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Molecular Interactions Continuously Define the Organizer during the Cell Movements of Gastrulation

Katherine Joubin and Claudio D. Stern*
Department of Genetics and Development and
Center for Neurobiology and Behavior
Columbia University
New York, New York 10032

Summary

The organizer is a unique region in the gastrulating embryo that induces and patterns the body axis. It arises before gastrulation under the influence of the Nieuwkoop center. We show that during gastrulation, cell movements bring cells into and out of the chick organizer, Hensen's node. During these movements, cells acquire and lose organizer properties according to their position. A "node inducing center," which emits Vg1 and Wnt8C, is located in the middle of the primitive streak. Its activity is inhibited by ADMP produced by the node and by BMPs at the periphery. These interactions define the organizer as a position in the embryo, whose cellular makeup is constantly changing, and explain the phenomenon of organizer regeneration.

Introduction

Spemann's organizer (Hensen's node in birds) is defined by several properties that make it unique in the gastrulating embryo. These properties include the expression of characteristic molecular markers (such as *goosecoid*, *HNF3*β, *Not1*, *Otx2*, *chordin*, *ADMP*, and many others) and the abilities to dorsalize the mesoderm and to induce and pattern the nervous system (reviewed by Hara, 1978; Harland and Gerhart, 1997; Tam and Behringer, 1997; Smith and Schoenwolf, 1998). Spemann's organizer becomes established at the late blastula stage, when it is induced by signals from a neighboring region called the Nieuwkoop center. In turn, the Nieuwkoop center is defined by the localization of maternal determinants, which generate an intersection between two activated signaling pathways: TGFβ/Vg1 and Wnt (Nieuwkoop, 1969; Kodjabachian and Lemaire, 1998).

Although the organizer is generated early in development, its activity is required later, during gastrulation. At this time, cell populations in the embryo undergo massive reorganization to generate the three germ layers. During this process, cells continuously move into and out of the blastopore (in amphibians) or the primitive streak (in amniotes) before reaching their final destinations in the mesoderm or endoderm. Spemann's organizer/Hensen's node is situated at one end of the blastopore/primitive streak and appears to be included in these movements.

These two sets of observations appear very difficult to reconcile. During gastrulation, do the properties that define the organizer (including gene expression and inducing signals) move with a distinct population of cells, or do they mark a fixed position in the embryo, whose cellular composition is constantly changing?

Here, we address this question using a combination of dye labeling, embryological manipulations, and misexpression of secreted factors in the chick. We show that during gastrulation, cells are continuously moving into and out of the node and that they acquire and lose organizer properties according to their position. This suggests the existence of a region with Nieuwkoop center–like properties during gastrulation. We identify a "node inducing center" in the primitive streak, which, like the Nieuwkoop center, is defined by the overlap of the Vg1 and Wnt signaling pathways. This inducing center, as well as the misexpression of Vg1+Wnt, is sufficient to induce adjacent cells to become organizer. In addition, mechanisms exist to regulate the activity of the node inducing center: the organizer and the lateral edges of the embryo produce inhibitors that impose spatial constraints to the response. We conclude that migrating cells possess organizer properties transiently, according to their location at a particular time. The organizer is therefore not a committed cell population defined by its cellular ancestry, but rather a cell state, actively maintained and regulated by interactions with neighboring tissues.

Results

The Organizer Is Not a Fixed Cell Population, but a Transitory Cellular State

The axial mesendoderm (prechordal tissue, notochord, somite, and gut endoderm) is generally considered to arise from cells in the organizer—Hensen's node in amniotes (reviewed by Tam and Behringer, 1997; Smith and Schoenwolf, 1998). However, previous fate maps of chick embryos at the mid–primitive streak stage have revealed the presence of precursors for these tissues *outside* the organizer region (Rosenquist, 1966; Nicolet, 1970, 1971; Psychoyos and Stern, 1996a). During the migration of these precursors to their final destinations, do they pass through the organizer? Almost all previous fate maps have been made by analyzing only the final destination of labeled cells and therefore cannot provide an answer. We have carried out fate mapping experiments but have now also followed the movements of the labeled cells. Embryos at primitive streak stages (Hamburger and Hamilton, 1951; HH 3–4$^+$) were labeled with DiI at one of four positions (A–D; Table 1A), and the movements and ultimate fates of the labeled cells were followed either by fluorescence photography at intervals of 4–6 hr (Figures 1A–1E) or by time-lapse video microscopy. We find that at early stages (HH 3–3$^+$), descendants of cells from all four positions move into the node before giving rise to axial and paraxial mesoderm (Table 1A). At HH 4$^-$, only cells from positions A–C move into the node and contribute to these tissues. After HH 4, lateral cells no longer move into the node, and

*To whom correspondence should be addressed (e-mail: cds20@columbia.edu).

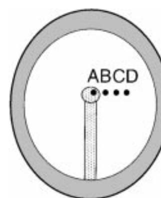
Table 1. Movements and Organizer Properties of Labeled Cells

(A) Fates of Cells from Different Regions

Stage	Fate	Region (See Inset)			
		A	B	C	D
3–3⁺	Notochord	9/9 (100%)	9/25 (36%)	4/21 (19%)	1/18 (6%)
	Somites	3/9 (33%)	7/25 (28%)	5/21 (24%)	10/18 (56%)
	Neural tissue	2/9 (22%)	15/25 (60%)	17/21 (81%)	9/18 (50%)
4⁻	Notochord	17/18 (94%)	7/23 (30%)	1/11 (9%)	0/9
	Somites	2/18 (11%)	5/23 (22%)	2/11 (18%)	0/9
	Neural tissue	5/18 (28%)	17/23 (74%)	9/11 (82%)	9/9 (100%)
4–4⁺	Notochord	8/9 (89%)	0/13	0/4	0/6
	Somites	5/9 (56%)	0/13	0/4	0/6
	Neural tissue	4/9 (44%)	13/13 (100%)	4/4 (100%)	6/6 (100%)

(B) Organizer Properties of Different Regions

Stage	Marker	Region (See Inset)	
		A	BCD
3–3⁺	*Sox3*	12/12 (199%)	0/12
	chordin	6/8 (75%)	0/8
4⁻	*Sox3*	6/6 (100%)	0/6
	chordin	6/6 (100%)	0/6
4–4⁺	*Sox3*	7/7 (100%)	0/7
	chordin	13/14 (93%)	0/14



(A) Epiblast cells were labeled with DiI at one of four positions (A–D), at different primitive streak stages (HH 3–4⁺). Position A corresponds to Hensen's node, and position D lies midway between the node and area opaca. At early stages (HH 3–3⁺), cells from all four regions move into the node and contribute to axial and paraxial mesoderm. At stage 4⁻, only cells from positions A–C move into the node. At stages 4–4⁺, lateral cells (positions B–D) no longer move into the node, but stay in the epiblast and give rise to neural tissue.
(B) Explants of region A or regions B+C+D taken from quail embryos were grafted into the area opaca of chick hosts. Following overnight incubation, embryos were processed for in situ hybridization and immunostained with anti-quail antibody to distinguish host and donor tissues. Notochord formation was assayed using *chordin* expression in the graft, and neural induction was assayed using *Sox3* expression in the host. At all stages (HH3–4⁺), only grafts of region A differentiate into notochord and induce neural tissue.

the only cells giving rise to axial and paraxial mesoderm are restricted to the node itself. We also observed that most labeled cells leave the node even before stage 4 (Figures 1D, 1J, and 1K). Therefore, during primitive streak stages, cells are continuously entering and leaving the node.

