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Twinning and Embryonic Left-Right Asymmetry

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Figure 1

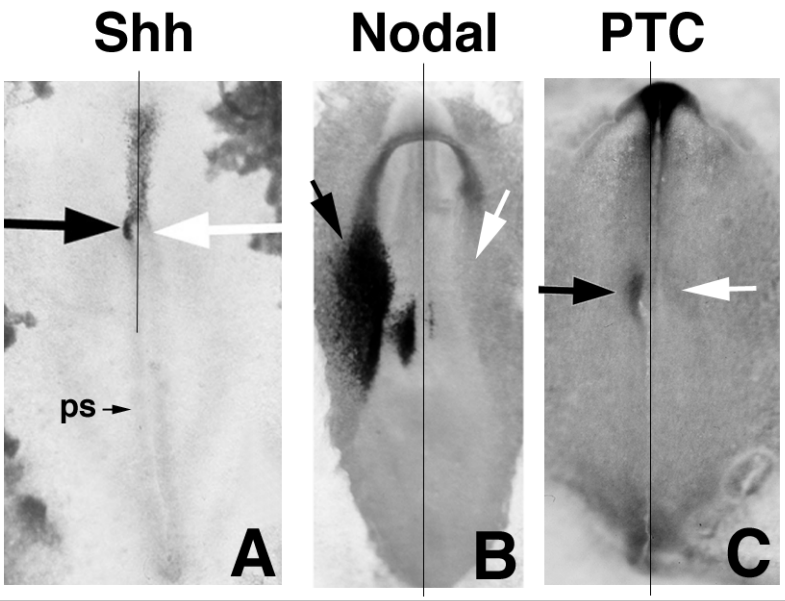


Figure 2

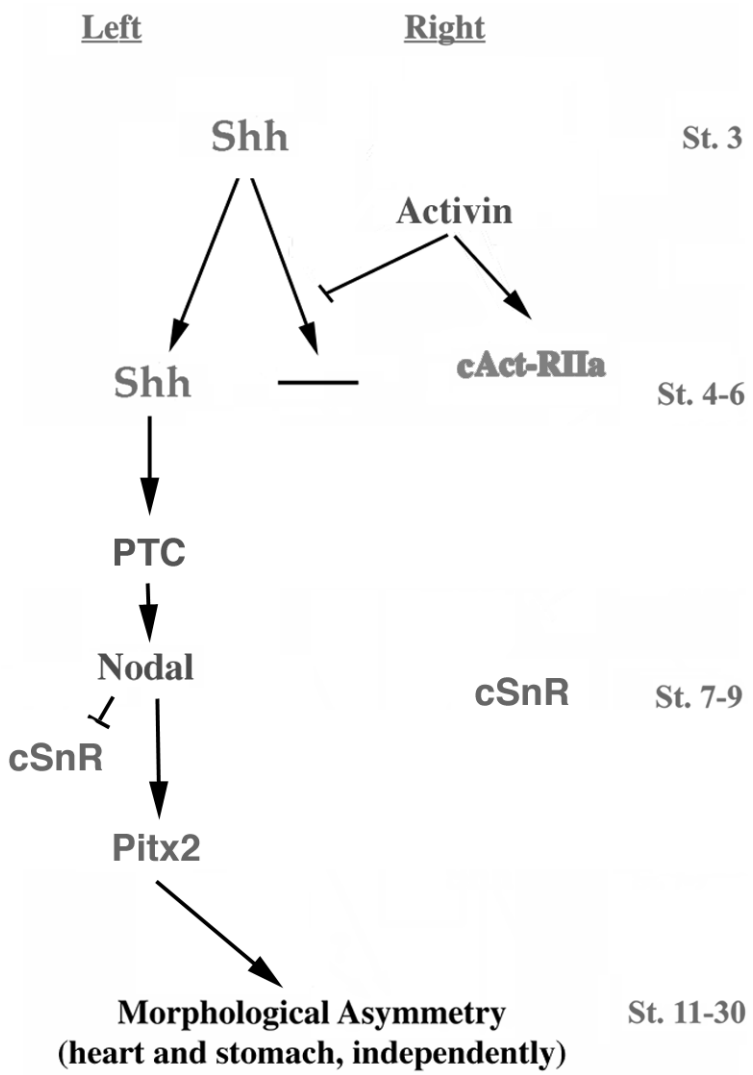
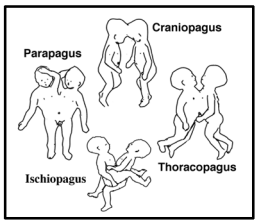

