Left-right asymmetry in vertebrate embryogenesis

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Summary

Embryonic development results in animals whose body plans exhibit a variety of symmetry types. While significant progress has been made in understanding the molecular events underlying the early specification of the antero-posterior and dorso-ventral axes, little information has been available regarding the basis for left-right (LR) differences in animal morphogenesis. Recently however, important advances have been made in uncovering the molecular mechanisms responsible for LR patterning. A number of genes (including well-known signaling molecules such as *Sonic hedgehog* and *activin*) are asymmetrically expressed in early chick embryos, well before the appearance of morphological asymmetries. One of these, *nodal*, is asymmetrically expressed in frogs and mice as well, and its expression is altered in mouse mutants exhibiting defects in laterality. In the chick, these genes regulate each other in a sequential cascade, which independently determines the situs of the heart and other organs.

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Introduction

The geometrical invariance known as symmetry is a striking feature of developmental morphology during embryogenesis. There are several types, such as translational symmetry (resulting in repeated units, such as embryonic somites or millipede segments) and reflectional symmetry (resulting from two or more sections of an organism looking the same to some level of detail on either side of a symmetry line). Animal body plans occur in a wide variety of symmetries: spherical (volvox), radial (starfish), chiral (snails, ciliates), bilateral (drosophila) and pseudo-bilateral (man). Vertebrates have a generally bilaterally symmetrical body plan, but this symmetry is broken further into a pseudo-symmetry by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen and gut, or an asymmetric development of paired organs (such as brain hemispheres or 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. In contrast, the sea-urchin develops from a bilaterally symmetric larva into an adult with a fivefold radial symmetry.

Arguably, the most interesting asymmetry in vertebrate development is that along the left-right (LR) axis. In this discussion, I limit LR asymmetry to include only invariant (i.e. consistent among all normal individuals of a given type) differences between the left and right sides of an animal's morphology. This specifically excludes pseudo-random characteristics such as coat colors, and minor stochastic deviations due to developmental noise. Thus, to count as true LR asymmetry, a feature has to appear consistently in all normal individuals. Here, I also purposefully neglect behavioral/sensory asymmetries (such as lobster claws, which are determined by neurological activity) and morphological/structural brain lateralization phenomena (reviewed in ref. 1). I avoid these issues because they are likely to be secondary (developmentally) to basic body situs; also, they are confounded by environmental influences, making them less tractable.

The LR axis itself follows automatically from the definition of the antero-posteroir (AP) and dorso-ventral (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 obvious macroscopic aspect of nature differentiates left from right. One possible way to use a fundamental force to orientate the LR axis relative to the other two axes was suggested by Huxley and deBeer[2]. 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 pointing right or left, if measured at the

dorsal or ventral sides. There is, however, no good evidence for such a mechanism.

Secondly, all normal members of a given species are asymmetrical in the same direction. However, animals with complete mirror reversal of internal organs can arise (*situs inversus*) and are otherwise phenotypically unimpaired. Thus, while it is possible to come up with plausible evolutionary reasons for why organisms might be asymmetric in the first place (optimal packing of viscera, etc.), 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 asymmetry (such as positive-feedback and amplification of stochastic biochemical differences) than for biasing it to a given direction. The left-right axis thus presents several unique and deeply interesting theoretical issues.

Besides the intrinsic interest to those working on fundamental morphogenetic mechanisms, LR asymmetry is also relevant to medical considerations of several fairly common human birth defects: syndromes such as Kartagener's and Ivemark's[3], dextrocardia, *situs inversus* (a complete mirror-image reversal of the sidedness of asymmetrically positioned organs and asymmetric paired organs), heterotaxia (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). Of these, only the complete (and rare) *situs inversus* is not associated with physiological difficulties. The rest, especially heterotaxia, often result in serious health problems for the patient. Laterality defects can arise in a single individual but are especially associated with monozygotic twinning[4-5].

Pre-molecular data

While molecular mechanisms underlying antero-posterior and dorso-ventral asymmetry have been studied in detail, the mechanistic basis for LR asymmetry was, until recently, completely unknown. The bilateral body plan is thought to have originated with the eumetazoa, the evolution of anterior-posterior body axis, unidirectional movement and cephalization probably occurring together, followed closely by the triploblastic architecture of the body plans[6]. The LR axis is probably specified after the anterior-posterior (AP) and dorso-ventral (DV) axes, and is determined with respect to them[7-8]. Echinoderms possess well-defined LR asymmetries, and *amphioxus*, considered to be the ancestor of vertebrates, exhibits many LR asymmetries[9]. Currently, several morphological markers of LR asymmetry are apparent in vertebrates: heart, direction of embryo rotation, gut, liver, lungs, etc. The organs possessing asymmetries, as well as the direction of their asymmetry, are evolutionarily well conserved. The heart is asymmetrically located in the molluscs[10]; the situs of the stomach and the liver[11] is the same among fish, reptiles, birds and mammals.

Neville[12] presents an extensive and fascinating survey of various animal asymmetries. Besides the above-mentioned internal organs, beetles consistently fold one wing under the other, many crustaceans have specialized right and left fore-limbs, some flatfish consistently settle on and undergo eye migration to one side, and there is even a species of parasite (the arthropod *Bopyrus*) which lives only on one side of host prawn and shrimp. Meanwhile, there has been little information shedding light on the mechanisms determining the sidedness of the asymmetries. Selection for LR asymmetries in *Drosophila*, in the hopes of generating a genetically tractable mutant, failed[13].

