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Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of *Shh* asymmetry in the node

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SUMMARY

Invariant patterning of left-right asymmetry during embryogenesis depends upon a cascade of inductive and repressive interactions between asymmetrically expressed genes. Different cascades of asymmetric genes distinguish the left and right sides of the embryo and are maintained by a midline barrier. As such, the left and right sides of an embryo can be viewed as distinct and autonomous fields. Here we describe a series of experiments that indicate that the initiation of these programs requires communication between the two sides of the blastoderm. When deprived of either the left or the right lateral halves of the blastoderm, embryos are incapable of patterning normal left-right gene expression at Hensen's node. Not only are both flanks required, suggesting that there is no single signaling source for LR pattern, but the blastoderm must be intact. These results are consistent with our previously proposed model in which the orientation of LR asymmetry in the frog, *Xenopus laevis*, depends on large-scale partitioning of LR determinants through intercellular gap junction channels (M. Levin and M. Mercola (1998) *Developmental Biology* 203, 90-105). Here we evaluate whether gap junctional communication is required for the LR asymmetry in the chick, where it is possible to order early events relative to the well-characterized left and right hierarchies of gene expression. Treatment of cultured chick embryos with lindane, which diminishes gap junctional communication, frequently unbiased normal LR asymmetry of *Shh* and *Nodal* gene expression, causing the normally left-sided program to be recapitulated symmetrically on the right side of the embryo. A survey of early expression of connexin mRNAs revealed that *Cx43* is present throughout the blastoderm at Hamburger-Hamilton stage 2-3, prior to known asymmetric gene expression. Application of antisense oligodeoxynucleotides or blocking antibody to cultured embryos also resulted in bilateral expression of *Shh* and *Nodal* transcripts. Importantly, the node and primitive streak at these stages lack *Cx43* mRNA. This result, together with the requirement for an intact blastoderm, suggests that the path of communication through gap junction channels circumvents the node and streak. We propose that left-right information is transferred unidirectionally throughout the epiblast by gap junction channels in order to pattern left-sided *Shh* expression at Hensen's node.

Key words: Left-right, Chick, Asymmetry, Gap junction, Blastoderm, Cx43, *Shh*, *Nodal*

INTRODUCTION

The elaboration of left-right (LR) asymmetry during animal embryogenesis involves cascades of asymmetrically expressed genes (Fujinaga, 1996; Levin, 1998b). These genes regulate each others' expression in sequential pathways consisting of inductions and repressions on the left and right sides of the embryo (Levin et al., 1995, 1997; Isaac et al., 1997; Levin, 1998a; Logan et al., 1998). Distinct left and right programs of gene expression have suggested that the left and right sides of the embryo function autonomously. This paradigm includes the notion of a midline barrier to prevent the inappropriate crossover of secreted signaling molecules (Danos and Yost, 1996; Levin et al., 1996; Harvey, 1998; Meno et al., 1998).

Hensen's node is an important signaling center for the orientation of LR asymmetry. During normal chick development, the left side of the node expresses higher levels of *Shh* which in turn activates a cascade of gene expression in the left lateral plate, eventually inducing *Nodal* and *Pitx2* (Harvey, 1998; Levin, 1998b). Several lines of evidence suggest that LR pattern does not originate in the node but, rather, that it responds to signals from elsewhere in the blastoderm. When the node is ablated, it regenerates with correct LR asymmetry, as assessed by expression of the left-sided gene *Shh* (Psychoyos and Stern, 1996). In contrast, portions of the blastoderm, when cultured separately, also regenerate the node, but without proper patterning of the LR axis (Levin and Mercola, 1998b; Yuan and Schoenwolf, 1998). One interpretation of these observations is that signals from potentially distant regions of the blastodisc may be required upstream of LR patterning of Hensen's node.

In this paper, we describe experiments directed at

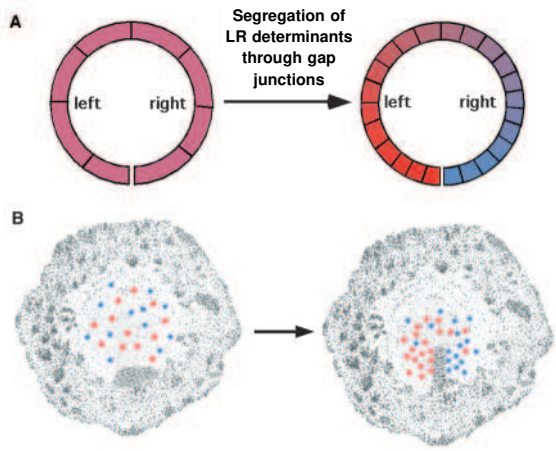


Fig. 1. LR asymmetric gap junctional communication in the early embryo. In a previous study, we showed that dorsoventral differences in GJC are likely to be involved in LR patterning of the early *Xenopus* embryo (Levin and Mercola, 1998). Introduction of gap junction communication ventrally by microinjection of wildtype connexin mRNA, or disruption of communication dorsally by injection of dominant negative connexins (but not the reverse) unbiased LR asymmetry. Affected embryos exhibited a heterotaxia syndrome characterized by mirror-image reversals of heart, gut and gall bladder development. These data suggested a model (A) in which low molecular mass determinants preferentially accumulate on one side of the midline (and later influence asymmetric gene expression). Asymmetric partitioning of the determinant(s) is facilitated by a zone of isolation that is relatively impermeable to the determinants (the ventral midline in the *Xenopus* embryo). Extended to the planar chick embryo (B), this model would suggest that LR determinants (red and blue dots) might segregate via gap junctions through the plane of the blastoderm. In the chick, the zone of isolation might be the streak and, in the simplest case, the flow of LR determinant(s) might circumvent the streak and node (as diagrammed).

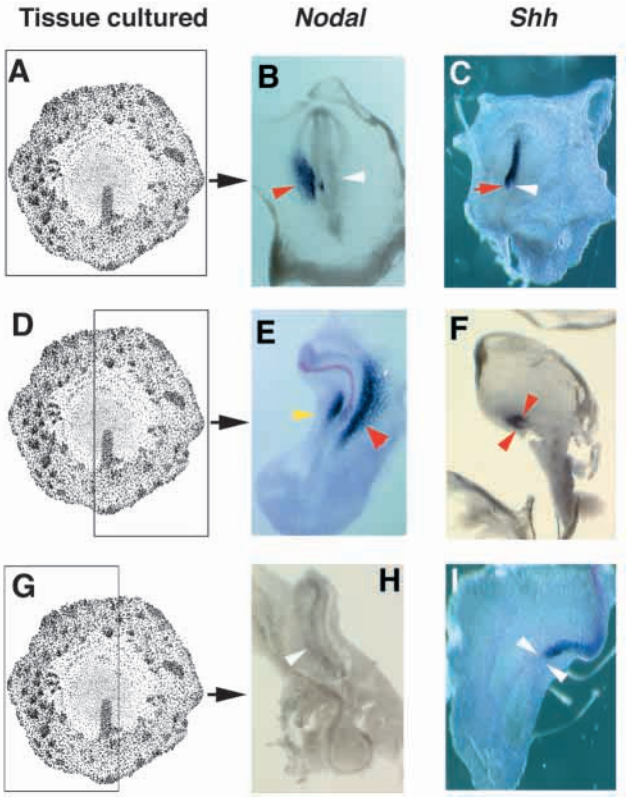


Fig. 2. Long-range signaling across the blastoderm is required for proper asymmetric expression of *Shh* and *Nodal*. (A-C) Control embryos cultured at stage 2 always developed proper left-sided expression of *Nodal* (B) and *Shh* expression (C). (D-F) In contrast, removal of the distal left-side of the blastoderm resulted in alterations of the normal asymmetry, such as ectopic expression of *Nodal* in the right LPM (E) and bilateral *Shh* in the node (F). Enough left-side tissue was left on during the explant to support endogenous *Nodal* expression on the left side (yellow arrow in E). (G-I) Likewise, removal of the distal right side of the blastoderm often resulted in a lack of normal *Nodal* expression in the left LPM (H) and symmetric (absent) *Shh* expression in the node (I). While other outcomes (such as bilateral, absent or right-sided *Nodal* or *Shh* expression) were present in each group (see Table 1 for numerical expression data and statistical analysis), only two sample scenarios are shown in E,F and H,I. Red arrows indicate expression; white arrows indicate lack of expression.

