BMP antagonism by Spemann’s organizer regulates rostral–caudal fate of mesoderm

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Abstract

Recent revisions to the Xenopus fate map challenge the interpretation of previous maps and current models of amphibian axial patterning (Lane, M.C., Smith, W.C., 1999. The origins of primitive blood in Xenopus: implications for axial patterning. Development 126 (3), 423–434.; Lane, M.C., Sheets, M.D., 2000. Designation of the anterior/posterior axis in pre-gastrula Xenopus laevis. Dev. Biol. 225, 37–58). We determined the rostralmost contributions to both dorsal and ventral mesoderm concomitantly from marginal zone progenitors in stage 6 embryos. Data reveal an unequivocal rostral-to-caudal progression of both dorsal and ventral mesoderm across the pre-gastrula axis historically called the dorsal–ventral axis, and a dorsal-to-ventral progression from animal-to-vegetal in the marginal zone. These findings support the proposed revisions to the fate and axis orientation maps. Most importantly, these results raise questions about the role of the organizer grafts and organizer-derived BMP antagonists in the induction of secondary axes. We re-examine both phenomena, and find that organizer grafts and BMP antagonists evoke caudal-to-rostral mesodermal fate transformations, and not ventral-to-dorsal transformations as currently believed. We demonstrate that BMP antagonism evokes a second axis because it stimulates precocious mediolateral intercalation of caudal, dorsal mesoderm. The implications of these findings for models of organizer function in vertebrate axial patterning are discussed. © 2004 Elsevier Inc. All rights reserved.

Keywords: Axial patterning; BMP antagonism; Dorsal–ventral patterning; Fate maps; Mediolateral intercalation; Rostral–caudal patterning; Spemann’s organizer

Introduction

Currently, there are contradictory fate maps for the amphibian Xenopus laevis, and the discrepancies in tissue distributions between the maps lead to conflicting assignments of the dorsal–ventral and rostral–caudal axes (summarized in Lane and Sheets, 2002b). The discrepancies involve primarily the mesoderm, and crucial points of contention between the two maps and the resulting axis designations are summarized diagrammatically in Figs. 1A and B. In the conventional fate map (Fig. 1A), the dorsal–ventral axis runs from Spemann’s organizer in the dorsal marginal zone (DMZ) to the meridian of sperm entry in the ventral marginal zone (VMZ). Mesodermal tissues are arranged notochord (most dorsal in the modern interpretation), muscle, pronephros, and blood (most ventral). The rostral–caudal axis is not assigned in the conventional map. In the revised map (Fig. 1B), it is the rostral–caudal axis that runs from Spemann’s organizer to the meridian of sperm entry. The dorsal–ventral axis of the mesoderm is reassigned to the animal/vegetal axis. As the region including and immediately surrounding Spemann’s organizer (which is historically called the DMZ) is the source of anterior mesoderm, it is renamed the rostral marginal zone (RMZ). The marginal zone on the opposite side of the embryo (historically called the VMZ) is renamed the caudal marginal zone (CMZ), as it is the source of posterior mesoderm. Thus, the two maps and the axis designations are incompatible. We believe the discrepancies between the maps must be resolved.
To this end, we report here the rostral–caudal and dorsal–ventral topographic projection from the stage 6 blastula marginal zone (i.e., the C- and B-tier blastomeres, Fig. 1C) to the mesoderm of the tadpole (stage 32–34). The results reported here support the revised map shown in Fig. 1B and contradict the old map diagrammed in Fig. 1A.

Fate maps are topographic projections of body plans that are used to assign the embryonic axes to embryonic stages at which the axes are not manifest. Significant discrepancies between fate maps and the ensuing mistakes in axis designations confound the interpretation of embryo phenotypes. To demonstrate how profound this impact is in amphibian embryology, we re-examined the seminal experiment of vertebrate axial patterning—the organizer grafting experiment of Spemann and Mangold (1924).

In this experiment, a small graft from above the upper blastoporal lip (i.e., the conventional DMZ, or the revised RMZ) of a gastrula stage amphibian embryo induced the formation of a partial secondary trunk when grafted above the lower blastoporal lip (i.e., the conventional VMZ, or the revised CMZ) of another gastrula stage embryo (Spemann and Mangold, 1924). The induced structures of the partial secondary axis included somitic mesoderm and neural tissue arranged as a normal vertebrate embryonic axis around graft-derived notochord, but lacking a second head. Only the region above the upper blastoporal lip induces a second axis in this assay. An analogous region that induces a partial secondary axis when grafted to an ectopic site has been described in all vertebrate embryos, and these regions are known as nodes or shields (Beddington, 1994; Saude et al., 2000; Shih and Fraser, 1996; Waddington, 1932). Of all the vertebrate organizers, Spemann’s organizer remains the best characterized. After 80 years of research, Spemann’s organizer is defined by four functions (Gerhart et al., 1991), some of which also characterize nodes or shields. It self-differentiates as notochord and prechordal (head) mesoderm; it regulates the morphogenesis of dorsal, axial structures; it induces/patterns the neural plate; and it patterns the mesoderm by dorsalization.

The modern concept of mesodermal “dorsalization” comes from Smith and Slack’s re-examination (1983) of Spemann and Mangold’s grafting experiment. Smith and Slack (1983) grafted a lineage-labeled organizer into the VMZ of a host, and the reciprocal experiment, VMZ into the organizer. They concluded that cells in the VMZ were specified to form blood, but converted into somites by the organizer graft. This conversion is “dorsalization”. Their conclusions led the authors to propose the “three-signal hypothesis of mesoderm induction”. A fate map subsequently indicated blood arose solely from the ventral marginal zone (Dale and Slack, 1987). Most molecular expression patterns and molecular models of axial patterning have been interpreted almost solely in the context of the three-signal hypothesis and the related fate map (as a recent example for zebrafish, see Sidi et al., 2003). Since the three-signal hypothesis was published, numerous revisions to the basic model have been proposed, but all retain two
central notions: that dorsal–ventral patterning is established across the horizontal axis of the marginal zone and that the non-organizer marginal zone is specified as ventral mesoderm in late blastula stage embryos. As no subsequent authors questioned these basic tenets (Dale and Jones, 1999; DeRobertis and Sasai, 1996; Graff, 1997; Harland and Gerhart, 1997; Heasman, 1997; Kimelman et al., 1992; Moon and Kimelman, 1998), we include all of the revised models under the umbrella name of the “three-signal hypothesis” throughout this paper. It is our premise that these central tenets of the three-signal model are incorrect and lead to numerous erroneous assumptions about the amphibian embryo.

The new *Xenopus* fate map paints a different picture of the marginal zone and suggests a very different interpretation of the organizer grafting experiment. The new map reveals that the organizer graft is moved from the region of rostral, dorsal mesoderm to caudal, dorsal mesoderm, and thus may cause caudal-to-rostral fate transformations of dorsal mesoderm rather than ventral-to-dorsal mesoderm transformations. In this report, we re-examine the role of the organizer in the caudal-to-rostral fate transformations of dorsal mesoderm and interpret this as evidence for precocious differentiation of caudal somites as axes formed by organizer grafts in the marginal zone. This report, we re-examine the role of the organizer in the caudal-to-rostral fate transformations of dorsal mesoderm rather than ventral-to-dorsal mesoderm transformations. In this report, we re-examine the role of the organizer in the “induction” of secondary axes and show that the secondary axes formed by organizer grafts in the marginal zone represent precocious differentiation of caudal somites as rostral somites. This demonstrates that the fate change is from caudal to rostral, not ventral to dorsal. We also examine the role of BMP antagonists secreted by the organizer, since an extensive body of experimental evidence concludes that BMP antagonists from the organizer “dorsalize” mesoderm (reviewed in Harland and Gerhart, 1997). The BMP antagonist noggin behaves like an organizer graft, stimulating precocious differentiation of caudal tissue. We further show that BMP antagonists provoke secondary axis formation by mediating precocious mediolateral intercalation behavior. Several lines of experimentation reveal that the marginal zone is prepatterned into animal and vegetal morphogenetic domains, which correspond to the prospective dorsal and ventral mesoderm fields, respectively. When considered in combination with the revised mapping data, these results alter the modern view of amphibian and vertebrate axial patterning. They challenge investigators to rethink all experiments and interpretations based on the old fate maps, and to devise a new working model of vertebrate axial patterning.