Is this migration into and out of the node accompanied by changes in the expression of organizer markers? To answer this, we labeled cells in positions B–D, as described above, and fixed the embryos when labeled descendants had reached the node. In other embryos, cells in position A (the node) were labeled and the embryos fixed when the fluorescent cells had left the node (still before HH 4⁺). Following fixation, the DiI fluorescence was photoconverted with diaminobenzidine (DAB), and the embryos were processed for in situ hybridization with *chordin*. Examples of the results are shown in Figures 1F–1M. Labeled cells arising outside the node express *chordin* only while in the node (Figure 1F–1I), whereas labeled cells that have left the node downregulate their expression of *chordin* (Figures 1J–1M). In conclusion, during their passage through the node region, cells acquire or lose their expression of organizer markers, as appropriate to their location at the time.

Does the passage of cells through the node correlate with their acquisition of other organizer properties? We assessed the formation of notochord and the ability to induce a nervous system. Cells from either region A (the node) or from regions B–D (lateral) from quail donor embryos were explanted into the peripheral area opaca of host chick embryos. After overnight incubation, only the node region produces notochord expressing *chordin* and induces the neural marker *Sox3* in host epiblast, regardless of the stage (HH 3⁺–4) of the donor (Table 1B).

Taken together, these results demonstrate that at primitive streak stages, cells continuously move into and out of the node. During these movements, they possess organizer properties only while in Hensen's node itself. The organizer is therefore not a fixed population of cells, but a state associated with a particular position in the embryo, whose cellular constitution is constantly changing.

Pattern of Reappearance of Organizer Markers after Node Ablation Reveals a Node Inducing Center

The findings presented above suggest the existence of mechanisms present in primitive streak stage embryos that provide cells with positional information, instructing them to become organizer only when in the appropriate location. To locate the source of these instructive signals, we took advantage of previous findings that chick embryos can compensate for the removal of the organizer (Hensen's node) at the primitive streak stage (Grabowski, 1956; Butros, 1967; Gallera and Nicolet, 1974; Yuan et al., 1995a, 1995b; Psychoyos and Stern, 1996b; Yuan and Schoenwolf, 1998). We examined the spatial pattern of reappearance of organizer markers at different times during regeneration to reveal the site at which expression of organizer genes begins. We surgically removed a large portion of the stage 4⁻ embryo (the entire anterior third of the primitive streak), which contains all

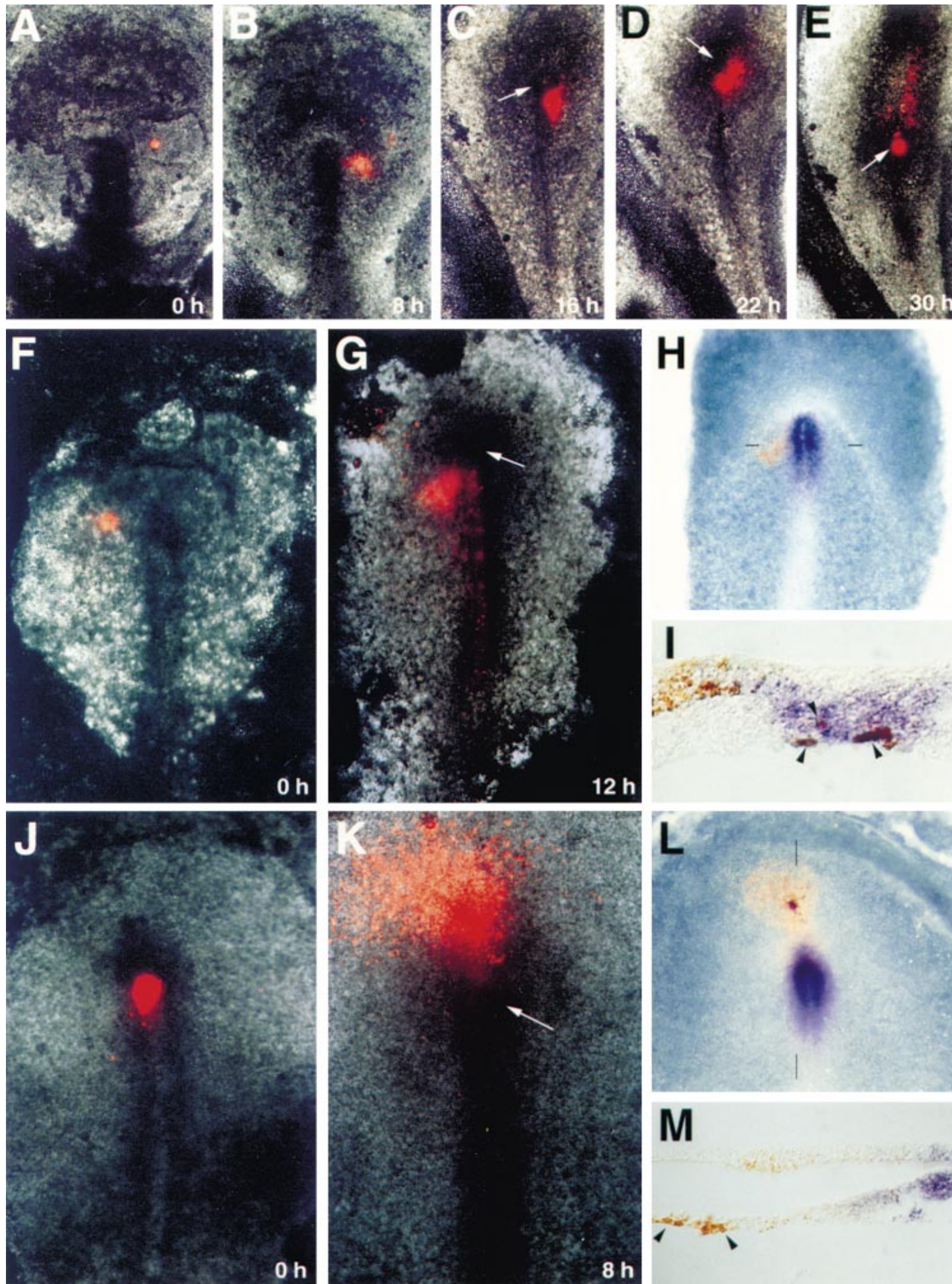


Figure 1. Cell Movements and Organizer Gene Expression during Gastrulation

(A–E) Sequence showing the movement of cells in a single embryo. DiI was applied to a region lateral to the node (A). By 16 hr (C), some of the cells have entered the node, and some of their progeny have left the node by 22 hr (D). Arrows in (C)–(E) point to the node.

(F–I) Cells entering the node acquire expression of organizer markers. Cells were labeled lateral to the node (F) and the embryo fixed when some cells had reached the node (G). (H) After photoconversion of DiI (brown), the embryo was processed for in situ hybridization with *chordin* (purple). (I) Section of the embryo in (H) at the level indicated. Arrowheads point to labeled cells that have moved into the node and now express *chordin*.

(J–M) Cells leaving the node lose expression of organizer markers. Cells were labeled in the node (J) and the embryo fixed when some descendants had left the node (K). After photoconversion and in situ hybridization with *chordin* (L and M), labeled cells that have lost their expression of *chordin* are seen in the endoderm (arrowheads in [M]). (M) is a parasagittal section at the level indicated in (L).

primitive streak cells destined to contribute to the node and its derivatives (Rosenquist, 1966; Psychoyos and Stern, 1996a). Operated embryos were fixed at 3 hr intervals after ablation (up to 12 hr), and the patterns of expression of the organizer markers *chordin*, *cNot1*, *HNF3*β, and *ADMP* (see below) were examined by whole-mount in situ hybridization. *chordin* (Figures 2A–2E) and *cNot1* (Figures 2F–2J) are the earliest to appear, by 3 hr after excision. *HNF3*β (data not shown) and *ADMP* (Figures 2K–2O) appear by 6 hr. Although the

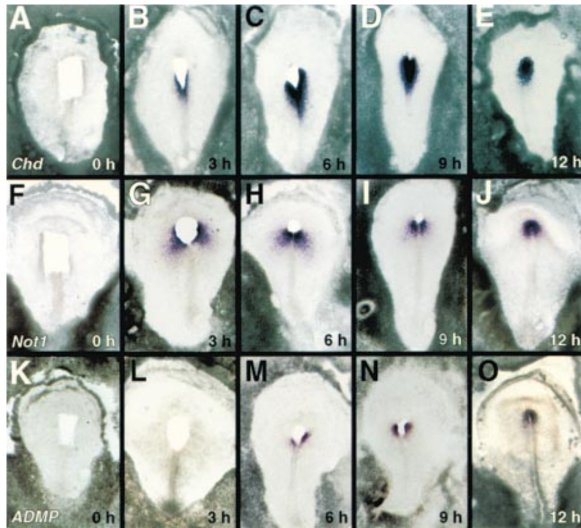


Figure 2. Time Course of Reappearance of Organizer Markers after Node Ablation

(A–E) Time course of expression of *chordin* after removal of Hensen's node and the anterior third of the primitive streak.

