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Activin can generate ectopic axial structures in chick blastoderm explants

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Summary

We have recently shown that activin can induce the formation of axial structures from chick blastulae and that activin beta-B is transcribed, in the hypoblast of the chick, at the same stage that axial mesoderm is being induced. It was not clear, however, whether activin was merely allowing the central epiblastic cells to express a differentiated phenotype for which they were already prepared. This report shows that activin-containing medium (ACM) can act as an instructive inductor, which can change the fate of competent cells and bring about the formation of an ectopic embryonic axis. Furthermore, we show data that suggest that during normal development only one axis is obtained as a result of a carefully controlled inhibitory process.

Key words: chick development, mesoderm induction, pattern formation, activin, blastoderm, axial structure.

Introduction

The mechanisms involved in the generation of axial structures in the chick are well documented. Between stage X and stage XIII (Eyal-Giladi and Kochav, 1976) the one-layer-thick chick blastoderm, containing the peripheral area opaca (AO), the marginal zone (MZ) and the central epiblastic area, gradually forms a second hypoblastic layer underlying the central epiblastic region. Normally, through the inductive action of the hypoblast, the embryonic axis starts to develop from the posterior end of the epiblast (Azar and Eyal-Giladi, 1979; Mitrani and Eyal-Giladi, 1981).

In normal development, an isolated central epiblast disk cannot generate axial structures in the absence of the hypoblast. Furthermore, the hypoblast can also change the fate of central epiblastic cells if applied ectopically; i.e. rotation of the hypoblast with respect to the epiblast will bring about the formation of an ectopic axis from the new epiblastic cells that are now in contact with the hypoblast in its new position (Waddington, 1932; Azar and Eyal-Giladi, 1981).

Mitrani et al. (1990) have recently shown that the TGF-beta-related factor, activin, can induce the formation of axial structures from chick central epiblastic disks and that activin beta-B is being transcribed, in the chick, at the same stages that axial mesoderm is being induced. The possible role of activin as inducer of axial mesoderm leads us to investigate whether activin can also induce ectopic axial structures if applied at 90º from the posterior end of central disks derived from chick embryos at stage XI. Two different approaches are taken. In the first (A series), synthetic AG1-X2 beads, presoaked in activin-containing medium (ACM), are applied at 90º from the posterior end of the central disk. In the second approach (B, C and D series), different central disk fragments, preincubated in ACM, are implanted at 90º from the posterior end of central disk hosts explants. We show that application of ACM in a localized manner can generate the formation of ectopic axes, which include in some cases a notochord, segmental somites and a rudimentary head.

Vertebrate axis formation is a highly regulative process (Green and Cooke, 1991; Melton, 1991). Cooke has performed interesting experiments, which indicate that, in the developing *Xenopus* embryo, regulation takes place ensuring an appropriate pattern of differentiation after removal, addition or transposition of material at a sufficiently early stage (Cooke, 1981). It is important to take this complexity into consideration when trying to identify molecules involved in mesoderm induction. The possible role of activin is discussed here in terms of the regulatory processes that must take place during normal axis formation.

Materials and methods

Preparation of activin-containing medium (ACM)

XTC-CM (Smith, 1987; Smith et al., 1990) and PIF (Sokol et al., 1990) were used as sources of activin. ACM was prepared either by diluting XTC-CM 1:4 in RPMI as previously described (Mitrani and Shimoni, 1990), or by taking partially purified PIF, at a 1:100 dilution in RPMI, as previously described (Mitrani et al., 1990).

Experimental series

Four different series of experiments were performed.

A series

Dowex AG1-X2 ion-exchange resin beads were incubated in RPMI culture medium in the presence or absence of ACM for two hours.

The beads were then rinsed (3×3 minutes) and implanted at 90º from the posterior end (P) of central disks obtained by removing the area opaca (AO) the marginal zone (MZ) and the hypoblast of stage XI blastoderms (Fig. 1).

B series

Donor central disks, obtained by removing the marginal zone, the area opaca and the hypoblast of stages XII-XIII blastoderms, were preincubated in ACM or in RPMI. The central disks were incubated at 37ºC for 4 hours after which they were removed and three posterior Pi sections: the most posterior P section and the two contiguous lateral P1 sections, were dissected and used as donors. Central disks obtained by removing the MZ, the AO and the emerging hypoblastic sheet of blastoderms at stage XI, were marked with vital dye at the anterior end and used as hosts. Each host received either a P, or a P1 region, which was implanted at 90º from the posterior end as shown in Fig. 1.

