Left/Right Patterning Signals and the Independent Regulation of Different Aspects of Situs in the Chick Embryo

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Recently, a pathway of genes which are part of a cascade regulating the side on which the heart forms during chick development was characterized (M. Levin et al., 1995, Cell 82, 1-20). Here we extend these previous studies, showing that manipulation of at least one member of the cascade, Sonic hedgehog (Shh), can affect the situs of embryonic rotation and of the gut, in addition to the heart. Bilateral expression of Shh, which is normally found exclusively on the left, does not result in left isomerism (a bilaterally symmetrical embryo having two left sides) nor in a complete situs inversus phenotype. Instead, misexpression of Shh on the right side of the node, which in turn leads to bilateral nodal expression, produces a heterotaxia-like condition, where different aspects of laterality are determined independently. Heart situs has previously been shown to be altered by ectopic Shh and activin. However, the most downstream gene identified in the LR pathway, nodal, had not been functionally linked to heart laterality. We show that ectopic (right-sided) nodal expression is able to affect heart situs, suggesting that the randomization of heart laterality observed in Shh and activin misexpression experiments is a result of changes in nodal expression and that nodal is likely to regulate heart situs endogenously. The first defined asymmetric signal in the left-right patterning pathway is Shh, which is initially expressed throughout Hensen’s node but becomes restricted to the left side at stage 4. It has been hypothesized that the restriction of Shh expression may be due to repression by an upstream activin-like factor. The involvement of such an activin-like factor on the right side of Hensen’s node was suggested because ectopic activin protein is able to repress Shh on the left side of the node, as well as to induce ectopic expression of a normally right-sided marker, the activin receptor Act-RIIa. Here we provide further evidence in favor of this model. We find that a member of this family, Activin βB, is indeed expressed asymmetrically, only on the right side of Hensen’s node, at the correct time for it to be the endogenous asymmetric activin signal. Furthermore, we show that application of follistatin-loaded beads eliminates the asymmetry in Shh expression, consistent with an inhibition of an endogenous member of the activin-BMP superfamily. This combined with the previous data on exogenous activin supports the model that Activin βB functions in the chick embryo to initiate Shh asymmetry. While these data extend our understanding of the early signals which establish left-right asymmetry, they leave unanswered the interesting question of how the bilateral symmetry of the embryo is initially broken to define a consistent left-right axis. Analysis of spontaneous chick twins suggests that, whatever the molecular mechanism, left-right patterning is unlikely to be due to a blastodermal prepattern but rather is initiated in a streak-autonomous manner.

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INTRODUCTION

During development, embryos acquire complex patterns along each of the three axes. The resulting morphologies can display a variety of symmetry types, including spherical (as in volvox), radial (as in starfish), chiral (as in snails), bilateral (as in Drosophila), and pseudobilateral (as in man). Most vertebrates have a generally bilaterally symmetrical body plan, but this symmetry is broken into pseudosymmetry by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen, and gut, and an asymmetric development of certain paired organs such as brain hemispheres or lungs. While the specification of anterior–posterior (AP) and dorsal–ventral (DV) axial asymmetries has been studied in some detail (reviewed in Hunt and Krumlauf, 1992), far less is known about the molecular mechanisms underlying left–right (LR) asymmetry.

The LR axis is probably specified after the anterior–posterior and dorsal–ventral axes and is determined with respect to them (McCain and McClay, 1994; Danos and Yost, 1995). Until recently, almost all available information on LR asymmetry centered around four lines of inquiry: a phenomenological literature describing various asymmetries (Neville, 1976, presents an extensive and fascinating survey), the genetics of chirality in snails (an unidentified cytoplasmic factor determines dextrality; Freeman and Lundelius, 1982), several drugs which cause alterations in LR patterning (for example, an adrenergic pathway is implicated by Fujinaga and Baden, 1993a), and mammalian mutants which have phenotypes associated with LR asymmetry, such as randomization or total reversal of internal situs (Brueckner et al., 1989; Yokoyama et al., 1993). Selection for LR asymmetries in Drosophila, in hopes of generating a genetically tractable mutant, have been unsuccessful (e.g., Tuinstra et al., 1990).

The discovery of signaling molecules asymmetrically expressed prior to overt morphological LR asymmetry in vertebrates (Levin et al., 1995) has opened the way to understanding how the LR axis is regulated. In the chick embryo Sonic hedgehog (Shh) is initially expressed throughout Hensen's node; however, it is subsequently expressed exclusively on the left, perhaps due to the influence of an adrenergic-like activity on the right side of the node. Subsequently, the resulting asymmetric expression of Shh induces the chick homologue of the mouse gene nodal (a member of the TGF-β family, previously called cNR-1), which spreads throughout the lateral plate mesoderm on the left side. Experimental manipulation of these asymmetrically localized signals verified that they form a linear pathway (Levin et al., 1995). Implanting a source of activin on the left side of the node can repress Shh on the left; together with the normal absence of Shh in the right, this results in a lack of nodal expression. Moreover, implanting a source of Shh on the right side, where it is normally repressed, results in bilateral expression of nodal. Either manipulation leads to randomization of heart situs, strongly suggesting that this molecular cascade is involved in the regulation of morphological asymmetry.

