, which is detected at somite stages. None of the early genes known to be asymmetric during chick gastrulation (*Shh*, *cAct-RIIa*, *cHNF-3β*, *Follistatin, cWnt-8C,* etc.) has been reported to be asymmetric in *Xenopus* despite searches by a number of labs (Ekker et al. 1995; Stolow and Shi 1995). 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. It is possible that the asymmetry in expression exists but has not been detected; it may also be that in *Xenopus* the asymmetries in Hedgehog signaling exist at the level of protein, and not mRNA. 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. 1999), but no consistent asymmetric gene expression has been reported upstream of *Nodal* (although the Notch pathway is known to direct *Nodal* laterality in mice [Krebs et al. 2003; Raya et al. 2003]).

A difference in mechanisms upstream of *Nodal* may exist between chicks and *Xenopus*. Although in chick embryos, the default state is lack of *Nodal* expression (Shh signaling is required to induce *Nodal* transcription on the left side [Levin et al. 1995]), it was reported that explants of right lateral mesoderm from *Xenopus* embryos turn on *XNR-1* expression (Lohr et al. 1997), arguing for an endogenous repressive influence from the midline. However, it was later demonstrated that explanted lateral

tissue induces ectopic notochord-like structures containing Shh (in both frog and chick embryos), suggesting that an inductive pathway upstream of *Nodal* may actually be conserved in both species. Regardless of the details of this possible difference between chick and frog embryos, other asymmetric factors definitely exhibit reversed laterality among species. Asymmetry of *FGF-8* is opposite in chicks versus mice, as are some downstream events such as asymmetry of *Nkx3.2* expression (Meyers and Martin 1999; Schneider et al. 1999).

Vertebrates thus initiate left/right asymmetry by various mechanisms that all, nonetheless, converge on the apparently invariant mechanism of left-sided Nodal signaling. In other words, Nodal may be a "stable point" in the establishment of pattern along the vertebrate left/right axis, whereas the pathways leading to that expression pattern have been free to diverge. This may seem an unlikely result; however, there are other examples of apparent stable points reached by different pathways. One example is the three-layered embryo created by the wide variety of gastrulation movements: The generation of three germ layers is a given, but the suites of movements that result in that organization vary significantly. It is as if stabilizing selection acted on a midpoint, but the means to that midpoint were free to evolve, as were the subsequent events. Of course, each pattern of gastrulation has become important; that is, the characteristic magnitude, direction, and rate of the movements are necessary for normal development of any given species. It is, nonetheless, interesting to speculate as to why having three distinct layers is the sine qua non of this stage of development.

Another well-known example of stable points is the pharyngula stage of vertebrate development (Collazo 2000). Despite very different patterns of cleavage and gastrulation, all vertebrates pass through what Gilbert has called a "bottleneck" in the period following neurulation during which diverse species have a very similar appearance regardless of the mechanisms by which they achieved that appearance (Gilbert 2000). This similarity is at the root of von Baer's principles. Raff has suggested that the pharyngula stage is less able to evolve (i.e., is more stable) because only at that stage in development are there whole-embryo-scale inductive events, and thus a need for a whole-embryo-scale geometry that puts inducing and induced tissues in the correct relative orientation. Prior to gastrulation, there are few inductive events; after early organogenesis, induction occurs, but on a localized scale (Raff 1994). The pharyngula stage of vertebrates is particularly interesting in the context of left/right asymmetry, because universal left-sided *Nodal* expression overlaps with the pharyngula stage. It is tempting to ask whether the stability of *Nodal* laterality is linked to the morphological stability of this stage in development. It does seem to be an example of a molecular bottleneck.

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