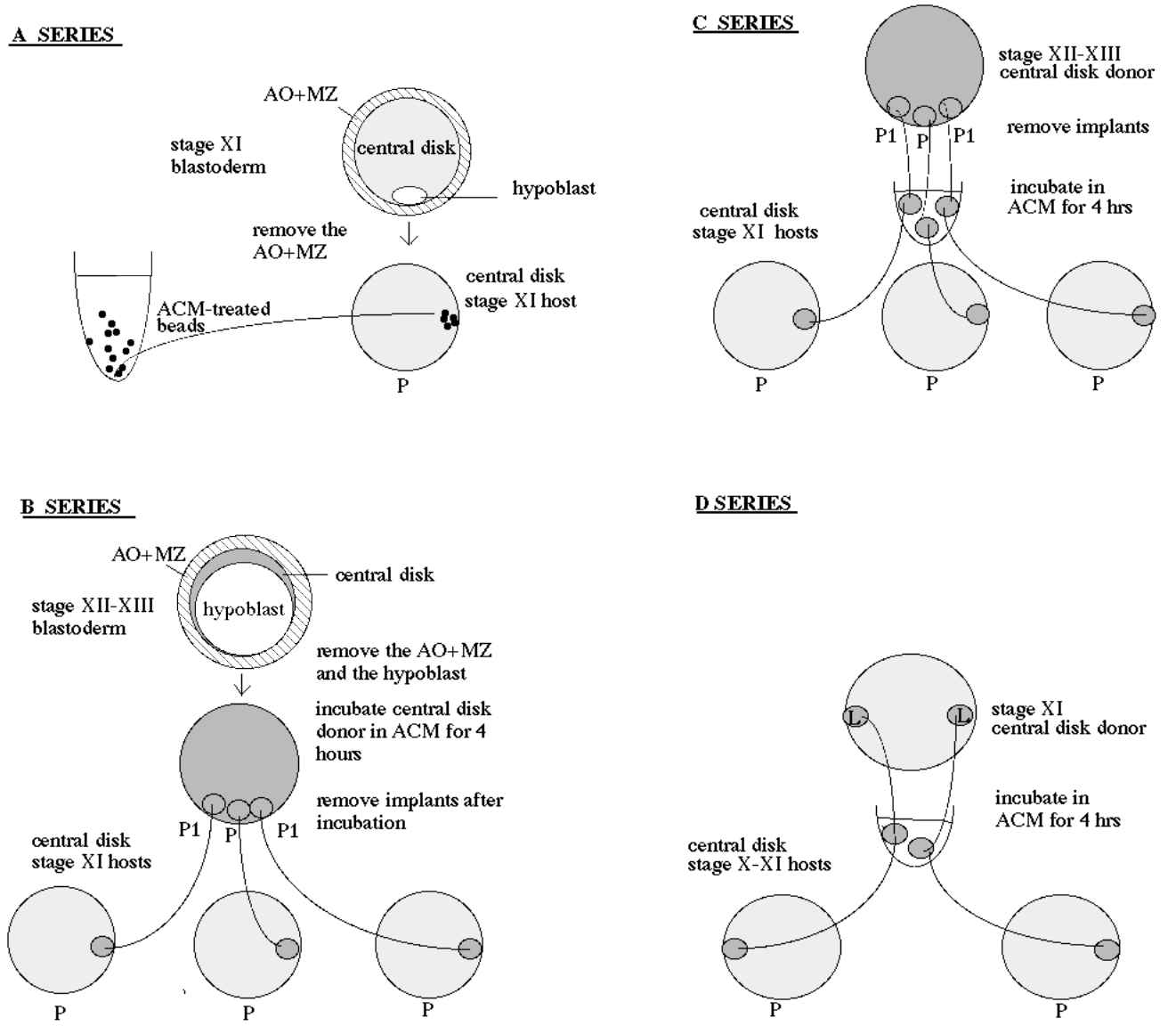

Fig. 1. A series Dowex AG1-X2 ion-exchange resin beads were incubated in RPMI culture medium in the presence or absence of ACM for two hours. The beads were then rinsed and implanted at 90º from the posterior end (P) of central disks, obtained by removing the area opaca (AO) and the marginal zone (MZ), from stage XI blastoderms. **B series** Central disks were obtained by removing the area opaca (AO), the marginal zone (MZ) and the hypoblast of stage XII-XIII blastoderms and were incubated in RPMI culture medium in the presence or absence of ACM for four hours. The central disks were then rinsed and three regions: the most posterior P region and the immediately lateral regions P1 were removed and implanted at 90º from the posterior end (P) of central disks from stage XI blastoderms. **C series** Central disks were obtained by removing the area opaca (AO), the marginal zone (MZ) and the hypoblast of stage XII-XIII blastoderms. Three regions of these central disks, the most posterior P region and the immediately lateral regions P1 were removed and were incubated in RPMI culture medium in the presence or absence of ACM for four hours. The fragments were then rinsed and implanted at 90º from the posterior end (P) of central disks from stage XI blastoderms. **D series** Central disks were obtained by removing the area opaca (AO), the marginal zone (MZ) and the hypoblast of stage XI blastoderms. Two lateral L regions of stage XI blastoderms were removed and were incubated in RPMI culture medium in the presence or absence of ACM for four hours. The fragments were then rinsed and implanted at 90º from the posterior end (P) of central disks from stage XI blastoderms.

C series

Donor central disks were obtained by removing the marginal zone, the area opaca and the hypoblast of stages XII-XIII blastoderms. Three posterior Pi sections: the most posterior P section and the two contiguous lateral P1 sections, were dissected and preincubated in ACM or in RPMI at 37ºC for 4 hours. Central disks obtained by removing the MZ, the AO and the emerging hypoblastic sheet of blastoderms at stage XI, were marked with vital dye at the anterior end and used as hosts. Each host received either a P, or a P1 region, which was implanted at 90º from the posterior end as shown in Figs 1 and 3A.

D series