Several important aspects of this pathway remained unclear, however. For example, while heart situs is clearly one endpoint of this pathway, it is unknown whether it is a heart-specific cascade or whether other aspects of laterality (such as situs of the gut) utilize the LR information inherent in the cascade. If this is the case, it becomes interesting to ask whether perturbation of this pathway results in: (a) all of the organs making coordinated, albeit randomized, situs choice (situs inversus; Hummel and Chapman, 1959), (b) the organs making independent laterality decisions in response to the signals (heterotaxia), or (c) an embryo formed symmetrically with respect to the LR axis (isomerism). Situs inversus, heterotaxia, and isomerism all occur at a significant incidence in many vertebrates, including man (Winer-Muram, 1995). Each of these conditions, in theory, could result from symmetrical signaling. For example, left-isomerism (exemplified by polysplenia syndrome; Iversen, 1955) might be predicted to result from double-sided Shh expression, since Shh is a left-specifying factor. In this study, we examine this question and address other upstream and downstream steps in the LR-determining pathway.

MATERIALS AND METHODS

In Situ Hybridization

After being fixed in 4% paraformaldehyde overnight, chick embryos were processed for whole-mount in situ hybridization as described in Levin et al. (1995). The clones used in the Shh and nodal in situ hybridizations are as described in Levin et al. (1995). The nodal gene was previously called chick nodal-related-1 (cNR-1). The nomenclature was changed to match that of the mouse gene (1995). The DIG antisense probe for Activin B (a gift from K. Patel) covers a 341-bp fragment of the Activin B/B clone. Embryo staging was according to Hamburger and Hamilton (1951).

Nodal and Shh Misexpression

All experimental manipulations were performed on standard pathogen-free white Leghorn chick embryos obtained from SPAFAS (Norwich, CT). At the time these experiments were conducted our clone of chick nodal did not include the entire N-terminal pro portion of the protein which is removed in processing. To obtain a construct that would encode a protein which would be processed correctly and secrete a normal, mature nodal protein, we fused the BMP-4 pro region (including the cleavage site) to the cNR-1 mature region. For misexpression, this construct was inserted into the RCAS-BP(A) vector, which encodes a replication-competent retrovirus (Hughes, 1987). Chick embryonic fibroblast (CEF) cells were infected with this construct (as in Riddle et al., 1993) and pelleted. Pellets were implanted between the epiblast and the hypoblast on the right side of stage 5–6 embryos in New culture (New, 1955). In situ hybridization of tissue (including the grafted pellet) to a cNR-1 riboprobe was used to ensure that the cells produce cNR-1 mRNA (data not shown). Shh was misexpressed by implanting protein-soaked beads (270 μg/ml of N-termi- minus of human Shh protein produced in bacteria) on the right side of stage 4–5 embryos in ovo.
Extirpation of Presumptive Heart Region

The region of the 30-hr chick embryo fated to give rise to the heart has been well mapped (Stalsberg and DeHaan, 1969). By this time, these cells express several cardiac-specific markers, including Nkx-2.5, but they have not yet started to form a morphological heart tube. Embryos with 6–7 segmented somites (ca. 30 hr of incubation) were explanted into New culture (N ew, 1955), but with the stretched vitelline membrane left flattened, underlaid by only a small amount of albumen medium and overlaid by a 2-mm layer of 1:1 Hanks BSS/Liebovitz air-buffered TCM mixture. Under this condition, groups of embryos could be assembled and age matched for experimental vs sham versions of the operation, which were carried out with tungsten needles. Splanchnic mesoderm and overlying endoderm/hypoblast of the precardiac regions were bilaterally excised up to their midline junction at the developing gut pocket. Sham-operated controls received transverse cuts across these regions anteriorly and posteriorly, but without removal of tissue. The space above the embryo within the ring was then drained and that beneath the vitelline membrane further filled with albumen to give convexity of the cultured blastoderm. The embryos were then allowed to grow in culture to the 18- to 20-somite stage, 20–24 hr later.

Follistatin Application

Heparin acrylic beads were soaked in follistatin protein (obtained from the National Hormone and Pituitary Program) at approximately 0.05 mg/ml in water or PBS. Control beads were soaked in water or PBS only. Beads were implanted between the epiblast and hypoblast of stage 3* embryos in New culture (N ew, 1955) and processed for in situ hybridization at appropriate later stages.

Analysis of Twins

Twins occur spontaneously in chick eggs at a rate of approximately 1–2%. Of such twins, approximately 5–10% are in the 180° head-to-head orientation required for this experiment.