**Materials and methods**

**Embryo culture, injections, and fate mapping**

Eggs were fertilized and regularly cleaving embryos were injected at stage 6 with 35 ng mini-ruby dextran as described previously (Lane and Sheets, 2002a,b). All four C-tier or B-tier blastomeres were injected from the same clutch of eggs, such that at least three replicates of all four blastomeres were generated for each experimental trial. Data were collected from at least three different mothers for each tier. The injected embryos were cultured to stages 32–34, when they were fixed for in situ hybridization, probed for globin (blue, using BCIP only for the colorimetric reaction), stained for mini-ruby with streptavidin horseradish peroxidase (black-brown, using diaminobenzidine and NiCl₂ for the colorimetric reaction), and finally immunostained for the somite marker 12–101 (orange-brown, detected by diaminobenzidine) as described in Lane and Sheets, 2002a. The rostralmost, ruby-labeled somite was scored in embryos cleared in BB: BA and observed in a compound microscope using 10x or 20x objectives. At stage 32, the first head somite has already disintegrated and it is ignored in the somite scoring. To determine the rostralmost contribution to the primitive blood field, the cleared embryos were positioned with their blood islands clearly visible and photographed. The distance from the rostral tip of the globin-expressing field to the rostralmost labeled cell within the field was measured on the photographs.

To demonstrate the entry of C4 and B4 progeny into the somites, regularly cleaving stage 6 embryos were injected into either blastomere with 250 pg Lac Z mRNA. The embryos were cultured and collected at various stages. Fixed embryos were stained for Lac Z activity as described (Lane and Sheets, 2002a) and specimens younger than stage 18 were probed for Xbra by in situ hybridization and those older than stage 18 were stained for 12–101 to reveal somites. Embryos were positioned for photography using a nitex screen.

**Spemann’s graft**

Approximately 30 regularly cleaving stage 6 embryos were selected, and 10 each were injected with 35 ng of mini-ruby in blastomere C4 (hosts), or 35 ng AF 488 into blastomere C1 (Spemann donors) or into C4 (negative control donors). The embryos were cultured to stage 10 (when the upper blastoporal lip forms), when the clone of fluorescent cells was removed from either type of donor. Spemann and Mangold (1924) took grafts from the region described as “at some distance above the upper blastoporal lip”. As the lip crossed the vegetal region of the clone, we used only the animal half of the labeled clone as the graft. We removed the animal portion of the ruby-labeled C4 clone in the host, and replaced it with the AF488-labeled donor tissue. A small fragment of coverslip glass was placed over the region to facilitate healing and removed after 20–30 min. The embryos were cultured until stages 32–34 when they were fixed and photographed. Several specimens were embedded in paraffin and sectioned at 12 μm.

**Noggin misexpression**

One C4 or B4 blastomere was injected with 1 nl containing 35 ng depc-treated mini-ruby dextran ± 180 pg of capped noggin mRNA, synthesized from plasmid pNOGGIN-A3 (gift of R. Harland and W. C. Smith). The embryos were cultured to stages 33–34, fixed in MEMFA,
stained for mini-ruby followed by immunostaining for 12–101 as described above. The embryos were mounted in BB: BA and the rostralmost somite in both the endogenous and induced axes scored.

**Explant preparation and filming**

Stage 6 regularly cleaving embryos were injected with 35 ng AF488 into one C1 blastomere, 35 ng mini-ruby dextran into one B4 blastomere, and 200 pg of GFP mRNA ± 180 pg noggin mRNA into the ipsilateral C4 blastomere. The embryos were cultured to stage 10.5, and dissected to give matching rostral and caudal 180 ° marginal zone explants, from which the crawling leading edge mesoderm was removed with a hair knife. Matched explant sets were placed deep surface down (toward the objective) on fibronectin-coated coverslips (Davidson et al., 2002), in an acrylic chamber containing DFA + BSA (Sater et al., 1993).

Multi-position, multi-wavelength time-lapse sequences were collected using an inverted compound microscope (Olympus IX70) with an X-Y stage positioner (Prior) and excitation filterwheel (Sutter Instruments) controlled by image acquisition software (Metamorph, Universal Imaging Corp.) running on a computer (Dell). Once movie sequences were collected, green and red emission channels were merged using image-processing software (ImageJ; Wayne Rasband, Research Services Branch, National Institutes of Mental Health).

**Results**

A rostral-to-caudal topographic projection runs from column 1 to column 4 blastomeres

The marginal zone forms mesoderm by definition in amphibians, and the bulk of the marginal zone forms from the B- and C-tier blastomeres of stage 6 embryos. We previously examined the dorsal–ventral topographic projection of B- and C-tier blastomeres to the tadpole by fate mapping the origins of somites (dorsal mesoderm) and primitive blood (ventralmost mesoderm). Here, we determine simultaneously the rostral-to-caudal and dorsal-to-ventral topographic projections of the marginal zone by lineage tracing from stage 6 to stages 32–34, and scoring the rostralmost contributions to somites and primitive blood.

The summary from three trials mapping both tiers is shown in Table 1 and representative examples are shown in Fig. 2. We consider primitive blood first. We measured the distance between the rostral tip of the globin-expressing blood islands and the rostralmost ruby-labeled cell in the blood islands. C1 forms the rostral tip of the ventral blood islands; C2’s mean rostralmost contribution is at 23 μm, C3’s at 265 μm, and C4’s at 485 μm caudal to the rostral tip. The B-tier did not contribute to the ventral blood islands. We next consider the somites. For the C-tier, the mean rostralmost somite labeled by C1 is somite 1.3, by C2 is somite 2.3, by C3 is somite 5.7 and by C4 is somite 8.3. For the B-tier, the mean rostralmost somite labeled by B1 is somite 5.0, by B2 is somite 4.9, by B3 is somite 7.2 and by B4 is somite 14.9. Thus, there is a distinct rostral-to-caudal topographic projection for both the B- and C-tiers running from columns 1 to 4, for the dorsal mesoderm. This is also true for the ventral mesoderm.

The B-tier contributes to dorsal mesoderm, while the C-tier contributes to both dorsal and ventral mesoderm. We therefore orient the dorsal–ventral axis in the animal-to-vegetal direction. By demonstrating that there is no dorsal-to-ventral topographic projection running from Spemann’s organizer to the meridian of sperm entry, but that there is a rostral-to-caudal projection, we strengthen our earlier arguments for the revision of the pre-gastrula embryonic axes as shown in Fig. 1B (Lane and Sheets, 2000; Lane and Smith, 1999).

**Cells from the caudal (formerly ventral) marginal zone populate trunk and tail somites**

Blastomeres C4 and B4 form what has traditionally been called the “VMZ” and have long been considered the source of blood (i.e., ventral mesoderm). Lane and Smith (1999) demonstrated that these blastomeres contribute to caudal somites as well as blood (also see Fig. 2). To demonstrate the temporal and spatial progression by which C4 and B4 progeny enter the somites, we injected nuclear Lac Z mRNA into a C4 or B4 blastomere at stage 6 and grew the embryos to gastrula through tadpole stages. The embryos were stained for Lac Z activity (detected as red nuclei) to locate the progeny, followed by either in situ hybridization for Xbra (for stages 10.5 through 18), or directly immunostaining muscle with 12–101 (which first reveals somites at stage 17). Representative stages are shown in Fig. 3.

