DEVELOPMENTAL GENETICS 23:185–193 (1998)

Evolutionary Conservation of Mechanisms Upstream of Asymmetric *Nodal* Expression: Reconciling Chick and *Xenopus*

MICHAEL LEVIN* **AND MARK MERCOLA**
Cell Biology Department, Harvard Medical School, Boston, Massachusetts

ABSTRACT Recent experiments have suggested a pathway of genes that regulate left-right asymmetry in vertebrate embryogenesis. The most downstream member of this cascade is *nodal* (XNR-1 in frogs), which is expressed in the left-side lateral mesoderm. Previous work in the chick [Levin, 1998] suggests that an inductive interaction by *Shh* (*Sonic hedgehog*) present at the midline was needed for the left-sided expression of *nodal,* which by default would not be expressed. Interestingly, it has been reported [Lohr *et al.,* 1997] that in *Xenopus*, right-side mesoderm that is explanted at st. 15 and allowed to develop in culture, goes on to express *nodal,* suggesting that lateral mesoderm expresses this gene by default and that a repression of *nodal* by the midline is needed to achieve asymmetry. Such a contradiction raises interesting questions about the degree of conservation of the mechanisms upstream of *nodal* asymmetry and, in general, about the differences in the LR pathway among species. Thus we examined this issue directly.

We show that in the chick, as in the frog, explanted mesoderm from both sides does, indeed, go on to express *nodal,* including both the medial and lateral expression domains. Ectopic nodal expression in the medial domain on the right side is not sufficient to induce an ectopic lateral domain. We also show that explanted lateral tissue regenerates node/ notochord structures exhibiting *Shh* expression. Furthermore, we show that *Xenopus* explants done at st. 15 also regenerate notochord by the stage at which *XNR1* would be expressed. Thus explants are not isolated from the influence of the midline. In contrast to the midline repressor model previously suggested [Lohr *et al.,* 1997] to explain the presence of *nodal* expression in explants, we propose that the expression is due to induction by signals secreted by regenerating node and notochord tissue (*Shh* in the chick). Thus our results are consistent with *Shh* being necessary for *nodal* induction in both species, and we provide an explanation for both sets of data in terms of a single conserved mechanism upstream of *nodal* expression. Dev. Genet. 23:185–193, 1998. © 1998 Wiley-Liss, Inc.

Key words: left-right asymmetry; *nodal,* regulation; regeneration; notochord

INTRODUCTION

Left-right (LR) asymmetry is a key feature of vertebrate embryogenesis [Fujinaga, 1996; Levin, 1997; Wood, 1997; Levin, 1998; Levin and Mercola, 1998]. Within the last few years, some understanding of the molecular basis for LR patterning has been gained through the characterization of a cascade of asymmetrically expressed genes in the chick [Levin *et al.,* 1995, 1997; Isaac *et al.,* 1997]. The most downstream member of this cascade, a *TGF-β* family member called *nodal* (*XNR-1* in frogs), is expressed in the left lateral plate mesoderm (LPM) of gastrulating chick, frog, and mouse embryos [Collignon *et al.,* 1996; Lowe *et al.,* 1996]. When misexpressed on the right, in both chick and *Xenopus,* this gene causes changes in the *situs* of the heart and other organs [Levin *et al.,* 1997; Sampath *et al.,* 1997].

In the chick, *Sonic hedgehog* (*Shh*) is expressed on the left side of Hensen's node, prior to the appearance of asymmetric *nodal* expression, in cells that are directly adjacent to cells expressing *nodal* [Levin *et al.,* 1995; Levin, 1997]. Furthermore, misexpression of *Shh* on the right results in ectopic right-sided *nodal* expression and a randomization of heart *situs.* Likewise, abolishing *Shh* expression with activin bead implants [Levin *et al.,* 1995] or anti-Shh antibodies [Pagan-Westphal and Tabin, 1988] leads to a loss of *nodal* expression. These data have been interpreted [Levin *et al.,* 1995] to suggest that *Shh* is an inducer that lies upstream of *nodal* (Fig. 1A). Thus lateral plate mesoderm by default would not be expected to express *nodal.* The presence of *Shh* on the left side, itself a consequence of earlier asymmetric events, induces *nodal* in the left LPM,

Contract grant sponsor: NIH; Contract grant number: HL59502; Contract grant sponsor: American Heart Association; Contract grant number: 97400521 (to M.M.); Contract grant sponsor: Helen Hay Whitney fellowship (to M.L.); Contract grant number: F-773.

*Correspondence to: Cell Biology Dept., Bldg. C1, Room 403, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. E-mail: mlevin@fas.harvard.edu

Received 15 April 1998; Accepted 12 June 1998

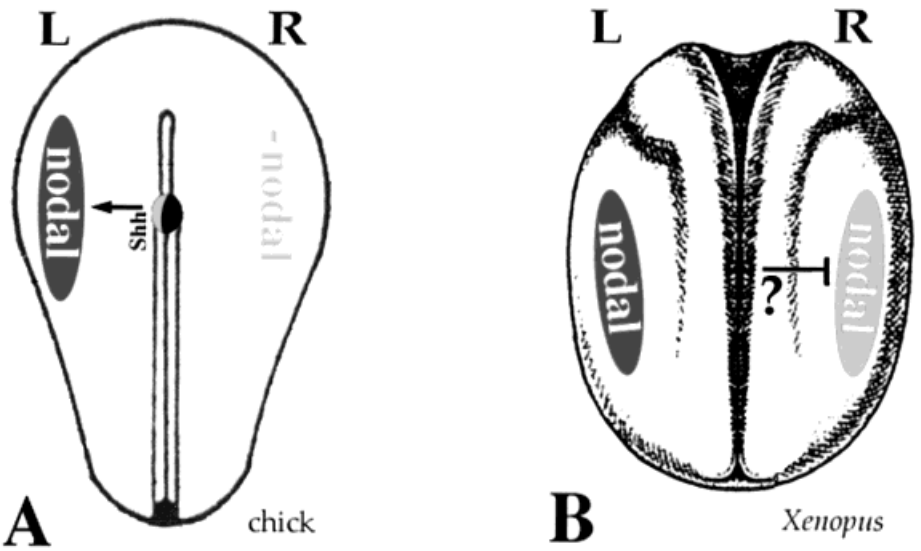

Fig. 1. Two competing models of events upstream of *nodal* asymmetry. Nodal (*Xnr-1* in *Xenopus*) is expressed in left lateral mesoderm in chicks, frogs, and mice. Studies in the chick [Levin, 1998] support the model that *Shh,* present in the left half of Hensen's node, is necessary to induce *nodal* on the left. In the absence of such an induction, nodal is not expressed on either side. In contrast, recent experiments in *Xenopus* [Lohr *et al.,* 1997] have been interpreted to suggest that both sides are normally committed to express *nodal* and that a midline repressor is needed to prevent right-sided expression. Such a discrepancy would be very difficult to understand in evolutionary terms.