understanding whether communication between left and right halves of the chick embryo is necessary for the establishment of correct LR asymmetry in the node. Indeed, we found that removal of either left or right lateral tissue unbiased the LR program of gene expression, frequently leading to symmetrical expression of both *Shh* in Hensen's node and *Nodal* in lateral mesoderm. Instead of indicating a discrete signaling source of LR pattern upstream of Hensen's node, these studies are more consistent with a model in which communication between the left and right halves of the embryo is required for the LR asymmetrical patterns of gene expression. Further experiments in which slits were cut in distant portions of the blastoderm also resulted in anomalous asymmetric gene expression (described below) suggesting that communication between distant halves of the embryo does not take place exclusively through the node and streak but rather involves the whole blastoderm.

One model postulating such long-range information transfer as a means to orient LR asymmetry involves gap junctional communication (GJC) between cells in early embryos (Levin and Nascone, 1997). A gap junction channel is formed by assembly and docking of hexamers (one hexamer of each apposing cell) of proteins from the connexin family (Goodenough et al., 1996). The intercellular channels are permeable to molecules of less than 1000 M_r and have been implicated in many aspects of development and physiological regulation (Fraser et al., 1987; Guthrie and Gilula, 1989; Davies et al., 1996b; Lo, 1996). In previous work, we have shown that specifically disrupting the endogenous spatial pattern of differences in GJC in *Xenopus* embryos results in heterotaxia (Levin and Mercola, 1998c). Importantly, the observed heterotaxia was not the effect of altering individual organ morphogenesis per se but, instead, suggested that the means for orienting overall LR asymmetry to the embryonic DV and AP axes had been perturbed. To explain these results, we postulated a directed circumferential movement of LR determinants through the early embryo (Fig. 1), which can propagate single-cell chiral information (Brown and Wolpert,

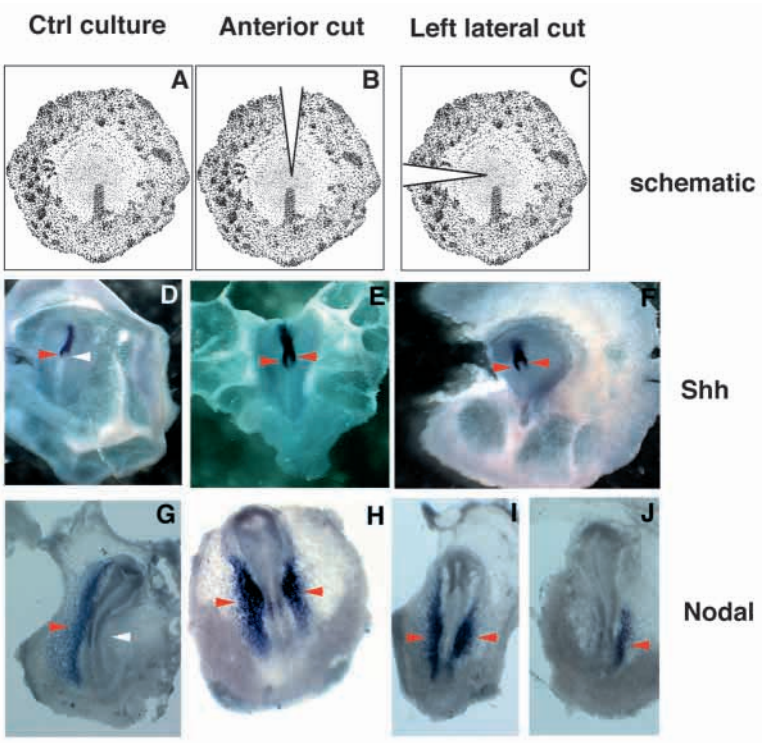


Fig. 3. Circumferential contiguity of the blastoderm is required for correct LR patterning. Control embryos cultured at stage 2 (A) always developed proper left-sided expression of *Shh* (D) and *Nodal* (G) expression. A single slit introduced through the anterior blastoderm resulted in frequent bilateral expression of *Shh* (E) and *Nodal* (H). Likewise, a single slit in the left lateral blastoderm induced ectopic domains of *Shh* (F) and *Nodal* (I,J) on the right side. Red arrowheads indicate expression; white arrowheads indicate a lack of expression. The numerical data is presented in Table 2.

1990) into cell fields of asymmetric gene expression. While we demonstrated that GJC plays a role during early stages of development (stage 5-12 in *Xenopus*) and is upstream of asymmetric *XNR-1* (the *Xenopus* homologue of *Nodal*) expression, the lack of earlier asymmetric markers in *Xenopus* made it impossible to place GJC more precisely within the known LR pathways. Thus, in order to determine whether GJC was upstream or downstream of the asymmetric information within Hensen's node, and to extend our model to the avian system, we explored whether the circumferential GJC model applies to the chick blastoderm, where gap junction-based transfer has been previously observed (Sheridan, 1966).

Our results suggest that GJC is an early component of the process that communicates LR pattern to Hensen's node. We find that *Connexin 43* (*Cx43*) is expressed in a circumferential pattern throughout the chick blastoderm at stage 2-3. Interfering with GJC in the early embryo by means of pharmacological agents, antisense oligonucleotides or antibodies directed against *Cx43* disrupts correct LR patterning. Introducing discontinuities in the circumferential path by making slits in the chick blastoderm likewise affects LR asymmetry. We conclude that (a) a system of gap-junctional communication is involved in left-right patterning in chicks as well as frogs, (b)

