

The roles of activin and follistatin signaling in chick gastrulation

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ABSTRACT *Activin*, a TGF- β family member, and *follistatin*, an activin antagonist, encode signaling proteins which have been implicated in fundamental events in early vertebrate embryogenesis, such as mesoderm and neural tissue induction, and axial patterning. In this study I examine the roles of *activin* and *follistatin* in gastrulation in the chick. *Activin* βB is found to be expressed at the base of the primitive streak prior to its formation, consistent with a role in streak induction. *Follistatin* has a more complex and dynamic expression in Hensen's node, and exhibits a left-right (LR) asymmetry. Antagonizing endogenous activin by ectopic application of follistatin protein causes the partial dissolution of the primitive streak and node, both morphologically and as assayed by loss of expression of molecular markers. This suggests that activin is necessary for the maintenance of streak morphology, and that follistatin may be involved in termination of the anterior progress of streak growth or in suppression of supernumerary streaks. Cell ingression through the node following follistatin application is normal, suggesting that it does not depend on the pit-like morphology of the wild-type node. Finally, follistatin temporally extends the asymmetric pattern of expression of *HNF3*- β , this, as well as the stronger right-sided expression of *follistatin*, suggests a possible role in LR patterning.

KEY WORDS: *follistatin*, *activin*, *HNF3*- β , *streak*, *left-right asymmetry*

Introduction

Axis formation is the most fundamental event in embryonic development, as it lays the foundation for all later morphogenetic processes. This process has been extensively studied in birds because of the accessibility of the gastrulating embryo to embryological and molecular techniques (Khaner, 1993). In bird embryos, the dorso-ventral (DV) axis is formed first, as the one-cell-layer-thick blastodermal disk forms a second hypoblastic layer underneath itself (Eyal-Giladi and Kochav, 1976). Through what is probably the inductive action of the hypoblast, the AP axis is defined by the point on the periphery of the disk where the primitive streak begins to grow towards the center (Azar and Eyal-Giladi, 1979, 1981; Khaner and Eyal-Giladi, 1989). Cells contribute to the mesodermal layer as they migrate through the streak and node. Interestingly, the early blastoderm is an integrative system in that it is programmed to form one and only one primary axis, and can regulate its activity to achieve this despite bisection into separate regions (Khaner, 1996); this has been suggested to be due to a balance of inductive and repressive activities present within the blastoderm. The molecular nature of factors which mediate these interactions has not been ascertained.

Activins, dimeric members of the transforming growth factor- β (TGF- β) superfamily (Vale *et al.*, 1990), have been implicated in mesoderm formation in studies on amphibians: activin is present in the egg (Asashima *et al.*, 1991; Dohrmann *et al.*, 1993), and exogenous activin is able to induce mesoderm (Smith *et al.*, 1990), while dominant-negative activin receptors abolish axis formation and mesoderm induction (Hemmati-Brivanlou and Melton, 1992). Similarly, in the chick, activin has been reported to specifically induce formation of axial structures (Cooke *et al.*, 1994), and *activin* βB mRNA has been detected in early embryos (Mitrani and Shimoni, 1990; Mitrani *et al.*, 1990). These observations suggest that activin may have a role as an endogenous inducer of the AP axis.

Follistatin is a secreted protein, initially identified as an antagonist of activin protein (Connolly *et al.*, 1995). It is expressed in the Spemann organizer in frogs, and is able to block the action of activin in embryonic explants (Hemmati-Brivanlou *et al.*, 1994). Furthermore, application of follistatin protein leads to neural induc-

Abbreviations used in this paper: DV, dorso-ventral; AP, antero-posterior; LR, left-right; PBS, phosphate-buffered saline.