which then signals further to asymmetric organs such as the heart.

Although this model (including *activin* as a factor thought to be upstream of *Shh*) fits the chick data, it is unclear to what extent the postulated pathway upstream of *nodal* applies to other species. No asymmetric expression of *Shh* has been observed in mice [Collignon *et al.,* 1996]. Likewise, null mutations in activin ligand do not result in a laterality phenotype [Matzuk *et al.,* 1995]. However, *activin receptor IIB* null mutant mice exhibit isomerism [Oh and Li, 1997], and mice with ectopic expression of *Shh* do show ectopic *nodal* expression [C. C. Hui, personal communication].

Interestingly, Lohr *et al.* [1997] reported that right LPM from *Xenopus* embryos, when cultured in explant from st. 15, goes on to express *XNR-1,* the frog homolog of chick *nodal.* This result can be taken to imply that *XNR-1* is expressed in lateral tissue as a default and that right-sided expression is normally inhibited by midline structures (Fig. 1B). This interpretation would contradict the model of the chick LR pathway; thus the frog data have interesting implications for understanding the asymmetric regulation of *nodal* expression. A priori, one can see at least two possible interpretations of the data that resolve this contradiction. Perhaps the pathway model needs to be modified in a way consistent with both the frog and chick data, e.g., instead of inducing *nodal* expression, perhaps *Shh* represses the expression of a midline repressor of *nodal* expression. Alternatively, perhaps the regulatory steps upstream of *nodal* asymmetry differ in frogs and birds. The latter possibility would be especially surprising given the conservation of *nodal* expression in several species. We explored this issue by an investigation of the fates of both chick and *Xenopus* explants.

MATERIALS AND METHODS

Chick Explants

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 PBS or Pannett-Compton medium. Embryos were explanted under a dissecting scope and trimmed of tissue anterior and posterior to the ends of the *area pellucida.* Then, the entire area to the right or left of the primitive streak was cut away and placed ventral side upward on a Costar 1 μm filter (catalog #110410) floating on top of 3 ml of medium (10% Fetal Calf Serum, 2% chick extract, 1% penicillin/streptomycin, 1% L-glutamine, in Alpha-MEM medium). In the control experiment, the explant was done similarly except the node was allowed to remain with the explant. Explants were cultured at 38°C with 5% CO_2 for 10–20 hours.

Chick In Situ Hybridization

Filters containing explants were transferred to 4% paraformaldehyde, and the explants were carefully detached and fixed overnight. Explants were processed for in situ hybridization in scintillation vials as previously described [Levin *et al.,* 1995].

Chick *Nodal* Viral Implants

Chick embryonic fibroblast (CEF) cells were infected with the *nodal* virus described previously [Levin *et al.,* 1997]. Briefly, the *BMP-4* pro region (including the cleavage cite) was fused to the *cNR-1* mature region and inserted into the RCAS-BP(A) vector. CEF cells infected with this virus pelleted, and the pellets were implanted between the epiblast and hypoblast on the

right side of st. 5–6 embryos in New culture [New, 1955].

Xenopus Explants

Xenopus embryos obtained by standard methods were grown to st. 16 in 0.1× MMR. They were then transferred to 0.75× MMR in a dish whose bottom was covered by 1% agarose in 0.75× MMR and de-vitellinized by forceps under a dissecting microscope. Using a sharp pair of forceps, the embryos were cut into portions comprising the left and right lateral pieces and a strip of dorsal tissue including the neural plate and notochord (~3 notochord widths). All explants contained underlying mesoderm and endoderm. Explants were then cultured in 0.75× MMR. Explants healed in ~30 minutes.

Xenopus In Situ Hybridization

In situ hybridization was performed according to a standard protocol [Harland, 1991].

Xenopus Antibody Staining

Embryos and explants were fixed in 4% formaldehyde in MEM salts for 1 hour. They were then dehydrated into methanol and stored. Prior to antibody staining, explants were rehydrated into PBS, blocked with 20% sheep serum in PBST (PBS + 0.1% Triton X-100 + 2 mg/ml BSA) for 1 hour, and incubated with a 1:1,000 dilution of primary MZ15 [Salisbury and Watt, 1988] antibody overnight at 4°C. Explants were then washed 5× in PBST, and a 6th wash in PBST overnight. Secondary antibody detection was done with an anti-mouse alkaline-phosphatase conjugated antibody overnight at 1:1,500 dilution in PBST + 20% sheep serum. Explants were then washed 5× in PBST and a 6th wash in PBST overnight. Detection was done with NBT and BCIP as for in situ hybridization and lasted 1.5 hours. Explants were then fixed with 4% paraformaldehyde, washed in PBS, and scored under a dissecting scope.

RESULTS

Both Left and Right Chick Explants Express *Nodal*

To investigate the possible discrepancy between the model of the inductive events thought to lead up to *nodal* expression in chick, and the midline repression model suggested by recent experiments in *Xenopus,* the first set of experiments were designed to recapitulate the explant experiments of Lohr *et al.* [1997] in the chick. Thus we wanted to look for *nodal* expression in cultured lateral tissue when explanted away from the primitive streak and Hensen's node at a stage before the asymmetric expression of *Shh* (St. 4).