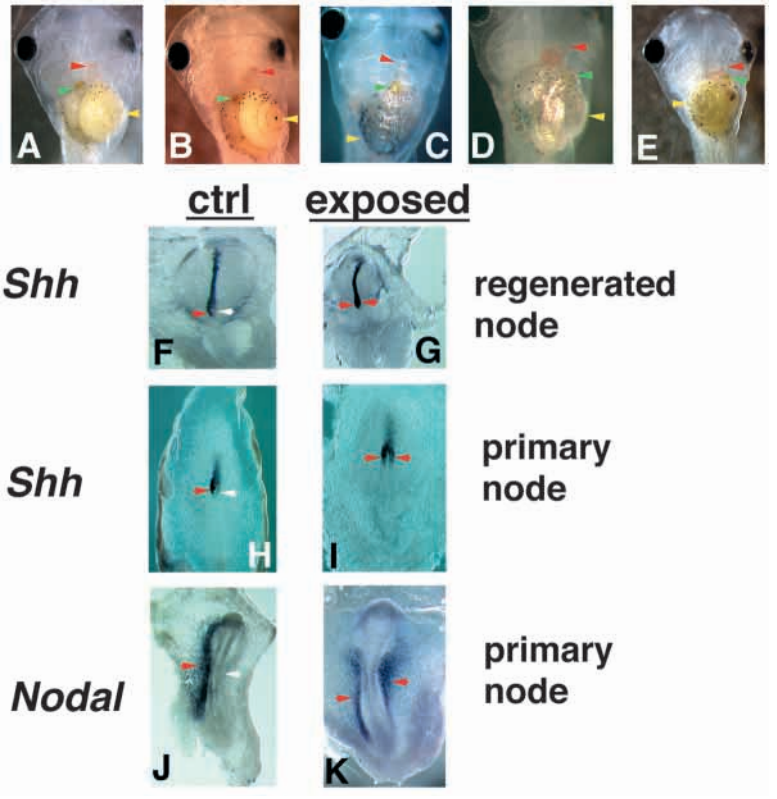


Fig. 4. Gap-junctional transfer is upstream of *Shh* and *Nodal* expression in chick embryos. Exposure to lindane, which causes closure of gap junctions, specifically led to heterotaxia in *Xenopus* embryos at doses which did not incur other defects (see Table 3 for numerical data). In contrast to situs solitus, where the heart loop points to the embryos' right, the gall bladder is on the embryos' right, and the gut apex points to the embryos' left (A), exposure to lindane caused inversions of the heart alone (B), the stomach alone (C), the heart and gall bladder (D), or full situs inversus (E). Red arrowheads indicate heart loop; yellow arrowheads indicate gut apex; green arrowheads indicate gall bladder. All *Xenopus* embryos are shown in ventral views. In chick embryos, when the node is excised at stage 3$^-$ and the embryos were allowed to develop in culture, normal left-sided expression of *Shh* was observed (F). Likewise, control cultured embryos with no surgery showed normal left-sided expression patterns of *Shh* (H) and *Nodal* (J). In contrast, following excision of the node, regenerated nodes of embryos cultured on medium containing lindane frequently had symmetrical expression of *Shh* (G). Moreover, embryos receiving no surgery, and cultured on medium containing lindane also show ectopic symmetrical expression of *Shh* (I) and *Nodal* (K). Red arrows indicate expression; white arrows indicate lack of expression. All chick in situ hybridizations are shown as dorsal views (such that the embryo's left is to the left of each panel). See Table 4 for numerical data. Red arrowheads indicate expression; white arrowheads indicate lack of expression.

its role is upstream of the asymmetric signals *Shh* and *Nodal*, and (c) *Cx43* is the likely candidate for gap junctions constituting the open GJC paths.

MATERIALS AND METHODS

Chick culture

All experimental manipulations were performed on standard pathogen-free White Leghorn chick embryos obtained from SPAFAS (Norwich, CT). Eggs at the stage indicated were cracked into a pan containing Pannett-Compton medium. Embryos were explanted under a dissecting scope. The embryo or tissue was placed ventral side upwards on a Costar 1 μm filter (catalog #110410) floating on top of 2 ml of Alpha-MEM medium containing no serum or any other additives except as indicated below. Embryos were then cultured at 38°C with 5% CO_2 until the required stage of development.

Surgeries

For whole-embryo culture, the entire embryo (including at least half of the radius of the area opaca) was cultured. Slits made in the embryo were made as single cuts with iridectomy scissors, taking care not to nick the node or streak. The slits healed and never resulted in twin embryos or duplicated structures. In experiments involving slits or cutting lateral sections of the blastoderms, the cuts were made no closer than 1 streak width to the streak or node.

Drug exposure

Xenopus embryos were exposed to 0.5 μg/ml lindane or 1 mg/ml EM12 in 0.1× MMR from fertilization to stage 16, washed thoroughly and allowed to develop to stage 43. Chick embryos were exposed to 25 μg/ml lindane or 1 mg/ml EM12 in the culture medium and harvested at stage 5 (to be examined for *Shh* expression) or stage 8 (to be examined for *Nodal* expression).

Oligonucleotide exposure

Antisense Cx43 and control oligonucleotides were prepared unmodified, exactly as in Makarenkova and Patel (1999). Oligodeoxynucleotides were included in the culture medium at a concentration of 0.1 mg/ml; embryos were cultured from stage 1 through to either stage 3 (to be examined for *Cx43* expression) or to stage 8 (to be examined for *Nodal* expression).

Blocking antibody exposure

Anti-Cx43 antibody (characterized in Wiens et al., 1995) at a dilution of 1:200 was added to the medium of whole embryos in culture. Embryos were cultured from stage 1 through to stage 8.

Chick in situ hybridization

Filters containing explants were transferred to 4% paraformaldehyde, and the explants were carefully detached and fixed overnight. Control embryos to be examined for connexin expression were fixed in 4% paraformaldehyde overnight. Control embryos and explants were processed for in situ hybridization in scintillation vials as previously described (Levin et al., 1995). Probes were as described: cRNA probes for *Shh* and *Nodal* (Levin et al., 1995); *Cx42* and *Cx45* cRNA probes (Beyer, 1990); *Cx43* probe (Musil et al., 1990). Histological sections were obtained by embedding embryos after in situ hybridization in JB4 and sectioning as previously described.

Scoring *Xenopus* embryonic situs

The laterality phenotype of embryos was determined by scoring the situs of the heart, stomach and gall bladder under a dissecting microscope in embryos immobilized with tricaine at stage 45. Only embryos with normal dorsoanterior development (DAI=5) and clear left-sided or right-sided organs were scored. A heterotaxic embryo was considered to be one in which any of those three organs was reversed in its position.

Statistical analysis

All quantitative data was analyzed using the Chi-squared test with Pearson correction (a more stringent version of the Chi-squared test).

RESULTS

Presence of lateral tissue is required for correct LR patterning of opposite side

In contrast to previous models that view the left and right sides of embryos as autonomous spatial fields isolated by a midline barrier, we asked whether long-range information transfer was part of the early processes underlying LR patterning in chicks. To determine whether events on one side of the embryo can affect markers of laterality on the opposite side, we removed the lateral tissue of stage 2 embryos in culture, cultured the remaining embryo and examined the expression of the asymmetric genes *Shh* and *Nodal*. The results are summarized in Table 1.

When whole embryos are cultured (Fig. 2A), normal left-sided expression of *Nodal* (Fig. 2B) and *Shh* (Fig. 2C) is observed in almost all cases. Surprisingly, when the left lateral tissue is removed (Fig. 2D), leaving the streak, node, and right lateral tissue intact, ectopic induction of *Nodal* on the right side is observed (46%, Fig. 2E). Likewise, *Shh* is seen to be symmetrically expressed in the node (61%, Fig. 2F). Similarly, when the right lateral tissue is removed (Fig. 2G), 33% of the embryos fail to develop the normal *Nodal* domain (Fig. 2H), while 88% of embryos show symmetrical (either bilateral or absent) *Shh* expression (Fig. 2I). These results are highly statistically significant to $P<3.6\times10^{-6}$ for *Shh* and $P<3.2\times10^{-17}$ for *Nodal*. Thus, the lack of either left or right lateral tissue can result in deviations from normal left-sided *Shh* or *Nodal* expression, often leading to bilaterally symmetrical expression or absence of the left-sided program of gene expression. Importantly, we conclude that the presence of lateral tissue on either side of the midline affects asymmetric gene expression on the contralateral side.

Circumferential contiguity of blastoderm is required for correct LR patterning

The dependence of correct asymmetric gene expression on the presence of lateral tissue on the opposite side of the midline could be due to signaling from each side to or through the streak and/or node tissue. Alternatively, it could result from long-range information transfer across the rest of the blastoderm (such as in a path that circumvents streak and node tissue). We asked whether such long-range signaling was taking place by culturing stage 2 embryos after slits had been made in the blastoderm outside of the streak and node. The slits were made as single cuts (no tissue was excised) along radii and the embryos were allowed to develop to appropriate stages to be analyzed for expression of the asymmetric markers *Shh* and *Nodal*. Slits were made either along the midline above the streak or on the left side, thus, ectopic gene expression would not arise from damage to the right lateral mesoderm where ectopic *Nodal* would be expressed. The results are summarized in Table 2.