Donor central disks were obtained by removing the marginal zone and the area opaca of stage XI blastoderms. Two lateral (L) sections were dissected and preincubated in ACM or in RPMI at 37ºC for 4 hours. Central disks, obtained by removing the MZ, the AO and the emerging hypoblastic sheet of blastoderms at stage X-XI, were marked with vital dye at the anterior end and used as hosts. Each host received an L region, which was implanted at 90º from the posterior end as shown in Fig. 1.

Blastoderm explant cultures

Host central disks were grown under defined culture conditions (DCM) as recently described (Mitrani and Shimoni, 1990) for a period of 44-50 hours.

Labeling experiments

Fluorescein isothiocyanate- (FITC) conjugated latex spheres (1μm) (Polysciences Inc.) were diluted 1:10000 from a 2.5% stock, and were added to the donor implant at the beginning of the preincubation with ACM. After 4 hours the donors were rinsed several times in RPMI and implanted as shown in Fig. 3A.

Results

A series

20% of the central disk hosts that received ACM-treated beads developed ectopic axes at the site of beads application (Fig. 2A,C). 13% of the hosts developed double axes, one from the posterior end and one from the site of beads application. Ectopic axes hardly ever developed further than a primitive streak. No segmented somites were observed. In contrast, no control hosts that received RPMI-treated beads developed axes at the site of beads application (Table 1).

B series

In series B, ACM-treated central disk cells were used rather than inert beads. This was done to test whether treated epiblast cells, as they are being induced, themselves become inducers. In series B, the donor central disks were preincubated in ACM or RPMI. After incubation, three posterior Pi sections: the most posterior P section and the two contiguous P1 sections, were dissected out and implanted on stage XI central disk hosts as shown in Fig. 1. Ectopic axes developed in 26% of the central disk hosts that received Pi implants from ACM-treated donors and only in one (4%) control explant that received a Pi implant from RPMI-treated donors (Table 1). 86% of the ectopic axes included a full-length notochord, segmental somites and a neural tube (Fig. 2B,D). No double axes were obtained. Axes from the posterior end developed in 14% of central disks that received implants from ACM-treated donors as compared to 52% that developed in central disks that received implants from RPMI-treated donors. In all cases except one, only one of the three