RESULTS

Shh Affects Situs of Multiple Organ Systems

It has been previously proposed (Waddington, 1937) that heart looping mechanically sets the situs of other morphological aspects of laterality. To test this possibility we surgically removed the prospective heart region from six- to seven-somite chick embryos and scored embryonic rotation. The stage when the heart tissue was removed was prior to the formation of the heart tube, later after the looping of the tube could exert physical forces on other organ primordia. Inspection at stage 13–14, when embryonic rotation was assessed, verified that the heart was completely removed by the surgical procedure. The results are summarized in Table 1. It is seen that the incidence of correct embryonic rotation in embryos receiving a sham operation, 88% (Fig. 1A), is not significantly altered ($\chi^2 = 0.126, P > 0.5$) in embryos having no heart whatsoever (76% correct embryonic rotation, Fig. 1B). While this experiment does not eliminate the possibility that cardiac cells signal other organs to instruct laterality, it does argue strongly against the possibility that the mechanical stress imparted by the bending heart tube has this effect, as following our surgical manipulations, no heart tube ever forms.

If the situs of organs other than the heart is not determined mechanically, then it could be set by a response to signaling molecules. The asymmetric signals described in early chick development have previously been shown to affect heart situs (Levin et al., 1995). In principle, this signaling cascade could lie after the regulatory branch point specifically controlling heart morphogenesis or it could lie upstream, playing a more fundamental role in establishing LR asymmetry of the body plan. The original studies of this signaling pathway were carried out in vitro using the technique of New culture. This procedure allowed a large number of embryos to be surgically manipulated, since in ovo surgery is much more difficult. However, using New culture, embryos do not survive long enough to assay the morphology of organs other than the heart.

To determine whether the activin–Shh–nodal pathway is heart-specific or a general LR determination system, we implanted beads soaked in Shh protein on the right side of Hensen’s node (opposite its normal expression) in ovo. We find that the in vivo procedure results in a much higher mortality rate than in vitro. Moreover, a lower percentage of surviving embryos show alterations in heart laterality than in vitro (12.1% instead of 50%; Table 2). Both the mortality and the decreased incidence of effect on heart morphogenesis among survivors are likely to be due to the deleterious long-term effects of successfully implanted Shh beads over the extended time of incubation required to allow other organs to develop.

We assayed surviving embryos for heart situs and for situs of the stomach as a representative second asymmetric organ. In addition, we examined the direction of embryonic rotation within the egg, a property which has previously been suggested to be mechanically linked to the bending of the heart tube (Waddington, 1937). For the purposes of this study, inverted heart situs is defined as the heart tube’s bending to the left, inverted stomach situs as the stomach on the right, and inverted body rotation as the embryo turning to the left. Embryos were scored as inverted only when unambiguous, not including, for example, embryos which failed to rotate in either direction. This conservative approach results in underestimating rather than overestimating the effects of Shh on laterality decisions. In addition, the only embryos scored were those which survived long enough to assay other organs in addition to the heart.

Seventy-four experimental animals which survived the procedure were examined 4 to 6 days after implantation. We find that all three scored aspects of laterality (heart situs, gut situs, and direction of embryonic turning) are affected by the presence of ectopic Shh (Table 2). Approximately 10% of the treated embryos showed each individual type of situs alteration. In a similar number of surviving embryos implanted with control beads we observed only a 1.5% incidence of reversal in the direction of body rotation (Embryo) and no examples of reversal in heart or stomach situs. Those controls were consistent with the low rates of spontaneous
reversal in body rotation (about 1%) and reversal of internal organ situs (less than 0.1%) we have observed in our laboratory. These results demonstrate that, in addition to heart situs (Levin et al., 1995), bilateral exposure to Shh signaling has a significant effect on rotation of the embryo ($\chi^2 = 73$, $P < 0.005$) and stomach situs ($\chi^2 = 3.8$, $P < 0.05$). Thus the asymmetric cascade lies upstream of the branch point leading to laterality decisions for each individual organ.

Interestingly, the situs of the heart and stomach and direction of rotation appear to be influenced independently by Shh signaling, resulting in a heterotaxic phenotype. We found examples of treated embryos where each was the only aspect of laterality which was reversed. Moreover, only 2 of the 16 embryos showing laterality defects were concordant for all three aspects being reversed (true situs inversus). This is particularly surprising in the case of the direction of heart looping and embryonic rotation as these have been previously reported to be linked. However, in response to Shh application we observed 5 cases where both the heart and the embryonic rotation were reversed, 4 cases where the heart but not embryonic rotation was reversed, and 4 cases where rotation but not heart looping was altered. Thus, in our experiments, these two processes were affected independently.