Left
Right
Shh
St. 3
Activin
Shh
cAct-RIIa
St. 4-6
PTC
Nodal
cSnR
St. 7-9
cSnR
Pitx2
Morphological Asymmetry
(heart and stomach, independently)
St. 11-30

Figure 3

Some classes of conjoined twins have extremely high levels of situs inversus



Instances where one twin has inverted heart

Parapagus:	**13/21 cases**
Thoracopagus:	**15/38 cases**
Craniopagus:	**0/90 cases**
Ischiopagus:	**0/8 cases**

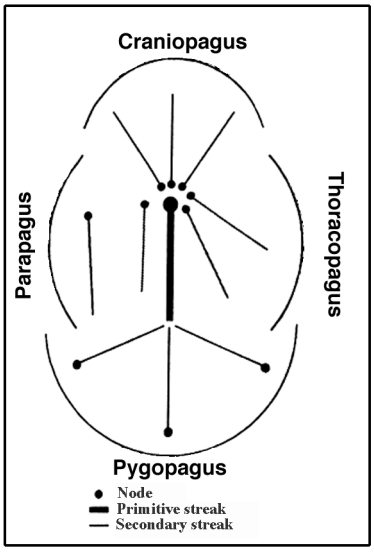
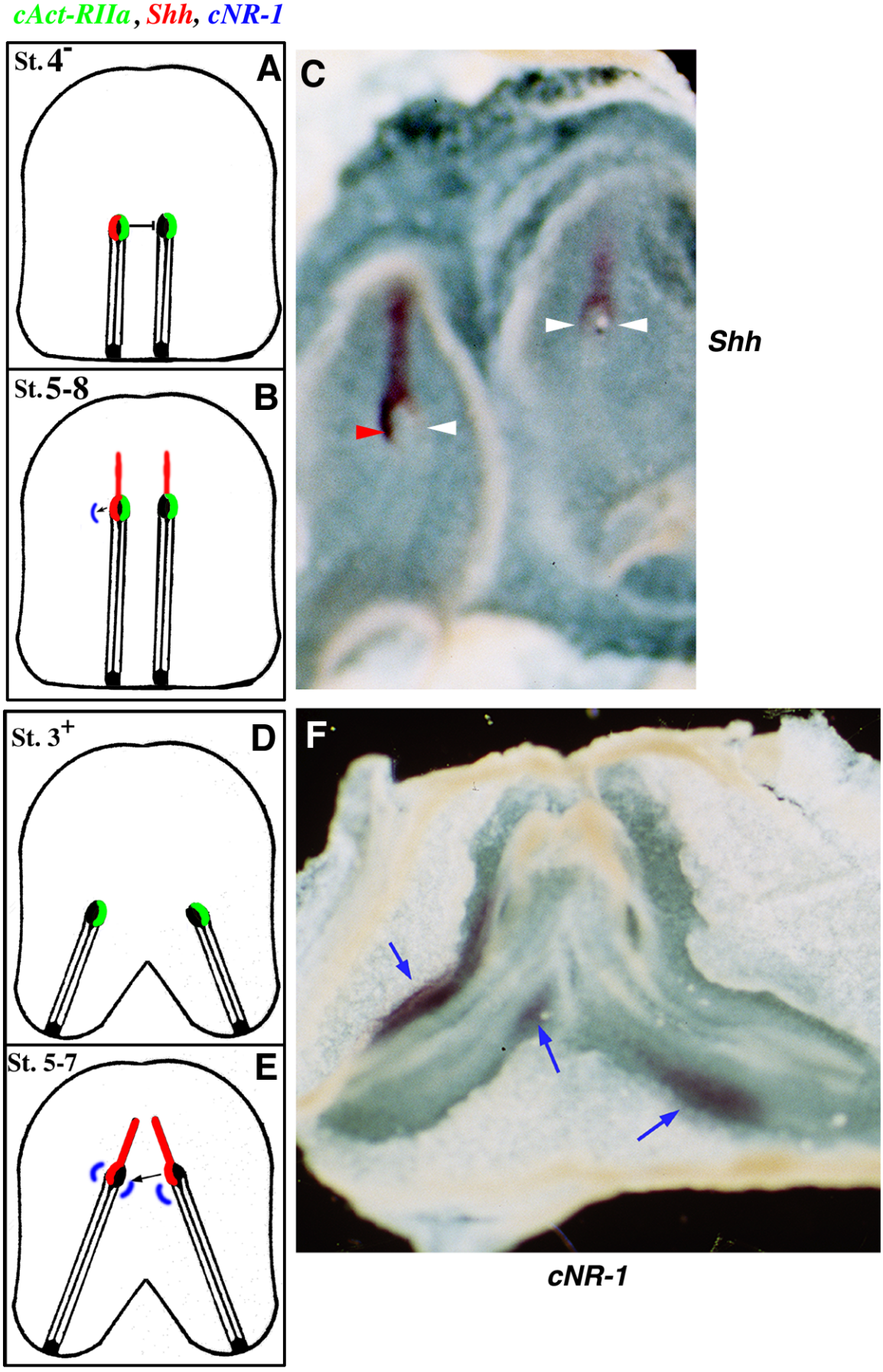