At stage 10.5, a vegetal view shows the clones of C4 progeny are situated in the marginal zone opposite Spe mann’s organizer and the prospective notochord field (Fig. 3A). In a lateral view, these C4 progeny lie within the Xbra expression domain, as well as both vegetal and animal of the
domain (Fig. 3B). At stage 20 (Fig. 3C), no cells from the clone yet contribute to the head somites or the segmental plate, but they lie lateral to the forming posterior notochord and segmental plate. By stage 26 (Fig. 3D), some C4 progeny have entered the paraxial mesoderm and label is seen in at least somite 8 in this embryo. By stage 34 (Fig. 3E), many C4 progeny populate trunk/tail somites, with somite 8 the rostralmost labeled somite in this embryo. C4 progeny are also present in ventral mesodermal structures (Fig. 2), and we know from previous mapping that this includes the caudal blood islands (Lane and Sheets, 2002a; Lane and Smith, 1999).

At stage 10.5, B4 clones lie opposite Spemann’s organizer, but are animal of the Xbra expression domain (Fig. 3F). By stage 12, B4 progeny are near the closing blastopore and many B4 progeny now lie within the Xbra expression domain opposite the forming notochord (Figs. 3G, H). At stage 20 (Fig. 3I), few if any B4-derived cells contribute to the somites, although numerous B4 progeny occupy the circumblastoporal collar (Fig. 3J), which is the source of the tail somites. By stage 34 (Fig. 3K), many B4 progeny populate trunk/tail somites, with somite 15 the rostralmost labeled somite in this embryo. Some B4 progeny remain undifferentiated in the remnant of the circumblastoporal collar around the proctodeum (rec in Fig. 3K); these cells continue to serve as a reservoir for tail somites yet to form. Comparing the spatial and temporal sequence by which C4 and B4 progeny differentiate as muscle, we see that both blastomeres contribute to the somites, but B4 progeny initiate expression of Xbra later than C4 progeny, and they first differentiate as somites at more caudal levels of the body plan.

Tracing the progeny of blastomeres B4 and C4 from the marginal zone into the forming somites reveals several important aspects of amphibian development. It demonstrates that the rostral-to-caudal progression of development known from chick and mouse development should be taken into account when interpreting both descriptive and experimental data in amphibians. In addition, tracing the cells from the marginal zone into axial structures reveals that the Xbra expression domain is more dynamic than previously published. Prospective paraxial mesoderm cells in the marginal zone expressing Xbra at the early gastrula stage turn this gene off during involution (Smith et al., 1991). Our data show that other cells, situated more animaly in the marginal zone in early gastrula, turn on Xbra as they radially intercalate into the circumblastoporal collar in mid-gastrula stages. This result indicates that the Xbra expression ring seen at stages 9 and 10 reveals only a subset of the prospective posterior, dorsal mesoderm. This confirms that the Xbra ring should not be thought to indicate all of the mesoderm, and further demonstrates that it does not indicate all of the prospective posterior, dorsal mesoderm.

These observations demonstrate unequivocally that the region opposite Spemann’s organizer that historically is called the ventral marginal zone is the caudal marginal zone—the source of posterior mesoderm, both dorsal and ventral. We will refer to this region as the caudal marginal zone (CMZ) throughout this report.

A re-examination of the grafting experiment of Spemann and Mangold

This new understanding of the CMZ leads us to reconsider the grafting experiment of Spemann and Mangold (1924). Spemann and Mangold believed that cells at the host site that received the graft were indifferent. The three-signal model (and all other current models of amphibian axial patterning) posits that cells at the site that receives the graft are both specified and fated to form blood. The revised fate map...
indicates that the region is the caudal primitive streak and the source of caudal somites, and molecular data indicates this region expresses Xbra and XmyoD at stage 10, two markers of posterior, dorsal mesoderm, before contact with the organizer. The true nature of the site has implications for the interpretation of the organizer grafting experiment and hence for vertebrate axial patterning.

We performed the organizer grafting experiment in *Xenopus* with several modifications designed to reveal more about the embryo. First, we labeled the donor and the host site separately to assess both. Second, we performed the control homotypic graft of the caudal marginal zone to determine the normal fate of the host caudal marginal zone and rule out the effects of surgery itself. Finally, we cultured embryos to older stages to allow differentiation of caudal tissues. Previous investigators (Smith and Slack, 1983; Spemann and Mangold, 1924) cultured to relatively young stages when caudal tissues remain undifferentiated and could erroneously be designated as blood.

We consider the homotypic control graft first. We labeled a C4 blastomere of a stage 6 donor embryo with AF 488, a green fluorescent dextran, and we labeled one C4 blastomere of a stage 6 donor embryo with AF 488, a green fluorescent dextran, and we labeled one C4 blastomere with β-galactosidase activity (red) to occupy the caudal marginal zone opposite Spemann’s organizer (white arrows). (B) Lateral view of two stage 10.5 embryos, positioned within nitex cages. C4 progeny at stage 10.5 lie in the marginal zone opposite the organizer (white arrows). (C) Dorsal view of stage 20 embryo. C4 progeny lie lateral of the posterior somites, forming somites (arrows), and not within the forming somites. (D) Dorsal view of stage 26 embryo. C4 progeny populate posterior somites (arrow) formed since stage 20. In this specimen, somite 8 is the rostralmost labeled somite. (E) Lateral view of stage 34 embryo. Many C4-derived cells (arrow) populate posterior somites. The rostralmost labeled somite is somite 8 in this embryo. (F) Caudal (upper panel) and lateral (lower panel) views of two stage 10.5 embryos, positioned within nitex cages. B4-labeled cells lie animal to the Xbra expression ring in the marginal zone (black arrows). (G) Vegetal view of stage 12 embryo, looking at the circumblastoporal collar and forming notochord (up). B4-derived cells express Xbra in the circumblastoporal collar (arrow). (H) Cross-section through the closing blastopore in G. Lac Z staining lies within the deep mesodermal layer of the collar (arrow). The forming notochord (N) and archenteron (ar) are indicated. (I, J) Dorsal view of stage 20 embryo and optical section through the circumblastoporal collar (arrow). The B4 clone has not contributed to somites at stage 20, but red nuclei are present in the circumblastoporal collar, shown in an optical cross-section. (K) By stage 34, B4 progeny populate the posterior somites (arrow). The rostralmost contribution of B4 progeny is in somite 13 of this embryo, much further posterior than the rostralmost contribution of C4 progeny (compare K to E).
mere in a stage 6 host embryo with mini-ruby, a red fluorescent dextran (diagrammed in Fig. 4A). At stage 10, a graft from the animal region of the labeled clone in the donor was removed and placed into the animal region of the red clone of the host. (By targeting the animal region of the labeled clones, we followed Mangold’s description of her experiment; see Spemann and Mangold, 1924). At stage 30, the host had both red and green trunk somites (Figs. 4B–F). Thus, the fate of the homotypic graft matches the fate predicted by the revised fate map, indicating that no change

Fig. 4. A graft from the Spemann organizer results in precocious differentiation of caudal dorsal mesoderm, not a conversion of ventral to dorsal mesoderm. (A) Schematic diagram of the experiment. (B–F) A control graft from a C4-derived animal marginal zone (green dextran) into a C4 animal region caudal marginal zone (red dextran). (B) The host site differentiates as caudal somites and caudal ventrolateral mesoderm, and in some specimens as neural crest invading the branchial arches. (C) A C4 animal region graft differentiates primarily as somitic mesoderm. Surgery has not altered the fate of the host or the graft. (G–K) A C1-derived Spemann graft (green dextran) placed into the animal region of a C4-clone (red dextran) induces a second axis. The donor forms primarily notochord (H), while the surrounding host cells differentiate as somites at rostral levels of the secondary axis (I, J, K). Thus, the organizer stimulated precocious differentiation of caudal somitic mesoderm as rostral somitic mesoderm, rather than the conventional view that blood was converted to muscle. Abbreviations: 1°, primary axis; 2°, secondary axis; N, notochord; S, somite.
in either dorsal–ventral nor rostral–caudal fate has occurred simply as a result of surgery.