Several experiments have shed light on the timing of LR asymmetry specification. Chick heart sidedness has been experimentally demonstrated to be determined during gastrulation[14]; studies on LR inversions induced by drugs likewise suggest that in mammals, a critical period in LR biasing occurs before late gastrulation[15].

Several kinds of molluscs undergo spiral cleavage and secrete an exoskeleton shaped like a conical spiral. In 3-D 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[16] found that the direction of coiling of *P. suturalis* is maternally inherited and sinistrality is dominant to dextrality. Freeman and Lundelius[17], studying a different species, found that dextrality is dominant; interestingly, the dextral gene apparently functions *via* a cytoplasmic product since it is possible to transfer (by micropipette) cytoplasm from the dextral variant of the snail into the sinistral variety, and thus rescue the phenotype. The biochemical nature of this activity has not yet been identified.

There are a variety of drugs which, within defined dose windows, cause defects in a LR-asymmetric manner or randomize asymmetry (Table 1). These form a basically unrelated group, which includes even such simple substances as cadmium. The drugs which cause worse limb defects on one side were suggested[18] 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[19-20], while cardiac anatomy and relative vessel size of both species are extremely similar (no known differences exist between rats and mice in this respect; W. Scott and M. Fujinaga, personal communication). This suggests a fundamental difference between left and right limbs. The pharmacology of these drugs has not yet suggested anything about the normal mechanisms of LR patterning, except that an adrenergic pathway may be involved[21].

Several mammalian mutants are known which display either defects in basic LR patterning or phenotypes which differentially affect the left or right sides of the body (Table 2). For example, *iv*[22] results in racemic offspring (50% being phenotypically *situs inversus*), while *inv*[23] mice have 100% of the offspring showing mirror image inversions of the internal organs. Mutants such as *legless*[24] exhibit limb phenotypes, which are more pronounced on one side of the body.

Table 1. *Drugs with effects relevant to LR asymmetry*

Substance	Type	Species	Phenotype	Reference
Cadmium	Element	Rat	Left limb deformities	19
Cadmium	Element	Mouse	Right limb deformities	20
Acetazolamide	Carbonic anhydrase inhibitor	Rat	Right limb deformities	a
MNNG	Alkylating agent	Mouse	Left ectodactyly	b
Acetoxymethyl-methylnitrosamine	Alkylating agent	Mouse	Left limb deformities	c
Xyloside	Proteoglycan synthesis inhibitor	Frog	No cardiac looping	d
Nitrous oxide	Anesthetic	Rat	*Situs inversus viscerum*	e
Retinoic acid	Teratogen	Hamster	*Situs inversus*	f
Phenylephrine	Adrenergic agonist	Rat	*Situs inversus viscerum*	g
Methoxamine	Adrenergic agonist	Rat	*Situs inversus* and heterotaxia	h
Staurosporine	PKC inhibitor	Rat	*Situs inversus*	i
Lidocaine	Local anesthetic	Rat	*Situs inversus*	j
Nitrofurazone	Anti-microbial agent	Rat	Right-sided hypoplasia	k
RGD polypeptides	Blocks ECM attachment	Frog	*Situs inversus viscerum*	l

a, Layton and Hallesy (1965), *Science* **149**, 306-308; b, Inouye and Murakami (1978), *Teratology* **18**, 263-268; c, Bochert *et al.* (1985), *Arch. Toxicol.* **56**, 139-155; d, Yost (1990), *Development* **110**, 865-874; e, Fujinaga *et al.* (1990), *Teratology* **41**, 131-135; f, Shenefelt (1972), *Teratology* **5**, 103-118; g, Fujinaga and Baden (1991), *Dev. Biol.* **143**, 203-205; h, McCarthy *et al.* (1990), *Teratology* **42**, 33A; i, Fujinaga and Baden (1993), *Teratology* **47**, 419; j, Fujinaga *et al.* (1993), *Teratology* **47**, 418; k, Greenaway *et al.* (1986), *Toxicol. Appl. Pharmacol.* **82**, 307-315; l, Yost (1992), *Nature* **357**, 158-161.

In crosses with *iv*, the side affected is shown to reverse with the organ situs. As none of the genes responsible for these phenotypes have been cloned yet, studies of these mutants have indicated only that there is a genetic (and more specifically, zygotic) component to LR biasing in development.

Asymmetric gene expression

Any mechanism for generating consistently biased LR asymmetry is likely to involve differential gene expression. Interestingly, Randy Johnson observed that *Sonic hedgehog* (a limb patterning gene[25]) was expressed asymmetrically in the gastrulating chick embryo. Likewise, activin receptor *cAct-RIIa* was found to be asymmetric slightly earlier (Claudio Stern, personal communication). Guided by these findings and experiments on the timing of LR asymmetry specification[14-15], Levin *et al.*[26] (M. Levin *et al.*, manuscript submitted) screened developmentally important genes for their expression during the equivalent period of chick development (stages 3+ to 7). While most genes have expression patterns which are symmetric about the LR axis (e.g. Fig. 1A), several important signaling molecules (see Table 3) show consistently asymmetric patterns of mRNA expression in the chick. *Activin βB* (Fig. 1B) and *cAct-RIIa* (Fig. 1C) are right-sided, while *Sonic hedgehog* (Fig. 1D), *HNF3*-β (Fig. 1E) and *nodal* (Fig. 1F) are left-sided, in and around Hensen's node. *Activin* and *nodal* are members of the TGF-β family and thus encode secreted signaling molecules. Another TGF-β family member, *Lefty* (asymmetrically expressed in the mouse nervous system[27]), as well as *cWnt-8C, PTC* and *follistatin* (Fig. 1G,H,I, respectively), are also asymmetrically expressed.