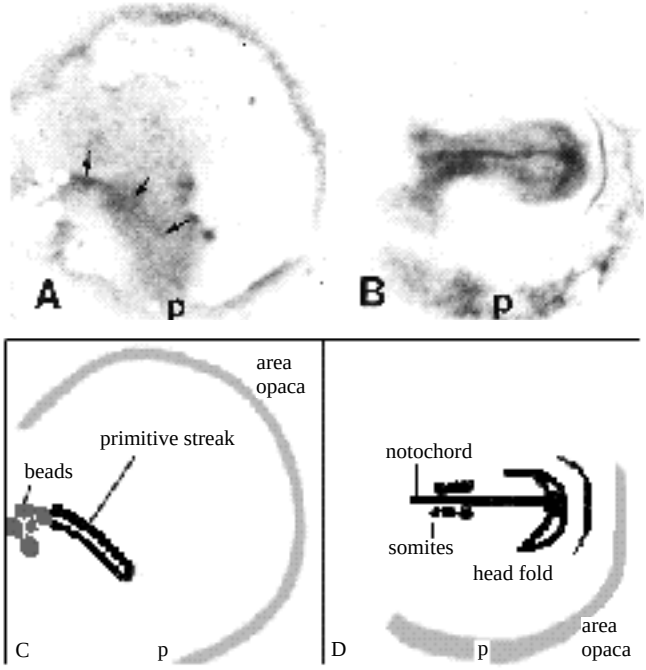

Fig. 2. Ectopic axes can develop from ACM-treated stage XI central disks. (A) ACM-treated beads were implanted at 90º from the posterior (p) end of a stage XI central disk. The explant was cultured for 24 hours. A primitive streak (outlined by arrows) develops from the site of beads application. Magnification ×25. (B) An ACM-treated P region was implanted at 90º from the posterior (p) end of stage XI central disk. The explant was cultured for 48 hours. An ectopic embryonic axis, which includes a notochord, somites and a clear fold from the emerging head develops from the site of cell implantation. Magnification ×25. (C) The periphery of the blastoderm (area opaca) and the primitive streak of Fig. 2A have been outlined in this diagram. (D) Only the embryonic structures of Fig. 2B have been outlined in this diagram.

Table 1. *Percentage of axial structures that developed at different angles from stage XI hosts. Each series was repeated between 4 and10 times*

Series	Treatment	Ectopic axes at 90º	Axes only at 0º	Explants without axes	Total no. of explants
A	Beads+ACM	20 (13)	37	43	30
	Beads+RPMI	0	41	59	12
B	Pi+ACM	26 (0)	14	60	46
	Pi+RPMI	4	52	46	25
C	Pi+ACM	40 (9)	17	43	35
	Pi+RPMI	0	38	62	13
D	L+ACM	40 (5)	35	25	20
	L+RPMI	0	54	46	13

Pi = implants from the posterior region of stage XII$^+$-XIII central disks.
L = implants from the lateral region of stage XI central disk.
The percentage of double axes are indicated in parenthesis.

Pi implants derived from each central disk donor was capable of inducing the formation of an ectopic axis.

C series

The results of series B suggested that, during the period that the central disk donor is incubated in ACM, only a small group of cells become dominant as a response to ACM stimulus while other regions become inhibited. To examine this possibility, series C experiments were performed. In this series, the central disk fragments were first dissected and only then incubated in ACM.

Ectopic axes developed in 40% of the stage XI central disk hosts that received ACM-treated implants (Fig. 3C). 75% of these ectopic axes included a full-length notochord, segmental somites and a neural tube. In several experiments, in which each Pi fragment was individually marked, more than one fragment from each donor was found to induce the formation of ectopic axes. In contrast, no control explant that received an RPMI-treated implant, developed axial structures at the site of donor implant (Table 1). Axes that developed only from the posterior end were obtained in 17% of central disks that received ACM-treated implants as compared to 38% that developed in central disks that received RPMI-treated implants. Double axes were observed in 9% of cases.

D series

Series D was performed to examine more closely the behavior of the cells that were involved in forming the ectopic axes. Two lateral (L) fragments were dissected out and incubated in ACM or RPMI, prior to implantation onto stage X-XI central disk hosts (Fig. 1). Ectopic axes developed in 40% of the stage XI central disk hosts that received ACM-treated implants (Fig. 3B,D). 75% of the ectopic axes included a full-length notochord, segmental somites and a neural tube. In contrast, no control explant that received an RPMI-treated L implant, developed axial structures at the site of donor implant (Table 1). Axes from the posterior end developed in 40% of central disks that received ACM-treated implants as compared to 54% that developed in central disks that received RPMI-treated implants. Double axes were observed in 5% of cases.