**Nodal Influences Heart Morphogenesis**

The results indicate that Shh signaling can influence the situs of several asymmetric organs. This creates a paradox however, since Shh is only asymmetrically expressed in a very limited spatial and temporal domain in Hensen's node and is no longer asymmetrically expressed at the time when the organs are formed. This suggests that the asymmetric signaling by Shh must be mediated by a secondary signal. Nodal is an excellent candidate for such a secondary signal. Its asymmetric expression is induced by Shh (Levin et al., 1995), correlating with the inductive effects of Shh on organ laterality. Alteration in nodal expression has similarly been correlated with changes in organ situs in the murine iv (Lowe et al., 1996) and inv mutants (Collignon et al., 1996). Moreover, the expression domain of nodal is quite broad, initiating (in the chick) in the anterior lateral plate mesoderm and subsequently spreading posteriorly while retracting rostrally (Levin et al., 1995). At stage 8, it is expressed in a domain directly adjacent to cells expressing Nkx-2.5, a marker of cardiac progenitor cells (Schultheiss et al., 1995), consistent with nodal's providing an asymmetric signal to lateralize the heart primordia.

To test directly whether nodal is indeed capable of influencing heart situs, pellets of CEFs infected with a retroviral vector expressing nodal were implanted into the right side of embryos at stage 6–7, the stage at which endogenous nodal is induced by Shh in the left side. To obtain higher frequency of survival, the experiments were carried out in vitro, in New culture (New, 1955). The results are summarized in Table 3. Under these conditions, in embryos receiving no implant or an implant of cells infected with a nonspecific control virus (alkaline phosphatase), 81% of the developing heart tubes bend to the right, as normal (Fig. 2A), while 19% were either inverted (Fig. 2B) or bilaterally symmetric (Fig. 2C), as previously seen under New culture conditions. There was no significant change in the percentage of hearts bending to the right (82%) when nodal-expressing cells were implanted in the left side, where nodal is normally expressed; however, when nodal was misexpressed

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**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>wt embryonic rotation</th>
<th>Reversed embryonic rotation</th>
<th>Ambiguous or absent embryonic rotation</th>
<th>N</th>
<th>Statistics</th>
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<tr>
<td>Sham operation</td>
<td>88%</td>
<td>6%</td>
<td>6%</td>
<td>15</td>
<td>$\chi^2 = 0.126$, $P &gt; 0.5$</td>
</tr>
<tr>
<td>Heart excision</td>
<td>76%</td>
<td>5%</td>
<td>19%</td>
<td>21</td>
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Note. Heart territories from stage 8 to 9 chick embryos in New culture were surgically removed (heart excision group) or transected without removal of tissue (control group). Embryos were allowed to develop and the situs of the embryonic rotation was scored.

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**FIG. 1.** The Shh pathway independently controls situs of organs other than heart. The prospective heart region was removed from embryos at stage 6–7. Embryos were allowed to develop until embryonic rotation took place. Embryos receiving a sham operation (A) rotate to the correct side in 88% of the cases; embryos whose heart region has been extirpated (B) likewise rotate correctly in 76% of the cases. Dark gray arrowheads show torsion, light gray arrowhead shows heart, and white arrowhead shows lack of heart.
on the opposite side, approximately half that number bent to the right (38%), with a corresponding increase in both inverted and bilaterally symmetric hearts. This effect is significant to $P = 0.005$ ($\chi^2 = 23.9$). It should be noted that approximately twice as many affected hearts were bilaterally symmetric, looping in both directions (right-isomerism, 43%) as were inverted (19%). Since ectopic nodal expression can alter heart situs, these data implicate nodal as part of the functional cascade determining cardiac laterality. The ability of nodal to affect LR specification has been independently demonstrated in Xenopus (Sampath et al., 1997).

### Signaling Upstream of Shh in the LR Asymmetric Cascade

Identifying a series of signals directing the asymmetric morphogenesis of the visceral organs is important, in part, because it provides the opportunity to work backward toward addressing the origin of LR asymmetry during embryogenesis. The first described signal in the asymmetric cascade is Shh, which is uniformly expressed at Hensen’s node at stage 4, but then is asymmetrically repressed on the right side at stage 4. An upstream activin-like signal mediating this repression was suggested by the finding that an activin-inducible marker (the activin receptor cAct-RIIa) is expressed on the right side of Hensen’s node concomitant with Shh repression (Levin et al., 1995). Consistent with an activin-like activity playing such a role, ectopic activin protein applied to the left side of the node can repress Shh and induce cAct-RIIa.

The model that an activin-like protein is involved in LR determination thus depended heavily on the effects of applying ectopic activin. If an endogenous activin-like signal is indeed critical for establishing the later asymmetric expression of Shh and nodal, then interfering with such signals at stage 3–4 should alter Shh and nodal expression. To test this, we implanted beads loaded with follistatin, an antagonist of signaling by activin and related molecules, including some BMPs (Hemmati-Brivanlou et al., 1994; Yamashita et al., 1995), on the right side of the forming node at stage 3. In 5 of 20 cases, Shh, which is normally expressed on the right side of Hensen’s node (Fig. 3A), was symmetrically expressed on both sides of the node following follistatin application (Fig. 3B). Preliminary experiments indicate that symmetrical nodal expression can also result from follistatin treatment (data not shown). Neither Shh nor nodal was ever expressed bilaterally following control bead implants ($n = 23$).