In the chick, *nodal* is expressed in lateral plate mesoderm [Levin *et al.,* 1995]. Thus, in order to investigate *nodal* expression in cultured explants, it was first necessary to show that mesodermal precursors had already left the streak and were present in explanted tissue, since otherwise a negative result could be attributed to lack of cells able to express *nodal.* It is generally believed that mesodermal precursors have already begun to ingress into lateral tissue away from the streak at stages 4 [Rosenquist, 1966; Vakaet, 1970; Nicolet, 1971; Schoenwolf *et al.,* 1992]. To show this conclusively, at the stages at which our explants were to be made (an example is shown in Fig. 2A), we examined by wholemount in situ hybridization the expression of the chick gene *Brachyury* (*cBra*), which is a marker for mesodermal cells [Knezevic *et al.,* 1997]. It is seen that at st. 4 *cBra* expression is detected at a significant distance away from the streak in whole embryos (Fig. 2B). To be sure that our explants contained mesodermal cells, explants (containing no streak or node tissue) made at st. 4 were immediately fixed and hybridized to a probe to *cBra.* The expression pattern shows (Fig. 2C) that such explants do indeed contain mesodermal precursors.

Having established the presence of mesodermal precursors in explants, it was necessary to show that they are present in sufficient abundance to provide *nodal* expression. Thus we made explants of left lateral tissue containing the node (which would provide the left-sided *Shh* signal needed to induce *nodal* expression), but excluding the primitive streak (the source of lateral mesodermal cells) [Psychoyos and Stern, 1996a]; this is schematized in Figure 2D. When cultured, such explants go on to display *nodal* expression (Fig. 2E), showing that sufficient numbers of mesodermal cells have already left the streak by the time our explants were done.

We next wished to show that our culture conditions recapitulate the normal progression of events leading up to *nodal* induction. Thus we explanted left and right sides of a st. 6 blastoderm including the primitive streak and node and cultured these for 6 hours. When these explants were fixed and probed with a *nodal* probe, it was observed that, as in the intact embryo, the left side (Fig. 2F) goes on to express *nodal* (6 out of 7 cases, Fig. 2G), whereas the right side (Fig. 2H) does not (0 out of 10 cases, Fig. 2I). Taken together, these data show that our culture system allows the induction and subsequent expression of *nodal* with correct sidedness, when the midline is present.

The pathway proposed for events leading up to LR asymmetry in the chick would suggest that in the absence of a source of *Shh* expression (Hensen's node), *nodal* would not be expressed. To ask whether chick lateral tissue would express *nodal* when cultured in isolation from the node and streak, as has been seen in *Xenopus,* we explanted left and right halves of a st. 4 embryo, just adjacent to the primitive streak, and cultured these separately for 12–18 hours (Fig. 3). Surprisingly, *nodal* expression was observed in 38% of left (n = 31, Fig. 3A) and 40% of right (n = 44, Fig. 3B)

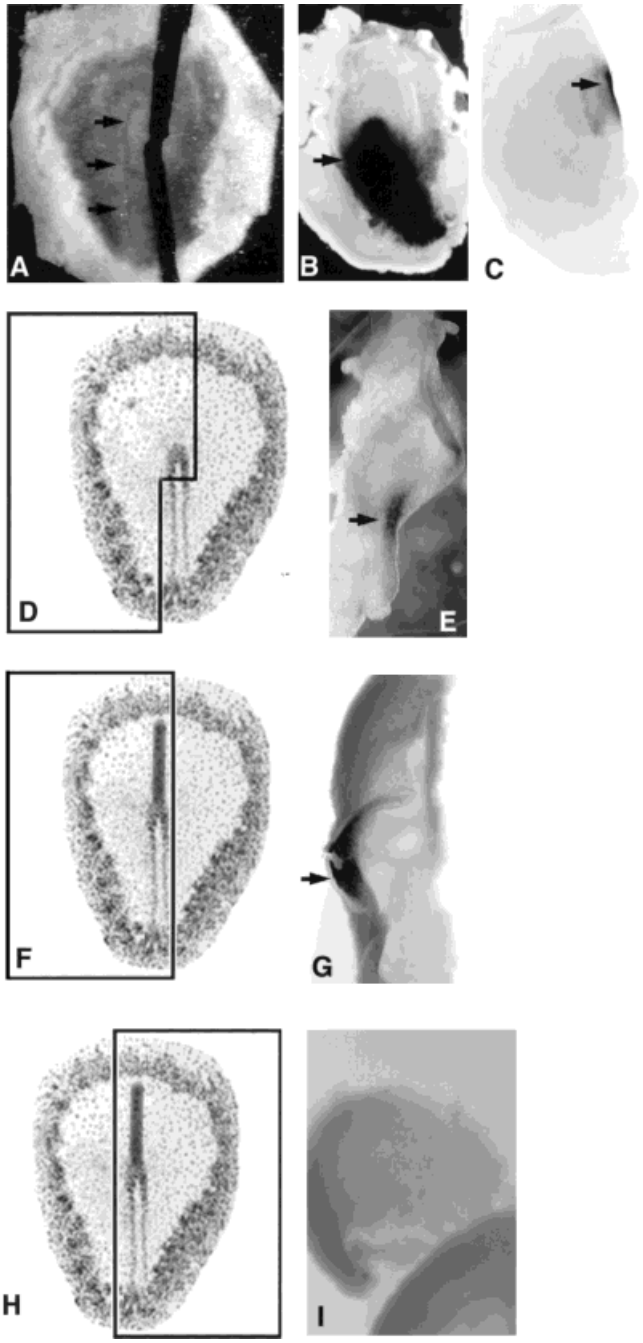

Fig. 2. Explant and culture conditions allow *nodal* expression in presence of midline. **A.** Explants were made by cutting halves of blastoderms, immediately adjacent to the primitive streak, at st. 4; arrows indicate primitive streak. **B.** Embryos at this stage show Brachyury (a mesoderm marker) stain lateral to the primitive streak. **C.** Stain is also seen in the explant (arrow). Thus mesodermal precursors are present in explants. **D.** When the node but not the streak is included in left explants, the explants go on to express *nodal* (**E,** arrow indicates expression), showing that sufficient numbers of mesodermal cells have left the streak by st. 4 to support *nodal* expression in the presence of signals from the node. **F.** Left lateral explants including the streak and node made at st. 6 go on to express *nodal* (**G,** arrow indicates expression). **H.** Right explants including the streak and node at st. 6 do not express nodal **I.** Thus culture conditions allow the proper sequence of events upstream of *nodal* expression.