To perform the experimental graft that recapitulates the Spemann and Mangold graft, we labeled a C1 blastomere in a donor embryo with green fluorescent dextran and a C4 blastomere in a host embryo with red fluorescent dextran (Fig. 4A). At stage 10, a clone of green cells from above the upper lip was grafted into the animal region of the red clone of the host, mimicking the graft described by Spemann and Mangold (1924). We observed a second partial axis (Figs. 4G–K), which was separated from the primary axis at its rostral end, but lacked head structures such as the eye and cement gland. The second axis shared a common tail with the host axis, and the tail was always defective and misshapen. In the primary axis, red label was confined to the posterior of the embryo, including the disorganized tail somites. In the induced secondary axis, the notochord and a few somite cells were green (donor-derived, Fig. 4J), while the bulk of the rostral somites were red, indicating they were host-derived from the immediate vicinity of the implant.

We interpret the results as follows. In both control homotypic grafts and organizer grafts, the caudal somites are always both red and green. However, in control grafts the tail is normal in structure, while in Spemann organizer grafts, the tail shared by the two axes is always deficient with abnormal somite morphology. By tracing the lineage of the host site, we know that cells that should contribute to tail somites form trunk somites in response to the organizer. This demonstrates that there has been no change in dorsal–ventral fate of cells at the host site, as the experiment is interpreted in modern times. The grafted organizer stimulates cells normally fated to form caudal somites to differentiate precociously and form somites at a more rostral level of the body plan. It evokes a rostral duplication of trunk structures, but without a second head.

A BMP antagonist mimics the organizer graft by altering rostral–caudal fate and not dorsal–ventral fate

Spemann’s organizer secretes antagonists that pattern the embryo. Several lines of evidence suggest that one class of secreted protein, the BMP antagonists (e.g., noggin and chordin), evokes the partial secondary axis observed by Spemann and Mangold (1924). We reasoned that interpretations of these data based on the three-signal hypothesis are correct, a BMP antagonist (e.g., noggin) will “dorsalize” mesoderm, and ectopic expression of noggin in the C4 blastomere should convert all C4 mesodermal progeny into dorsal mesoderm. To test this, we injected 180 pg noggin mRNA with the lineage tracer mini-ruby dextran into one C4 blastomere at stage 6 and cultured the embryos to stages 32–34. (This concentration of noggin mRNA gave 95% second axes and 5% head-only, with no trunk or tail, in three trials.) The embryos were stained for mini-ruby distribution and somites, and the rostralmost-labeled somite in both the primary and secondary axes scored. Controls injected with lineage tracer only into C4 have a single axis with all label confined to the posterior of the embryo; the mean rostralmost labeled somite is somite 10.1 (Fig. 5A; Table 2, Trial 1). In C4 noggin-injected embryos, there are two axes (Figs. 5B, C and Table 2, Trial 1). The mean rostralmost labeled somite is somite 9.7 in the primary axis (so this axis is nearly unaffected), and somite 1 in the secondary axis (Fig. 5C). At the rostral end of the secondary axis, there is a stream of labeled mesoderm into the ventral region of the secondary axis in all cases (Fig. 5C). Thus, expression of noggin by C4 progeny results in a distribution of labeled cells that resembles C1 progeny, except there are no secondary head structures and therefore no contributions to head structures, but there is always both dorsal and ventral mesodermal label. This distribution of label in C4-noggin injected embryos contrasts with the results for a second control, which is injection of noggin mRNA plus mini-ruby into the B4 blastomere. B4 normally forms dorsal but not ventral mesoderm (Table 1). Over-expression of noggin by B4 results in a second axis, but label is restricted to dorsal structures only and no stream of labeled ventral mesoderm is observed (Fig. 5D). However, label is distributed throughout the rostral–caudal axis of the secondary axis, and in the caudal region only of the primary axis, indicating that B4 progeny expressing a BMP antagonist now behave like B1 progeny in a normal embryo.

These results demonstrate that the dorsal–ventral fates of both C4 and B4 progeny are not altered by the BMP antagonist noggin, but the rostral–caudal fates of both blastomeres are affected. This experiment also provides evidence for a dorsal–ventral prepattern within the C4 blastomere—some cells in the C4 clone “realize” they are dorsal and some that they are ventral, and expression of a BMP antagonist does not alter this information.

BMP antagonism evokes a secondary axis by stimulating precocious mediolateral intercalation behavior

During construction of the primary embryonic axis in Xenopus, a subpopulation of cells known as the vegetal alignment zone forms across the rostral notochordal and somitic domains in the region of Spemann’s organizer during stage 10.5 (Lane and Keller, 1997). This group of cells is the first to undertake mediolateral intercalation behavior, and through cell–cell contact, this behavior spreads progressively into the caudal (posterior) notochordal and somitic domains (Domingo and Keller, 1995; Shih and
Keller, 1992). One result of mediolateral intercalation behavior in the embryo is convergent extension of the primary embryonic axis. A second result is extensive mixing of the cells derived from the animal region of the organizer with the cells derived from the rest of the animal marginal zone of the early gastrula embryo. Morphogenesis of the prospective rostral notochord and somites is studied in explant culture of the RMZ (formerly called DMZ, or Keller openface explants), which contains the animal region of Spemann’s organizer and nearby neighbors, and undergoes autonomous convergent extension (Keller and Danilchik, 1988; Shih and Keller, 1992).

In contrast to the RMZ, explants of the CMZ (formerly VMZ) do not undergo elongation and do not make notochordal nor somitic mesoderm (Keller and Danilchik, 1988) when explanted before mid-gastrula stages. Ectopic expression of BMP antagonists or the dominant-negative BMP receptor, or treatment with exogenous noggin protein, induces caudal marginal zone explants to form muscle (Graff et al., 1994; Maeno et al., 1994a,b; Smith and Harland, 1992) and undergo elongation proposed to be convergent extension.

We determined whether a BMP antagonist initiates mediolateral intercalation behavior and recruitment of cells into axial structures by filming cell behavior in CMZ’s misexpressing noggin mRNA. Although chordin is believed to be the critical BMP antagonist secreted by the organizer (Oelgeschläger et al., 2003), we tested noggin mRNA because it gave more consistent results than chordin mRNA. We co-injected GFP mRNA with 180 pg noggin mRNA in one C4 blastomere, and mini-ruby dextran into one B4 blastomere (diagrammed in Figs. 6A–C). Monitoring C4 behavior allows us to determine the autonomous effects of noggin expression while monitoring B4 behavior allows us to see whether neighboring cells are recruited into axial structures by noggin-expressing C4 cells. We cut 1808 caudal marginal zone explants and filmed cell behavior from stage 10.5 to approximately stage 28. The explants were fixed at the conclusion of filming, and triple stained for somites, notochord and mini-ruby dextran.

In RMZ explants, which express BMP antagonists autonomously, C1 AF488-labeled cells are randomly oriented at the start of filming (Fig. 6D and Movie 1 in Supplementary Material). Mediolateral intercalation begins within 1 h, and by 4-1/2 h, cells are mediolaterally elongated (yellow circle in Fig. 6E). Intercalation behavior spreads through the explant, from vegetal to animal, and the notochord/somite boundary forms (Fig. 6F), all as described previously (Domingo and Keller, 1995; Shih and Keller, 1992).