Figure 4



Introduction

The geometrical invariance known as symmetry is a striking feature of developmental morphology during embryogenesis. The left-right axis of an animal's body plan is often thought of as being fundamentally different from the dorso-ventral and antero-posterior axes because of the symmetry which it exhibits when viewed from the outside. Interestingly though, the internal organs of most animals reveal an individually and evolutionarily conserved asymmetry which requires patterning of the same order of complexity as the other two axes. Animal body-plans occur in a wide variety of symmetries such as spherical, radial, and bilateral. 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). I limit this discussion 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 exclude pseudo-random characteristics such as animal coat colors, and minor stochastic deviations due to developmental noise.

The LR axis itself follows automatically from the definition of the AP and DV axes, as it is perpendicular to both; however, consistently imposed asymmetry across it is fundamentally different from patterning along the other two axes. Firstly, while the AP and DV axes can be set by exogenous cues such as gravity, or sperm entry point, there is no independent way to pick out the left (or right) direction, since no obvious macroscopic aspect of nature differentiates left from right. 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 in 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.

Several model systems have contributed to the study of LR asymmetry (see refs (Fujinaga, 1996; Levin, 1997; Wood, 1997; Levin and Mercola, 1998a) for general reviews on LR asymmetry). It has long been known ((Burn, 1991; Winer-Muram, 1995)) that human beings occasionally present loss of LR asymmetry (isomerism), complete reversals of the normal asymmetry (*situs inversus*), or partial reversals of symmetry (heterotaxia). Several strains of vertebrates and invertebrates which recapitulate these syndromes (Hummel and Chapman, 1959; Yokoyama et al., 1993; Supp et al., 1997; Mochizuki et al., 1998) as well as a list of pharmacological agents which cause *situs* defects in mammalian embryos are known (Levin, 1997). *Drosophila,* which has provided molecular entry points into so many other biological phenomena through classical genetics, has not been a factor in LR research because it has a very low degree of asymmetry (manifest only as the chiral nature of the rotation of the male sex organ and the testicular outflow tract ((Polani, 1996), but see (Martin-Blanco and Garcia-Bellido, 1996)), and selections for external LR asymmetry have not been successful (Tuinstra et al., 1990).