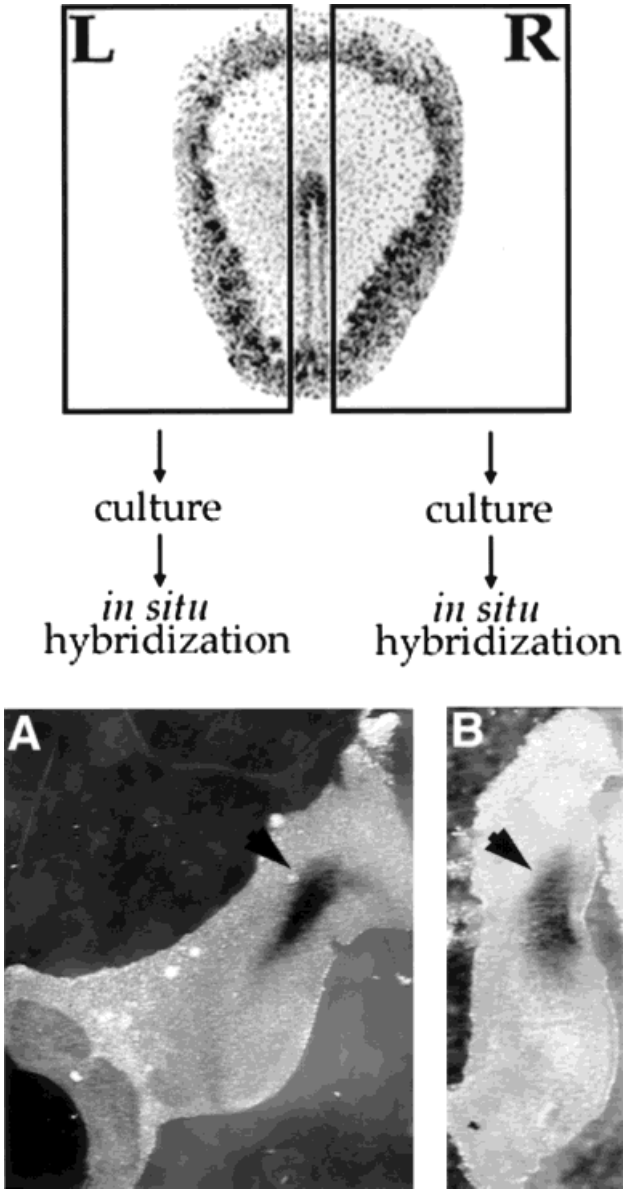

Fig. 3. As in *Xenopus,* chick lateral tissue expressed *nodal* when explanted away from the midline. **A.** When left lateral tissue, not including primitive streak or Hensen's node, is explanted at st. 4 and grown for 12–20 hours, *nodal* expression can be detected in 38% of the cases (n = 31). **B.** Right side tissue likewise expresses *nodal,* in 40% of the cases (n = 44). Arrowheads indicate expression.

explants. Taking into account some attrition due to imperfect culture conditions, this result shows that lateral tissue does express *nodal* when isolated from the primitive streak and Hensen's node. A similar result was observed by Yuan and Schoenwolf [1998].

Chick Explants Regenerate a Node and Notochord Without Correct LR Pattern

The expression of *nodal* in lateral tissue explanted away from Shh present in Hensen's node seemed to