Table 2
Mean rostralmost labeled somites in 1° and noggin-induced 2° axes

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>N</th>
<th>Mean rostralmost labeled somite in 1° axis</th>
<th>Induced axis present</th>
<th>Mean rostralmost labeled somite in 2° axis</th>
</tr>
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<tbody>
<tr>
<td>C4 ruby</td>
<td>7</td>
<td>10.1 ± 2.0</td>
<td>0</td>
<td>N.A.</td>
</tr>
<tr>
<td>C4 ruby + nog</td>
<td>7</td>
<td>9.7 ± 2.2</td>
<td>7</td>
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<th>N</th>
<th>Mean rostralmost labeled somite in 1° axis</th>
<th>Induced axis present</th>
<th>Mean rostralmost labeled somite in 2° axis</th>
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<td>C4 ruby + nog</td>
<td>5</td>
<td>9.5 ± 1.8</td>
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Fig. 5. Ectopic BMP antagonism alters the rostral–caudal fate of mesoderm, not the dorsal–ventral fate. (A) C4-derived, ruby-labeled progeny occupy both dorsal and ventral mesodermal tissues in the posterior half of the embryo. (B) Noggin-expressing C4 progeny occupy rostral and caudal regions of the secondary axis, and comprise both dorsal and ventral mesodermal tissue. (C) A higher magnification view of the rostral end of the secondary axis, showing that all of the somites are labeled. (D) In contrast to C4 noggin expression, B4 noggin-expressing cells populate only dorsal mesodermal tissues, but both rostral and caudal structures are labeled. B4 normally populates the posterior half of the embryo. Abbreviations: 1°, primary axis; 2°, secondary axis; D, dorsal; S, somite; V, ventral.
In control CMZ explants expressing GFP but not noggin, no mediolateral intercalation was apparent at stage 10.5 when the explant was cut (Fig. 6G and Movie 2 in Supplementary Material), and little or none commenced during the 20-h filming period (green cells, Figs. 6H, I; 1/7 explants positive for MIB in Table 3). Progeny of B4 did not undertake mediolateral intercalation behavior (red cells, Figs. 6H, I; Table 3).

When noggin is expressed in the CMZ, mediolateral intercalation behavior (MIB) is underway when filming begins (stage 10.5, see elongated, bipolar cells in Fig. 6J and Movie 3 in Supplementary Material). MIB commences near the vegetal end of the explant (white circle) and progresses animalward and laterally within the GFP-expressing zone (arrowheads in Figs. 6K, L; 9/11 explants in Table 3). By 5 h (Fig. 6L), ruby-labeled progeny from B4 are recruited to undertake MIB (asterisk, 4/11, Table 3). The observed intercalation pattern is the same as that seen in control rostral explants in which C1 progeny were labeled with the green fluorescent dextran AF 488 (Figs. 6D–F), and the same pattern reported by Keller et al. We conclude that expression of a BMP antagonist in the caudal marginal zone evokes a duplication of rostral trunk structures because the BMP antagonist permits animal marginal zone cells to initiate MIB prematurely. Noggin-expressing cells subsequently recruit their neighbors to undertake mediolateral intercalation and form axial structures.

In the CMZ explants, GFP-expressing cells are found at the vegetal edge of the explant (i.e., the green cells marked by double asterisks in Figs. 6G–I, J–L). These cells crawl “vegetally” onto the fibronectin substratum, away from the mass of the explant, during filming. These crawling cells are leading edge mesoderm (LEM, i.e., the lateral plate/true ventral mesoderm), and their behavior in explant culture was documented previously (Davidson et al., 2002). In normal development, these cells do not crawl vegetally, but crawl toward the animal pole and form lateral plate derivatives. Those shown in Figs. 6J–L correspond to the ventral stream of migrating cells seen in Fig. 5B when noggin mRNA and mini-ruby dextran were injected into blastomere C4. Thus, noggin expression in the CMZ does not alter normal morphogenetic behaviors of the LEM cells. They do not undertake MIB but continue crawling behavior indicative of ventral-type morphogenesis, although they may be undertaking this behavior sooner than normal caudal LEM cells.

This experiment provides two types of evidence of prepatterned behavior within the CMZ. First, we observe that the CMZ is prepatterned to respond to BMP antagonists emitted by the organizer, with only the animal marginal zone initiating mediolateral intercalation, while the vegetal marginal zone continues crawling behavior. Second, we observe that noggin expression does not promote simultaneous mediolateral intercalation behavior throughout the animal C4 progeny (i.e., all of the green cells do not initiate mediolateral intercalation behavior and adopt elongate morphology at the same time). Instead, mediolateral intercalation behavior commences within the animal marginal zone near the animal/vegetal boundary and spreads animaly and laterally through the animal marginal zone by cell–cell interaction, as it does from the organizer.

The explants were immunostained for muscle (with antibody 12–101, in brown, Fig. 7) and notochord (with antibody Tor 70, in blue). We detected the B4 progeny by staining for mini-ruby (in black). All of the noggin-expressing caudal explants (n = 7) formed muscle, and 6 of 7 formed a central notochord flanked by 2 blocks of muscle (Fig. 7A, Table 3). B4 progeny are recruited into both muscle and notochord. Caudal marginal zones expressing only GFP did not form muscle nor notochord (n = 3; Fig. 7B, Table 3), while control explants from the RMZ formed both tissues in 100% of the explants (n = 6; Fig. 7C; Table 3). Thus, in the explants, ectopic noggin expression leads to mediolateral intercalation of cells in the CMZs at the same time as mediolateral intercalation occurs in rostral marginal zones, and in a similar spatial sequence. Mediolateral intercalation was prerequisite in these explants for the formation of somites and ectopic notochord.

**Discussion**

The dorsal–ventral axis of the mesoderm in amphibians is portrayed historically as the horizontal axis running from Spemann’s organizer to the meridian of sperm entry on the opposite side of the embryo. The mapping data presented in Table 1 demonstrates that this horizontal axis in pre-gastrula embryos corresponds to the rostral–caudal axis of the tadpole. C1 progeny contribute to the most rostral tissues while C4 contributes to caudal tissues. The data also reveal that the animal/vegetal axis of the pre-gastrula marginal zone corresponds to the dorsal–ventral axis of the mesoderm. B-tier-derived mesoderm is almost exclusively dorsal, while C-tier-derived mesoderm is both dorsal and ventral. Thus, the dorsal–ventral axis within the mesoderm is rotated 90° from its conventional designation, and the upper blastoporal lip comprises rostral mesoderm (both dorsal and ventral) while the lower blastoporal lip comprises caudal mesoderm, again both dorsal and ventral. These findings are supported by a careful re-examination of the data underlying several fate maps for amphibians including *Xenopus* (Lane and Sheets, 2002b) and warrants reassignment of the embryonic axes as shown in Fig. 1B.

**Cells in the caudal marginal zone are neither naive nor specified as blood**

The new fate map raises questions about the differentiation status of cells in the non-organizer marginal zone. Spemann believed that the upper lip of the blastopore was the first region of the amphibian embryo to become
determined (Spemann and Mangold, 1924), and he believed organizer grafts instructed naive tissue at the host site to differentiate as somites and neural tissue. The modern interpretation of the organizer grafting experiment (i.e., the three-signal hypothesis of Smith and Slack, 1983, and all subsequent models based on the hypothesis) posits that at late blastula/early gastrula stages, the non-organizer marginal zone is specified as blood/ventral mesoderm, while the organizer sector of the marginal zone is specified as dorsal mesoderm. The organizer releases diffusible signals that “dorsalize” cells specified to form blood, instructing them to form muscle. Thus, the prevailing view is that the organizer sends instructive signals to surrounding tissues to generate somitic mesoderm.