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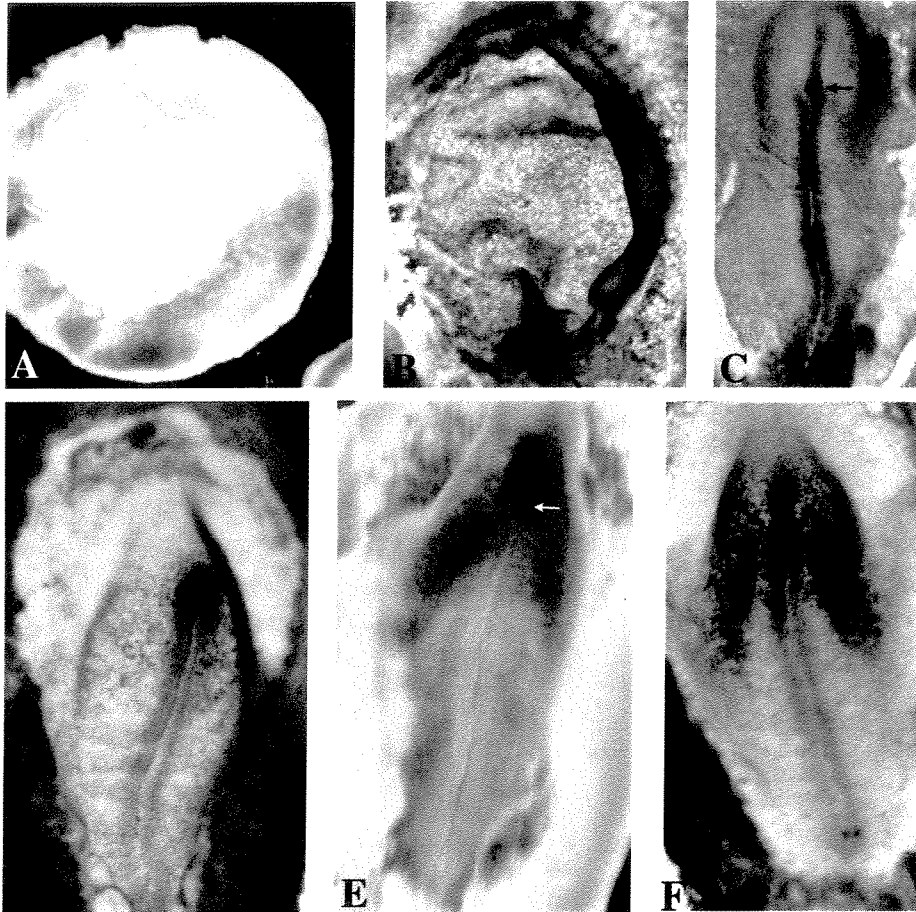


Fig. 1. Endogenous expression of *activin betaB* and *follistatin*. Whole-mount *in situ* hybridization with a sense probe (control) reveals no background staining (A). *Activin betaB* mRNA is first detected with an antisense probe in stage 1 embryos at the base of the nascent primitive streak (black arrow, B). It is then detected in the right side of Hensen's node at stage 4 (black arrow) and more weakly in the streak and notochord (C). *Follistatin* mRNA is first detected by whole-mount *in situ* hybridization in stage 4 embryos, as a horse-shoe running symmetrically around the top of Hensen's node (D). It then becomes expressed throughout Hensen's node and briefly exhibits a tail of expression in the right primitive ridge just under the node (E) analogous to the left-sided tail found in *HNF3-beta* expression (compare Fig. 5A). By stage 5, it is more strongly expressed on the right side (black arrow) than the left side (gray arrow) of Hensen's node (F), as well as in the notochord and lateral precardiac areas similar to the domains of *cAct-R11a* expression (Levin *et al.*, 1995).

tion in explants, as does inhibition of activin receptor signaling (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994). Together, these observations suggest that *activin* and *follistatin* may play opposing roles in early embryonic development.

In this study, the roles of *activin* and *follistatin* were examined in several aspects of chick gastrulation by determining in detail their early expression patterns, and by applying follistatin protein to chick embryos *in vitro*.