A LR-patterning cascade

The identification of a set of asymmetrically expressed genes made it possible to formulate and test hypotheses about regulatory interactions among these genes, based on the spatio-temporal patterns of their expression (summarized in Fig. 2A). For example, earlier-expressed genes could be misexpressed on the opposite side, to determine

Table 2. *Mutants with LR patterning defects*

Name	Species	Phenotype	Reference
Mgat1 k.o.	Mouse	Randomized turning, and heart	a
ft	Mouse	Randomized turning, wild type heart	b
inv	Mouse	100% of offspring have *situs inversus viscerum*	23
iv	Mouse	50% of offspring have *situs inversus viscerum*	22
Legless	Mouse	Right limb defects	c
Heterotaxia	Man	Independent situs of internal organs	54
Dh	Mouse	*Situs inversus viscerum*	d
Hyd	Rat	*Situs inversus viscerum*	e
py	Mouse	Right limb defects	f
Roller	*C. elegans*	Left or right twisted helical morphology	g
glp-1(e2072)	*C. elegans*	Almost true isomerism	57

a, Metzler *et al.* (1994), *EMBO J.* **13**, 2056-2065; b, van der Hoeven *et al.* (1994), *Development* **120**, 2601-2607; c, Singh *et al.* (1991), *Genes Dev.* **5**, 2245-2255; d, Biddle *et al.* (1991), *Teratology,* **44**, 675-683; e, Torikata *et al.* (1991), *Am. J. Pathol.* **138**, 341-347; f, Kochar and Bocher-Becker (1980), *Teratology of the Limbs* (ed. Merker *et al.*), pp. 259-272; g, Higgins *et al.* (1977), *Mol. Gen. Genet.* **150**, 63-72.

Table 3. *Genes expressed in embryos*

Gene	Product/Role	Sidedness	References
cNot	Specifies notochord identity	Symmetric	a
Activin βB	A TGF-β-family signaling molecule	Right	b
cAct-RIIa	Activin receptor	Right	c
Shh	Signaling molecule	Left	25
HNF3-β	Winged-helix transcription factor	Left	d
nodal	TGF-β-family signaling molecule	Left	e
cWnt-8C	wnt-family member signaling molecule	Right	f
PTC	Receptor	Left	g
follistatin	Signaling molecule	Right	h

a, Stein and Kessel (1995), *Mech. Dev.* **49**, 37-48; b, Slack (1994), *Curr. Biol.* **4**, 116-126; c, Stern *et al.* (1995), *Dev. Biol.* **172**, 192-205; d, Weinstein *et al.* (1994), *Cell* **78**, 575-588; e, Conlon *et al.* (1994), *Development* **120**, 1919-1928; f, Hume and Dodd (1993), *Development* **119**, 1147-1160; g, Goodrich *et al.* (1996), *Genes Dev.* **10**, 301-312; h, Connoly *et al.* (1995), *Dev. Genet.* **17**, 65-77.

whether the expression of any of the later genes would change. If so, the earlier gene could then be hypothesized to normally function up-stream of the later one. Thus, using retroviral strategies and protein-coated beads to misexpress several of these genes in whole embryo culture, followed by *in situ* hybridization to assay for effects on downstream genes, a sequential pathway has been worked out[26] (M. Levin *et al.*, manuscript submitted).

This cascade (summarized in Fig. 2B) begins when *activin βB* becomes expressed on the right side of Hensen's node (stage 3). This soon induces the expression of *cAct-RIIa* in the right side, and shuts off the right-side expression of *Shh* (which was previously expressed throughout the node). Indeed, activin-coated beads implanted into the left side of the node are able to reproduce both effects there, and antagonizing the action of endogenous activin by applying follistatin beads results in bilateral *Shh* expression, strongly suggesting that activin functions *in vivo* to set up *Shh* asymmetry. By stage 6, *Shh* (which at that point is expressed only on the left side of the node and in the notochord) induces *nodal* in a small domain of cells adjacent to the left side of the node. This is soon followed by a much larger domain in the lateral plate mesoderm. That *nodal* is endogenously induced by *Shh* was demonstrated by showing that ectopic *Shh* applied to the right side is able to induce *nodal* in the right lateral plate mesoderm, and conversely, that removing left-sided *Shh* by early applications of activin to the left side of the node result in a lack of *nodal* expression.

The genetic cascade controls morphological asymmetry

Most importantly, the early asymmetrically expressed genes are not merely markers of inherent laterality, but play an active role in LR patterning. Misexpression of *activin* or *Shh* (which result in missing or bilateral *nodal* expression, respectively) specifically randomize heart situs in the chick[26]. Moreover, *nodal* (the most downstream member of the cascade), which is in direct contact with cardiac precursor cells, can reverse heart situs or cause symmetric hearts (M. Levin *et al.*, manuscript submitted). Thus, though there is no consensus on what causes cardiac looping in the first place, it is plausible that *nodal* is instructing heart looping by providing an asymmetric signal to one side of the cardiac primordia, and affecting the proliferation, migration or cytoskeletal organization of cardiac precursors.