The new fate map and molecular expression patterns indicate that the marginal zone is spatially patterned into two circumferential rings. The lower or vegetal ring lies deep to the constricting bottle cells (i.e., toward the interior of the embryo) and is the site where the blastopore lip forms, while the upper or animal ring lies several cells diameters animal of the forming lip. The vegetal ring expresses menf (Kumano and Smith, 2002), while the animal ring expresses Xbra (Zoltewicz and Gerhart, 1997). In addition, the animal marginal zone excluding the organizer also expresses the myogenic determinant MyoD (Frank and Harland, 1992; Harvey, 1992). The two rings form separate morphogenetic domains within the mesoderm during normal development: the vegetal ring exhibits

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**A**

B3 B4 B4 B3

C3 C4 C4 C3

**B**

180° CMZ

B4 C4

**C**

180° RMZ

C1

**D**

C1 AF488

an 0:00

vg

**E**

4:30

**F**

12:30

N

**G**

C4 GFP

B4 ruby

an 0.00

**H**

1:30

**I**

9:00

**J**

an 0.00

**K**

1:40

**L**

5:00

C4 nog GFP

B4 ruby

**M**

**N**

**O**

**P**

**Q**

**R**

**S**

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crawling behavior while the animal ring undertakes mediolateral intercalation. The vegetal ring is called leading edge mesoderm and ultimately forms lateral plate derivatives such as blood and heart, as well as head mesoderm. The animal ring ultimately forms notochord and somitic mesoderm. These morphogenetic domains will be discussed below.

When the CMZ (formerly VMZ) receives an organizer graft, the cells at the implant site are not naive, nor are they specified as blood. The vegetal cells express menf, while animal cells express MyoD and Xbra, two markers of posterior, dorsal mesoderm. Isolated caudal marginal zones do not form blood nor muscle autonomously. They form blood only when explanted with animal pole tissue, which emits blood-inducing signals (Maeno et al., 1994a,b). Indeed, blood markers are expressed only when leading edge mesoderm migrates away from the vegetal marginal zone and toward the animal cap (e.g., GATA2, Bertwistle et al., 1996). The blood widely reported in cultured “VMZs” likely results from cutting very large explants that encompass both the caudal marginal zone and this source of blood-inducing signals in the animal cap. Despite expressing MyoD, isolated CMZs do not form muscle autonomously. They are suppressed by BMPs and require derepression to differentiate as muscle. Thus, the CMZ consists of two domains, one dorsal and one ventral, neither of which realizes its potential without additional signals from elsewhere in the embryo. The dorsal domain gets additional signals from the organizer to continue through the muscle differentiation pathway, and the ventral domain gets additional signals from the animal pole to continue through the blood differentiation pathway. The FGF receptor pathway is implicated in distinguishing between the dorsal (animal) and ventral (vegetal) domains of the marginal zone (Kumano and Smith, 2000).

Proposed activity gradients do not conform to the revised fate map

With the advent of molecular techniques, investigators proposed the following molecular model of mesodermal patterning (Dale and Jones, 1999; DeRobertis et al., 2000). The organizer marginal zone is high in BMP antagonists (and therefore low in BMP activity), while BMP activity is highest on the opposite side. A gradient of BMP activity arises across the horizontal aspect of the marginal zone that determines dorsal–ventral mesodermal fates, with low activity generating dorsal tissue and high activity determining ventral tissue. This model affirms the belief that the organizer is the dorsal marginal zone and the opposite side is the ventral marginal zone.

Other pathways are proposed to control dorsal–ventral mesodermal patterning across the same horizontal axis of the embryo. Various investigators propose that activin-like, nodal or β-catenin gradients across the horizontal aspect of the marginal zone determine dorsal–ventral mesodermal fates (Gurdon and Bourillot, 2001; Moon and Kiemelman, 1998). The conclusions from all of these studies rely heavily on the underlying premise of the three-signal hypothesis that dorsal tissues arise from the region of Spemann’s organizer and ventral tissues arise from the opposite side of the embryo.
The revised Xenopus fate map challenges these molecular models of axial patterning (Lane and Sheets, 2000; reviewed in Lane and Sheets, 2002b). The revised fate map demonstrates that dorsal and ventral mesoderm arise from all 45° sectors of the marginal zone, and not solely from the regions of the upper and lower blastoporal lips respectively. The fact that every 45° sector of the pre-gastrula marginal zone yields dorsal and ventral mesoderm argues against gradient models where high BMP antagonist activity or high TGF-β/nodal activity in the organizer region determines dorsal fates and low activities determine ventral fates. The region experiencing low BMP activity and high nodal activity in early gastrula forms both rostral, dorsal and rostral, ventral mesoderm, which is why we call it the rostral marginal zone. We propose that the reported spatial and/or temporal activity gradients of BMP/Smad1, TGFβ/Smad2, and β-catenin running from Spemann’s organizer to the meridian of sperm entry (Faure et al., 2000; Lee et al., 2001; Schohl and Fagotto, 2002), affect how much tissue is devoted to head/rostral structures (both dorsal and ventral), versus trunk and tail/caudal structures (again, both dorsal and ventral), and are not indicators of dorsal–ventral signaling. Signaling pathways determining dorsal–ventral fates within the mesoderm should show temporal or spatial activity differentials across the animal/vegetal axis of the marginal zone. (As an example, see the summary diagrams for P-MAPK signaling at stages 8.5 to 10 of Schohl and Fagotto (2002). P-MAPK signaling is high in the region that corresponds to the animal circumferential ring and low in the region corresponding to the vegetal circumferential ring. As Schohl and Fagotto interpret all of their data in the light of the old fate maps and the three-signal model, they do not connect their observations on P-MAPK signals with a new model of dorsal–ventral mesoderm determination. Kumano and Smith (2000) propose differential FGFR signaling determines dorsal–ventral mesoderm patterning across the animal/vegetal aspect of the marginal zone. Their model is supported by the P-MAPK pattern observed by Schohl and Fagotto). Likewise, the earliest signaling pathways determining dorsal–ventral fates within the endoderm should show differential activity across the animal/vegetal axis of the endoderm.

Two circumferential morphogenetic domains, one vegetal and one animal, correspond to the true ventral and dorsal marginal zones

To understand the induction of incomplete secondary axes resulting from organizer grafting experiments, we must first understand normal development. With an improved fate map and information on morphogenetic movements during gastrulation and neurulation, we understand the frog embryo better than preceding generations of investigators. Furthermore, we have molecular information that reveals details about the status of different cell populations in early development. This information reveals a gastrula stage frog embryo that, while morphologically fairly simple, internally is highly complex and patterned, with a variety of expression domains. Some of these expression domains ultimately lead to the adoption of different cellular behaviors, which leads to different morphogenetic activities by groups of cells. We propose that the upper lip “organizes” construction of the amphibian body plan by controlling the temporal sequence in which some domains exhibit their specific morphogenetic activities.

For this discussion of mesoderm morphogenesis, we divide the late blastula/early gastrula marginal zone into two circumferential rings, one vegetal and one animal (Figs. 8A, B). The vegetal ring primarily forms ventral mesoderm and is the true “ventral marginal zone”, while the animal ring primarily forms dorsal mesoderm and is thus the true “dorsal marginal zone”. The two rings are further subdivided into four regions as follows (Fig. 8A): the animal region of Spemann’s organizer is RD, the rostral, dorsal marginal zone; the vegetal region of Spemann’s organizer is RV, the rostral, ventral marginal zone; the animal region of the non-organizer marginal zone is CD, the caudal, dorsal marginal zone; and the vegetal region of the non-organizer marginal zone is CV, the caudal, ventral marginal zone.

These divisions are based on morphogenetic behaviors observed during normal development and molecular expression patterns. Cells in both the RV and CV regions of the vegetal marginal zone (orange) express menf (Kumano and Smith, 2002) and crawl toward the blastocoel roof; cells in the RD and CD regions of the animal marginal zone (red) express...
Xbra and intercalate mediolaterally. Cells in the RD marginal zone (i.e., animal region of the organizer) express Xnot while cells in the RV region of the organizer express goosecoid (Zoltewicz and Gerhart, 1997). Cells in both RD and RV express BMP antagonists such as noggin, chordin, Xnr3, and follistatin (reviewed in Harland and Gerhart, 1997), while cells in the CD and CV regions express BMPs (crosshatched).