Several experiments have shed light on the timing of LR asymmetry specification. Chick heart sidedness has been experimentally demonstrated to be determined during gastrulation (Hoyle et al., 1992); studies on LR inversions induced by drugs likewise suggest that in mammals, a critical period in LR biasing occurs before late gastrulation (Fujinaga and Baden, 1991). Thus it is clear that decisions fundamental to LR asymmetry are made long before any overt signs of morphological asymmetry, and long before the morphogenesis of asymmetric organs.

The Molecular Left-Right Pathway

A number of asymmetrically-expressed genes have now been described (Levin, 1997). These include a variety of signaling molecules and transcription factors. Figure 1

illustrates the expression pattern of three such genes as assayed by *in situ* hybridization with riboprobes to the relevant genes: *Sonic Hedgehog* (Shh), *Nodal*, and *PTC* (Levin et al., 1995). *Shh* is expressed only on the left side of Hensen's node in the gastrulating chick embryo. Shortly thereafter, *Nodal* and *PTC* are expressed also on the left side.

Once a set of asymmetrically-expressed genes was identified, their location and relative timing of expression suggested a possible pathway of sequential inductions and repressions. Using artificial retroviruses bearing the gene of interest or protein-coated beads, a model for the LR pathway was constructed. For example, it was found that misexpressing the normally left-sided gene *Shh* on the right side caused the ectopic right-sided expression of *Nodal*, which is normally also confined to the left side. This cascade (summarized in Figure 2) begins when *activin _B* becomes expressed on the right side of Hensen's node (st. 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). Soon thereafter, *Shh* (which at that point is expressed only on the left side of the node and in the notochord) induces *PTC* in cells adjacent to the node, and *nodal* in a small domain of cells adjacent to the left side of the node. This is soon followed by a much larger domain of *nodal* expression in the lateral plate mesoderm. *Nodal* then induces the expression of *Pitx-2* also on the left side (Logan et al., 1998). *cSnR* is expressed on the right side, and is suppressed by *Nodal* on the left (Isaac et al., 1997).

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 (Levin et al., 1995). Moreover, *nodal*, which is in direct contact with cardiac precursor cells, can reverse heart *situs* or cause symmetric hearts (Levin et al., 1997). The same is true of *Pitx-2* (Logan et al., 1998). Thus, though there is no consensus on what causes cardiac looping in the first place, it is plausible that *nodal* or *Pitx-2* 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 provide a pivotal influence determining the handedness of the heart. Interestingly, the other organs besides the heart likewise take their cues from this genetic cascade (Levin et al., 1997). Some members of this cascade (such as *Nodal* and *Pitx-2*) are clearly involved in LR decisions in the frog and mouse, as well as chick (Collignon et al., 1996; Lowe et al., 1996; Ryan et al., 1998), though the extent of evolutionary conservation of the other parts of the pathway is still unknown (Levin and Mercola, 1998b).

Several other genes which are not asymmetrically expressed but play a role in laterality decisions have been described, but it is currently unknown where in the pathway they fit (Supp et al., 1997; Hyatt and Yost, 1998; Levin and Mercola, 1998c; Mochizuki et al., 1998).

Models for Conjoined Twins and Laterality Defects

Twins and the twinning process have had a relevance for laterality research ever since the tantalizing experiments of Spemann (Spemann and Falkenberg, 1919). It has been noted (Newman, 1916; Morrill, 1919; Schwind, 1934; Burn, 1991; Winer-Muram, 1995) that conjoined twins in human beings, mice, and frogs tend to exhibit laterality defects. While the *Xenopus* system makes it very easy to induce twins on demand (Hyatt et al., 1996; Nascone and Mercola, 1997), modern workers in the chick system have relied on spontaneous twins.

In reviewing the human literature on conjoined twins, it was observed that only parapagus and thoracopagus twins tend to exhibit *situs* abnormalities (Levin et al., 1996); these are twins thought to originate from two adjacent embryonic streaks developing side by side, either in parallel or obliquely (Fig. 3B). Guided by the LR pathway, Levin *et al*. (1996) examined the expression of the asymmetric genes in analogously positioned chick twins and proposed two models explaining laterality defects found in conjoined twins. These are both based on molecules in the LR pathway crossing some distance in the blastoderm and affecting the conjoined embryo.