Results

Endogenous expression of Activin betaB

Activin expression is known to be present during early mouse embryogenesis (Albano *et al.*, 1994; Feijen *et al.*, 1994). Likewise, *Activin betaB* mRNA was detected in gastrula-stage chick embryos (Mitrani *et al.*, 1990); however, no spatial expression pattern was reported in that study. For the purposes of these experiments, I examined its expression by *in situ* hybridization. *Activin betaA* was not detected by *in situ* hybridization at these stages (data not shown). In contrast, *Activin betaB* is first detected in stage 1 embryos at the base of the nascent primitive streak (Fig. 1B, compare to sense probe control in Fig. 1A), consistent with a possible role in primary axis initiation. It is later observed throughout the streak and in the right side of Hensen's node [Fig. 1C, see also (Levin *et al.*, 1997)].

Endogenous expression of follistatin

The expression of *follistatin* in the early chick embryo has been partially characterized (Connolly *et al.*, 1995). In order to ascertain the possible role of *follistatin* in gastrulation, its expression pattern was examined in greater detail in gastrulating chick embryos by *in situ* hybridization. Endogenous *follistatin* has a complex expression pattern during gastrulation. It is not detected prior to stage 4⁻ (data not shown), and can first be seen as a horse-shoe pattern around both sides of the node at stage 4⁻ (Fig. 1D). Sectioning reveals stain mainly in the endoderm and mesoderm (data not shown). *Follistatin* then becomes expressed throughout the node, but somewhat more strongly on the right side, displaying a consistent left-right asymmetry at stage 4 (Fig. 1F). Expression is then observed in the head process, and neural plate. Interestingly, during a brief phase of stage 4, its expression includes a "tail" on the right primitive ridge, just under the node (Fig. 1E). This domain is a mirror image of a tail of *HNF3-beta* expression on the left primitive ridge [see Fig. 5A, (Levin *et al.*, 1995)].

Morphological effects of follistatin

Since follistatin has been shown to antagonize activin at the protein level (Ying *et al.*, 1987; Hemmati-Brivanlou *et al.*, 1994), it should be possible to study the endogenous role of activins by examining the effects of applying ectopic follistatin (which would be expected to inhibit activin protein once it is secreted). Beads soaked in follistatin protein were applied to stage 3 embryos in New

culture (Fig. 2) by implanting them between the epiblast and hypoblast just posterior and lateral to the node. This resulted in morphological changes to the streak and node (n=6). The node was converted from the normal pit morphology (Fig. 2D) into a solid bump (Fig. 2G). At the same time, the primitive streak lost its normal grooved morphology (Fig. 2A,E), sometimes becoming completely invisible (Fig. 2B,H). Gross patterning anterior to the node seemed to be unaffected (note somite and head development in Fig. 4D). None of these effects were observed when control beads (soaked in PBS) were implanted (n=35).

Follistatin and streak markers

Morphologically, follistatin application appeared to abolish the primitive streak. In order to examine this streak-destroying activity of follistatin in more detail, embryos which had received follistatin beads at st. 3 were harvested at stages 5-7 and processed for *in situ* hybridization with probes for genes expressed in the primitive streak. Molecular streak markers such as *FGF-4* (Fig. 3A-B), *notch* (Fig. 3C,D) and *delta* (data not shown) were markedly reduced in expression. This result demonstrates that follistatin is able to cause partial destruction of the primitive streak, both in terms of morphology and in terms of characteristic streak gene expression.

Cell migration and node morphology

The node is the chick organizer, and is functionally homologous to the dorsal lip of the amphibian blastopore (Streit *et al.*, 1994). Epiblast cells migrate through Hensen's node and the primitive streak and contribute to the mesodermal layer between the epiblast and hypoblast. To test whether the alteration in node morphology would disrupt cell migration through the node, st. 3 embryos in New culture received a follistatin bead implant. When cells in the node of such embryos (n=9) were labeled with Dil (Fig. 4B) and allowed to develop to stage 8, label could be seen in notochord (Fig. 4D), just as in control embryos (n=11, Fig. 4A,C). Thus, large-scale cell ingressation and contribution to the head process is disrupted neither by follistatin, nor by the morphological changes it causes. These results demonstrate that it is possible for a node to exist without a streak, and that the grooved node morphology is not required for proper cell migration. The fact that anterior patterning of later stage embryos is apparently normal following follistatin bead implants (e.g., Fig. 4D) may indicate that later development is not dependent on the events occurring posterior to the node after stage 5.