Labeling experiments

In order to determine the origin of the ectopic axes, fluorescein isothiocyanate (FITC)-conjugated latex beads were used to label the donor implant (Fig. 2A). Most labeled cells were found first to be located behind the emerging primitive streak. As the embryonic axis developed, labeled cells were found distributed sparcely in the vicinity of the axis but only few of the cells that formed the ectopic axial structures were found to be labeled (Fig. 3B).

Discussion

In the present work, we have applied ACM in a localized manner and shown that it can generate the formation of ectopic axes, which include in some cases a notochord, segmental somites and a rudimentary head. Ectopic axes were invariably found to develop from the point of ACM application.

In preliminary experiments (data not shown), hosts at different stages of development were tested. Younger stage XI hosts developed a higher number of ectopic axes and attained a higher degree of development than later stage XII hosts. Furthermore, stage XII hosts that received ACM-treated beads were not able to generate ectopic axes. Clearly, the competence of the host cells decreases as development progresses in agreement with previous findings (Azar and Eyal-Giladi, 1981; Eyal-Giladi, 1984).

There have been attempts at obtaining ectopic axes in *Xenopus* embryos by localized application of activin. Cooke et al. (1987) have shown that small pieces of animal pole tissue previously exposed to XTC MIF, developed secondary axes when grafted to the ventral marginal zones of stage-9 embryos. In such experiments, it is likely that the implanted mesodermal graft not only induced neural structures but caused pattern respecification by producing further inductive signals since a significant portion of the axis developed from host tissue. Ruiz i Altaba and Melton (1989) have obtained similar results when tissue was pretreated with XTC-MIF and then grafted into the blastocoel but could only get tail-like structures when the grafted tissue was pretreated with bFGF.

ACM can induce the formation of ectopic axes in chicks

In series A, ACM-treated beads were capable of inducing the formation of an ectopic primitive streak from the point of beads application. In series B, C and D, ectopic axes, which included a full-length notochord and segmented somites were observed. Series A and D show that ACM can change the fate of central epiblastic cells and thus act as an instructive inductor.

The results presented here are consistent with a model postulating that mesoderm induction is mediated through morphogens distributed in a gradient manner

The experiments described above suggest that mesoderm induction can be mediated through morphogens distributed in a gradient manner. In such a model, cells along a gradient would respond to different morphogen concentrations with alternative differentiation pathways (Green and Smith, 1990). The morphogen, acting as an activator, would stimulate a more rapidly diffusing inhibitor, which would spread rapidly and prevent activator accumulation in the distal part of the field. Other gradients would be stopped from forming because of this process of lateral inhibition (Meinhardt, 1982; see also Green and Cooke, 1991).

During axis formation in the chick, the gradient could be established through a simple process of diffusion or by autocatalysis. Autocatalysis is appealing in the present context since it has been previously shown that TGF-beta1 positively regulates its own expression through an autocrine process (Van Obberghen-Schilling et al., 1988). It is also difficult to conceive that the complex regulatory behavior found in early embryos could be determined by a fixed morphogen gradient. Series B to D suggest that ACM-induced implants form a

new organizing center which generates a gradient along which the ectopic axis develops. Regulation of the system normally takes place so that formation of more than one axis is inhibited.

Lateral inhibition may take place during axis formation

In chick development, evidence for inhibition as part of the regulatory mechanism of axis formation has been suggested previously (Kahner and Eyal-Giladi, 1989; Mitrani and Shimoni, 1989). In *Xenopus*, failure of injected XTC-MIF into the blastocoel of a host embryo to induce axial mesoderm has been taken as some evidence for the existence of inhibitors (Cooke et al., 1987).

The increase in the number of ectopic embryonic axes that developed in C as compared to B series indicate that, within a normal central epiblastic disk, several regions can become inducing centers if each is treated independently with ACM. Presumably, in series B, other central disk regions, which clearly have the potency to generate an axis, become inhibited. These results suggest that, in normal conditions, a regulatory process that includes inhibition takes place, which prevents more than one region from responding to the inductive stimulus normally provided by the inducing hypoblastic layer (Fig. 4).