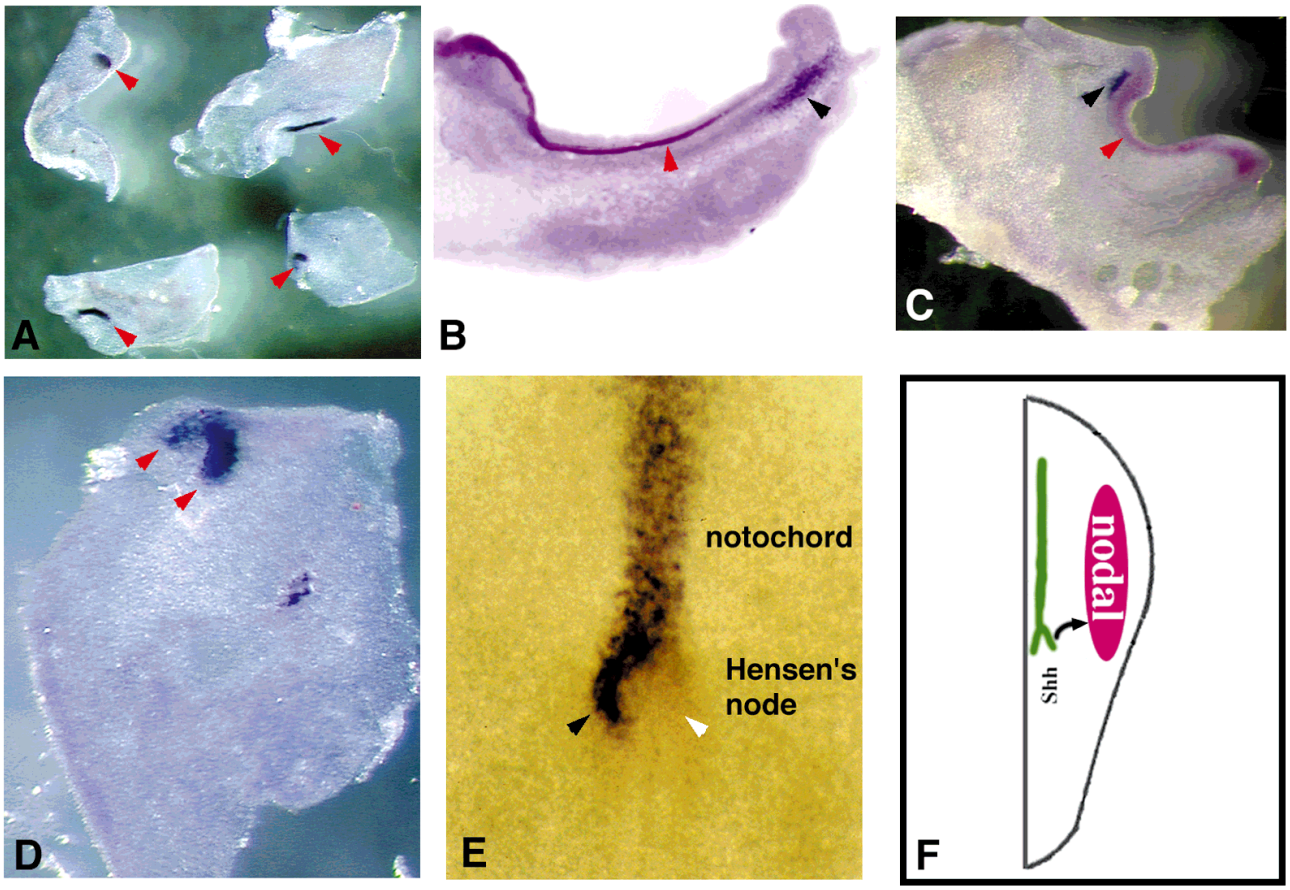

Fig. 4. Chick explants regenerate node without correct LR asymmetry. **A.** Left and right explants, when cultured for 10–20 hours, exhibit *Shh* expression. The expression domains range from spots or horseshoes (node-type pattern) to straight lines (notochord-type pattern). **B.** Left and (**C**) right explants express *nodal* adjacent to *Shh* expression domains. **D.** The regenerating *Shh* domain often exhibits no left-right asymmetry, in contrast to endogenous w.t. expression (**E**), where *Shh* is expressed only on the left side of Hensen's node. **F.** These results are consistent with *nodal* expression in lateral explants being a result of induction by newly regenerating *Shh* expression. Red arrowheads indicate *Shh* expression; black arrowheads indicate *nodal* expression.

suggest that a modification of the chick *Shh* → *nodal* pathway model was necessary. However, it had been reported that Hensen's node and notochord can regenerate [Yuan *et al.,* 1995b,c; Psychoyos and Stern, 1996b; Yuan and Schoenwolf, 1998] and express several specific markers. Thus we asked whether our lateral explants regenerate a source of *Shh* signal [Yuan and Schoenwolf, 1998] and, if so, whether its LR polarity was correct. As in the previous experiment, left and right sides of st. 4 embryos were explanted, cultured, and probed for *Shh* expression. Indeed, *Shh* expression was detected in 58% of left explants (n = 31), and in 67% of right explants (n = 34). The pattern of expression ranged from a round spot or horseshoe shape similar to expression in Hensen's node of intact embryo, to an extended line of expression similar to expression in notochord tissue (Fig. 4A). In both left (Fig. 4B) and right (Fig. 4C) explants, *nodal* expression was detected proximal to *Shh* expression, as in intact embryos (see Fig. 5A). Thus we conclude that cultured lateral tissue does eventually contain midline structures and, specifically, regenerates sources of *Shh* expression similar to the node and notochord.

In the intact embryo, *Shh* expression is asymmetric in Hensen's node (Fig. 4E), being expressed only on the left side. Likewise, ablated nodes in cultured embryos regenerate with proper left-right asymmetry [Psychoyos and Stern, 1996b]. Since our right-sided explants contained *nodal* expression, whereas normally *nodal* is left-sided, we asked whether the node regenerated in explants has normal LR asymmetry. By itself, the presence of *nodal* expression in right explants does not prove that the regenerated node loses correct asymmetry, since it can be argued that the left half of a correctly patterned regenerating node would be expected to induce *nodal* in the left half of the right-side explant. Thus we examined closely the expression of *Shh* in regenerating nodes in left and right explants. In all cases where the expression was not a straight line (corresponding to a later stage of *Shh* expression in notochord and floor plate, which is symmetric in intact embryos), *Shh* expression was seen to be symmetrical,

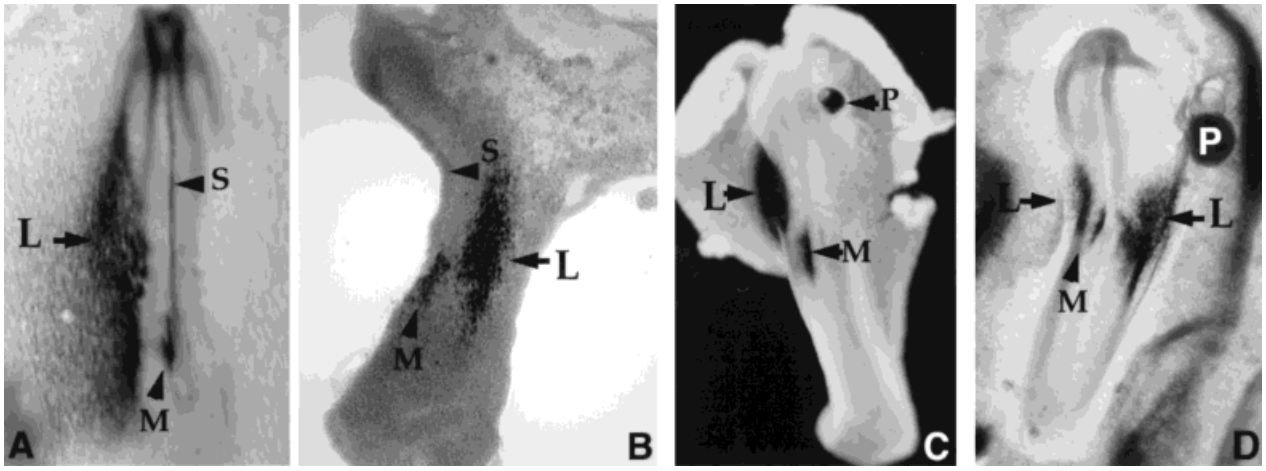

Fig. 5. *Nodal* does not induce *nodal.* **A.** In intact embryos, there are two domains of *nodal* expression: a small medial domain (M arrowhead) directly adjacent to *Shh* expression (S arrowhead), and a larger lateral domain some distance away (L arrowhead). **B.** Explants sometimes recapitulate this pattern of expression exactly. **C.** To test whether the large *nodal* domain could result from *nodal* expression in the medial domain, nodal-expressing cells were implanted on the right side of the node in st. 6 embryos. Ectopic right-sided *nodal* expression was never observed outside of the nodal-expressing cell pellet (P arrowhead). **D.** In contrast, Shh-expressing cell pellets (positive controls) do induce ectopic *nodal* domains (L arrowhead). P—*Shh* cell pellet.