(F–J) Time course of expression of *cNot-1* after the same operation.

(K–O) Time course of reappearance of *ADMP*. Note that although the spatial domains of expression are characteristic for each of the genes, expression in all cases spreads from a common region at the stump of the primitive streak. Time after ablation in hours is indicated on each panel.

timing and shape of the initial domain of gene expression differ slightly among the different genes, in all cases expression starts very close to the stump of the primitive streak, from where it gradually spreads to surround the site of ablation (Figure 2). These observations point to the stump of the primitive streak as a source of organizer inducing signals.

To examine more directly whether the middle third of the primitive streak acts as a node inducing center during gastrulation, we tested the ability of grafts from different regions of a donor embryo to induce ectopic expression of organizer markers (*chordin* and *cNot1*) when transplanted to a host embryo, halfway between the node and the lateral edge (Figure 3A). We used donor quail and host chick embryos and combined in situ hybridization with immunostaining with a quail-specific antibody (QCPN) to monitor the origin of the expressing cells. Of three sources of tissue tested (Figure 3A), only the middle of the primitive streak induces organizer markers in cells of the host in this assay (10/21, 48% with *chordin*; 4/8, 50% with *cNot1*; Figures 3A–3C). Therefore, the middle of the primitive streak can act as a node inducing center, inducing expression of organizer markers when grafted to an ectopic site.

Vg1+Wnt Mimics the Node Inducing Center

The above findings are surprising because previous research in amphibians suggested that the organizer is specified only very early in development, before gastrulation, under the influence of signals from a region called the Nieuwkoop center (reviewed by Harland and Gerhart, 1997; Kodjabachian and Lemaire, 1998). The activity of the Nieuwkoop center involves interplay between two signaling pathways: Vg1/activin and Wnt (Watabe et al., 1995; Harland and Gerhart, 1997; Kessler, 1997; Kodjabachian and Lemaire, 1998). If the middle of the chick primitive streak acts as a "late" inducer of the organizer during gastrulation, does it emit these signals? At stage 3^{+}–4^{-}, both *cVg1* (Figure 3D; Shah et al., 1997) and *cWnt8C* (Figure 3E; Hume and Dodd, 1993) are expressed in the posterior two-thirds of the primitive streak and, at a lower level, slightly lateral to it (Figures 3D and 3E).

To test the involvement of these factors in node induction, we misexpressed them by grafting pellets of secreting cells into the embryo. Vg1-secreting COS cells were produced as described by Shah et al. (1997). We were unable to achieve efficient secretion of Wnt8C from transfected COS cells. However, since Wnt-1 and Wnt-8 belong to the same functional subclass of Wnt proteins in several different *Xenopus* assays (the tests include mesoderm dorsalization, alterations in gap junctional communication, and direct binding to Frzb1; Olson et al., 1991; Sokol et al., 1991; Du et al., 1995; Torres et al., 1996; Leyns et al., 1997; Wang et al., 1997a, 1997b), we used instead a stable fibroblast cell line secreting Wnt1 (Shimizu et al., 1997). When Vg1 was misexpressed alone (about one-third of the distance between the node and the lateral edge), organizer markers (*chordin*, *HNF3*β, *cNot1*, and *ADMP*) were induced adjacent to the grafted cells in 7/26 (27%) cases (Figure 3F). In contrast, Wnt1-secreting cells did not induce ectopic expression of markers (0/16) (Figure 3G). We also tested the combined effects of Wnt1 and Vg1: organizer markers were induced in 16/35 (46%) of cases (Figures 3H and 3I), and the intensity of expression was sometimes increased as compared to misexpression of Vg1 alone. In conclusion, a source of combined Vg1+Wnt1 can behave as a node inducing center.

The Node Emits an Inhibitory Signal

The existence of such a node inducing center suggests that some mechanism prevents the formation of ectopic organizer tissue during normal development. To test the possibility that the node secretes an inhibitor, we compared the inducing ability of grafts of the middle of the primitive streak in intact host embryos and in embryos from which the anterior third of the primitive streak (including the node) had been removed. We find that removal of the node and anterior primitive streak significantly enhances the response of the host to grafts placed close to the normal site of the node (Figure 4, site A, also D). Removal of the node also enhances the response of the host to Vg1+Wnt1. In the absence of the node, Vg1+Wnt1 induces expression of organizer markers in 23/34 cases (68%; compare with 46% in the presence of the node; $p < 0.04$). These results suggest that the node emits an inhibitor that regulates the activity of signals from the node inducing center, or the responses of cells to them.

ADMP: An Inhibitor Produced by the Node

The above results suggest that an inhibitor, secreted by the node, hampers the formation of ectopic organizer tissue adjacent to the node. In *Xenopus*, the BMP family member antidorsalizing morphogenetic protein (ADMP; Moos et al., 1995) has the paradoxical properties of