Follistatin and HNF3-β

Shh, a signaling molecule implicated in many developmental events (Riddle *et al.*, 1993; Johnson *et al.*, 1994; Chiang *et al.*, 1996), is normally expressed exclusively on the left side of Hensen's node after stage 4. It was previously observed that application of follistatin beads caused symmetric expression of *Shh* (Levin *et al.*, 1997). To test for other effects on markers of laterality, embryos which had received follistatin bead implants were processed for *in situ* hybridization with an *HNF3-β* probe. This resulted in a sharpening and prolonging of the asymmetry in *HNF3-β* expression. The normal left-sided "tail" of *HNF3-β* expression [Fig. 5A, (Levin *et al.*, 1995)] is quite subtle and brief, lasting less than 1 h. In control embryos, it is always gone by stage 5 (n=17, Fig. 5B); in contrast, embryos exposed to follistatin on the right side of the node often maintain asymmetric *HNF3-β* expression to stage 5 (n=5, Fig. 5C).

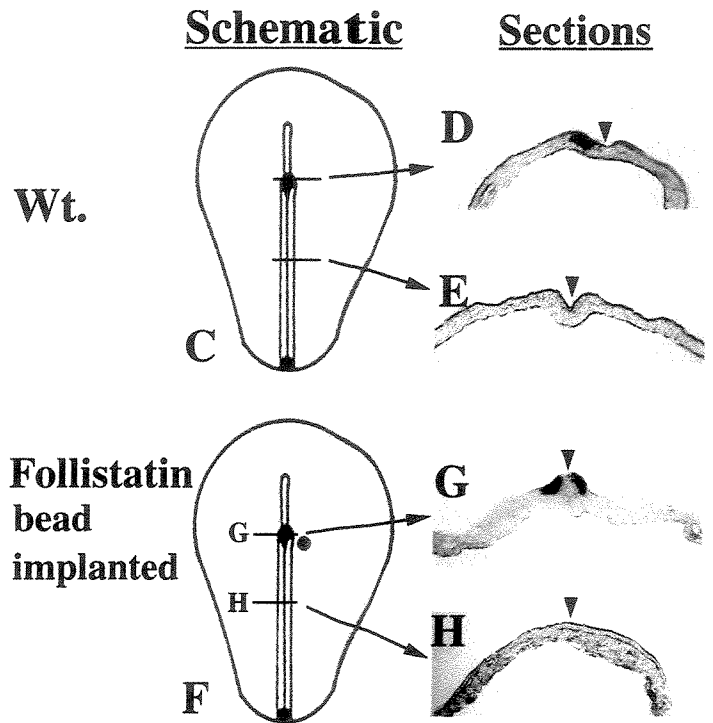
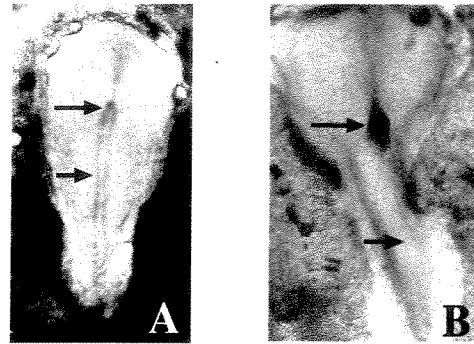


Fig. 2. Ectopic follistatin destroys the normal morphology of the primitive streak and node. When a bead soaked in follistatin protein was implanted near the node of st. 3 embryos, the node acquired the shape of a bump (B) instead of the normal pit-like morphology (A) while the streak often became invisible (compare B to A, red arrow). In sections, taken at levels indicated by horizontal lines in schematics C and F, it is apparent that application of ectopic follistatin protein (F) has caused the node to lose its pitted morphology – it is no longer hollow (red arrowhead, G), while the primitive streak is no longer a groove (H), compare to sections through the node (D) and streak (E) of control embryos. Embryos in panels D and G were also hybridized with a probe for *Shh* (purple stain within node) to show that *Shh* expression becomes symmetrical (G) due to the action of follistatin.