To identify a candidate for the endogenous activin-like signal, we examined the expression of various activin and related BMP genes at stage 4 (including BMP-2, 4, 6, and 7 and Activin βA and βB), all of which except one either were

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<th>TABLE 2</th>
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<tr>
<td><strong>Control beads</strong></td>
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<tr>
<td>Heart situs</td>
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<td>Heart situs</td>
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<tr>
<td>Stomach situs</td>
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<td>Embryonic rotation</td>
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<td>No. of embryos examined</td>
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Note. Beads loaded with Shh protein by equilibrating for 24–48 hr were implanted into the right side of Hensen’s node of stage 4 embryos in ovo. Embryos were allowed to develop and the situs of the heart, gut, and embryonic rotation was scored.

<table>
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<th>TABLE 3</th>
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<tr>
<td><strong>Control cells</strong></td>
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<td><strong>on right side</strong></td>
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<tr>
<td>Right-sided (wt) hearts</td>
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<tr>
<td>Left-sided (reversed) hearts</td>
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<tr>
<td>Bilaterally symmetric hearts</td>
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<td>No. of embryos examined</td>
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$\chi^2 = 5.3, P = 0.07 \quad \chi^2 = 23.9, P = 0.005$

Note. Pellets of CEFs infected with the nodal virus were implanted into the right side of Hensen’s node of stage 6 embryos in New culture. Embryos were allowed to develop and the situs of the heart was scored.
Activin most clearly at stage 4 Shh (1990) suggests that LR asymmetry is determined within organ systems by initially acting on one, such as the heart expression in the chick embryo. At least two models for (e.g., Afzelius, 1976).

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The Shh Pathway and Heterotaxia

Activin /B is currently the earliest molecular marker of LR asymmetry in the chick. This leaves unexplored the factors responsible for initiating LR asymmetries in gene expression in the chick embryo. At least two models for this have been proposed. One theory (Brown and Wolpert, 1990) suggests that LR asymmetry is determined within each cell (perhaps by a chiral molecule which is oriented with respect to the AP and DV axes, which are well established by stage 2 in the chick). An alternative hypothesis is that LR information arises from the maternal localization of a determinant in a LR asymmetric manner within the blastoderm (Wilhelmi, 1921). The analysis of spontaneous twin chick embryos gives a possible indication of which of these two hypotheses about the initial origin of LR asymmetry is more likely to be correct. The cell-autonomous theory predicts that in twin embryos that are oriented in a head-to-head fashion (180°), each twin will be correctly patterned along the LR axis because each node will contain cells which know left from right with respect to their own AP orientation (Fig. 4A). The prepattern theory, however, predicts that the twins will be mirror images of each other since the LR factors localized in the blastoderm will determine left and right sides regardless of the AP orientation of the embedded streaks, resulting in one correct and one reversed embryo (Fig. 4B). Examination of four such embryos probed with Shh (Fig. 4C) and nodal (data not shown) as markers of laterality, as well as six such sets of twins assayed by the morphology of the node at stage 5 (Fig. 4D; Cooke, 1995) and two sets of twins examined for embryo turning at stage 22 (Fig. 4E), shows that, in every such case, each embryo is correctly patterned with respect to its own orientation. This suggests that LR information is initiated with respect to the AP and DV axes of each embryo and is due neither to a maternally derived prepattern within the blastoderm nor to a zygotic decision prior to the establishment of the AP and DV axes.

DISCUSSION

The Shh Pathway and Heterotaxia

Shh has been previously shown to randomize heart situs (Levin et al., 1995). We now find that when ectopic (right-sided) Shh protein is applied to the node in ovo, reversals of the situs of the heart, gut, and embryonic rotation are observed. Thus, Shh does not lie upstream of a heart-specific pathway, but rather provides a left-right reference by which multiple organs assess their laterality during morphogenesis.

In normal development, the morphogenesis of different organs is a tightly coordinated process. For the asymmetric organs to have an invariant orientation relative to each other, their primordia must respond to a common LR asymmetric set of cues. This is verified by the existence of situs inversus mutants (e.g., the inv mouse; Yokoyama et al., 1993) where all of the internal organs are in an absolute reverse orientation but maintain the same relative configuration. On the other hand, ultimately each organ forms independently, and other, presumably downstream, mutations result in a randomization of asymmetric placement of each organ. This is seen in human heterotaxia syndromes (e.g., Afzelius, 1976).