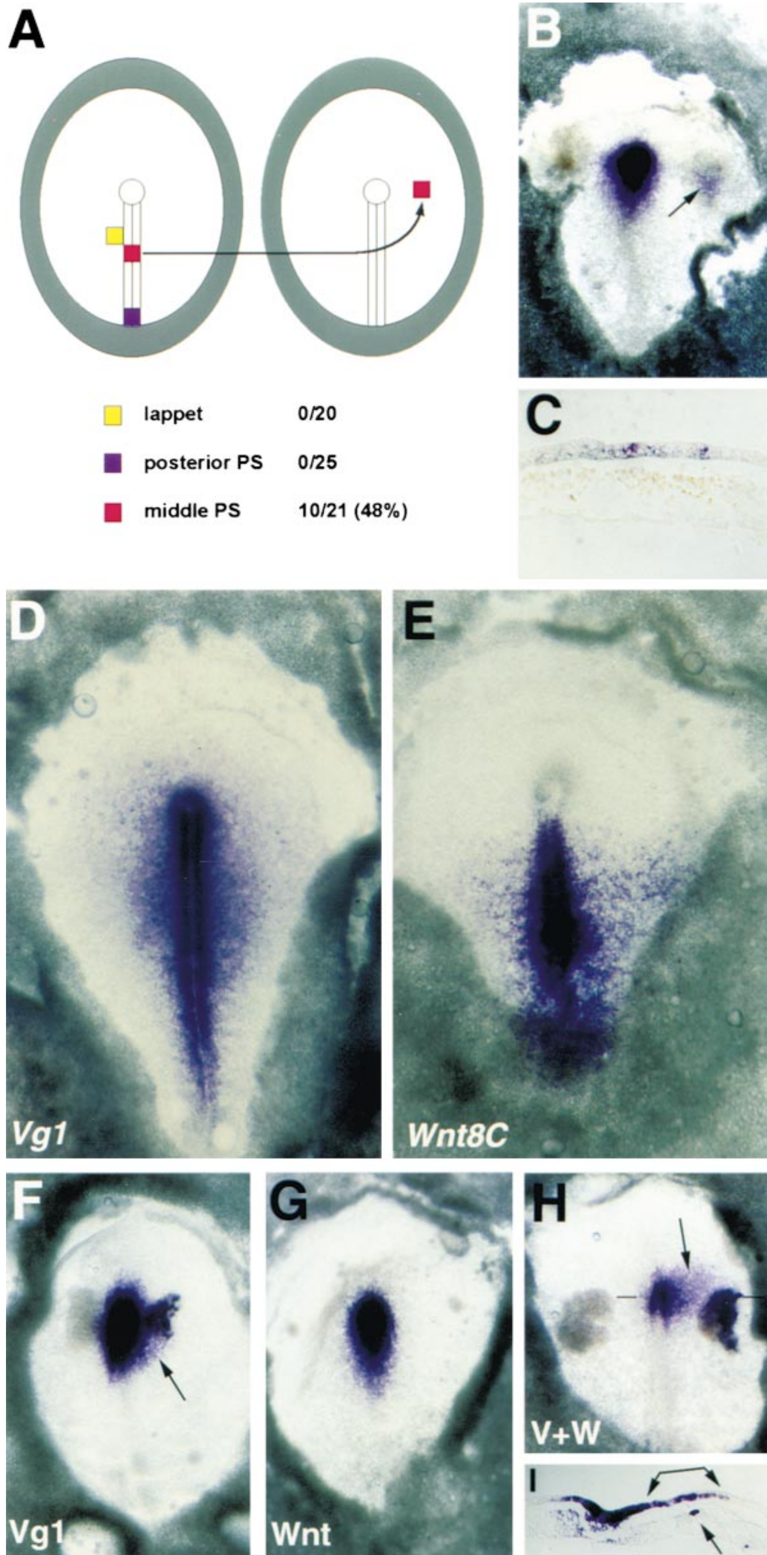


Figure 3. The Middle of the Primitive Streak and Vg1+Wnt1 Act as a Node Inducer

(A) Explants were taken from three regions of donor quail embryos: the middle of the primitive streak (red), posterior primitive streak (purple), and a domain lateral to the primitive streak (corresponding to the inducer lappet as defined by Yuan et al. [1995a, 1995b] and Yuan and Schoenwolf [1998]; this lies 0–250 μm lateral to the primitive streak and 500–750 μm posterior to the node; yellow). Each was grafted into a chick host, halfway between the node and the lateral edge of the area pellucida. The numbers indicate the proportion of embryos in which ectopic expression of *chordin* was induced in host cells 6 hr after grafting. Of these three regions, only the middle of the primitive streak can induce ectopic organizer markers.

(B) A graft of middle primitive streak induces ectopic expression of *chordin* (arrow).

(C) Section through the embryo in (B), showing the grafted quail cells (brown nuclei) and the induced *chordin* expression in the host (purple).

(D and E) Normal expression of *Vg1* (D) and *Wnt8C* (E) at stage 4. The expression of these factors overlaps in the middle of the primitive streak.

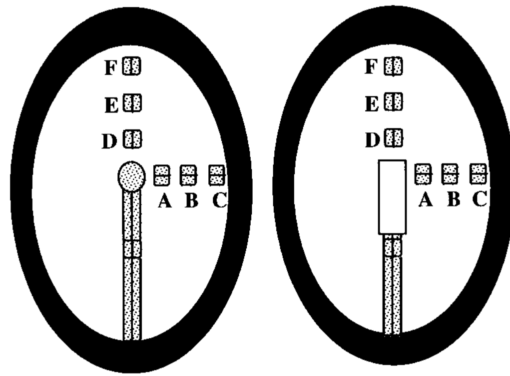
(F) COS cells secreting Vg1 induce ectopic expression of *chordin* (arrow) after 6 hr. Each embryo received 1000 *Vg1*-transfected cells on the right and 1000 mock-transfected cells on the left.

(G) Neither Wnt1-secreting cells (right) nor control cells (left) induce organizer markers (*chordin*).

(H) Grafts of Vg-1- and Wnt-1-secreting cells (right) and mock-transfected COS cells and control fibroblasts (left). The factors strongly induce expression of organizer markers (*ADMP*).

(I) Section of the embryo in (H), showing the expansion of the expression domain in host epiblast on the side that received Vg1+Wnt (bracket arrows). Bottom arrow points to the grafted COS cells.

being a ventralizing molecule (that is, it inhibits the formation of dorsal structures, including those derived from the organizer), yet being expressed in the organizer itself. Injection of *ADMP* RNA into the ventral side of the embryo has no effect, while injection near its normal site of expression (dorsally) inhibits dorsal and anterior development (Moos et al., 1995). These properties make ADMP a good candidate inhibitor produced by the node, and we therefore undertook the cloning of a chick homolog (Figure 5; see Experimental Procedures). In situ hybridization revealed that *cADMP* expression begins at stage 2^+ in a small patch of cells just anterior to the



position	w/HN	w/o HN
A	10/25 (40%)*	16/21 (76%)*
B	10/21 (48%)	6/16 (38%)
C	3/15 (20%)	1/21 (5%)
D	3/18 (17%)	7/17 (41%)
E	2/12 (17%)	4/13 (31%)
F	1/7 (14%)	2/10 (20%)

Figure 4. Spatial Response to Grafts of the Node Inducer

Explants of the middle primitive streak (node inducing center) from quail were grafted into six host sites (A–F) in a chick host, both in the presence and absence of the host node. After 6 hr incubation, the response of the host was assayed by in situ hybridization with *chordin* followed by immunostaining with QCPN. The response decreases with distance from the host node and is enhanced by ablation of the node. The asterisk indicates a statistically significant difference ($p < 0.02$).

primitive streak (data not shown). When the streak reaches its full extent (HH 3^+–4^-), it is strongly expressed in the epiblast layer of the node (Figures 6A and 6B). As the head process starts to form (HH 4^+), expression is quickly downregulated (data not shown), and no expression is detected at later stages (to at least HH 20), in contrast to *Xenopus ADMP*, which is maintained in the prechordal plate and posterior neuroectoderm at an equivalent stage (Moos et al., 1995). To test for functional conservation of chick ADMP, 100 pg of synthetic mRNA or 1 pg of *cADMP* DNA was injected into dorsal or ventral blastomeres of *Xenopus* embryos at the four-cell stage; the ventralizing effect (DAI 2.2 for dorsal injections, DAI 4.1 for ventral injections; Kao and Elinson, 1989) was identical to that caused by injection of *Xenopus ADMP* (Figure 6C).

We then investigated whether ADMP can inhibit the action of the node inducing center by implanting COS cells transfected with *cADMP* together with an explant from the middle of the primitive streak into a host embryo, one-third of the distance between the node and the lateral edge of the area pellucida (between positions A and B in Figure 4) in embryos from which the node and anterior streak had been removed. On the contralateral side, the primitive streak was grafted with mock-transfected cells as a control. ADMP reduced the induction of *chordin* by the primitive streak from 11/19 (58%) in control sides to 4/19 (21%) in the experimental sides of the same embryos ($p < 0.02$; Figures 6F and 6I).

Is the activity of Vg1+Wnt1 similarly regulated by ADMP? Pellets of COS cells containing both Vg1- and ADMP-expressing cells were grafted along with pellets of cells expressing Wnt1 into host embryos from which the node and anterior primitive streak had been removed. On the contralateral (control) side, mock transfected cells were substituted for the ADMP cells. Vg1+Wnt1+ADMP induced ectopic expression of *chordin* in 4/31 (13%) of cases, in contrast with 11/31 (35%) with Vg1+Wnt1+control cells ($p < 0.03$; Figures 6G and 6J). The lower frequency of induction by Vg1+Wnt+control cells (35%) as compared to that by Vg1+Wnt1 alone (68%) correlates with the number of Vg1-expressing cells grafted in the two experiments (500 versus 1000, respectively; see Experimental Procedures).

In conclusion, ADMP is a BMP family member expressed in the node, which can antagonize the organizer when misexpressed in *Xenopus* embryos and repress the induction of ectopic organizer markers by the node inducing center and by Vg1+Wnt in the chick.