The fact that morphologically normal hearts form in the absence of *Shh* and *nodal* expression (albeit with randomization of heart situs) indicates that the genes in this cascade are neither responsible for inducing heart formation nor for instructing its morphogenesis. Rather, they seem to act to provide a pivotal influence determining the handedness of the heart. Likewise, the finding that the morphology of the heart and embryonic development in general was not disturbed by the ectopic expression of such powerful inducing factors such as *Shh* and *activin*, suggests further that these molecules play specific roles in providing LR information to tissues and organs whose development in other

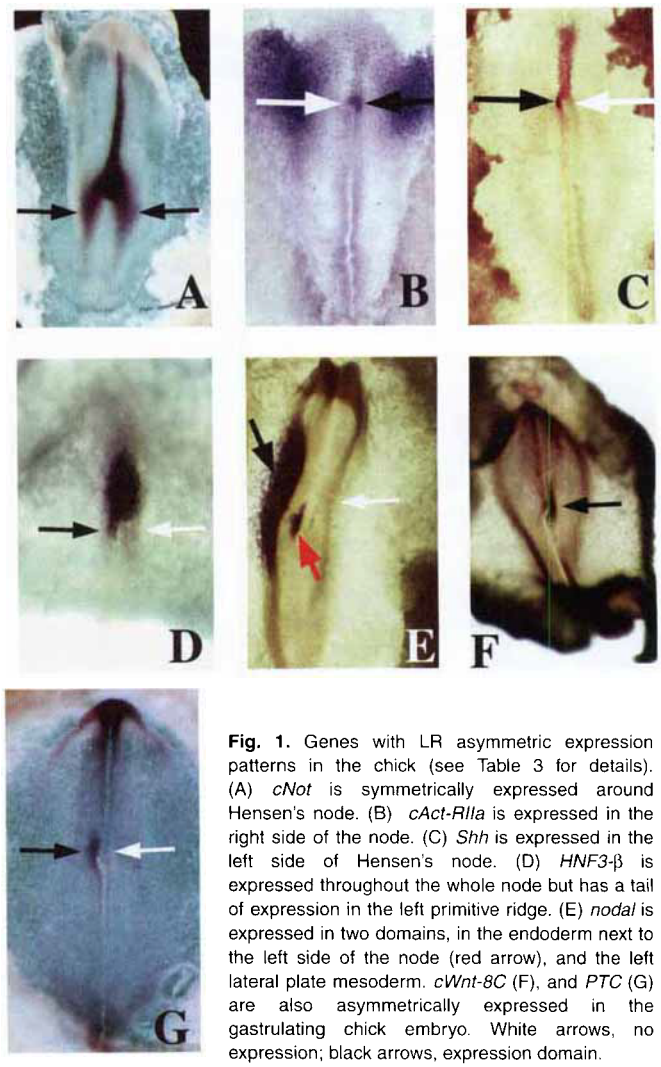

Fig. 1. Genes with LR asymmetric expression patterns in the chick (see Table 3 for details). (A) *cNot* is symmetrically expressed around Hensen's node. (B) *cAct-RIIa* is expressed in the right side of the node. (C) *Shh* is expressed in the left side of Hensen's node. (D) *HNF3-β* is expressed throughout the whole node but has a tail of expression in the left primitive ridge. (E) *nodal* is expressed in two domains, in the endoderm next to the left side of the node (red arrow), and the left lateral plate mesoderm. *cWnt-8C* (F), and *PTC* (G) are also asymmetrically expressed in the gastrulating chick embryo. White arrows, no expression; black arrows, expression domain.

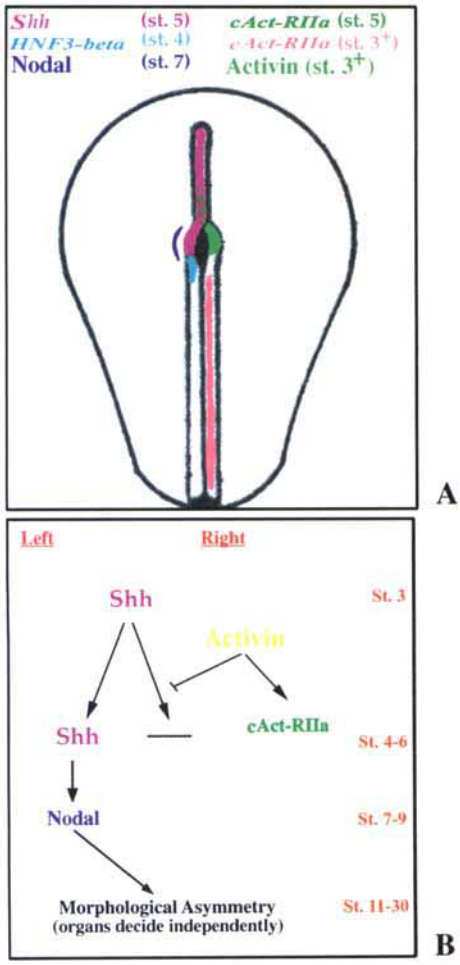

Fig. 2. A LR pathway. (A) Diagram of asymmetrically expressed genes in a gastrulating chick embryo. (B) Diagram of the regulatory interactions between known members of the LR pathway.

respects is regulated by other means. Finally, the exactly 50%/50% randomization of heart situs by most likely far-differing amounts of ectopic activin and Shh strongly suggest that looping is, at some point in the cascade, a binary decision, influenced by the presence or absence of a signal, not the relative amount of the signal between the two sides.