Table 1. Presence of lateral tissue affects distant LR patterning events

Phenotype	Control culture	Removed L side	Removed R side
(A) Effects of removal of lateral tissue on expression of *Shh* in the node			
Left side	16	11	2
Right side	0	0	0
Absent	0	9	11
Bilateral	0	8	3
Total:	16	28	16
% incorrect:	0%	61%	88%

Removal of left side causes aberrant *Shh* expression to χ^2=13.37, P<0.00026.

Removal of right side causes aberrant *Shh* expression to χ^2=21.46, $P<3.6\times10^{-6}$.

(B) Effects of removal of lateral tissue on expression of Nodal in the LPM			
Correct	140	61	10
Incorrect	2	51	5
Total:	142	112	15
% incorrect:	1%	46%	33%

Removal of left side causes ectopic *Nodal* expression in the right side to χ^2=71.19, $P<3.2\times10^{-17}$.

Removal of right side causes a lack of *Nodal* expression in the left side to χ^2=25.4, $P<4.7\times10^{-7}$.

Table 2. Circumferential contiguity of blastoderm is required for correct LR patterning

Phenotype	Control culture	Cultured with anterior slit
(A) Anterior slits in blastoderm disrupt correct *Shh* expression		
Left side	17	4
Bilateral	0	11
Right	0	0
Absent	0	1
Total:	17	16
% incorrect:	0%	75%

Anterior slits in the blastoderm disrupt correct *Shh* expression to χ^2=16.93, $P<3.9\times10^{-5}$.

(B) Anterior slits in blastoderm disrupt correct *Nodal* expression		
Left side	68	23
Bilateral	0	6
Right	0	1
Absent	2	3
Total:	70	33
% incorrect:	3%	30%

Anterior slits in the blastoderm disrupt correct *Nodal* expression to χ^2=13.85, $P<2.0\times10^{-4}$.

Phenotype	Control culture	Cultured with left lateral slit
(C) Left lateral slits in blastoderm disrupt correct *Shh* expression		
Left side	17	9
Bilateral	0	10
Right	0	0
Absent	0	4
Total:	17	23
% incorrect:	0%	61%

Left lateral slits in the blastoderm disrupt correct *Shh* expression to χ^2=13.36, $P<2.6\times10^{-4}$.

(D) Left lateral slits in blastoderm disrupt correct *Nodal* expression		
Left side	126	32
Bilateral	2	21
Right	0	2
Absent	3	5
Total:	131	60
% incorrect:	4%	47%

Left lateral slits in the blastoderm disrupt correct *Nodal* expression to χ^2=49.9, $P<1.6\times10^{-12}$.

Control embryos (Fig. 3A) in culture showed normal left-sided expression of *Shh* (Fig. 3D) and *Nodal* (Fig. 3G). Surprisingly, when slits were made in the blastoderm 180° opposite the streak (Fig. 3B), the resulting embryos often had aberrant symmetric expression of *Shh* (75%, Fig. 3E) and *Nodal* (30%, Fig. 3H). Likewise, when slits were made lateral to the streak and node on the left side (Fig. 3C), symmetrical expression of *Shh* (61%, Fig. 3F) and *Nodal* (47%, Fig. 3I,J) were often observed. These results are highly statistically significant to P values between 2.0×10^{-4} and 1.6×10^{-12}.

Based on the requirement of contiguity of the blastoderm outside the streak and node for proper LR patterning, we conclude that some signaling between the left and right halves of the embryo takes place well outside of the streak and distant to sites of *Shh* and *Nodal* gene expression. The possibility of circumferential communication between left and right sides is similar to the pattern of gap-junctional communication (GJC) which we proposed is required for the establishment of LR asymmetry in *Xenopus* (Levin and Mercola, 1998c). We then used chick embryos to test several predictions of this model and to order gap junctional communication relative to the established hierarchies of sided gene expression.

Pharmacological disruption of junctional communication disrupts LR patterning of the blastoderm

The GJC model postulates that early differences between the left and right sides are set up by communication via gap junctions and predicts that disrupting GJC by pharmacological means will result in aberrant asymmetric gene expression in chick embryos. Thus, we asked whether GJC is necessary for proper LR patterning in the chick blastoderm.

Several drugs have been shown to decrease GJC and induce heterotaxia in *Xenopus* embryos (Levin and Mercola, 1998c). Some of these proved toxic for chick embryos (data not shown). Lindane, a proven inhibitor of GJC (Li and Mather, 1997), is well-tolerated by both frogs and chicks in culture. Specifically, lindane has been shown to affect Cx43 (Guan and Ruch, 1996), which is expressed in early *Xenopus* (Gimlich et al., 1990), zebrafish (Wagner et al., 1998) and mammalian embryos (Ruangvoravat and Lo, 1992).

To first characterize the effects of lindane in *Xenopus* embryos were exposed between stages 5 and 12 (Table 3). When cultured to stage 43, control embryos always exhibited normal right-sided heart looping and gall bladder situs, as well as leftward coiling guts (Fig. 4A). In contrast, exposure to lindane during early stages (stage 5-13) induced complete heterotaxia in 29% of the embryos, including individual reversals of the heart (Fig. 4B), gut (Fig. 4C), heart and gall bladder (Fig. 4D), and complete situs inversus (Fig. 4E). Induction of heterotaxia by lindane is highly statistically significant to $P<3.9\times10^{-9}$.

Having shown that the gap-junction inhibitor lindane causes heterotaxia in frogs, we asked whether LR patterning in the chick blastoderm was likewise dependent on GJC. It had

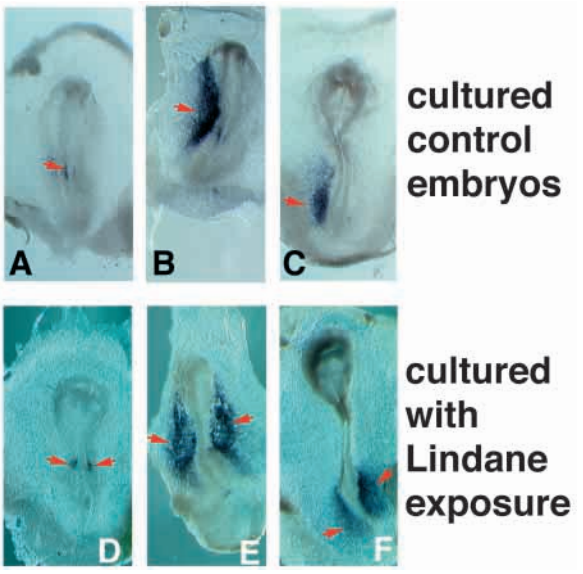


Fig. 5. Ectopic *Nodal* domain induced by lindane recapitulates normal progression of *Nodal* expression. The expression of *Nodal* starts out as a small domain proximal to Hensen's node at stage 7 (A) and a larger lateral domain appears soon afterwards (B), which later migrates posteriorly (C). These three phases of expression are identical to the pattern observed in embryos that are not cultured but harvested directly. When embryos are cultured in the presence of lindane, an ectopic domain of *Nodal* expression is induced on the right side in a pattern which exactly recapitulates the dynamic profile of expression of *Nodal* mRNA on the left side (D-F). Red arrowheads indicate expression.

previously been shown that when Hensen's node is excised from early embryos, it regenerates with correct LR asymmetry (Psychoyos and Stern, 1996). To determine whether this process is dependent on GJC, nodes were excised in chick embryos at stage 3$^-$ and were then cultured in the presence of lindane. Control embryos cultured without lindane regenerated nodes with correct left-sided *Shh* expression (Fig. 4F). In contrast, embryos cultured on medium containing lindane frequently exhibited symmetrical *Shh* expression in the newly regenerated node (Fig. 4G). We conclude that patterning of regenerating nodes is dependent on GJC.