Competence to Respond to the Node Inducing Center Decreases at the Periphery

Can any region of the embryo respond to signals from the node inducing center? To address this, we grafted the middle of the primitive streak to sites progressively more distant from the host chick node along the mediolateral or anteroposterior axes. We find that the effectiveness of induction decreases with distance from the host node along both axes (Figure 4). In addition, only proximal sites (sites A and D in Figure 4) are sensitive to the inhibitory presence of the host node. The ability of the embryo to respond to Vg1+Wnt1 also diminishes at the periphery. In these more lateral locations (at site C in Figure 4), no induction of *chordin* was seen by Vg1 (0/11), Wnt1(0/10), or Vg1+Wnt1 (0/10). These findings suggest either that the competence to respond to signals from the node inducing center decreases toward the periphery or that additional inhibitory signals emanate from the edge of the embryo.

BMPs at the Periphery Restrict Induction of Ectopic Organizers to the Center of the Embryo

The patterns of expression of chick *BMP2*, *BMP4*, and *BMP7* (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Tonegawa et al., 1997; Streit et al., 1998) and their ventralizing activity in *Xenopus* (Dale et al., 1992; Jones et al., 1992, 1996; Smith and Harland, 1992; Sasai et al., 1994; Steinbeisser et al., 1995; Piccolo et al., 1996; Hoppler and Moon, 1998) point to these secreted factors, produced at the periphery of the embryo, as possible inhibitors of node induction. At stage 4, *BMP4* is expressed strongly in the epiblast as a ring that starts adjacent to the posterior half of the primitive streak and continues laterally and anteriorly around the center of the area pellucida (Figure 6D). The pattern of expression of *BMP2* is spatially similar to that of *BMP4* at this stage but is restricted to the underlying mesendoderm (Schultheiss et al., 1997). *BMP7* is expressed in the epiblast, in a domain that overlaps the posterior quarter of the primitive streak (Figure 6E). Together, these factors are expressed over the posterior primitive streak and encircle the area pellucida but are excluded from the vicinity of the node (Figures 6D and 6E).

To test whether BMP4 can inhibit induction by the

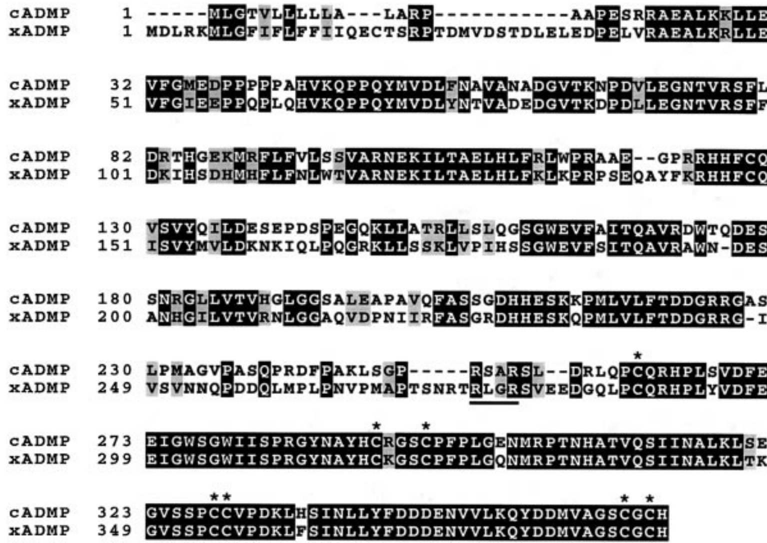


Figure 5. Chick ADMP

Alignment of the amino acid sequence of chick ADMP and its *Xenopus* ortholog. The putative cleavage site is underlined; the asterisk indicates the seven conserved cysteines. The mature domain of ADMP lies to the right of the cleavage site.

node inducing center, we implanted a pellet of *BMP4*-transfected COS cells together with the middle of the primitive streak into the area pellucida of a host embryo, midway between the host node and the lateral edge. Induction of *chordin* was never seen (0/18; data not shown), indicating that BMP4 is an inhibitor of node induction, whose expression pattern can explain the failure of lateral regions to respond to grafts of the node inducing center. As a further test for the endogenous role of BMPs as inhibitors of node induction in the lateral area pellucida, we transplanted the node inducing center together with an aggregate of cells producing Noggin, a BMP inhibitor, into the lateral edge of a host embryo. On the contralateral (control) side of each embryo, Noggin cells were implanted alone. No induction was seen on the control side (0/22), while 12/22 (55%; compare with the 20% induction by primitive streak in region C; Figure 4, $p < 0.03$) of experimental cases showed ectopic expression of *chordin* (Figures 6H and 6K).

Finally, we tested whether Noggin can relieve the ability of host tissue to respond to misexpression of Vg1+Wnt1 at the periphery, as it does with grafts of the node inducing center. Vg1+Wnt1 cells were grafted at the periphery along with two aggregates of Noggin-expressing cells. In the presence of Noggin, Vg1+Wnt1 induces ectopic expression of *chordin* in 7/27 (26%) of embryos, in contrast with 1/22 (5%) with Vg1+Wnt1 alone ($p < 0.04$, Figure 6L). Taken together, these results indicate that BMPs expressed at the lateral edge of the embryo restrict the response to grafts of the node inducing center to the middle of the embryo.

Discussion

Regulation after Removal of Hensen's Node

Our findings challenge the notion that the organizer is a fixed cell population, defined only once during very early development by the action of localized maternal determinants. Instead, they indicate that the organizer property is associated with a particular position in the embryo, whose cellular composition is perpetually changing during gastrulation. This conclusion is consistent with the observation that chick embryos develop normally when Hensen's node is extirpated or exchanged with other neighboring structures (Abercrombie and Bellairs, 1954; Grabowski, 1956; Butros, 1967; Gallera and Nicolet, 1974; Yuan et al., 1995a, 1995b; Psychoyos and Stern, 1996b; Yuan and Schoenwolf, 1998, 1999). A new organizer forms even when the ablation includes not only the node itself but also the entire region of the primitive streak that contains organizer-derived cell types (Psychoyos and Stern, 1996b), or when the lateral part of the embryo is isolated to exclude the primitive streak and node (Yuan and Schoenwolf, 1998, 1999). Reconstitution of the organizer has been considered "an auxilliary system," "a fail-safe mechanism", or "a compensatory response to injury" (Smith and Schoenwolf, 1998). However, it seems unlikely that the phenomenon of organizer regeneration represents a mechanism specifically designed during evolution to compensate for such precise accidental injury of the embryo. Our results make it much more likely that it is a consequence of interactions between signaling cells and molecules that take place during normal development.

Since cell movements bring new cells into the organizer territory during gastrulation, it is conceivable that cells that will later pass through the organizer have an intrinsic cellular clock that programs them to express organizer properties at a particular time. Our findings rule out this possibility: when cells lateral to the node (which would normally pass through the node) are isolated, they do not express markers of the organizer or its derivatives, nor do they develop the ability to induce neural tissue (Table 1B).