Fig. 8. Morphogenesis of the marginal zone, illustrating construction of the rostral–caudal axis of the mesoderm during normal gastrulation and neurulation. All illustrations depict primarily the mesodermal germ layer. Prospective dorsal mesoderm (expressing Xbra) is shown in red and prospective ventral mesoderm is shown in orange. Morphogenetic domains are designated 1–4. Regions populated primarily by the progeny of stage 6 C-tier blastomeres are designated C1 through C4. Crosshatching indicates regions under BMP repression, while colored areas devoid of crosshatching express BMP antagonists, or are derepressed by BMP antagonists expressed by neighboring cells. (A, B) Late blastula stage. Surface view and sagittal view, respectively. The four regions of the marginal zone are RV, RD, CV and CD, as defined in the text. RV and RD comprise Spemann’s organizer. During gastrulation, region 1 undergoes vegetal rotation, region 2 undergoes crawling behavior, region 3 undergoes mediolateral intercalation behavior and region 4 undergoes epiboly. The rostral termini of all three germ layers lie along the prime meridian (0°). (C–F) Normal development, illustrated in sagittal section. (C) Early gastrula. Vegetal rotation and crawling of the leading edge mesoderm begins in the rostral mesendoderm along the prime meridian. This both inverts the rostral marginal zone and internalizes it. Only the progeny of C1 and C4 (black lettering) are visible in sagittal section, C2 and C3 progeny populate parasagittal regions. Red lettering shows their approximate positions. The early lip of the blastopore is indicated by a black arrowhead, and consists predominantly of C1 progeny. (D) Mid-gastrula. Rostral mesendoderm migrates closer to the animal pole. Progeny of C2 escape BMP repression, undertake mediolateral intercalation and join C1 progeny in the forming dorsal mesoderm. If we consider the lateral view of the embryo as the face of a clock, we see that the convergence motion of C2 progeny is not directed toward 3 o’clock, but is directed toward 5 o’clock. This causes the eccentric closing of the blastopore, and results in elongation of the dorsal, axial structures, indicated by red arrow. Vegetal rotation and crawling of leading edge mesoderm begins in the caudal marginal zone at this stage, but the prospective dorsal mesoderm in the animal caudal marginal zone is still repressed by BMPs. (E) Late gastrula. Rostral mesendoderm now underlies rostral ectoderm, establishing the head anlagen (blue box). C3 progeny escape BMP repression, undertake mediolateral intercalation behavior and join C1 and C2 progeny in forming dorsal mesoderm. Note that convergence of C3 is toward the original vegetal pole at 6 o’clock. The blastopore continues to close eccentrically (toward the point “X”), lengthening the forming rostral–caudal axis (red arrow). Most C4 animal progeny remain repressed within the circumblastoporal collar, while C4 vegetal progeny migrate toward the animal pole. The late blastoporal lip, designated by a black arrowhead, is composed of both organizer progeny and progeny of the original caudal marginal zone, including C3 and possibly C4. (F) Early neurula. The vanguard of the rostral leading edge mesoderm crawls beyond the animal pole to settle and form the anterior end of the ventral blood islands, the liver and the heart. Some C4 animal progeny escape BMP repression and join the forming somite files while some remain repressed by BMPs within the circumblastoporal collar. Convergence of C4 progeny is directed toward 8 o’clock, further lengthening the rostral–caudal axis. Thus, in normal development, the true “dorsal midline” of the tadpole becomes manifest during gastrulation, and the prime meridian, (which has historically and erroneously been called the dorsal midline) is more accurately considered the rostral midline. This figure illustrates why failure of convergence extension movements results in embryos with a normal head and open blastopore over the yolk plug where the dorsal midline structures should normally form.
Gastrulation movements begin along the prime meridian, first building the head anlagen (Figs. 8C–F; the prime meridian 0° passes through the center of Spemann’s organizer and was formerly called the dorsal midline; we believe it is the embryo’s rostral midline). Two morphogenetic activities in late blastulae bring cells from the vegetal and equatorial prime meridian underneath the animal prime meridian (i.e., bring the prospective rostral mesoderm and endoderm under the prospective rostral ectoderm, diagrammed in Figs. 8B–D, early to late gastrula). Vegetal rotation along the prime meridian shifts RV cells animalward beneath the animal region of the organizer (Winklbauer and Schurfeld, 1999), and inverts the rostral marginal zone. Goosecoid-expressing mesendoderm cells, also part of the RV region, next initiate crawling migration toward the animal pole. As a result, the rostral ends of all three germ layers lie in close proximity (blue box in Figs. 8E, F) and secondary inductions can now drive head formation along the prime meridian.

With the cells of all three germ layers fated to form head structures grouped together and out of the way, trunk formation begins. We consider ventral mesodermal morphogenesis first, and then dorsal mesodermal morphogenesis.

CV morphogenesis begins after RV morphogenesis, and also involves both vegetal rotation and crawling migration. These behaviors spread primarily along the horizontal axis from column 1 to 4 progeny during gastrulation, until all 360 degrees of the vegetal marginal zone (orange) participates. Thus, cells from throughout the vegetal marginal zone move toward the animal pole (Figs. 8C–F) and end up in lateral plate derivatives. During their migration, the vanguard of these cells receives signals from the animal cap that specify them to form the ventral blood islands (Maeno et al., 1994b). As they crawl, they maintain the spatial projection such that C1 progeny form the anterior blood islands and C4 progeny form the posterior blood islands (Table 1, Fig. 8F).

Dorsal mesoderm morphogenesis, which constructs the dorsal, axial structures of the trunk and tail, begins within the RD domain. During stage 10.5, a subset of the cells in the RD region forms the vegetal alignment zone by initiating mediolateral intercalation (mid-gastrula stage; Lane and Keller, 1997). As the cells in the organizer intercalate mediolaterally, they recruit cells within the organizer and eventually in region CD to participate in mediolateral intercalation and axis formation. The revised fate map reveals that recruitment spreads in the embryo as it does in explants: we find C1 progeny in the most rostral somite (Fig. 8C), C2 progeny next joining the axis (Fig. 8D, at the level of somite 2 in our embryos, Table 1), C3 progeny undertaking mediolateral intercalation later (Fig. 8E, and first contributing to somites 6–7 in our embryos), and C4 progeny undertaking mediolateral intercalation much later (Fig. 8F, and not contributing to somites until the 9th somite is made). Thus, during normal development, cells enter the forming rostral–caudal axis in a progression that starts in the marginal zone in C1 descendents and more cells are recruited in both time and space from BMP-repressed C2, C3 and C4 progeny (Figs. 8C–F). As C-tier progeny involute and join the nascent axial tissues, some B-tier progeny move into the closing circumblastoporal collar by radial intercalation/epiboly, in essence replacing the departing C-tier progeny. These B-tier progeny eventually come into contact with organizer cells, undertake MIB and enter the somite files (not shown, as the diagram is already too complicated). B1 and B2 progeny enter at about the same time, followed by B3 and finally B4 progeny. Because B-tier progeny enter the circumblastoporal collar later than their corresponding C-tier progeny (e.g., B2 later than C2, B4 later than C4, etc.), they also enter the somite file later and end up in more caudal positions.