We next asked whether the LR patterning of the embryo's original, primary node is likewise dependent on GJC. The results are summarized in Table 4. Chick embryos were cultured from stage 2 in medium containing lindane. Control embryos in culture always displayed the normal left-sided pattern of *Shh* (Fig. 4H) and *Nodal* (Fig. 4J). In contrast, embryos cultured in the presence of lindane frequently exhibited bilateral expression of *Shh* (45%, Fig. 4I) and *Nodal* (55%, Fig. 4K). We conclude that GJC is required upstream of *Shh* asymmetry in Hensen's node.

The expression profile of *Nodal* in embryos is dynamic and

Table 3. Lindane exposure causes heterotaxia in *Xenopus* embryos

Organ inverted	Control embryos	Lindane-exposed embryos
w.t. embryos (no inversion)	92	256
Heart	0	8
Stomach	0	1
Gall bladder	0	1
Heart and stomach	0	0
Heart and gall bladder	0	0
Stomach and gall bladder	0	46
Complete situs inversus	1	50
Total:	93	362
% heterotaxia:	1%	29%

Lindane causes heterotaxia in frog embryos to χ^2=34.7, P<3.9×10^{-9}.

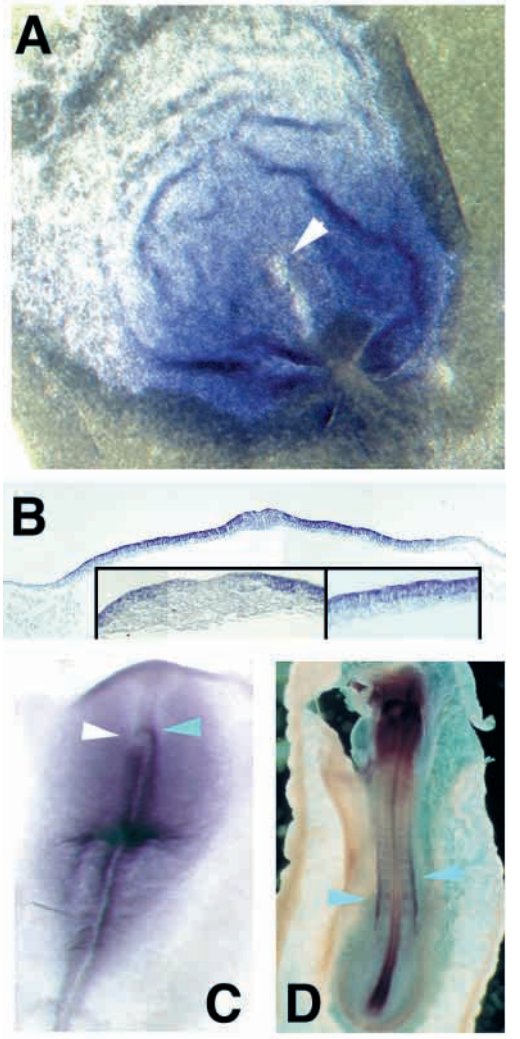


Fig. 6. *Cx43* is expressed in early chick embryos in a circumferential pattern. Wild-type embryos were processed for in situ hybridization with a probe to *Cx43* at early stages of development. (A) At stage 2, *Cx43* mRNA is present in a radial pattern throughout the blastoderm but is specifically excluded from the primitive streak (white arrowhead). (B) Sectioning reveals that expression is ectodermal. Insets show magnified views of lateral tissue (same embryo) and streak (from a different embryo). (C) At stage 5, *Cx43* is expressed in the streak and somewhat lateral to it, and shows an asymmetry, being present on the right side of Hensen's node (blue arrowhead indicates expression in the node; white arrowhead indicates lack of expression). (D) At later stages, *Cx43* is expressed in the tail bud and regressing streak, as well as tissues lateral to posterior somites (blue arrowheads).

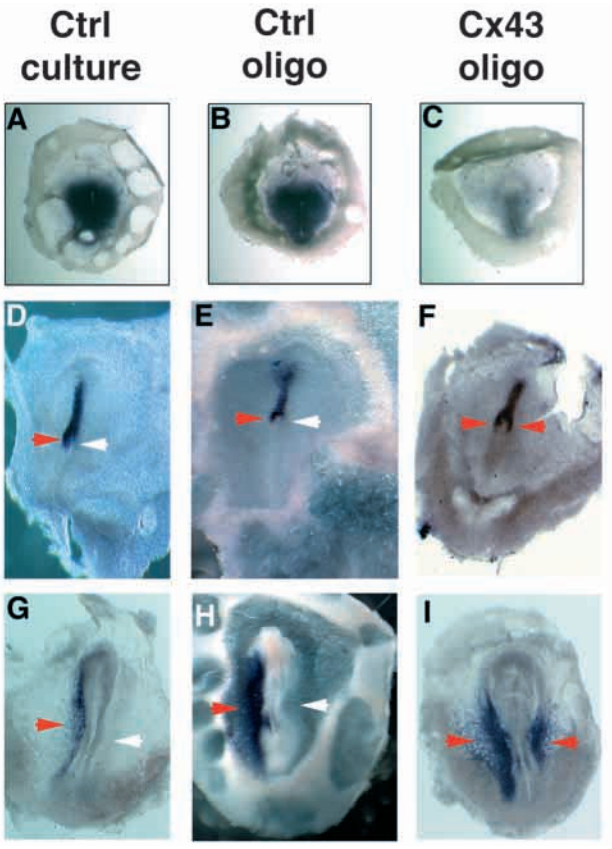
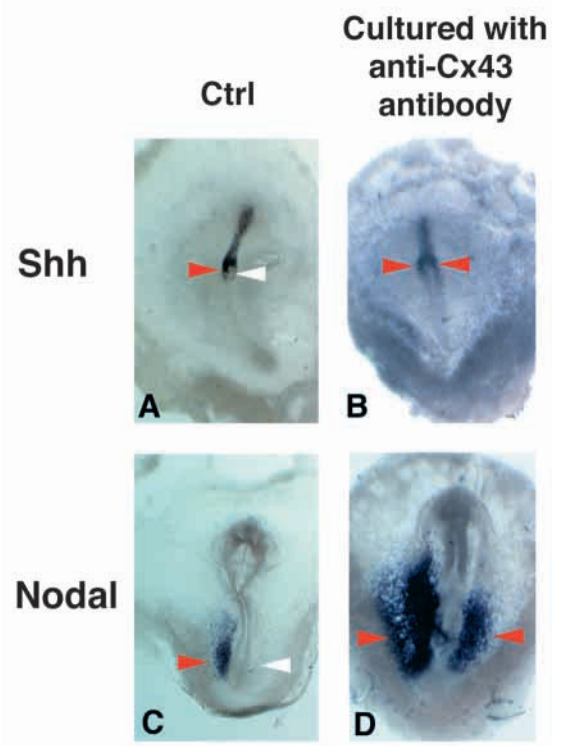