The phenomenon of regeneration of the organizer is most likely not unique to the chick, but rather is a general principle in vertebrate embryos, all of which are characterized by extensive cell movements during gastrulation. In teleost fish, both classical (Nicholas and Oppenheimer, 1942) and more recent (Shih and Fraser, 1996) observations have shown that ablation of a large region including the embryonic shield is followed by normal

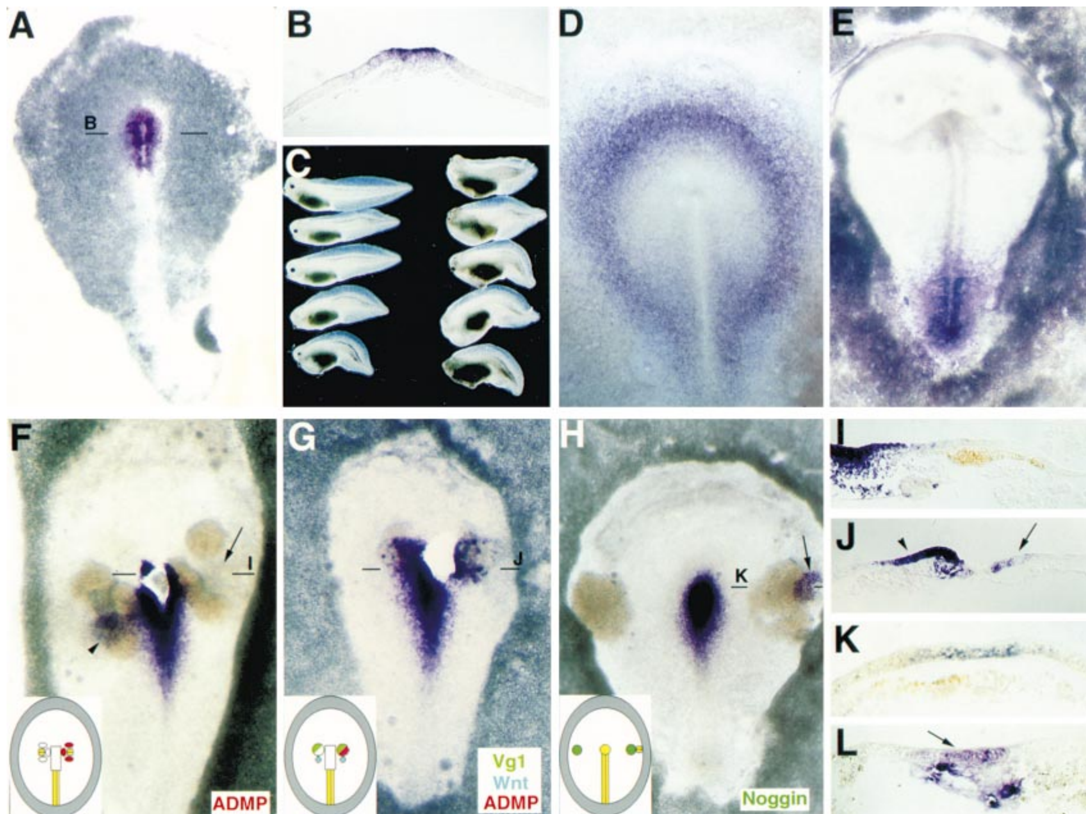


Figure 6. ADMP and BMP4 Inhibit Organizer Induction

(A and B) Expression of chick *ADMP* at stage 4 in whole-mount (A) and transverse section (B). Transcripts are restricted to the epiblast layer of the node.

(C) Effects of injection of RNA encoding chick ADMP into the ventral (left) or dorsal (right) side of *Xenopus* embryos at the four-cell stage. Injection to the ventral side has little or no effect (DAI 4.1), while dorsal injection produces ventralized and caudalized embryos (DAI 2.2).

(D) Expression of *BMP4* at stage 4. Transcripts describe a ring and are absent from the center of the embryo.

(E) Expression of *BMP7* at stage 4. Expression overlaps the posterior primitive streak.

(F) ADMP inhibits induction of organizer markers by the node inducer. The inset shows the experimental design. The middle of the primitive streak is grafted together with mock-transfected COS cells (left) or ADMP-secreting COS cells (right) into an embryo from which the node was ablated. The embryo was fixed after 6 hr. Ectopic expression of *chordin* is seen on the control (left) side (arrowhead), but not on the side that received ADMP (arrow).

(G) ADMP inhibits induction of organizer markers by Vg1+Wnt1.

(H) The BMP inhibitor Noggin uncovers the ability of the lateral area pellucida to respond to the node inducer. Noggin-secreting cells were grafted alone (left) or together with the middle third of the primitive streak (right). Induction of ectopic *chordin* is seen on the right (arrow).

(I) Section of embryo in (F). Quail cells stained brown.

(J) Section of embryo in (G). Arrowhead points to ectopic expression of *chordin* induced in host epiblast by Vg1+Wnt1. This induction is greatly diminished by ADMP (arrow).

(K) Section of embryo in (H); quail cells stained brown.

(L) Section of an embryo that received a lateral graft of Noggin together with Vg1+Wnt1. *chordin* is induced (arrow).

development of axial structures normally derived from the shield. In amphibians, Cooke (1973, 1975; see also Gerhart, 1980) showed that removal of the dorsal lip results in a developmental delay, but subsequently the notochord and other structures normally derived from the lip appear.

In conclusion, our findings suggest that the apparent regenerative behavior of the vertebrate organizer is a consequence of normal inductive cell interactions whose function is to position the organizer within a field of moving cells.

Transiting and Resident Cells

Previous results in both chick and mouse (Selleck and Stern, 1991, 1992; Beddington, 1994; Nicolas et al., 1996) have suggested the presence of resident cells in the epiblast of the node, which may behave as asymmetrically dividing stem cells and contribute progeny to the notochord and somites. Such a resident population is likely to be very small. Our experiments reveal that most node cells are continuously replenished; even when a large region of the node is marked at early primitive streak stages, almost all labeled cells have left the node before the start of neurulation (see Figures 1J and 1K). Some of the few remaining cells appear to regress with the node at later stages, contributing to notochord and/or somite (data not shown). Therefore, the bulk of the node consists of transiting cells. Resident cells appear to be confined to the epiblast (Selleck and Stern, 1991, 1992), yet all layers of the node are able to induce a

secondary axis upon transplantation (Storey et al., 1995). These results argue that organizer properties are associated with the site of the node rather than with any particular cell population. In addition, the finding that embryos can regenerate the node and anterior primitive streak further suggests that no specific node cell population is absolutely required for the inducing and patterning functions of the organizer.

Inducing Cells and Molecules

Our results suggest that the inducing signals emanate from a node inducing center, located in the middle sections of the primitive streak. *Vg1* and *Wnt8C* are expressed in this region, and misexpression of Vg1+Wnt can mimic grafts of the inducing center. Recent molecular studies of the 5′ regulatory region of the organizer-specific gene *goosecoid* have identified two distinct enhancers regulating its expression in frog and mouse embryos (Watabe et al., 1995; see also Sokol and Melton, 1992; Brannon and Kimelman, 1996; Cui et al., 1996; Kessler, 1997; Crease et al., 1998; Zorn et al., 1999). One of these elements is activated by transcription factors downstream of activin/Vg1-like signals, while the other responds to the Wnt pathway. These two pathways have been proposed to act synergistically to activate organizer markers, explaining the activity of a region of the amphibian embryo known as the Nieuwkoop center (Nieuwkoop, 1969; see Harland and Gerhart, 1997 for a recent review). The Nieuwkoop center acts before gastrulation to induce the organizer but does not contribute cells to it. It is generally believed that the inducing activity of the Nieuwkoop center is confined to these very early stages of development.

A recent study in the chick embryo (Bachvarova et al., 1998) ascribed a Nieuwkoop center–like activity to the posterior marginal zone of the pre–primitive streak stage chick embryo: it can induce a complete axis when transplanted ectopically, and this includes the formation of a new organizer from cells that were not destined for this fate. The posterior marginal zone expresses both *Vg1* and *Wnt8C* (Hume and Dodd, 1993; Shah et al., 1997; I. Skromne and C. D. S., unpublished observations), and the organizer arises from cells that initially lie adjacent to the posterior marginal zone (Izpisúa-Belmonte et al., 1993; Hatada and Stern, 1994). Misexpression of Vg1 in the anterior marginal zone can initiate the formation of an ectopic organizer and subsequently a complete axis (Seleiro et al., 1996; Shah et al., 1997), but responsiveness is lost as the host embryo reaches the very early primitive streak stage (Shah et al., 1997). Therefore, in both amniotes and amphibians, cells of the Nieuwkoop center/posterior marginal zone act before gastrulation to induce an organizer in neighboring cells without making a cellular contribution to it, and the activity of the Nieuwkoop center seems to rely on the intersection of activin/Vg1 and Wnt signaling pathways. However, it is important to note that the posterior marginal zone does not make a direct cellular contribution to the primitive streak (Hatada and Stern, 1994; Bachvarova et al., 1998). Therefore, the node inducing center is made up of a different cell population than the posterior marginal zone (Nieuwkoop center equivalent). In conclusion, although the signaling pathways that initially establish the primitive streak and organizer may well be the same as those that regulate the organizer during gastrulation, our results argue against the idea that the overlap of *Vg1/Wnt8C* expression marks a conserved population of signaling cells that spans both stages of development.