The precise details of geometric arrangement and timing determine which members of the LR cascade affect the twin, and thus control which twin exhibits the *situs* anomaly.

For example, in primitive streaks which grow parallel to each other from early stages (Fig. 4A), right-sided sided *activin* in the left streak would inhibit the normal left-sided expression of *Shh* in the right twin. This would lead to absence of *nodal* expression (Fig. 4B) in the right twin, which causes morphological *situs* defects (Levin et al., 1995). When spontaneous chick twins with parallel primitive streaks are examined by *in situ* hybridization for *Shh* expression, as predicted it is seen that the left twin has normal *Shh* expression but the right twin has a lack of *Shh* expression in the node (Fig. 4C).

In contrast, when primitive streaks arise far apart, but grow towards each other during gastrulation (Fig. 4D), *Shh* expression proceeds normally in both twins. However, during head-fold stages, the *Shh* expression of the right twin induces aberrant *nodal* expression on the right side of the left twin (Fig. 4E). When head-fold stage spontaneous chick twins with oblique streaks are examined for *nodal* expression (Fig. 4F), as predicted it is seen that the right twin has *nodal* expression only on the left side (i.e., normal expression), while the left-most twin has expression (black arrows) on both the left and right sides (which leads to laterality defects, Levin *et al.*, 1995).

The accompanying paper by Dr. von Kraft represents a careful and painstaking set of experiments on the relationship between twinning and LR asymmetry. Taking advantage of the fact that conjoined twins can be readily created in *Triturus* by fusing embryos during development, Dr. von Kraft examined morphological laterality of twins conjoined in different orientations. Thus, this work represents further, specific experimental tests of the models described above. A great advantage of this paradigm for mechanistic studies of LR patterning over systems where twins are created by inducing ectopic axes in an existent embryo (Hyatt et al., 1996; Nascone and Mercola, 1997) is that it avoids additional complexities caused by splitting an embryonic field which already may contain left-right information. Additionally, it allows finer control over the relative ages of the twins.

The studies in *Triturus* present several interesting conclusions. The most fundamental observation is that fusion of embryos results in laterality defects; this clearly demonstrates that splitting of an embryo's left-right field is not required for laterality defects. This is consistent with the chick models which suggest that the asymmetry phenotypes are the result of interference between rather mature streaks, and not a fundamental result of the splitting of the organizer prior to streak formation. Secondly, the phenotype of the resulting twins is heterotaxia, including all possible permutations of the viscera (many more morphological markers of laterality were scored than in most *Xenopus* or chick studies). Heterotaxia is precisely what is caused by introducing ectopic sources of several of the genes in the LR pathway in chick embryos (Levin et al., 1997). Thirdly, heterolateral fusion is more effective in causing heterotaxia than homolateral fusion. This result is also predicted by the chick pathway models which specifically postulate ectopic downstream gene expression caused by influence of left-sided upstream genes on the right side (and vice versa). Fourthly, it was found that gastrula and early neurula stages are the most sensitive to laterality defects. That is precisely the stages during which asymmetric genes have been described in the chick. Fifthly, which side is seen to be dominant depends on which aspect of laterality one scores. Finally, the effect occurs even when the embryos are of different families of amphibians. This is not surprising given that the asymmetric genes discussed above are well-conserved signaling molecules present in most animal systems (see (Levin and Mercola, 1998b) for a discussion of conservation of LR signaling among species).