Fig. 7. Reduction of *Cx43* mRNA correlates with a loss of normal asymmetric expression of *Shh* and *Nodal*. (A) Embryos cultured to stage 3 showed abundant *Cx43* expression in the blastoderm. (B) Likewise, embryos cultured in the presence of a control, random antisense oligodeoxynucleotide also showed abundant *Cx43* message. (C) In contrast, a fraction (25%, see text) of embryos cultured in the presence of an antisense oligodeoxynucleotide to *Cx43* had a much reduced level of *Cx43* expression. (D,G) Untreated cultured embryos developed correct left-sided expression of *Shh* (D) and *Nodal* (G). (E,H) The same is true of embryos cultured with the control oligodeoxynucleotide. (F,I) In contrast, bilateral expression of *Shh* (F) and *Nodal* (I) was observed in embryos cultured with the antisense oligodeoxynucleotide to *Cx43*. Numerical data is summarized in Table 5. Red arrowheads indicate expression; white arrowheads indicate lack of expression.

complex, involving two different domains of left-sided expression, as well as a posterior movement of the larger domain despite anterior migration of cells at that time. Since the application of lindane in our previous experiment was systemic and not spatially specific, we sought to rule out simple induction of *Nodal* by lindane. Thus, we followed the spatial expression pattern of *Nodal* mRNA over developmental time. We examined stages 7 through 8$^+$ in embryos exposed to lindane. Embryos in culture first show a small left-sided domain of *Nodal* expression proximal to the node (Fig. 5A). This is followed by a larger distal domain (Fig. 5B), which later moves posteriorly (Fig. 5C). Similarly, the stereotypic, highly regulated, spatial profile of *Nodal* mRNA expression is duplicated on the right side of embryos when GJC is disrupted by lindane (Fig. 5D-F, compare Fig. 5A-C). We conclude that lindane is not simply an inducer of *Nodal* expression but rather functions upstream of the complex control mechanisms underlying the dynamic *Nodal* expression profile.



Connexin43 is expressed in a radial pattern throughout the blastoderm

Gap junctions are formed by apposing hexamers of connexin proteins inserted in to the plasma membranes of neighboring cells. Having seen that correct LR patterning of the blastoderm is disrupted by pharmacologically closing gap junctions, we sought to determine whether a connexin is expressed at early stages of chick development. The GJC model predicts expression of at least one connexin family member, which would be present in a radial pattern around the blastodisc. Since the node is patterned by stage 4$^+$ (Levin et al., 1995; Pagan-Westphal and Tabin, 1998), and lindane affects asymmetry when present from stage 2, the GJC model suggests expression between those two stages. Also, since asymmetric genes expressed in the node are ectodermal (Levin et al., 1995), the simplest model would predict the connexin to be present in the ectodermal cell layer.

Fig. 8. Application of antibody to Cx43 alters normal asymmetric expression of *Shh* and *Nodal*. (A,C) Embryos in culture with a control antibody showed correct left-sided expression of *Shh* (A) and *Nodal* (C). (B,D) In contrast, embryos exposed to anti-Cx43 antibodies showed bilateral expression of *Shh* (B) and *Nodal* (D). Numerical data is summarized in Table 6. Red arrowheads indicate expression; white arrowheads indicate lack of expression.

Table 4. Lindane exposure alters normal asymmetry of *Shh* and *Nodal* expression

Phenotype	Control culture	Exposed to Lindane
(A) Effects on expression of *Shh* within the node		
Left side	17	23
Right side	0	0
Absent from node	0	5
Bilateral	0	14
Total:	17	42
% aberrant	0%	45%
Lindane destabilizes *Shh* asymmetry to χ^2=9.36, $P<2.2\times10^{-3}$.		
(B) Effects on expression of *Nodal* in the lateral mesoderm (LPM)		
Left side	35	24
Right side	0	0
Absent from LPM	2	16
Bilateral	0	13
Total:	37	53
% aberrant	0%	55%
Lindane destabilizes *Nodal* asymmetry to χ^2=21.33, $P<3.9\times10^{-6}$.		

Table 6. Antibodies to Cx43 proteins disrupt LR patterning

Phenotype	Control culture	Cultured with Cx43 antibody
(A) Effect of Cx43 antibodies on expression of *Shh*		
Left side	12	4
Bilateral	0	2
Right side	0	0
Absent	0	2
Total:	10	8
% incorrect:	0%	50%
This effect is significant to χ^2=4.7, P=0.03.		
(B) Effect of Cx43 antibodies on expression of *Nodal*		
Left side	12	7
Bilateral	0	4
Right side	0	0
Absent	1	9
Total:	13	20
% incorrect:	7%	65%
This effect is significant to χ^2=8.37, P=0.0038.		

We surveyed expression of various connexin family members by in situ hybridization. Neither *Cx42* nor *Cx45* transcripts could be detected between stages 2 and 4^+ (data not shown). In contrast, *Cx43* (Wiens et al., 1995; Minkoff et al., 1997), which can perturb LR asymmetry upon overexpression in *Xenopus* (Levin and Mercola, 1998c), is broadly expressed in the blastoderm at stage 2 (Fig. 6A). Note that it is specifically excluded from the streak and node at stage 2-3. Very similar expression was observed in duck embryos (data not shown). Sectioning revealed that the expression is ectodermal (Fig. 6B). Later, *Cx43* is expressed in the right side of Hensen's node at stage 5^- (Fig. 6C), as well as in tissues lateral to the somites at stage 12 (Fig. 6D). Based on the mRNA in situ hybridization, we conclude that *Cx43* is expressed in a pattern that is consistent with providing a circumferential open GJC path for signaling between the L and R sides.

Connexin43 is functionally involved in LR patterning upstream of *Shh*

We tested the possibility that *Cx43* propagates LR information by diminishing expression with antisense oligodeoxynucleotides and blocking antibody. Modified antisense oligonucleotides have been previously demonstrated to be capable of blocking Cx43 function in the chick by diminishing mRNA levels (Makarenkova and Patel, 1999). Untreated embryos cultured in parallel to the treated embryos have normal expression patterns of *Cx43* (Fig. 7A), as do embryos cultured with the control (nonspecific) oligonucleotides (Fig. 7B). In contrast, 25% (n=12) of embryos cultured on medium containing antisense oligonucleotides to *Cx43* have a drastically reduced level of *Cx43* mRNA expression (Fig. 7C) as determined by in situ hybridization (several more embryos were observed to have less severe reductions in *Cx43* signal). We conclude that culture with antisense oligonucleotides significantly reduced the levels of *Cx43* mRNA present in a portion of the embryos.

We next asked whether reduction of *Cx43* mRNA might affect the LR pattern of the blastoderm. Control embryos in culture, as well as embryos cultured with the nonspecific antisense oligonucleotide, both displayed the normal left-sided patterns of *Shh* (Fig. 7D,G) and *Nodal* (Fig. 7E,H) expression. In contrast, embryos cultured on medium containing antisense oligonucleotides to *Cx43* often exhibited aberrant *Shh* (Fig. 7F) and *Nodal* (Fig. 7I) transcripts. The data is summarized in Table 5. Though only approximately 25% of the embryos were affected by the antisense oligonucleotide treatment, an almost equal number (22%) showed alterations in normal asymmetry of *Nodal* expression, suggesting that LR symmetry was affected in most of the instances where the abundance of *Cx43* mRNA was significantly decreased.

We next sought to determine whether blocking Cx43 protein directly affects LR asymmetry. Antibodies to chick Cx43 have previously been shown to work immunohistochemically (Wiens et al., 1995). Embryos cultured on medium containing non-specific antibody always display the normal left-sided expression of *Shh* (Fig. 8A) and *Nodal* (Fig. 8C). In contrast, embryos cultured on medium containing antibody to Cx43 displayed aberrant symmetric profiles of *Shh* (Fig. 8B) and *Nodal* (Fig. 8D) in 50% and 65% of the cases, respectively. The data is summarized in Table 6. Based on the oligonucleotide and antibody data, we conclude that Cx43 is functionally involved in LR patterning upstream of *Shh*.