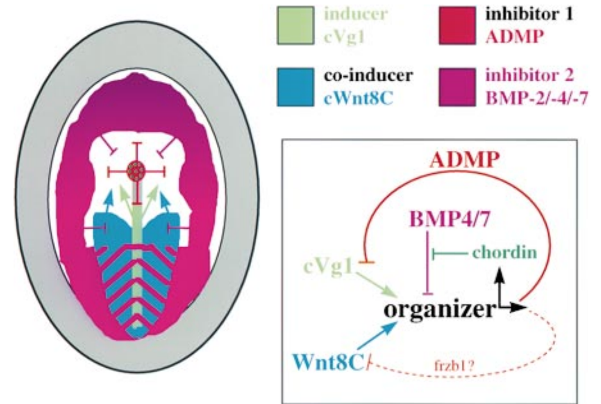


Figure 7. Model of Molecular Interactions that Position the Organizer during Gastrulation

Inducers (Vg1 and Wnt8) are shown in green/blue; inhibitors are shown in red/purple. The expression patterns in the embryo and interactions between cells are diagrammed on the left; the inset summarizes a proposed pathway of molecular interactions. Vg1 and Wnt8 secreted by the middle of the primitive streak synergize to induce organizer. One result of this induction is the production by the organizer of ADMP (red), which acts as a feedback inhibitor. BMPs (purple) secreted by the periphery of the embryo obliterate the activity of the inducers Vg1 and Wnt8 over the posterior primitive streak and can block induction of organizer in regions remote from the center of the embryo. Expression of the BMP inhibitor Chordin by the organizer (dark green dots) may serve to keep BMP expression clear of the center. "Frzb1?" refers to a hypothetical chick ortholog of *Xenopus* Frzb1 (Leyns et al., 1997; Wang et al., 1997a, 1997b; Hoang et al., 1998), expressed in the organizer, which may bind and inhibit Wnt8.

The overlapping expression of *Vg1* and *Wnt8C* in the middle of the primitive streak of later gastrulation-stage embryos suggests that this region too can act as a Nieuwkoop center. It was recently reported (Yuan et al., 1995a, 1995b; Yuan and Schoenwolf, 1998) that lateral isolates of chick blastoderms (excluding the entire primitive streak, and therefore the node inducing center) can form an ectopic organizer only if a region defined as the "inducer lappet" (see Figure 3A) is included. *Vg1* and *Wnt8C* are both expressed in this region, albeit at a lower level than in the node inducing center, which may account for the lack of inducing activity of the inducer lappet in our assay. Our results therefore suggest that the strongest source of node inducing activity is the middle of the primitive streak itself.

Inhibitors

The spatial pattern of expression of *Vg1* and *Wnt8C* in normal embryos should induce an organizer in adjacent epiblast cells. However, this does not happen unless the node is removed, suggesting that the organizer produces an inhibitor, ADMP, which forms part of a feedback mechanism that represses induction by the node inducing center and prevents cells adjacent to the node from becoming organizer prematurely. Figure 7 presents a model for these interactions.

The expression and activity of BMP-2, -4, and -7 also suggest their involvement in defining the organizer during gastrulation. In *Xenopus*, BMP signaling "ventralizes" the whole embryo before gastrulation, and just the mesoderm during the gastrula stage (Dale et al., 1992; Jones et al., 1992, 1996; Smith and Harland, 1992; Sasai et al., 1994; Steinbeisser et al., 1995; Piccolo et al., 1996; Hoppler and Moon, 1998; Hsu et al., 1998; see Harland and Gerhart, 1997 for review). In the chick, the expression of *BMP-2*, *-4*, and *-7* describes a ring, overlapping the posterior primitive streak and surrounding the center of the embryo such that the vicinity of the organizer is devoid of signal. Their expression in the posterior streak could account for the inability of this domain to act as a node inducer (Figure 3A). At the lateral edges of the embryo, inhibition of endogenous BMP activity by misexpression of Noggin is not sufficient to generate an ectopic organizer but can unmask the ability of this region to respond to a graft of the node inducing center and to Vg1+Wnt (Figures 6H, 6K, and 6L). By contrast, BMP inhibition by Chordin at earlier stages (up to stage 3) is sufficient to induce the formation of a primitive streak including the organizer (Streit et al., 1998). Taken together, these findings suggest that, by the late primitive streak stage, the function of BMP expression at the edges of the embryo is no longer connected with an inhibition of primitive streak formation (analogous to ventralization of the embryo in *Xenopus*), but rather contributes to prevent the formation of ectopic organizers other than in the center.

The node itself expresses *chordin* from the time of primitive streak formation and maintains this expression at later stages of development both within the node and in its derivative, the notochord (Streit et al., 1998). *noggin* (Connolly et al., 1997; Streit and Stern, 1999) and *follistatin* (Connolly et al., 1995) are expressed weakly, if at all, in the node at primitive streak stages and increase in the remnants of the node thereafter. Since BMP activity may be required to maintain *BMP* transcription (Biehs et al., 1996; Streit and Stern, 1999), it seems likely that at least one function of the expression of its antagonist, Chordin, by the node is to keep the central region of the embryo free of BMP expression. Following node ablation, *chordin* is among the earliest markers to be expressed (Figure 2), which takes place 3 hr or less after surgery. This suggests that early reexpression of *chordin* may be important to prevent BMPs from advancing toward the regenerating node. By contrast, the inhibitor *ADMP* is not expressed at this time, but it is detected in the same region 6 hr following surgery.

Do the inhibitory signals act on the inducing cells by repressing expression of *Vg1* and *Wnt8C*, or are positive and negative signals integrated by the responding cells? In the posterior third of the primitive streak, expression of *Vg1* and *Wnt8C* overlaps with expression of the inhibitors *BMP-2*, *-4*, and *-7* (compare Figures 3D and 3E with Figures 6D and 6E; see Figure 7), suggesting that these BMPs do not affect transcription of the inducing factors in the primitive streak. The idea that positive and negative signals are integrated by the responding cells is supported by the signal transduction machinery involved in the TGFβ pathway. Members of the TGFβ superfamily that have ventralizing activity in *Xenopus* (including the BMP family) act through activation of the transcription factor Smad1 (for review, see Kretzschmar and Massagué, 1998; Whitman, 1998), and it is likely that ADMP, which belongs to the BMP subfamily, is no exception. On the other hand, TGFβ superfamily members with dorsalizing activity (such as Vg1) activate Smad2 (see Kretzschmar and Massagué, 1998; Whitman, 1998). This suggests that the activities of the inducer Vg1 and of the inhibitors (BMPs and ADMP) are integrated at the level of the responding cells, by measurement of the relative activity of Smad1 and Smad2 complexes.

Protecting the Organizer against Itself?

Even though our results provide a novel function for ADMP in the node, some features of this molecule remain paradoxical. Overexpression of ADMP at the dorsal (organizer) side of the *Xenopus* embryo (its normal site of expression) effectively ventralizes the embryo by preventing further development of the organizer. What protects the organizer from the inhibitory action of this molecule during normal development? ADMP is a member of the BMP family. Although it is conceivable that ADMP is inactivated by the BMP antagonist Chordin, which is expressed in the organizer at this stage (Streit et al., 1998), we have been unable to demonstrate inhibition of ADMP activity by Chordin.