Interestingly, this pathway is not heart-specific, but also controls the laterality of the gut and embryonic rotation in chick. *A priori*, several things could happen when a left-sided factor such as *Shh* (for example) is misexpressed, resulting in symmetrical domains: all of the organs could make coordinated, albeit randomized, situs choice (*situs inversus*[22]), organs could make independent laterality decisions (heterotaxia), or isomerism could result, where the embryo would form symmetrically with respect to the LR axis. It was found (M. Levin *et al.*, manuscript submitted) that misexpression of Shh *in ovo* results in independent situs randomization of the various aspects of morphological asymmetry (thus producing a heterotaxia-like phenotype, contrary to the previous suggestion that heart situs sets the laterality of the other organs[28]. It is currently unknown whether the other organs derive their sidedness information from *nodal* expression, or whether other asymmetric molecules become induced by *Shh*, which then direct the situs of the other systems.

The pathway and existing mouse mutants

The availability of known genes which participate in LR asymmetry suggests attempts to place the mouse mutant genes into the chick LR pathway. Analyses of the *iv* and *inv* mutants indicate that they are both upstream of *nodal* and *lefty* in the LR pathway[27,29-30]. It was previously thought that the *iv* mutant would be simpler to explain than the *inv*, since it is easier to envisage a disruption of the biasing mechanism (which would result in randomization of situs, as seen in *iv*) than a 100% reversal of such biasing (as seen in *inv*). Two recent papers, however, have examined the expression of *nodal* in these mouse mutants, with some surprising results.

Lowe *et al.* find[29] that all $inv^{-/-}$ homozygotes have *nodal* expression on the right side. While it is still unclear how an insertion into the *inv* gene would reverse the LR biasing, its effects on the expression of the *nodal* gene represent a straightforward explanation for the observed subsequent inversions of internal situs. In contrast, $iv^{-/-}$ mice display roughly 25% incidences of all four possibilities with respect to *nodal* expression: no *nodal* expression at all, double-sided expression, right-sided expression and normal left-sided expression. This probably invalidates the initially plausible hypothesis that the *iv* mutation simply uncouples the biasing LR mechanism from the generation of asymmetry, because this would have resulted in equal numbers of offspring with purely left- and right-sided *nodal* expression.

The mechanism by which a defect in the *iv* gene could result in all four possibilities of *nodal* expression is unclear. One possibility is that *iv* directs the very early localization of LR determining factors, which are initially distributed randomly. If a positive-feedback or amplification process was used after the action of *iv* to sharpen the differences, a lack of *iv* activity could result in the amplification of random stochastic distributions and amounts of these determinants. It is then plausible that two left sides (double-*nodal*), two right sides (no *nodal*), or two sides of mixed identity (which would function as L or R, depending on which was dominant) would result. Many other models are possible, however (for example, that *iv* controls the directed migration of cells important in asymmetric induction of some gene; its disruption could cause random wandering and potentially result in all four types of *nodal* expression).

How conserved is the *Activin-Shh-nodal* pathway?

Morphological asymmetry is well conserved[9,11], but to what extent is the underlying pathway, as found in chicks, conserved in other species? Except for *nodal*, which is similarly

left-sided in mice and frogs[29], no genes have been reported to be consistently asymmetrically expressed in any species other than birds. Furthermore, several mice have been generated which are null mutants for *activin* and its receptors[31], but which appear to have no phenotype associated with LR patterning. However, the mutants had no phenotypes associated with mesoderm induction either, suggesting perhaps that the embryos were somehow compensating for the deletion or that maternal activin is involved[32]. Mice have also been generated carrying homozygous mutations in *Shh*, and they exhibit no laterality defects[33]. The activin receptor IIb knockout, however, does exhibit laterality defects (En Li, personal communication), suggesting that activin may likewise be involved in LR patterning in mammals.

It has been reported that no asymmetry in *Shh* expression can be detected in mice[30]. The mouse node is very small, and it is possible that in these species a transient asymmetric distribution of *Shh* exists for a very short time window and has been missed. Or, perhaps there is another member of the hedgehog family which fills *Shh*'s role in mice. Finally, it is possible that differential proteolytic processing of Shh protein may occur on the left and right sides of the mouse node. The conserved expression of *nodal* between several vertebrate species, the interactions between *nodal* and *HNF3-β*[30], and the finding that *nodal*'s expression is modified in mouse mutants exhibiting laterality defects[29,30] strongly suggests that *nodal*, at least, is a common point in LR patterning in vertebrates.

Laterality disturbances in twins and the midline barrier

The identification and characterization of several players in LR patterning has led to models explaining the finding that conjoined twins of armadillo[34], fish[35], frog[36] and man[3-4], often exhibit alterations of situs in one of the twins. As early as 1919, Spemann and Falkenberg[37] reported that producing conjoined twins by tying a hair between the two blastomeres of amphibian eggs results in *situs inversus*, usually in the right twin. Levin *et al.*[5] suggest that an explanation for the rather surprising right-twin bias in laterality defects might be found by considering interactions between signaling molecules in two closely aligned primitive streaks. Analysis of spontaneous twins by *in situ* hybridization supports such models[5]; these studies promise to provide an understanding of laterality defects in human conjoined twins[38,39].