Table 5. Depletion of *Cx43* mRNA disrupts LR patterning

Phenotype	Control culture	Culture with control oligo	Culture with Cx31 oligo	Culture with Cx43 oligo
Left side	108	23	21	32
Bilateral	1	0	0	4
Right	0	0	0	0
Absent	2	0	1	5
Total:	111	23	22	41
% incorrect:	3%	0%	5%	22%
Antisense oligodeoxynucleotides to *Cx43* disrupt correct asymmetry of *Nodal* expression to χ^2=4.19, $P<0.04$.				

Different mechanisms underlie the GJC isolation zone in chicks and frogs

The GJC model as drawn from the *Xenopus* data predicts a

Table 7A. EM12 exposure causes heterotaxia in *Xenopus* embryos

Organ inverted	Control embryos	EM12-exposed embryos
w.t. embryos (no inversion)	102	74
Heart	0	12
Stomach	0	3
Gall bladder	0	1
Heart and stomach	0	0
Heart and gall bladder	0	4
Stomach and gall bladder	0	12
Complete situs inversus	0	34
Total:	102	140
% heterotaxia:	0%	47%

EM12 causes heterotaxia in frog embryos to χ^2=62.7, $P<2.4\times10^{-15}$.

Table 7B. EM12 does not affect situs in chick embryos

Phenotype	Control culture	EM12-exposed
w.t. Nodal exposure	30	21
Aberrant Nodal exposure	0	0

zone of junctional isolation within the embryo that is crucial for proper LR asymmetry (Levin and Mercola, 1998c). The experimental introduction of functional connexins in this zone in *Xenopus* (which apparently lies on the ventral midline) results in heterotaxia. However, gap junctions can be regulated at several levels, from mRNA localization to post-translational mechanisms such as the phosphorylation-mediated gating of mature gap junctional complexes (Bruzzone et al., 1996b); thus, it is important to understand the level at which the isolation zone is regulated. As seen from the expression data of *Cx43* in chick embryos, the absence of *Cx43* mRNA in the streak could provide a barrier to the propagation of a putative LR determinant. The isolation zone in the frog embryo, in contrast, appears to be maintained at the protein level since exposure to drugs, such as melatonin or EM-12 (Nicolai et al., 1997), which cause the opening of existing gap junctions, also cause heterotaxia in *Xenopus* (Levin and Mercola, 1998c). This, combined with the induction of GJC prior to zygotic transcription at the mid-blastula transition, suggest that gap junction proteins are normally present in the isolation zone but are maintained in a non-functional state. To begin to investigate the basis of junctional isolation in chick embryos, we treated chick embryos with EM12 to evaluate whether alterations in the channel regulation could affect LR pattern.

When *Xenopus* embryos are cultured in the presence of EM12 during stages 5 to 13 of development, a 47% incidence of heterotaxia is observed, in the absence of other defects (see Table 7a). In contrast, when chick embryos are exposed to EM12, no aberrant expression patterns of *Nodal* are observed (Fig. 9, Table 7b). This result is consistent with the idea that junctional isolation of the chick streak results from an absence of mature connexin proteins that are permeable to the LR signal.

DISCUSSION

Large-scale communication in the blastoderm

Although many genes have now been positioned into distinct left- or right-sided hierarchies of gene expression (Isaac et al., 1997; Levin, 1998b; Logan et al., 1998; Meno et al., 1998; Ryan et al., 1998; Boettger et al., 1999), the upstream mechanisms responsible for initiating asymmetric gene expression during gastrulation are unknown. Thus, we have attempted to understand the nature of the signals that dictate the sidedness of early genes such as *Shh* in Hensen's node. It has been suggested that cells in the node (or their progenitors in the early streak) autonomously compute chirality from the AP and DV axes, perhaps utilizing dynein or other cytoskeletal mechanisms (Brown and Wolpert, 1990; Supp et al., 1997), as reviewed in (Levin and Mercola, 1998a). Alternatively, others have proposed that the node itself receives LR information by tissue immediately lateral to it (Pagan-Westphal and Tabin, 1998). Regardless, most models of LR patterning have viewed the embryo's left and right sides as independent regions, which are separated by a midline barrier impervious to LR signals (Levin et al., 1995). It was unexpected therefore that excising tissue on one side of the early embryo results in induction of asymmetric gene expression on the far contralateral side.

When lateral tissue is excised from the left side of the early chick embryo, leaving more than a streak-width of tissue next to the streak and node, *Nodal* is subsequently induced on the right. Fig. 2E (yellow arrow) shows that this happens even when sufficient lateral tissue is left intact to allow the endogenous left-sided *Nodal* domain to appear. The result that the gene expression on the right is affected by the disruption of tissue on the opposite side of the embryo and far from the streak is not predicted by models that focus on separate left and right programs of gene expression. Consequently, we propose that this result is evidence of an earlier developmental process and reflects large-scale communication between the left and right sides in the early embryo.

The notion that the orientation of LR asymmetry derives, in part, from the comparison of the embryo's left and right sides has been suggested by experiments in *Xenopus* (Hyatt and Yost, 1998). In previous work, we proposed a specific model in which long-range, LR information is transferred between the left and right sides of the early embryo by means of gap junctions (Levin and Mercola, 1998c). In *Xenopus*, we found that genetic or pharmacologic disruption of GJC dorsally, or introduction of communication ventrally, led to early alterations in LR patterning in the absence of other defects. In the pharmacologic experiments (in which stage of treatment could be controlled), embryos were particularly sensitive between stages 5 and 12. This suggested that an endogenous pattern of dorsal>>ventral GJC is crucial to subsequent orientation of LR asymmetry relative to the embryo's AP and DV axes, in agreement with measurements of dye transfer between blastomeres (albeit at stages 4-6; Guthrie, 1984; Guthrie et al., 1988; Olson and Moon, 1992). Based on these data, we proposed that low molecular mass determinants (the nature of which is currently unknown) traverse open junctional paths to preferentially accumulate on one side of the midline, and subsequently to induce asymmetric gene expression.

When applied to the chick blastoderm, this model makes very specific predictions, which are discussed individually below. We sought to test these predictions and to ascertain whether GJC might explain the surprising dependence of one side's asymmetric gene expression on events occurring on the opposite side. In addition to generalizing the model to chicks,

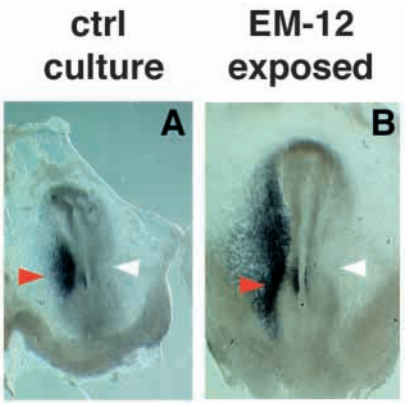


Fig. 9. EM12 does not alter LR asymmetry in chick embryos. (A) Embryos in culture showed correct left-sided expression of *Nodal*. (B) Chick embryos treated with EM12, which induces GJC and causes heterotaxia in *Xenopus*, also showed correct asymmetrical expression of *Nodal*.

our results indicate that GJC is required upstream of the known left- and right-sided cascades of gene expression.

The GJC model applies to LR patterning in early chick embryos

The first prediction of the GJC model in the chick (illustrated in Fig. 1) is that interruptions of the circumferential contiguity of the blastoderm should disrupt LR asymmetry. We found that introducing single slits in anterior tissue or left lateral tissue alter the normal asymmetric expression of *Shh* and *Nodal*. The fact that slits made on the left side result in the induction of *Nodal* on the right side (see Fig. 3J) is further evidence of the long-range signaling that must occur upstream of asymmetric gene expression.