Discussion

Activin βB is present at the point of initiation of the primitive streak in the chick blastoderm. Previous studies have shown that exogenous activin has been shown to be able to induce primary axes (Mitrani and Shimoni, 1990; Mitrani *et al.*, 1990; Cooke *et al.*,

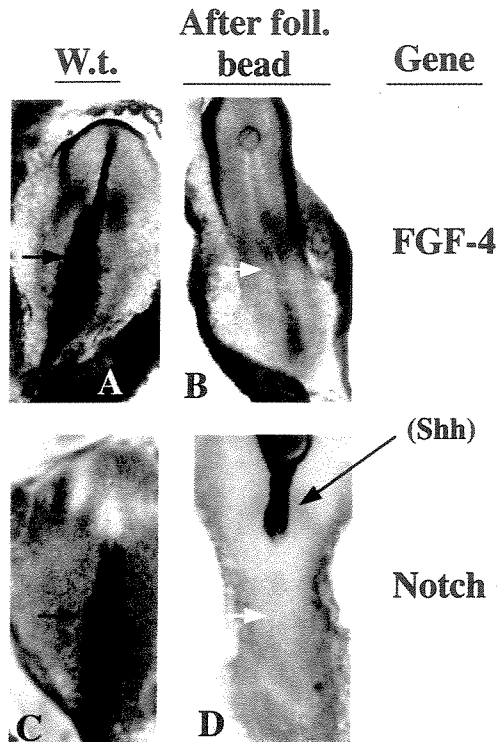


Fig. 3. Ectopic follistatin causes changes in expression of genes normally expressed in the streak. After receiving a follistatin bead implant at st. 3, embryos were allowed to develop further and processed for *in situ* hybridization. It is seen that FGF-4, which is normally strongly expressed in the streak (A), is almost absent as a result of follistatin exposure (B). Notch, which is expressed in the primitive ridges (C) is also absent following application of follistatin (D). The embryo in panel D was also hybridized to *Shh* probe; *Shh* signal on the right side of the node indicates that the follistatin protein is having its effect.

1994). These observations suggest that *Activin βB* is the factor which functions endogenously to specify the location of the primitive streak. Addition of Wnt-1 increases the efficacy of activin implants in causing ectopic axes (Cooke *et al.*, 1994), suggesting that *activin* may not be the only factor involved. Another candidate for this role is *Vg-1*, which has recently been shown (Seleiro *et al.*, 1996) to also be able to induce ectopic streaks in the chick. However, follistatin has been shown not to inhibit the activity of *Vg1* (Schulte-Merker *et al.*, 1994; Kessler and Melton, 1995), consistent with *activin*, and not *Vg1*, being the endogenous streak inducer.

Activin has been shown to have streak-inducing activity (Ziv *et al.*, 1992). The morphological and biochemical streak dissolution caused by ectopic application of follistatin, an antagonist of *activin* function, suggests that perhaps *activin* is also required for maintenance of the streak as well as its induction. The function of the horse-shoe expression of follistatin around the anterior-most tip of the primitive streak at the time of maximal streak elongation could be to halt the anterior progress of streak growth at the appropriate time.

Interestingly, the streak-disrupting effects of follistatin partially simulate a proposed endogenous streak-inhibiting activity. The development of only one primary axis in normal embryos has been

suggested to be due to competition between regions which could potentially generate nascent streaks (Khaner and Eyal-Giladi, 1989). In fact, early embryos often exhibit more than one streak (Fig. 6A); occasionally these persist and result in conjoined twins (Levin *et al.*, 1996), but almost invariably blastoderms resolve to one streak by stage 4 (Fig. 6B), the time at which follistatin expression begins (Fig. 1D). Accomplishing this by means of streak competition would have to involve mechanisms for streak initiation and suppression, perhaps through short-range induction and long-range inhibition by diffusible factors. *Activin βB* is a good candidate for the inductive factor. Follistatin is not expressed (at levels detectable by *in situ* hybridization) during the stages of streak initiation, so it is probably not the factor which suppresses additional streaks during the earliest phases of gastrulation. However, the effects of follistatin suggest that a related factor may be playing that role at stage 1.