The proposed models for the generation of laterality defects in conjoined twins require that signals such as *activin, Shh* and *nodal,* be able to cross considerable distances across the blastoderm (e.g. see ref. 40 for a discussion of distance in *Shh* signaling). In general, the gene cascade identified above suggests inductions over short and moderate distances, each limited to one side of Hensen's node, since *activin, Shh* and *nodal* can be expressed on one side of the node and have effects only on its own side of the embryo, without crossing over the very narrow width of the node and streak to affect the contralateral side. This suggests the presence of a barrier, possible candidates for which include the primitive pit and notochord. Consistent with this, removal of the notochord destabilizes LR asymmetry[41]. Likewise, the *flh* ('floating head') and *ntl* ('no tail') mutants have notochord defects that are often accompanied by cardiac inversions[42,43]. Moreover, Klessinger and Christ[44] show that the notochord is a LR barrier to endothelial cells in the chick.

Collignon *et al.* report[30] an interesting interaction of *nodal* with *HNF3-β* in controlling LR asymmetry. Mice containing a lac-Z reporter construct driven by the normal control sequences of one of the *nodal* alleles (*nodal*$^{lac-Z/+}$) are phenotypically normal, and display lac-Z staining which basically recapitulates normal *nodal* expression. In crosses, however, mice which were homozygous mutant for *HNF3-β* displayed no lac-Z staining at all. This may be due to lack of node development in *HNF3-β*$^{-/-}$ mice[45], which is the site of *nodal* induction by *Shh* in the chick. Even more interestingly, *HNF3-β*$^{+/-}$×*nodal*$^{lac-Z/+}$ mice show double-sided lac-Z signal and random embryonic rotation (as well as defects in positioning of abdominal viscera and heart). This may be due to the *HNF3-β* heterozygotes having a node which is sufficient to support *nodal* induction, but lacking a sufficiently developed notochord, which would otherwise act as a barrier to separate the L and R inductive signals.

While the notochord may serve this LR compartmentalization function in frogs or fish, and may be a barrier to the diffusion of the nodal protein in chick, it is clearly not able to separate the early asymmetric signals in the chick node. Whatever these structures are, they may serve to maintain LR compartments that are defined by the expression of various asymmetric genes.

Remaining puzzles

The identification of a cascade of asymmetrically expressed genes which play a role in LR patterning of a vertebrate embryo makes possible significant progress in this field. The genetic pathway characterized in these experiments, however, represents roughly the 'middle' third of LR patterning. In stage 1, a currently unknown mechanism is able to pick out a L (or R) side relative to the other two axes. Stage 2 represents the cascade of differential gene expression which can result from some activity in Stage 1. Stage 3 involves the interactions between the asymmetric expression domains and the various asymmetric organ primordia, whereby the individual organs read the LR information and pattern themselves accordingly. Several laboratories are currently working on identifying other asymmetric genes, working out the fine details of the regulatory interactions between known genes, and elucidating mechanisms by which organ primordia interact with such genes; thus, we are well on our way to understanding stages 2 and 3. The most fascinating questions, however, concern stage 1, and are still almost completely open.

Initial LR orientation

Firstly, however far backwards the gene cascade is followed, one must ask: whichever gene is asymmetrically expressed first, what is the cause of its asymmetry? *A priori*, there are three ways the asymmetric expression of the first asymmetric gene could be established. The asymmetry could be due to: a prepattern in the oocyte set up by the already asymmetric maternal organism (as in the AP axis in *Drosophila*), an external force (such as gravity for the DV axis) or a signal transduced by a chiral molecule. Based on the mammalian LR mutants, Brown and Wolpert[46] present an excellent theoretical analysis of the initial determination of LR asymmetry. They suggest that LR patterning occurs in three phases: (1) the generation of random asymmetry between L and R compartments; (2) the biasing of this asymmetry to a consistent direction with respect to the AP and DV axes; and (3) the interpretation of this information by the various organ primordia. In their initially cell-autonomous scheme, each cell contains a chiral molecule or structure, which, when properly anchored with respect to the other two axes, has some biochemical activity that always points to one direction (L or R). This activity is then transduced into differential gene expression on the R and L sides. This model has the problem of requiring that each cell know its orientation with respect to the other two axes. In the chick, this is plausible with respect to the DV axis (since the cells face the widely different environments of the yolky ventral side and the membrane-covered dorsal side), but it is harder to imagine how each cell knows which way is 'anterior', in order to properly orient the chiral molecule. The chiral molecule model is particularly attractive because many examples of chiral molecules are known, and because maternal prepattern models[47] are unlikely to be feasible in organisms such as mice, where a normal embryo can result after some serious disruption of morphology (*viz.*, allophenic mice). Evidence from twinning experiments[5,48] also argues against maternal prepattern, since it was shown that primitive streaks possess their own LR orientation, regardless of their position within the blastoderm.

In snails (which exhibit spiral cleavage), the biasing of LR asymmetry is known to be maternal, both from genetics, and from the fact that cleavage in one LR orientation or the other starts before the zygote transcribes its own genes[49]. Raven[50] suggests that this is due to localized structures in the egg cortex. There does not seem to be a cytoplasmic maternal determinant in mice, however, since *iv* eggs fertilized with wild-type sperm all result in normal embryos[51] (however, it is possible that *iv* is downstream of such a mechanism). In any case, it is unclear whether direct parallels between LR determination molluscs and mammals are likely to exist.