either as a round spot or as a complete horseshoe (Fig. 4D), in contrast to the one-sided sickle shape of wild-type expression (Fig. 4E). Taken together, these data suggest that *nodal* expression in explants is due to signaling from a regenerated node that has lost the ability to impose proper *Shh* asymmetry.

Nodal Does Not Induce *Nodal*

Intact embryos exhibit two asymmetric domains of *nodal* expression (Fig. 5A): a small domain proximal to *Shh* expression in the node (green arrowhead) and a large lateral domain (blue arrowhead). Interestingly, lateral explants often (but not always) recapitulated this pattern (Fig. 5B). Since nodal is a TGF-β family member and presumably represents a secreted signaling molecule, we asked whether perhaps the *nodal* expression of the medial domain induces the expression of *nodal* within the lateral lateral tissue. Thus we infected chick embryo fibroblast cells in culture with an avian retrovirus containing the mature portion of the *nodal* gene. Pellets of these cells were made and implanted to the right of Hensen's node in st. 6 chick embryos in New culture, to determine whether misexpression of *nodal* on the right was sufficient to cause lateral tissue to express *nodal.* The pellets clearly exhibit *nodal* signal (Fig. 5B, arrow labeled with "P"), thus showing that the cells are making *nodal* mRNA. These cell pellets are also known to make functional nodal protein, as they have been shown to induce reversed and symmetrical hearts in chick embryos [Levin *et al.,* 1997]. Following implantation of such pellets on the right side of Hensen's node, right-sided *nodal* expression was never observed to occur in the lateral plate mesoderm (n = 9). In contrast, control pellets infected with the *Shh* virus were able to induce ectopic *nodal* (Fig. 5C, yellow arrow).

Xenopus Explants Regenerate Notochord

Based on the results we obtained using chick explants, our model predicted that *Xenopus* lateral tissue would likewise have to regenerate an inducer of *nodal* (*Xnr-1*) expression. In contrast, the midline repressor model [Lohr *et al.,* 1997] requires the absence of midline structures in the explants. In order to test this in the frog, we duplicated the experiments of Lohr *et al.* [1997] and asked whether midline structures were regenerated. Left and right lateral tissue was explanted from *Xenopus* embryos at st. 15/16 (Fig. 6A). Such explants were cultured to ~st. 25 and probed for two markers of midline structures: MZ15 [Salisbury and Watt, 1988], an antibody that recognizes a mature notochord-specific epitope, and *Xnot,* a gene expressed in the early notochord [Dassow *et al.,* 1993]. Since in *Xenopus,* unlike in chick, it is unclear which member of the Hedgehog family (if any) is responsible for *Xnr-1* induction, we used these two different ways of identifying notochord cells. The results obtained by each marker were identical.

Whole embryos show stain in the notochord at st. 18 (*Xnot*) and at st. 26 (MZ15) (Fig. 6B,H). In contrast, explants fixed and probed immediately after surgery (st. 15/16) show no stain (Fig. 6C,I), showing that no original notochord cells are included in the explants. Tissue removed from the dorsal part of the embryo during the surgery does express the notochord markers (Fig. 6D,J); furthermore, it is seen that 1–2 notochord widths separate the notochord from the lateral tissue in our explants. This also demonstrates that lateral ex-