It should be noted that follistatin has been reported to interact with BMP-7 and BMP-4 (Yamashita *et al.*, 1995; Fainsod *et al.*, 1997), so it is still possible that a related molecule plays the streak inductive role instead of *activin*. BMP-7 can interact with *activin* receptors (Yamashita *et al.*, 1995), but while it is present in early embryos, it is not expressed in a manner consistent with such a role: it is located in the posterior streak and stripes along the edges of the embryo proper [(Schultheiss *et al.*, 1997) and Levin, unpublished].

The morphological effects of follistatin enable the study of cell behavior during gastrulation as dissociated from gross morphology of the streak and node. The fact that *Dil* labeled cells in the node are able to ingress and migrate normally, as well as contribute to

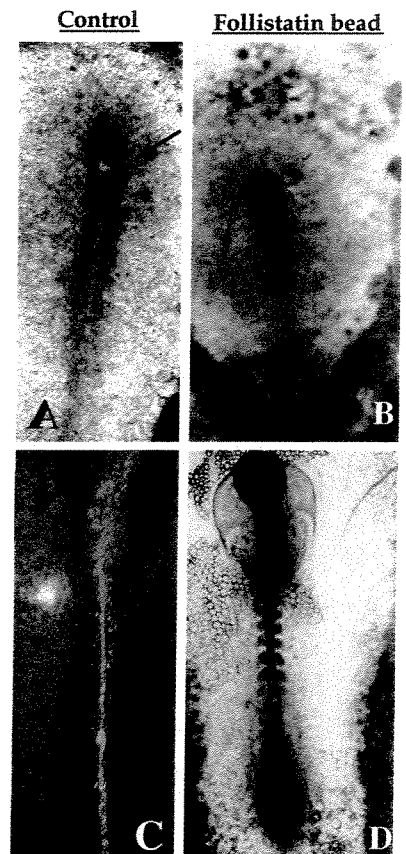


Fig. 4. Ectopic follistatin application does not interfere with cell ingress through node. When cells in the right side of a stage 4 embryo in new culture are labeled with *Dil* (A), label is observed along the length of the embryo [(C), fluorescent imaging] when the cells migrate anteriorly. Likewise, when a follistatin bead is implanted next to the node and the cells in the right side of the node are labeled with *Dil* (B), the same migration pattern is seen, as well as a phenotypically normal head and somites [(D), combined transmitted light and fluorescent imaging]. Black arrows in panels A and B show sites of *Dil* labeling. Blue arrow in panel B shows the follistatin bead.

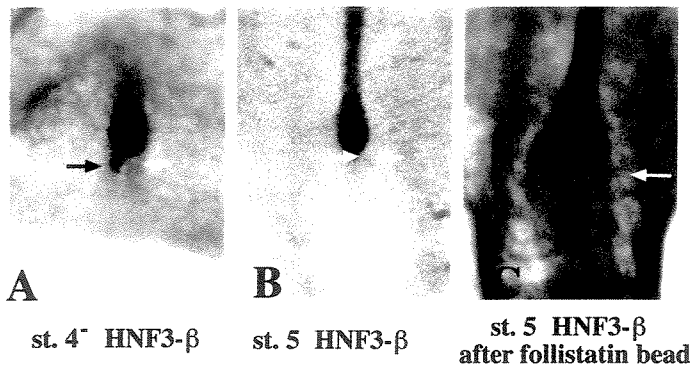


Fig. 5. Follistatin affects expression of *HNF3-β*. *HNF3-β* is normally expressed in the left primitive ridge [(A), black arrow] for a very short time, and is completely symmetrical by st. 5 [(B), white arrows indicate lack of expression in left and right primitive ridges]. After the application of a follistatin bead at st. 3, however, the asymmetric expression persists well through st. 5 [(C), black arrow]. Black arrows indicate expression, white arrows indicate lack of expression.

an apparently phenotypically normal head process (as well as somites and neural tube), despite drastic changes in the morphology of the node and streak, demonstrate that the pitted shape of Hensen's node is not required for normal migratory cell behavior during gastrulation.