The fact that mirror-image individuals are at no apparent phenotypic disadvantage, while, within any species, normal individuals are always LR biased in one direction (and this direction of asymmetries is so well conserved), suggests that the source of LR asymmetry is very old, and perhaps basic to cell function. One (purely speculative) model of initial LR determination could involve a cytoskeletal component, such as a centriole, which is chiral. It is oriented with respect to the AP and DV axes of the egg by means of other cytoskeletal filaments, and serves as a nucleation center for filaments or microtubules which run along the LR axis. The head-tail attractive feature of microtubule assembly[52] ensures that the chiral nature of the nucleating center is passed on as a directionality of the LR tracks. Consistent with this model, the mouse egg has no centriole (one forms anew after several cell divisions), so that defects in the origin of chirality would show up as zygotic; in contrast, the maternal mode of inheritance of chirality in snails may be explained by the fact that the snail egg's cytoskeletal components are formed by the mother.

Next, a microtubule motor, such as dynein, would ride the LR tracks carrying mRNA or protein determinants, which become localized on one side of the cell. Dynein is a viable candidate for this function[51] because it is a motor protein, is expressed in early embryos[53], and has been shown to be defective in patients with heterotaxia as part of Kartagener's syndrome[54]. This model can also subsume the various LR mutations: heterotaxia could result from a broken dynein motor which is unable to perform localization of determinants (thus the LR-determining factors would homogeneously accumulate in both halves of a cell), the *inv* mutant could result from a nucleation center that becomes oriented in the opposite orientation, and the *iv* mutant could represent a nucleation structure that was not tethered at all (and thus would face in different directions in different cells; depending on stochastic events, this would result in a mosaic of domains of cells that were oriented properly, adjacent to cells that were not, and when magnified by cell proliferation and lineage relationships could thus easily account for the full spectrum of *nodal* expression patterns observed in the mutant mice[29]).

The initial basis of LR axis determination is somewhat better understood in *C. elegans*[55]. While the AP axis apparently follows asymmetries already present in the egg, the LR (and DV) axis arises from cell/cell interactions[56]. The AB blastomere divides at 90° to the AP axis; its daughter cells are mechanically shifted, due to the constricting action of the egg shell, and assume different positions with respect to the EMS and P2 cells, thus defining the LR axis. While Aba and ABp are equivalent at birth, their fates become different at the next division due to interactions between the MS cell and the AB daughters surrounding it[57]. This interaction is likely to involve *glp-1*, a homologue of the *notch* cell surface receptor[58], suggesting that over- or under-expression studies of the notch-delta pathway in vertebrates should pay particular attention to possible LR phenotypes. Two further LR asymmetries arise at the 24-cell stage, also by interaction with the MS lineage[59].

Generation versus *biasing of asymmetry*

A condition known as isomerism is occasionally observed in human patients[3]. This condition, sometimes called Ivemark

syndrome, where the organism is bilaterally symmetric (polysplenia when two left sides are present, or asplenia when both are right sides), is especially interesting because because the LR pathway as characterized to date provides no obvious clues as to how this might happen. For example, as shown by the *Shh* misexpression studies, producing bilateral *nodal* expression does not result in two morphologically identical left sides, but rather causes a heterotaxic phenotype where each organ decides its situs randomly (M. Levin *et al.*, manuscript submitted). Isomerism is likely to shed clues on a mechanism quite different from that in which the genes and mutants discussed above are involved: the generation of LR asymmetry, as opposed to its biasing in an appropriate direction[46,51].

One possible candidate for a role in this process is the gap junction gene *connexin-43* (*Cx43*). Several human patients with isomerism have also been reported to carry mutations in *Cx43*[60]. Mice carrying homozygous deletions in this gene likewise show cardiac malformations[61], although these are not true situs anomalies. The difference may be due to the fact that the mutant mouse carries a null mutation whereas the human patient does not, often showing a Ser364-Pro change, which may result in more subtle changes in regulation of *Cx43* conductive properties[62]. Another potential player in this process is *glp-1*, since mutations in it can remove almost all asymmetries in the *C. elegans* embryo[57].

From chiral molecules to cell fields

However a cell determines its left from its right, this cell-autonomous information must get transduced into asymmetric domains of gene expression on the scale of multi-cellular fields. This can happen in several ways. Morgan[63] suggests that a morphogen gradient does not need to be asymmetric, if cells that know their left from their right can measure its first derivative, or direction: if cells in the middle of the embryo are the source of some diffusible substance, then cells on either side of the midline will, when measuring differences in concentration across themselves, be able to tell whether they are on the right or left side by the direction of the gradient. This of course would require a very steep gradient. Alternatively, one could postulate a non-diffusible signal generated symmetrically by the midline cells, which, if adjacent cells had receptors only on their left sides (for example), would be perceived only by cells on the right side of the embryo. They could then propagate the signal to the rest of the cells on the right side.