Clearly by stage XI a prepattern already exists in the central disks as shown by the embryonic axes that can develop from the posterior end in the absence of treatment. The difference in the portion of axes that developed at 90º in relation to those that developed at 0º could be explained as a competition between the two inducing centers that are being formed. In series A the growing hypoblast tends to form an inducing center at the posterior end, while the ACM-treated beads tend to form an inducing center at 90º from the posterior end. If one of them will get to a threshold level before the other, it will induce an axis and inhibit the other center. If both of them would get to the threshold level together, double axes will form. In series B, the donor central disk is incubated in ACM and forms an inducing center from the more competent cells. The other less competent cells would be inhibited by the newly formed inducing center. At the time of implantation, some of the implants will constitute a strong center of induction and will induce axes at 90º, the other implants would be already inhibited, and will allow formation of an axis from the original posterior end, no double axes will appear. In series C and D, the donor implants are incubated separately in ACM, so that even the less competent group of cells can develop an inducing center without being inhibited. At the time of implantation, the implants with the strong inducing center will induce an axis at 90º, while the implants that constitute a weaker center of induction will compete with the host natural inducing center, and either allow the axis to develop from the posterior or will form double axes.

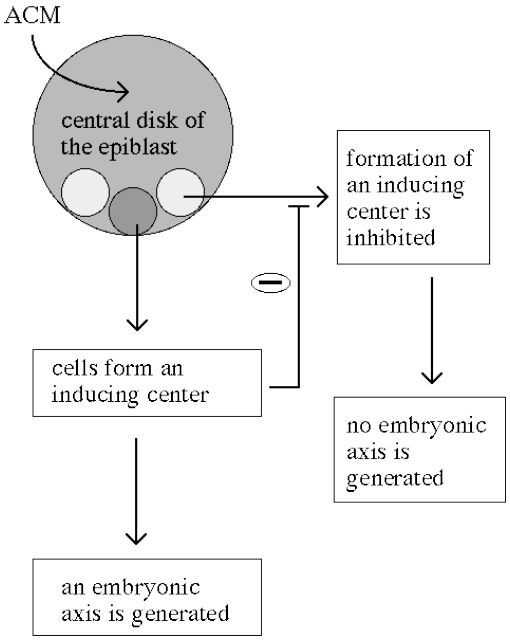

Fig. 4. Model illustrating that lateral inhibition may take place during the process of axis formation.

Induced cells may themselves become inducers

In all series, the lateral side of the central disk is the responding tissue. As shown in series D, treatment of lateral fragments with ACM can alter the cells not only to generate an axis but, in addition, to become a center of induction. It is possible that a similar process takes place during normal axis formation, cells that come in contact with the inducing hypoblastic tissue respond by either forming an axis, or by becoming themselves inducers and thus transmiting information to neighboring cells. It is also possible that a combination of both processes takes place.

Although we have shown here that activin can act as an instructive inductor of complete axial mesodermal structures, we still do not know whether activin itself is directly inducing or whether a separate set of substances are being triggered by activin (Cho et al., 1991) and they generate a gradient that would act as a putative morphogen.

In summary, the results presented above show that activin-containing medium (ACM) can act as an instructive inductor which can induce an ectopic axis. Furthermore we show data that suggest that during normal development only one axis is obtained because of a carefully controlled inhibitory process.

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Fig. 3. (A) An ACM-treated Pi region was implanted at 90º from the posterior (p) end of a stage XI central disk and examined immediately under UV light. FITC-conjugated latex spheres, used to label the donor implant, stain in green. Magnification ×50. (B) An ACM-treated L region was implanted at 90º from the posterior (p) end of a stage XI central disk. The explant was cultured for 24 hours. An ectopic primitive streak including a Hensen's node (Hn) develops from the site of cell implantation where most of the FITC-labeled cells can be seen. The extent of the streak is delineated by arrows. Magnification ×50. (C) An ACM-treated Pi region was implanted at 90º from the posterior (p) end of a stage XI central disk. The explant was cultured for 48 hours. An ectopic embryonic axis develops from the site of cell implantation. It includes a full-length notochord (arrows), segmental somites (s) and a clear fold from the emerging head (hf). FITC-labeled cells were found distributed sparcely in the vicinity of the embryonic structures. Magnification ×65. (D) An ACM-treated L region was implanted at 90º from the posterior (p) end of a stage XI central disk. The explant was cultured for 48 hours. An ectopic embryonic axis develops from the site of cell implantation. It includes a full-length notochord (arrows), and a clear fold from the emerging head (hf). Magnification ×65.