In principle, asymmetric signals could affect all of the organ systems by initially acting on one, such as the heart

FIG. 2. Nodal determines heart asymmetry. Ventral views: when nodal is misexpressed on the right side of the node (n = 21), 38% of the resulting hearts are wt (A), while 19% show left-sided looping (B) and 43% are symmetrical (C). However, when cells infected with a control virus (alkaline phosphatase) are implanted on the right side of the node (n = 31), 81% of the resulting hearts are wt, 10% exhibit left-sided looping, and 9% are symmetrical. This effect is significant to P = 0.005, \( \chi^2 = 23.9 \). When a nodal-expressing pellet is implanted on the left side of the node (n = 22), 82% of the hearts are wt, 0% are left-sided, and 18% are symmetrical. These phenotypes are not statistically significantly different from control implants (P = 0.07, \( \chi^2 = 5.3 \)). Arrows indicate looping of the heart tube.

Asymmetry Does Not Appear to Arise from a PrePatterned Blastoderm

Activin /B is not detectable at stage 4 or were expressed symmetrically (BMP-6 was not detectable, while BMPs 2, 4, and 7 were expressed in the posterior third of the primitive streak, neural folds, and lateral mesoderm, respectively, data not shown). Chick Activin /B was seen in whole-mount in situ hybridization to be specifically expressed on the right side of Hensen's node from stage 3 to stage 5. Unfortunately the Activin /B probe gives a high, uniform, nonspecific background at all stages examined. Nonetheless, the right-sided expression can be clearly seen above background, most clearly at stage 4* (Fig. 3C). To verify that this signal was real, and was present prior to the asymmetry in Shh expression, we sectioned several entire stage 3 embryos in a plane perpendicular to the primitive streak and hybridized all sections with an Activin /B probe. Hybridization was exclusively detected on the right side in sections through the anteriormost tip of the primitive streak (Fig. 3D). Thus, the Activin /B gene is specifically expressed on the right side of the node at the correct stage to influence Shh expression and is therefore a candidate for the endogenous activin-like signal in the LR cascade.

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FIG. 3. Endogenous asymmetric activin-like activity. Control beads (soaked in PBS) or beads carrying follistatin protein were implanted into the right side of Hensen's node at stage 3, and the embryos were harvested at stage 5 and processed for in situ hybridization with the Shh probe. Control beads never caused symmetrical expression patterns of Shh (n = 23, A). In contrast, when a bead loaded with follistatin protein was implanted in the same manner, symmetrical expression of Shh is observed (5/20 cases, B). This result is significant to P = 0.02. The beads move anteriorly from their original implant location because of the cell migration which occurs during incubation (see B, green arrowhead). The bead in A is not visible because it became dislodged during in situ hybridization processing. Stage 4 embryos in whole mount (C) and cryosections at the level of the forming node of stage 3 embryos (D), were processed for in situ hybridization with the Activin βB probe. Signal was detected in the right side of Hensen's node (black arrow), consistent with its proposed role in repressing Shh there. Black arrows indicate endogenous expression domain. Gray arrows indicate ectopic domain. L and R, left and right sides of the primitive streak, respectively.

FIG. 4. LR asymmetry is apparently streak autonomous. The maternal prepattern theory suggests that such twins should result in mirror image embryos because the blastodisc is divided into left and right domains (shown here as yellow and white) containing distinct positional information, such as an asymmetrically positioned factor which influences subsequent LR decisions (B). The chiral molecule theory predicts that embryos which are arranged head to head should have correct LR orientation with respect to their own axes (A). When such twins are hybridized to a Shh probe, it is seen that each embryo is correctly patterned with respect to itself (C). Red arrows point to wt left-sided Shh expression. The morphological asymmetry in the node, shown in section through a wt embryo (D, black arrowhead points to node asymmetry), as well as the direction of embryonic turning (shown in E, black arrowheads point to anterior) is likewise correct in each twin, with respect to its own AP and DV axes.

primordia, which could then mechanically influence the others (Waddington, 1937). In such a case, heterotaxia could be explained by a decoupling of different organs from this influence. Alternatively, each organ primordia could directly respond to the asymmetric signals, and heterotaxia would be a consequence of the failure to interpret these
cues (Brown and Wolpert, 1990). Our experiments support the latter scenario (consistent with the findings of Fujinaga and Baden, 1991b) since ectopic application of Shh results in independent alteration of situs of each of the properties we assayed.

In these experiments we placed Shh protein on the right side of Hensen's node resulting in bilateral Shh signaling and hence bilateral nodal expression (Levin et al., 1995). Bilateral nodal expression has also been correlated with independent segregation of heart and gut orientation in the frog, following ectopic Vg1 expression (Hyatt et al., 1996). It remains to be determined which organs respond to this nodal signal directly and which if any organs get their LR information from genes upstream of (Shh), downstream of, or parallel to nodal (for example, lefty; Meno et al., 1996). The fact that heterotaxia was observed in these experiments instead of left isomerism (which might have been expected to result from the double-sided expression of Shh, a normally left-sided gene), suggests that the Shh pathway is involved in the biasing of random asymmetry, not in its generation (Brown and Wolpert, 1990).