Another intriguing hypothesis for how this might happen involves *Cx43*, localized distribution of which can create patterned ionic currents. The idea that endogenous electric fields specify large-scale embryonic pattern is quite old[64-67]. The distribution of communication channels in the early *Xenopus* embryo shown in Fig. 3[68] immediately suggests a model for LR asymmetry generation by gap junctions: that cells a and/or h (Fig. 3) are a battery, which generates a potential difference (due to asymmetric placement of ion pumps on the cell surface). Since the other cells appear to be connected to each other by gap junctions, they represent an open circuit with respect to the current generated by the cells at a. Thus, charged LR determinant molecules, able to fit through gap junctions, would experience a net electromotive force and would tend to electrophorese to different halves of the embryo. A similar electrophoretic mechanism for directing the movement of maternal components has been characterized in egg-ovary systems[69,70]; likewise, endogenous electric fields have also been shown to be involved in symmetry breaking in the *Fucus* embryo[71]. The finding that placement of amphibian embryos in applied electric fields results in reversals of LR asymmetry[72] is also consistent with this model, which represents one possibility by which asymmetry at the level of the cell (possibly generated by a dynein-like mechanism) becomes transformed into asymmetric fields of gene expression.

Evolutionary questions

Why are so many organisms asymmetric? The lack of any macroscopic feature of the world that is LR asymmetric would suggest that an organism might take the economical route of not having to contain information and developmental processes to specify left and right sides, and simply let the left and right halves develop identically, on their own. This is consistent with the observation that the degree of left-right symmetry can be used to gauge the genetic and developmental 'robustness' of an animal, both by ecologists[73] and by other animals (as in the role symmetry plays in the human judgment of facial beauty and in non-human mate choice[74].

Pansera[75] suggests that LR asymmetrical placement of certain organs allows more efficient packing of internal organs. Perhaps asymmetry provides physiological benefits (in the mechanics of the heart, for example). Given that individuals with full *situs inversus* seem to have no phenotypic disadvantage[4,23], however, it probably does not matter which direction the asymmetry points, as long as all of the organs are biased as a unit. So, why we are all asymmetric in the same direction? It would seem to be more economical to dispense with the biasing mechanism and have all animals show random symmetry. The *iv* mice, however, which instantiate this possibility, do have a non-trivial incidence of heterotaxia[76], and perhaps it is not possible to consistently avoid this without a biasing mechanism (though it should be noted that more *iv* pups with heart malformations have *situs solitus* than *situs inversus*[76],

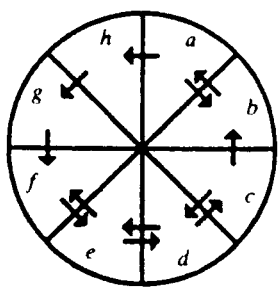

Fig. 3. Diagram (modified after ref. 68) of gap junctional communication in the early frog embryo.

suggesting that heart malformation is an additional effect of the *iv* mutation and is not a consequence of reversed situs itself). Alternatively, perhaps the LR bias comes from the chirality of a subcellular component (such as the cytoskeletal molecule element in the model described above). Thus, though changes in this mechanism are possible (cf. the *inv* mice), it is unlikely to be removed entirely by evolution, since it probably represents a very primitive component which is used for many other functions besides LR asymmetry biasing. Its chirality in turn may be a consequence of that of some enantiomer choice in biomolecular structure (handed amino acids, for example).

The issue of original chirality (i.e. why living organisms contain only L-amino acids and D-sugars) is also a very interesting one, and is bound up fundamentally with the origin of life. Pasteur[77] showed that *in vitro* synthesis invariably results in equal mixtures of enantiomer pairs of compounds, while biosynthetic processes were able to clearly separate such racemic mixtures. Several theories for this have been proposed. Perhaps, whatever type of isomer happened to have formed first biased the rest of evolution towards that type by competition[78]. The chirality of the first one could have been determined by chance, or by exogenous factors such as the Coriolis force, light[79] or even the geomagnetic field. Interestingly, the GMF seems to have a relationship with LR chirality[80]. The geological fossil record shows a clear correlation between flipping of the GMF polarity and reversals of the chirality of several types of molluscs such as *Globorotalia menardii*[81,82]. Thus, the determination of chirality may be one of the several roles the GMF probably plays in embryogenesis[83-87].

Alternatively, there may be a fundamental reason why biological forms prefer one type of molecule over its enantiomer. For example, Garay[88] has shown that when racemic mixtures of the amino acids alanine, tryptophan and tyrosine in alkaline solution are subjected to decomposition by radioactive decay of strontium-90, the D-isomers are destroyed more quickly than the L-isomer (see ref. 89 for a similar argument about sugars). There are also arguments[90-92] based on weak neutral currents, which show that the terrestrially dominant L-amino acids will predominate in a period of the order of 15,000 years. Thus, radioactive decay could plausibly have biased enantiomer choice in the pre-biotic environment. Likewise, the energy of the right-handed α-helix of poly-L-alanine is a few tenths of a kilocalorie per mole per residue lower than that of the left-handed helix, implying that over some length, the right-handed forms will be more stable[93]. Both asymmetries are presumably consequences of the non-conservation of parity in sub-atomic weak nuclear interactions[94].

Conclusion

LR asymmetry research is currently at a very exciting place, since important advances have been made, which point directly to feasible new approaches. Among these are characterizing the mouse mutations, elucidation of the nature of the midline barrier separating L and R compartments, and examination of possible novel players such as *Cx43* and dynein. LR asymmetry is a problem that will provide everyone from biophysicists to evolutionary biologists with deep and important lines of research, both theoretical and experimental. Its developmental mechanisms and ecological ramifications promise to shed light on the most fundamental issues of molecular embryology.

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