An alternative possibility is that the activity of ADMP, like that of other TGFβs (see Hoppler and Moon, 1998), is enhanced by Wnt factors. If so, the node could protect itself by producing an inhibitor of the Wnt pathway with a restricted range of diffusion. In mouse and *Xenopus*, the secreted factor Frzb1 is a specific inhibitor of Wnt8 and Wnt1 signaling and is expressed in the organizer during gastrulation (Leyns et al., 1997; Lin et al., 1997; Wang et al., 1997a, 1997b; Hoang et al., 1998). Since Wnt8 and Wnt1 are dorsalizing factors in *Xenopus*, the expression of their antagonist *frzb1* in the organizer is as paradoxical as that of *ADMP*. The conservation of sequence, activity, and expression pattern between frog and mouse suggests that a homolog expressed in the chick node at stages 3^+–4 should exist (Figure 7, dashed red line).

Conclusions

Our study emphasizes the dynamic nature of gastrulation and shows that during their movements, cells acquire and lose gene expression and functional attributes according to their position in the embryo. A Nieuwkoop center–like region, expressing *Vg1* and *Wnt8C*, exists in the middle of the primitive streak, which induces neighboring cells to become organizer. However, the cells of this center are not lineally derived from those of the posterior marginal zone (the Nieuwkoop center before gastrulation). The spatial and temporal control of organizer induction is further refined by the action of inhibitors (ADMP and BMPs). These cellular and molecular interactions also account for the phenomenon of organizer regeneration.

Experimental Procedures

Embryo Manipulations and DiI Labeling

Fertile White Leghorn chick eggs (SPAFAS, CT) and quail eggs (Karasoulas, CA) were incubated at 38°C for approximately 13 hr and

staged according to Hamburger and Hamilton (1951). Chick embryos were explanted in modified New (1955) culture (Stern and Ireland, 1981). Node ablations were done as described by Psychoyos and Stern (1996b). Quail embryos were dissected in Pannett-Compton saline (Pannett and Compton, 1924) with fine glass needles made from 50 μl capillaries (Sigma) in a vertical electrode puller (Kopf). DiI labeling was performed as described by Psychoyos and Stern (1996a).

In Situ Hybridization and Immunocytochemistry

Whole-mount in situ hybridization and antibody staining on chick embryos were performed as described previously (Streit et al., 1997). The anti-quail antibody QCPN was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biological Sciences, University of Iowa, Iowa City, IA under contract N01-HD-2-3144 from NICHD). The probes used for in situ hybridization were as follows: *chordin* (Streit et al., 1998), *cNot1* (Stein and Kessel, 1995), *HNF3*β (Ruiz i Altaba et al., 1995), *Vg1* (Shah et al., 1997), *Wnt8C* (Hume and Dodd, 1993), *BMP2*, *BMP4*, and *BMP7* (Liem et al., 1995).

Isolation of cADMP cDNA Clones

Degenerate primers (5′ = ATHGGITGGWSIGGITGGATHAT and 3′ = ATISWYTGIACIGTIGGRTGRTT), based on sequences common to *Xenopus* ADMP and other BMP family members, were used to amplify a 122 bp fragment from chick genomic DNA. One hundred picomoles of these primers was used in 50 μl reactions, which were cycled 25 times at 94°C (50 s), 50°C (50 s), and 72°C (2 min). PCR products were purified by agarose gel electophoresis, cloned into pGEM-T (Promega), and sequenced by the Sanger dideoxy method (Sequenase kit, USB). All products isolated encoded the same sequence, which had significant homology to *Xenopus* ADMP. This clone was used to screen a chick stage 3^+–4 Hensen's node cDNA library in λZap (Stern et al., 1995) under standard stringent conditions using a ^{32}P-labeled probe. Clones (10^6) were screened and, of approximately 35 positive clones, six were plaque purified and converted into pBlueScript plasmids. The longest clone, which was 1.4 kb, was sequenced and contained the entire 1.1 kb open reading frame. The deduced amino acid sequence is 94% identical to *Xenopus* ADMP in the mature domain after the first conserved cystein, and 51% identical in the prodomain (Figure 5).

DNA Constructs for Misexpression

The c-Myc epitope (EQKLISEEDL) was inserted four amino acids downstream of the putative cleavage site of cADMP by PCR using two internal primers, *IT-myc-1* (CGGAATTC*GATATC*CGAGGAG GACCTGCTGCAGCCGTGCCAGAGG) and *IT-myc-2* (GCGAATTC *GATATC*AGCTTCTGCTCGCGGTCCAGGGAGCGGTC), each containing half of the c-*myc* sequence and flanking *cADMP* sequence. The internal *myc* sequence required construction of the plasmid from two fragments. The N-terminal fragment was generated using T7 and *IT-myc-2*. The C-terminal fragment of the coding sequence was generated using *IT-myc-1* and the primer cADMP.b3′ (AGCGC GGCCGCATTGTCTCTGCATCAGTGGCAGC). These fragments were joined by their primer-derived EcoRV sites (italics above) and ligated into the pMT23 expression vector. When this construct is transfected into COS cells and conditioned medium analyzed on Western blots using anti-c-Myc antibody, very little cADMP is detected. To enhance the secretion of cADMP by COS cells, a chimeric protein containing the proregion and cleavage site of Dorsalin-1 and the active region of cADMP was generated by ligating the C-terminal PCR fragment into a *dorsalin-myc* expression construct whose C-terminal domain was removed (Basler et al., 1993; Shah et al., 1997). This construct, *dorsalin-myc-cADMP*, was used for all experiments.

COS-1 Cell Transfections and Noggin- and Wnt-Secreting Cell Lines

COS-1 cells were grown in DMEM containing 10% newborn calf serum. Transfection with *ADMP*, *BMP-4*, and *Vg1* was performed using lipofectamine (GIBCO-BRL). Twenty-four hours after transfection, pellets containing 500 or 1000 cells were generated by setting up hanging drop cultures. The pellets were transplanted into embryos 48 hr after transfection. For ADMP and BMP-4 experiments, three pellets of 500 cells were placed around the primitive streak graft. For Vg1 experiments, one pellet of 1000 cells was used, and for misexpression of both Vg1 and ADMP, one pellet containing 500 ADMP cells and 500 Vg1 cells was used. The presence of ADMP, BMP-4, and Vg1 in conditioned medium collected from COS cells was confirmed by Western blots using anti-Myc antibodies as described previously (Streit et al., 1998) (data not shown). As controls in experiments with transfected COS cells, we used mock-transfected cells.

A stable cell line secreting Noggin has been described previously (Lamb et al., 1993); as controls for these cells we used the parent CHO cell line (kind gift of Richard Harland). CHO cells do not spontaneously form aggregates in hanging drop culture. To produce aggregates suitable for grafting, a suspension of cells was centrifuged lightly (200 × g, 5 min) in an Eppendorf tube, and the resulting pellet was loosened with a steel needle and removed with a Gilson micropipette. It was then cut into suitably sized pieces for grafting (about 100–150 μm) using fine mounted steel pins.

A rat B1-fibroblast-derived stable cell line secreting Wnt1 and control parent fibroblasts (Shimizu et al., 1997; kind gift of J. Kitajewski) were grown in glutamine-supplemented DMEM containing 7.5% newborn and 2.5% fetal calf serum. For grafting into embryos, pellets of 2500 cells were made as described above for COS cells.

Xenopus Injections

cADMP was subcloned into pSP64TEN, and capped mRNA was produced using mMessage mMachine SP6 kit (Ambion). For DNA injections, *cADMP* was subcloned into pXEX and injected directly. Embryos were generated and injected as previously described (Wu and Gerhart, 1991; Moos et al., 1995) and grown until sibling embryos had reached tail bud stages. The dorso-anterior index (DAI) was scored according to Kao and Elinson (1989).

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