Nodal Is a Causal Determinant of Heart Situs

Randomization of heart situs caused by ectopic Shh or activin has been correlated with double-sided and absent nodal expression respectively (Levin et al., 1995). Similarly, recent studies showed that expression of nodal is altered in two mouse mutations (iv, Lowe et al., 1996; and inv, Collignon et al., 1996) which also display laterality defects. This placed the mutations upstream of nodal, but it did not distinguish between nodal's being a causal factor in heart situs determination and its expression being a parallel effect of earlier parts of the asymmetric gene cascade. Here, we show that ectopic nodal expression itself results in inverted and double-sided hearts. This strongly suggests that nodal endogenously controls the laterality of cardiac looping.

In these experiments, a significant percentage of embryos display right-isomerized symmetric hearts following bilateral nodal expression achieved by implanting nodal-expressing cells. This is a striking contrast to the phenotypes observed following bilateral nodal expression generated by implanting Shh-expressing cells, in which heart laterality was randomized but no symmetric hearts were observed (Levin et al., 1995). One possible explanation for this difference could be that nodal is but one component of the signals downstream of Shh necessary to fully specify heart situs. For example, lefty, another TGF-β family member, has been identified in mice (Meno et al., 1996), has an expression pattern similar to that of nodal, and may work in concert with it. An alternative interpretation is that ectopic and endogenous nodal domains, when induced by Shh, are only transiently expressed in the cardiac-forming region; in contrast, nodal-expressing cell implants come to lie next to the heart tube and provide a constant source of signal adjacent to the forming heart, which can in some instances disrupt its morphogenesis. Consistent with this explanation, we also observe an increase in bilaterally symmetric hearts when nodal cell implants are placed on the left side, where nodal is normally expressed.

Nodal, a member of the TGF-β superfamily, encodes a secreted factor (Zhou et al., 1993, and data not shown). Thus, it is plausible that it directly affects heart looping by providing an asymmetric signal to only one of the heart primordia. This could then affect heart morphogenesis by affecting the migration (Manasek, 1981), proliferation (Stalsberg, 1969), or cytoskeletal organization (Itasaki et al., 1989, 1991) of cells descended from the left-side cardiac precursors.

An apparent paradox arises: Hoyle et al. (1992) report a difference (in the ability to bias the heart tube) between the left and right precardiac mesoderm as early as stage 5–6, while nodal is not expressed until somewhat later. The resolution of this issue is likely to be that cells become committed to express nodal earlier, at the time when Shh is expressed asymmetrically (stage 4⁺). Consistent with this, when cell expressing Shh are ectopically implanted on the right side adjacent to Hensen’s node, the cell pellet migrates anteriorly prior to nodal expression. Nodal is then induced next to the location where the Shh cells were originally implanted, demonstrating a commitment to express nodal prior to its actual expression. This commitment is probably what Hoyle et al. observed, assayed by them as the ability to bias the heart tube when transplanted.

An Endogenous Activin-like Signal Is Upstream of Asymmetric Shh Expression

Shh is asymmetrically expressed in the early chick embryo and is capable of inducing asymmetric nodal expression. The identification of this pathway of genes which are asymmetrically expressed leads to the question of further upstream factors: what is responsible for the asymmetry in the expression of Shh? Several lines of evidence suggest that an activin-related signal may play a critical role. First, previous studies demonstrated that exogenously applied activin is sufficient to repress Shh on the left side of the node and thereby prevent nodal induction (Levin et al., 1995). In other reported experiments, exogenous activin had somewhat different consequences for Shh expression, resulting in some cases of reversed Shh expression as well as bilaterally expressed Shh (Isaac et al., 1997). The reason for this difference is unclear, but may reflect differences in timing or placement of the activin beads. In any case, both sets of experiments indicate that exogenous activin can act upstream of Shh. Here we provide evidence that this pharmacological effect likely reflects the action of a related endogenous signal normally present in the right side of the node, since ectopic follistatin, an inhibitor of activin and related factors, leads to symmetrical Shh expression. An excellent candidate for the endogenous Shh-repressing activity is Activin βB. Activin βB is expressed in the right side of the node just before and during the expression of cAct-RIIα there and, most importantly, before the disappearance of Shh expression from the right side. However, it should be noted that follistatin may also interfere with signaling by
related molecules such as BMP-7 (Yamashita et al., 1995, Wilson and Hemmati-Brivanlou, 1995) and hence the endogenous activin-like activity upstream of Shh may in fact be a related molecule. We examined early chick embryos for the expression of a number of related signaling molecules (including BMP-2, 4, 6, and 7) and none were expressed in the node, or in a manner that would indicate a role in LR patterning. However, it remains possible that another member of this family exists which is asymmetrically expressed on the right side of Hensen's node at the same time as Activin βB. In any case, asymmetric expression of Activin βB is the earliest currently known marker of left-right asymmetry in the chick, expressed asymmetrically long before major organ LR asymmetry. The events further upstream that initiate LR asymmetry and lead to right-sided Activin βB expression remain enigmatic.