My data suggest that a previously proposed role of follistatin – that of endogenous neural inducer (Hemmati-Brivanlou *et al.*, 1994) – is unlikely in the chick. It is expressed only after the zenith of the node's ability to induce ectopic neural tissue (Storey *et al.*, 1992). This is consistent with the finding that mice carrying a homozygous mutation in the *follistatin* gene have no phenotype related to neural induction (Matzuk *et al.*, 1995). Furthermore, mouse *follistatin* is not expressed in the node (Albano *et al.*, 1994), paralleling other differences in expression of LR patterning genes between mice and chicks (Levin *et al.*, 1995; Collignon *et al.*, 1996).

It has previously been shown (Levin *et al.*, 1997) that endogenous *activin βB* likely plays a role in left-right (LR) patterning. *Activin βB* is expressed in the right side of the node prior to the restriction of *Shh* from the right half of the node; exogenous *activin* protein applied to the left side of the node represses *Shh* expression there while inducing an *activin*-responsive gene (*cAct-R11a*), and application of follistatin protein to the right side of the node results in bilateral *Shh* expression (Levin *et al.*, 1995, 1997). While the endogenous expression of *follistatin* in chicks begins too late for it to participate in setting up asymmetries in *activin* activity, it may function to terminate *activin* activity in the node when it is no longer required.

The prolongation of asymmetrical expression of *HNF3-β* by ectopic follistatin raises the possibility that endogenous *activin* may play a role in repressing the left-sided tail of *HNF3-β* at stage 5. It is known that *HNF3-β* induces *Shh* in some tissues (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Weinstein *et al.*, 1994); likewise, *Shh* is able to induce *HNF3-β* in early chick embryos (M. Levin and C. Tabin, unpublished observations). This, in conjunction with the endogenous expression of *follistatin* shown above, and the previously established regulatory interactions between *activin βB* and *Shh* (Levin *et al.*, 1995) suggest that *activin*, *Shh*, *HNF3-β* and

follistatin form a complex network of interacting genes, involving four separate spatial domains: the right and left halves of the node, and the right and left anterior-most regions of the primitive ridge. The latter two components of this network are also likely to have a role in LR patterning, since *follistatin* can abolish the asymmetry in *Shh* expression (Levin *et al.*, 1997), while compound crosses between *nodal*^{+/−} and *HNF3-β*^{+/−} mice display *nodal* expression on both sides and frequently show alterations of *situs* (Collignon *et al.*, 1996). Taken together, these data indicate that *activin* and *follistatin* have opposing roles in the initiation and left-right patterning of the primary axis in birds.

Materials and Methods

In situ hybridization

Whole-mount *in situ* hybridization and sectioning of stained embryos was done as in Riddle *et al.* (1993). For simultaneous detection of multiple probes, one probe was labeled with digoxigenin and the other with fluorescein (Jowett and Lettice, 1994). Following detection of the digoxigenin-labeled probe, embryos were heat-inactivated at 70°C for 30 min. Detection of the fluoresceinated probe was carried out in exactly the same manner as for digoxigenin-labeled probes, except that an anti-fluorescein-alkaline phosphatase conjugate (Boehringer) was used to bind fluoresceinated label and alkaline phosphatase activity was revealed with the chromogen magenta-phos (Biosynth). After fixing in 4% paraformaldehyde/0.1% glutaraldehyde, some embryos were embedded in gelatin, and sectioned at 20 μm on a cryostat. All whole-mount embryo photomicrographs were performed with the dorsal side of the embryo uppermost, so that the embryo's right and left correspond to those of the photograph. The following cDNA clones were used to probe for *HNF3-β*, *Shh*, *FGF-4*, and *Notch* respectively: MS14 [1.2 kb, (Levin *et al.*, 1995)], pHH-2 [1.4 kb, (Riddle *et al.*, 1993)], pLN20 [600bp, (Niswander *et al.*, 1993)], cNotch-1 [1 kb, (Myat *et al.*, 1996)].