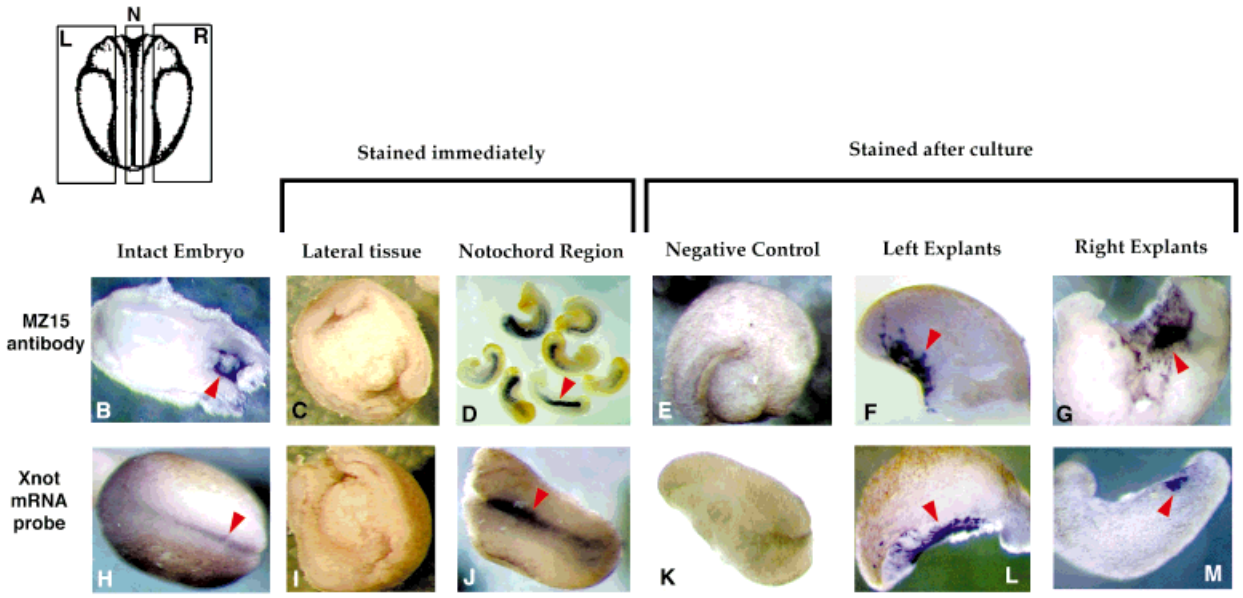

Fig. 6. Xenopus lateral explants regenerate notochord structures. **A.** Lateral tissue was explanted from st. 15/16 embryos and cultured to st. 22 (for *Xnot* stain) or st. 28 (for MZ15 stain). **B.** MZ15, an antibody to the notochord epitope keratan sulphate, stains the notochord sheath in control st. 16 embryos sectioned transversely. **C.** Explants fixed and stained with MZ15 immediately after explantation (i.e., without culture), exhibit no stain, showing that the explants contain no notochordal cells when they are put into culture. **D.** In contrast, dorsal tissue left over from the explants does show a stripe of notochordal staining; it is also seen that the dorsal tissue not included in the explants is approximately three times as wide as the notochord. **E.** Explants cultured overnight and processed for immunohistochemistry without the primary MZ15 antibody exhibit no staining (negative control). In contrast, left (**F**) and right (**G**) explants clearly show MZ15 staining, showing that they regenerate notochord cells. Analogous results were obtained with in situ hybridization to an *Xnot* probe. **H.** Whole st. 15 embryos probed with *Xnot* show signal in the notochord. **I.** Tissue remaining after the dorsal strip was removed (but before the remaining embryo was divided into left and right halves) exhibits no signal. **J.** Dorsal explants show signal in the midline. **K.** When explants are cultured and probed with a sense probe for *Xnot,* no signal is detected. In contrast, both left (**L**) and right (**M**) explants exhibit *Xnot* expression. Red arrowhead indicates expression.

plants do not contain residual notochordal tissue when explants are made. Left and right explants cultured overnight and probed without primary antibody or with *Xnot* sense probe (negative controls) show no signal (Fig. 6E,K). In contrast, both left (88%, n = 71, Fig. 6F,L) and right (87%, n = 66, Fig. G,M) explants when cultured and probed with MZ15 antibody or antisense *Xnot* probe, clearly demonstrate the presence of notochord cells. However, the regenerated notochord has the appearance of a scattered density of cells and does not seem to have the organized morphology of the original notochord.

DISCUSSION

In this set of experiments, we attempted to differentiate between two competing models of events upstream of asymmetric *nodal* expression. The left-sided inducer model [Levin *et al.,* 1995] proposes that a left-sided molecule (*Shh,* in chick) is necessary to induce *nodal* expression in left lateral mesoderm. The absence of this signal on the right is what accounts for the absence of *nodal* expression in right mesoderm. Thus the mesoderm would be considered naive tissue with respect to *nodal* expression. In contrast, the intriguing experiments on lateral mesoderm isolation in *Xenopus* [Lohr *et al.,* 1997] have suggested a midline repressor model, which holds that lateral tissue is fated to express *nodal* by default and that a repressor molecule secreted by the midline is what accounts for the lack of *nodal* expression in the right side.

Our results show, contrary to the simplest predictions from the proposed chick pathway, that chick lateral tissue behaves like *Xenopus,* in that both right and left lateral explants cultured away from the midline tend to express *nodal.* We detected no statistically significant differences in *nodal* expression between the left and right sides, suggesting that the lateral tissue is symmetric with respect to ability to express *nodal* and that asymmetries in this gene reflect prior asymmetries at the midline.

Importantly, we show that midline structures such as the node and notochord are regenerated in our explants, and express *Shh,* confirming the previous findings of Psychoyos and Stern [1996b], and Yuan *et al.* [1995a] and Yuan and Schoenwolf [1998]. As in intact embryos, *nodal* expression is always seen in proximity

to *Shh* expression in explants. Thus we propose that *nodal* expression in chick lateral tissue explanted away from the midline is due to an induction from the regenerated node. This is likely to explain an apparent discrepancy between our results and those of Pagan-Westphal and Tabin [1988], who found no *nodal* expression in chick lateral explants done at st. 5. Our explants were done at st. 4, which allows the node to regenerate, whereas theirs were done at a later stage which is likely to be less plastic. Moreover, heterochronic transplants indicate that the ability of adjacent tissues to pattern left-right *Shh* expression in grafted nodes wanes past st. 5 [Pagan-Westphal and Tabin, 1988].

As in the case in chick, we show that *Xenopus* lateral explants also regenerate notochordal cells. Thus such explants actually contain midline signals known to induce *nodal* in chick, as opposed to being isolated from the midline, as would be required for the midline repressor model.

Based on these data, which are consistent with all of the chick pathway experiments [Levin, 1998] as well as the *Xenopus* data [Lohr *et al.,* 1997], we conclude that *nodal* expression indeed requires an asymmetric inducer generated by the midline. This model is consistent with a HH protein being necessary for *nodal* induction in both species and provides an explanation for both sets of data in terms of a single conserved mechanism upstream of *nodal* expression. Previous chick data [Levin, 1998] and new data [Pagan-Westphal and Tabin, 1988] showing that anti-Shh antibodies specifically abolish *nodal* expression in chick embryos, suggest that SHH is the endogenous *nodal* inducer in chick. The specific nature of the inducer in *Xenopus* is less clear, since no asymmetric Hedgehog expression has been demonstrated in frogs. However, it is known that misexpression of Hedgehogs in *Xenopus* does result in *situs* abnormalities [Sampath *et al.,* 1997]; thus it is likely that some member of the family has a similar role in *Xenopus.*

The induction of *nodal* in explants frequently occurs in two domains, much as in intact embryos. The induction of the distal, lateral domain of expression may be due to signaling from the medial domain adjacent to the *Shh* expression in the node. However, we show that this signal is not mediated by nodal itself, since ectopic *nodal* expressed on the right side of Hensen's node in whole embryos does not induce ectopic lateral expression of *nodal.* Thus the mechanism by which asymmetric *Shh* expression induces two separate domains of *nodal* expression is unknown.