A second prediction is that GJC should be upstream of asymmetric gene expression. This prediction is likewise validated, as we show that exposure of chick embryos to lindane, a drug which closes gap junctions and specifically induces heterotaxia in *Xenopus* embryos, results in alterations in the normal expression of both *Shh* and *Nodal* (Fig. 4). It should be noted that fewer than 100% of the embryos are expected to be affected by this treatment (and all other experiments described in this paper), because (a) the drug was titrated to a level low enough not to cause toxic effects, and (b) even in completely randomized embryos, a 25% incidence of correct, left-side only gene expression would be expected to be observed by chance alone (thus at most 75% would exhibit aberrant [bilateral absent, bilateral symmetric, or right sided] gene expression).

These results place GJC upstream of *Shh* expression in chick LR patterning. Moreover, these manipulations recapitulate the normal dynamic and complex profile of *Nodal* expression on the right side (Fig. 5). This reinforces the idea that GJC is upstream of the left-sided cascade of gene expression, including the mechanisms that regulate the complex pattern of *Nodal* expression.

A third prediction of the model is that one or more members of the connexin family must be expressed broadly throughout the blastoderm to provide a basis for the circumferential junctional path. Furthermore, it must be expressed prior to stage 4 (when asymmetric gene expression is beginning). Indeed, we find that *Cx43*, which has been implicated in LR patterning in humans (Britz-Cunningham et al., 1995, but see Gebbia et al., 1996) and which can cause heterotaxia in *Xenopus* when misexpressed (Levin and Mercola, 1998c), is expressed throughout the ectoderm of the chick embryo at stages 2-3 (Fig. 6). *Cx43* expression is ectodermal and may, therefore, affect *Shh* expression without an extracellular signaling intermediate.

The expression pattern that we observed for *Cx43* is ideally suited to a role in providing circumferential GJC paths. Treatment of cultured embryos with either antisense oligonucleotides or blocking antibody specifically altered the normal asymmetric expression of *Shh* and *Nodal* (Figs 7, 8), strongly suggesting that *Cx43* is involved in mechanisms upstream of the *Shh* asymmetry. Embryos deficient in Cx43 activity appear grossly normal, but we cannot rule out subtler defects, which would be detected only by examining molecular markers. Thus, we cannot say whether the early expression of *Cx43* is involved in processes other than LR patterning. Interestingly, the homozygous $Cx43^{-/-}$ mutant mice do not have a LR patterning defect (Reaume et al., 1995). Although alterations in heart looping have been described (Ya et al., 1998), they have been attributed to altered neural crest cell migration (Huang et al., 1998). *Cx43* is indeed expressed at streak stages in the mouse (Ruangvoravat and Lo, 1992) but it is not known whether the expression pattern matches that found in the chick. Either compensation or redundancy could explain the lack of an early LR phenotype in the homozygous $Cx43^{-/-}$ mutant mice. At least eleven members of the connexin family are present in mice (Davies et al., 1996a), and it is known that different connexins can oligomerize into functional gap junction channels (Bruzzone et al., 1996a). Indeed, defects in early embryonic compaction were expected for the *Cx43* knock-out mouse and it was unexpected that compaction proceeded normally in these mice. As such, either compensatory or redundant connexins are likely explanations (DeSousa et al., 1997). In *Xenopus*, *Cx43* was reported (Gimlich et al., 1990) to be expressed only after LR asymmetry has been patterned (dependence on GJC has been determined to be between stage 5-12, (Levin and Mercola, 1998c)). While low abundance transcripts or maternal Cx43 protein would not have been detected, this finding is consistent with the idea that at least one additional connexin is involved in orienting LR asymmetry.

The open path of intercellular communication must be terminated by a zone of isolation in order to confer laterality information. Exclusion of *Cx43* mRNA in the streak and node of stage 2-4 embryos may provide such a zone. Gap junction channels are subject to complex regulation and we have suggested that the zone of junctional isolation in *Xenopus* is maintained by rendering existing channels impermeable to LR determinants (as discussed in Results above). Interestingly, the absence of *Cx43* mRNA in the chick streak suggests that, in birds, the zone of junctional isolation is maintained at the mRNA level, by spatial control of transcription. Consistent with this model, we show that EM12, a drug that opens gap junctions and induces heterotaxia in *Xenopus*, has no effect on the asymmetry of chick *Nodal* expression. Apparently, the general circumferential GJC scheme is conserved between frogs and chicks, but details, such as the connexin that

constitutes the active junctions or the means of establishing the isolation zone, might vary among species.

GJC and events upstream of node asymmetry in chick

Following extirpation, nodes that regenerate in intact embryos always display correct asymmetry of *Shh* and *Nodal* (Psychoyos and Stern, 1996). In contrast, lateral explants regenerate a morphologically correct Hensen's node but one without proper LR asymmetry (Levin and Mercola, 1998b; Yuan and Schoenwolf, 1998). We extend this observation to show that embryos in which either left or right lateral tissue, but not node, has been excised also do not reliably form correctly patterned nodes (Fig. 2). Moreover, disruption of blastoderm contiguity by microsurgical slits along radii also unbiases node LR asymmetry, frequently leading to bilateral *Shh* and *Nodal* expression. Based on experiments where an early node (stage 4-5), rotated 180° within the plane of the tissue, developed correct LR asymmetry (relative to the blastodisc), Pagan-Westphal and Tabin (1998) hypothesized that the node receives LR information from immediately adjacent tissue. Our data are in agreement with the notion that laterality information resides in adjacent lateral tissue; however, they are inconsistent with discrete, independent sources of inductive or repressive signals in lateral tissue on each side of the node. Instead, we propose that proper patterning of the node requires communication between the left and right sides, mediated by circumferential contiguity.

Our data argue for a role of GJC upstream of asymmetric gene expression in the streak and node. It has recently been proposed that chiral motion of node monocilia is an important step in LR patterning in mice (Nonaka et al., 1998). We speculate that GJC might act earlier than asymmetric ciliary movement since both Cx43 expression and the requirement for an intact blastoderm precede formation of Hensen's node. Testing this hypothesis directly is not yet feasible since asymmetric node ciliary motion has been observed only in mature nodes of mouse embryos and LR pattern dependence on GJC has not yet been characterized in mice. It will be interesting to order these events and determine whether and how GJC and ciliary motion are linked.

Our data are consistent with a model in which epiblast cells, beginning at stage 2-3, are connected by gap junctions and that LR information is propagated circumferentially around the developing node and streak (Fig. 1). Although the nature of low molecular mass LR determinants is unknown, we envision preferential accumulation within, or on one side of, the primitive streak. These molecules can then (probably indirectly) induce transcription, resulting in previously described cascades of asymmetric gene expression. It should be noted that this model provides a mechanism for linking the LR axis with the DV and AP axes, and transducing chirality at the cell level into multicellular asymmetry, which can result in large domains of asymmetric gene expression (Levin and Nascone, 1997; Levin and Mercola, 1998a). Our model does not provide a mechanism for 'step 1' of LR patterning – the very first differentiation of left from right, which is necessary for the circumferential path to propagate information in one direction. This step may occur in a few cells (or possibly in a single cell) and may involve the recently characterized products of the murine *inv* or *iv* genes (Supp et al., 1997; Mochizuki et al., 1998). Once oriented, a cell may propagate LR information unidirectionally through gap junctions, possibly by orienting assembly of biased ion channels (Werner et al., 1989; Barrio et al., 1991; Robinson et al., 1993). Experiments are currently underway to characterize the low molecular mass determinants that traverse gap junctions, and to identify the cause of the uni-directional transfer.

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