Vg1 has been reported to be a possible candidate for a signal upstream of activin (Hyatt et al., 1996). Vg1 is misexpressed on the right side of Xenopus embryos, or expression of a dominant-negative (truncated) activin receptor on the left side, produces situs defects in the resulting embryos. It should be noted however, that the truncated activin receptor interacts with other ligands (Kessler and Melton, 1995; Hemmati-Brivanlou et al., 1995); likewise, injected Vg1 may cross-react with receptors for other TGF-β family members. Thus it is possible that these results reflect manipulation of an activin signal, not endogenous Vg1 signaling. It is unlikely that the experiments presented here reflect the activity of an endogenous chick Vg1 homologue, as Vg1 signaling is not inhibited by follistatin (Kessler and Melton, 1995).

Activin and its receptors have not been reported to be asymmetric in any species other than chick. Moreover, several mice have been generated which carry null mutations for Activin-βB and Activin receptor IIA (Matzuk et al., 1995), and these mice appear to have no phenotype associated with LR patterning. However, null mutations in Activin receptor IIB do result in laterality defects (En Li, personal communication), suggesting that this part of the pathway may indeed be conserved in mammals. The target of asymmetrical activin signaling in chicks is Shh, but Shh does not appear to be asymmetric in the mouse node (Collignon et al., 1996), and mice carrying a homozygous deletion of Shh have no laterality defects (Chiang et al., 1996). These differences between chicks and mice may be due to different homologues playing the respective roles in LR signaling, or perhaps the early steps involving activin and Shh are specific to avian species.

There Is Not an Irreversible LR Determination Prior to the Induction of the Primary Axis

The series of experiments described here is concerned with the investigation of the middle part of LR patterning: the cascade of differential gene expression which lies between initial LR asymmetry determination (the cause of the restriction of the very first gene to be asymmetrically expressed in any given embryo) and the final asymmetric morphogenesis of organs. The two most commonly discussed mechanisms of initial LR determination, a blastodermal prepattern of maternal origin and a chiral molecule within each cell, make opposite predictions for the laterality of 180° head-to-head twins. We have examined 12 cases of such spontaneously occurring twins. Determination of situs by means of molecular markers of laterality, embryonic turning, and morphological asymmetry at Hensen's node revealed that each twin was correctly patterned with respect to itself, not to a hypothesized blastodermal prepattern.

Although it is not clear with spontaneously arising twins exactly when the secondary streak was formed, in all cases examined the 180° twins appeared to be of identical stage and size, indicating that they arose at nearly the same time. In principle such spontaneous twins could have arisen within a common blastodisc or in two separate blastodiscs which subsequently fused. However, experimental protocols which induce 180° twins from within the same blastodisc give similar results to those we obtained, at least as assayed morphologically. For example, mechanical transection of a blastoderm can lead to formation of such head-to-head twins, each of which has correct situs (Lepori, 1967). Likewise, induction of ectopic streaks with activin and Wnt proteins (Cooke et al., 1994) can produce such twins, which we found in preliminary experiments to give similar results to those described here. We chose not to pursue the experimentally induced twins because in that paradigm, the streak-inducing factors could themselves influence situs, making interpretation difficult.

Our examination of 180° twins suggests that their respective situs either is determined without regard to information in the blastodisc or is dominant to any underlying bias present as a blastodermal prepattern. This streak-autonomous model is consistent with the theory of Brown and Wolpert (1990) who propose that cells contain a tethered chiral molecule whose directed differential activity serves to produce LR asymmetries. The rarity of experimental material made it impossible to obtain sufficient numbers to confidently test a variety of other angular orientations in addition to 180°. This would have been useful to rule out more exotic prepattern geometries than the one set out in Fig. 4; however, the results of Lepori (1967), who produced duck twins in various angular orientations and observed very few cases of situs inversus, are consistent with our own. The one other relatively common class of spontaneous twins represents those arising in parallel or nearly parallel orientations. Under such circumstances the nodes and streaks of the two embryos are in close juxtaposition throughout their development and in those cases the asymmetric signals appear to be able to cross between embryos (Levin et al., 1996). In the 180° twins the two primitive streaks form at opposite ends of the blastodisc. The nodes never become closely juxtaposed because even at full extension the primitive streak does not reach the anterior limit of what will be the embryo. We do not see evidence for cross-signaling in the 180° twins as downstream asymmetric markers and morphology are normal in each twin.
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