In contrast to regeneration of ablated node in whole embryos, which happens with correct LR asymmetry of several markers [Psychoyos and Stern, 1996b], the node regenerated by explants seems to be unable to express correct asymmetry in *Shh* expression. The punctate and disorganized nature of the notochord that regenerates in *Xenopus* explants likewise is also consistent with a loss of normal asymmetry in midline structures. Thus distal lateral halves of the embryo need to be in contact via the midline for proper asymmetric gene expression. This suggests a view of the midline tissues as facilitating signaling necessary for asymmetry, in contrast to the predominating view of the midline as only an isolating barrier between the left and right compartments [Melloy *et al.,* 1998]. Thus the randomization of heart *situs* and bilateral or aberrant expression of *XNr-1* seen following extirpation of midline (floorplate + notochord) tissue [Danos and Yost, 1996] might be explained by improper (ectopic) regeneration of the midline signaling center, rather than a loss of a barrier.

ACKNOWLEDGMENTS

We thank Sylvia Pagan, Cliff Tabin, and Claudio Stern for helpful discussions and advice on explant culture conditions. Chick Brachyury plasmid was obtained from Gerhard Herrmann. Xenopus XNot-10 plasmid was obtained from Chris Kroll.

REFERENCES

Collignon J, Varlet I, Robertson E (1996): Relationship between asymmetric nodal expression and the direction of embryonic turning. Nature 381:155–158.

Danos M, Yost H (1996): Role of notochord in specification of cardiac left-right orientation in zebrafish and *Xenopus.* Dev Biol 177:96–103.

Dassow G v, Schmidt J, Kimelman D (1993): Induction of the Xenopus organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. Genes Dev 7:355–366.

Fujinaga M (1996): Development of sidedness of asymmetric body structures in vertebrates. Int J Dev Biol 41:153–186.

Harland RM (1991): In situ hybridization: An improved whole mount method for *Xenopus* embryos. In Kay BK, Peng HB (eds): "Xenopus Laevis: Practical Uses in Cell and Molecular Biology." San Diego: Academic Press, pp 685–695.

Isaac A, Sargent MS, Cooke J (1997): Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. Science 275:1301.

Knezevic V, Santo RD, Mackem S (1997): Two novel chick T-box genes related to mouse Brachyury are expressed different, non-overlapping mesodermal domains during gastrulation. Development 124: 411–419.

Levin M (1997): Left-right asymmetry in vertebrate embryogenesis. BioEssays 19:287–296.

Levin M (1998): Left-right asymmetry and the chick embryo. Sem Cell Dev Biol 9:67–76.

Levin M, Johnson R, Stern C, Kuehn M, Tabin C (1995): A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell 82:803–814.

Levin M, Mercola M (1998): The compulsion of chirality. Genes Dev 12:763–769.

Levin M, Pagan S, Roberts D, Cooke J, Kuehn M, Tabin C (1997): Left/right patterning signals and the independent regulation of different aspects of *situs* in the chick embryo. Dev Biol 189:57–67.

Lohr J, Danos M, Yost H (1997): Left-right asymmetry of a *nodal*-related gene is regulated by dorsoanterior midline structures during *Xenopus* development. Development 124:1465–1472.

Lowe L, Supp D, Sampath K, Yokoyama T, Wright C, Potter S, Overbeek P, Kuehn M (1996): Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. Nature 381:158–161.

Matzuk M, Kumar T, Vassalli A, Bickenbach J, Roop D, Jaenisch R, Bradley A (1995): Functional analysis of activins during mammalian development. Nature 374:354–356.

Melloy P, Ewart J, Cohen M, Desmond M, Kuehn M, Lo C (1998): No turning, a mouse mutation causing left-right and axial patterning defects. Dev Biol 193:77–89.

New D (1955): A new technique for the cultivation of the chick embryo in vitro. J Embryol Exp Morph 3:326–331.

Nicolet G (1971): Avian gastrulation. In Abercrombie M, Brachet J, King TJ (eds): "Advances in Morphogenesis." New York: Academic Press, pp 231–262.

Oh S, Li E (1997): The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymmetry in the mouse. Genes Dev 11:1812–1826.

Pagan-Westphal S, Tabin C (1988): The transfer of left-right positional information during chick embryogenesis. Cell 93:25–35.

Psychoyos D, Stern C (1996a): Fates and migratory routes of primitive streak cells in the chick embryo. Development 122:1523–1534.

Psychoyos D, Stern C (1996b): Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo. Development 122:3263–3273.

Rosenquist G (1966): A radioautographic study of labeled grafts in the chick blastoderm. In "Contributions to Embryology." Washington, DC: Carnegie Institution, pp 71–110.

Salisbury J, Watt F (1988): Lack of keratan sulphate in the human notochord. J Anat 157:175–179.

Sampath K, Cheng A, Frisch A, Wright C (1997): Functional differences among *Xenopus nodal*-related genes in left-right axis determination. Development 124:3293–3302.

Schoenwolf G, Garcia-Martinez V, Dias M (1992): Mesoderm movement and fate during avian gastrulation and neurulation. Dev Dyn 193:235–248.

Vakaet L (1970): Cinephotomicrographic investigations of gastrulation in the chick blastoderm. Arch Biol 81:387–426.

Wood W (1997): Left-right asymmetry in animal development. Ann Rev Cell Dev Biol 13:53–82.

Yuan S, Darnell D, Schoenwolf G (1995a): Identification of inducing, responding, and suppressing regions in an experimental model of notochord formation in avian embryos. Dev Biol 172:567–584.

Yuan S, Darnell D, Schoenwolf G (1995b): Identification of inducing, responding, and suppressing regions in an experimental model of notochord formation in avian embryos. Dev Biol 172:567–584.

Yuan S, Darnell D, Schoenwolf G (1995c): Mesodermal patterning during avian gastrulation and neurulation. Dev Gen 17:38–54.

Yuan S, Schoenwolf G (1998): De novo induction of the organizer and formation of the primitive streak in an experimental model of notochord reconstitution in avian embryos. Development 125:201–213.