Embryo culture

All staging was done according to (Hamburger and Hamilton, 1992). Chick embryos (from Spafas, CT) were grown *in vitro* (New, 1955). Briefly, eggs were cracked into a container of Panett-Compton saline (Stern and

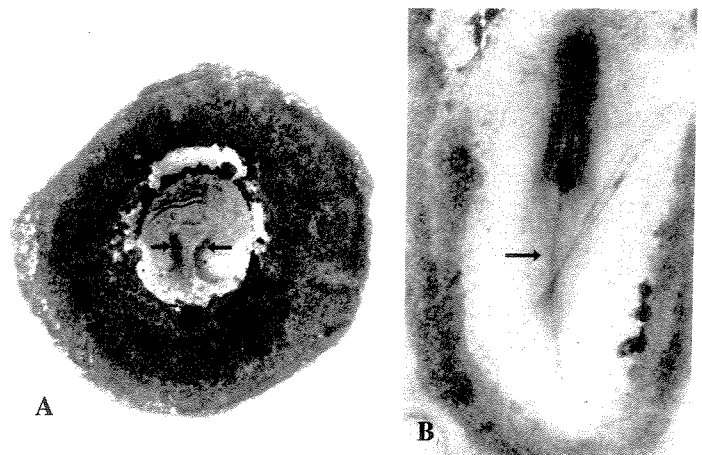


Fig. 6. Competition between streaks results in one primary axis. Early (younger than st. 4-) embryos frequently possess more than one streak (A); however, these almost always result in one single embryo (panel B shows the embryo from panel A after culturing to st. 7) due to the dissolution of all but one of the streaks. Black arrows indicate primitive streaks.

Holland, 1993) and cleared of heavy albumin. The vitelline membrane was cut around the equator of the floating yolk, and placed upside-down onto a watch-glass. A glass ring was placed on top of it, and the vitelline membrane was wrapped around its edges. The preparation was then taken out of the saline, and under a dissecting microscope the remaining liquid was removed from the ring, leaving the embryo, endoderm upwards, dry inside the ring. The ring was then placed in a petri dish of light albumen for culture.

Dil labeling

Dil (a fluorescent dye which intercalates into cell membranes, Molecular Probes) at a concentration of 0.5% w/v in 100% ethanol was diluted 1:9 in 0.3 M sucrose. Using a fine glass micropipette, groups of approximately 50 cells were labeled with Dil (Stern and Holland, 1993). This was done while the embryos were in New culture (New, 1955).

Follistatin bead implants

Heparin acrylic beads (from Sigma) were soaked in 20 μ l of follistatin protein (obtained from the National Hormone and Pituitary Program) at approximately 0.05 mg/ml in water or PBS. Control beads were soaked in water or PBS only. Beads were implanted near the node, between the epiblast and hypoblast, of st. 3⁺ embryos in New culture, and processed for *in situ* hybridization at appropriate later stages. Morphological data reported here for such embryos included only cases in which the bead position was such as to preclude the possibility of its presence disturbing the morphology of the streak and node.

Acknowledgments

I would like to thank Cliff Tabin for his support and helpful discussions. Follistatin protein was obtained from Philip Smith at the National Hormone and Pituitary Program. Delta, and Notch clones were obtained from Enrique Domingos; the activin β A and β B clones were obtained from Ketan Patel; the FGF-4 clone was obtained from Lee Niswander. This work was supported by a grant to Cliff Tabin from the March of Dimes.

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Received: November 1997
Accepted for publication: December 1997