Chirality Issues in Non-conjoined Twins

The models discussed above present plausible explanations of laterality defects in conjoined twins. There is, however, an interesting set of observations which suggest that they do not tell the whole story, and that even in mammals, chirality is determined as early as the first few cell divisions, and certainly before the streak appears. Non-conjoined monozygotic twins, while not exhibiting the kinds of visceral laterality defects that occur in conjoined twins, do manifest many subtler kinds of mirror-image asymmetry ("bookend" or enantiomer twin pairs). Pairs of such twins have been noted

to present mirror asymmetries in hand preference, hair whorl direction, tooth patterns, unilateral eye and ear defects, and even tumor locations and un-descended testicles (Newman et al., 1937; Gedda et al., 1981; Yager, 1984; Carton and Rees, 1987; Beere et al., 1990; Townsend and Richards, 1990; Morison et al., 1994; Cidis et al., 1997). Most healthy, non-conjoined twins presumably result from separation of cleavage, morula, or early blastocyst stage embryos (James, 1983). Thus, some chiral information may be present in the very early mammalian embryo, manifesting itself in hair whorls etc. if the cells are separated at an early stage. In contrast, the asymmetry of the major body organs seems to be unspecified (or at least, plastic enough to be re-specified) at those stages, and is developed correctly for both monozygotic twins. This may be related to the fact that heterotaxic reversals in hair whorls and tooth patterns would not be expected to be disadvantageous, while discordant *situs* for internal organs clearly is subject to negative evolutionary pressure.

Many interesting questions remain in the field of laterality research. The study of twins, human as well as those of other species, whether conjoined or not, present unique opportunities to address embryological and evolutionary issues in left-right asymmetry.

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Figure Legends:

Figure 1: Sample asymmetrically-expressed genes

(A) *Sonic Hedgehog* (*Shh*), a signaling protein involved in patterning the limb and neural tube in vertebrates, is expressed on the left side of Hensen's node as well in the notochord during gastrulation. PS = primitive streak. (B) *Nodal*, a member of the *BMP* superfamily of signaling molecules, is expressed in two domains on the left side of the midline in neurula stage chick embryos. (C) *PTC*, a receptor for the Shh protein is expressed adjacent to Hensen's node on the left side. The thin vertical lines indicate the axis of LR symmetry.

Figure 2: The pathway of LR-asymmetric genes

Shh is initially expressed throughout the node. Right-sided *Activin* expression induces expression of *cAct-RIIa* on the right, and represses *Shh* on the right side of Hensen's node, leaving left-only expression. It then induces left-sided expression of *PTC*, which probably functions to transduce the *Shh* signal to induce *Nodal*, which in turn represses the transcription factor *cSnR* (which stays expressed on the right, where *Nodal* is absent), and induces left-sided *Pitx2*. This leads to proper asymmetric organogenesis in the viscera.

Figure 3: Human conjoined twins and laterality defects

(A, modified from (Arey, 1965)) Parapagus and thoracopagus conjoined twins are the only ones associated with laterality defects. (B, modified from (Spencer, 1992; Kapur et al., 1994; Spencer, 1995)) Side-by-side and oblique arrangements of primitive streaks are thought to give rise to such twins, consistent with models of laterality defects caused by crossover of asymmetric signaling molecules between adjacent primitive streaks.

Figure 4: Two specific models for laterality defects in conjoined twins

(A) Schematic of primitive streaks which grow parallel to each other from early stages; this causes right-sided sided *activin* in the left streak to inhibit the normal left-

sided expression of *Shh* in the right twin. (B) This would lead to absence of *nodal* expression and thus morphological *situs* defects in the right. When spontaneous chick twins with parallel primitive streaks are examined by *in situ* hybridization for *Shh* expression, as predicted it is seen that the left twin has normal *Shh* expression (black arrow), but the right twin has a lack of *Shh* expression in the node (white arrows). In contrast, when primitive streaks arise far apart, but grow towards each other during gastrulation, *Shh* expression proceeds normally in both twins (schematized in panel D). However, during head-fold stages, the *Shh* expression of the right twin induces aberrant *nodal* expression on the right side of the left twin (E). When head-fold stage spontaneous chick twins with oblique streaks are examined for *nodal* expression, as predicted it is seen that the right twin has *nodal* expression only on the left side (i.e., normal expression), while the left-most twin has expression (black arrows) on both the left and right sides (which leads to laterality defects, as shown in Levin *et al.*, 1995).