Reinterpreting Spemann’s grafting experiment

The rostral-to-caudal construction of the dorsal, axial structures revealed by understanding the revised map raises questions about the interpretation of Spemann and Mangold’s organizer experiment (Smith and Slack, 1983; Spemann and Mangold, 1924). The expression of Xbra indicates that the cells in region CD “know” they should undertake dorsal-type morphogenesis—mediolateral intercalation behavior—but our results indicate they are suppressed by BMPs. MyoD expression in region CD indicates these cells are partway down the myogenic differentiation pathway. An organizer graft secretes BMP antagonists (Fig. 9A), precociously releasing host cells from suppression, and they prematurely initiate mediolateral intercalation and recruitment (Figs. 9B–D, mid-gastrula through neurula stages). They make somites prematurely and probably induce overlying ectoderm to form relatively posterior neural structures such as hindbrain and spinal cord, which may account for neural induction by the graft.1 As the caudal marginal zone under the influence of the organizer forms a domain of mediolateral intercalation separate from the endogenous organizer (Fig. 9B), two axes, separate at their rostral ends, forms. The two fields of mediolateral intercalation collide partway across the marginal zone (e.g., somewhere around the C3 territory). They merge into a single morphogenetic field of mediolaterally intercalating cells and construct a common tail (Fig. 9D, neurula stage). Thus, the endogenous primary axis is undisturbed rostrally, but shares its posterior trunk and highly defective tail with the secondary axis. The secondary axis is composed of cells that should have remained repressed until contact was established with intercalating cells from the endogenous organizer and should have formed somites caudal to somite 9 in the primary axis. Because these cells made rostral somites prematurely in the second axis, they are no longer present to make caudal somites in the primary axis. The fact that the shared tail is always

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1 Some of this neural induction is true induction; that is, cells from C4 and B4 (which normally make minor contributions to axonal fiber tracts in the nervous system, MCL and MDS, unpublished observation) make major contributions to the secondary hindbrain and spinal cord. Other cells (e.g., B3 progeny), which normally form caudal neural tissue in the primary axis, are recruited into more rostral positions in the secondary axis.
misshapen and deficient suggests that there is a limited competence group available to differentiate as somitic mesoderm, and that the organizer is not sending out “instructive signals” to become somitic mesoderm, but that it only releases permissive signals. Some other unidentified signal that activated Xbra and XmyoD in the animal marginal zone makes cells competent to form somitic mesoderm, but the cells are suppressed by BMPs from assuming somitic fate. A BMP antagonist from the organizer then “rescues” cells from suppression to assume a somitic fate. A testable prediction of this model is that BMP antagonists will not induce muscle if the signal that activates Xbra and MyoD expression in the late blastula animal marginal zone is abolished, or perhaps if Xbra is compromised (Conlon and Smith, 1999; Kwan and Kirschner, 2003).

The embryological and molecular evidence leads us to propose the following model. The non-organizer marginal zone is initially patterned independently of the organizer into two morphogenetic domains. An animal domain expresses Xbra (a posterior, dorsal marker) and MyoD (a myogenic determinant), but is repressed by BMPs from undertaking dorsal mesoderm-specific morphogenesis (i.e., mediolateral intercalation behavior). Animal domain cells near the organizer also express myf5, another myogenic determinant required to form muscle, but cells in the animal domain distant from the organizer do not express myf5 (Dosch et al., 1997). A vegetal domain, identified by menf expression (Kumano and Smith, 2002), undergoes ventral mesoderm-specific behavior, that is, crawling behavior toward the blastocoel roof. In the embryo, mediolateral intercalation begins within the organizer, and BMP antagonists secreted by organizer cells locally recruit neighboring cells to participate in dorsal mesoderm-specific morphogenesis. One aspect of this recruitment is that BMP repression by a BMP antagonist like noggin initiates myf5 expression by MyoD- and Xbra-expressing cells that did not previously express this myogenic determinant (Dosch et al., 1997). This suggests that cells in the caudal, animal marginal zone are suspended WITHIN the myogenic specification pathway (i.e., they are MyoD-positive but myf5-negative) until the organizer rescues them back into the myogenic specification pathway by derepressing BMP signaling. Derepression involves at least two activities: first, activation of myf5 expression restarts myogenic differentiation, and second, stimulation of mediolateral intercalation via Xbra. Thus, the signal from the organizer that leads to muscle differentiation is permissive rather than instructive—the animal marginal zone must already express Xbra and MyoD to respond to a BMP antagonist and make muscle.

We note that the animal cap, which does not express Xbra, responds to BMP antagonists by forming neural ectoderm, not somitic mesoderm. However, ectopic expression of Xbra in animal cap cells, either directly by Xbra mRNA injection or by other treatments that induce Xbra expression, promotes the differentiation of dorsal mesoderm in the presence of BMP antagonists (Cunliffe and Smith, 1994). Thus, BMP antagonism in gastrula stages is not instructive but permissive—it provokes context-dependent responses based on the state of the responding cell.
Our model helps to explain why a long list of seemingly disparate molecules “induce” partial secondary axes when expressed in the caudal marginal zone. For example, GATA factors are transcription factors involved in mesendoderm differentiation, while derriere is TGF-β growth factor family member. When a dominant-negative GATA2 construct (that includes an engrailed repressor domain) is expressed in the CMZ (formerly the VMZ), a partial second axis is “induced” (Sykes et al., 1998). Further investigation by the authors showed that dominant-negative GATA increased chordin (another BMP antagonist) expression in the CMZ, which likely acts as noggin does in our experiments. It likely stimulates precocious mediolateral intercalation of cells in the animal region of the CMZ, which leads to a partial trunk duplication with a shared tail. Derriere is a second molecule that causes partial secondary axes when expressed in the CMZ (Sun et al., 1999). The effects of derriere misexpression in the CMZ on BMP antagonist expression (e.g., chordin, noggin) were not reported, but other organizer molecules (e.g., goosecoid) were upregulated. Given that the authors reported observing only partial secondary axes, we suspect that a closer examination will reveal that derriere expression in the CMZ either locally upregulates BMP antagonist expression or down-regulates BMP signaling, as these are the common denominators behind partial secondary axes. Any manipulation that decreases BMP repression in an otherwise normal CMZ will result in a partial secondary axis because BMPs are preventing the animal cells of the CMZ from undergoing mediolateral intercalation.

**The role of BMP-mediated repression and BMP antagonism in amphibian embryos**

Experiments expressing noggin suggest that BMP antagonists from the organizer are one of the active molecular species that release caudal cells to participate in axial morphogenesis and differentiation. This reveals that one important role for BMP repression in the early gastrula, and what may turn out to be the first role for BMPs after the mid-blastula transition, is to prevent cells from entering and participating in axial morphogenesis. Frogs develop from egg to early tadpole with very little growth—cleavage divides the egg cytoplasm into thousands of cells, which construct the embryo until feeding begins 4 days after fertilization (at stage 45, Nieuwkoop and Faber, 1967). True growth occurs only after the embryo takes in outside energy stores. One consequence of this developmental strategy is that all of the materials necessary to construct the early tadpole are present early in development. However, only a few cells at a time should be utilized to build a particular rostral–caudal level of the body plan. Most cells must be held quiescent in a reservoir for incorporation at more caudal levels. For example, the cells that form somite pair 15 in the body plan are present in the embryo long before somite pair 15 is generated, and they must not differentiate prematurely if the entire rostral–caudal axis is to be properly assembled. Our experiments indicate that the frog blastula/gastrula uses BMPs as a widespread repressive mechanism to maintain and protect a reservoir of quiescent cells, and the BMP antagonists released by the organizer insure an orderly, progressive entry of a few dorsal mesodermal cells at a time so that the body plan is completed when the reservoir is emptied. (See Fig. 12, stages 9 and 9.5 in Schohl and Fagotto, 2002. Smad1 signaling, which should indicate BMP repression, is elevated everywhere except the animal region of the prime meridian.) If BMP expression is reduced inappropriately (e.g., as in the case of XBP2 overexpression, Mariani et al., 2001), large anterior somites result from premature recruitment of cells into the muscle differentiation pathway. A similar phenomenon may occur in the fish. We pointed out previously (Lane and Sheets, 2002b, and references therein) that zebrafish mutants categorized as “dorsalized”—somitabun, swirl, and snailhouse, all of which are mutations in the BMP signaling pathway—are more likely “rostralized”. These mutants present radialized rostral somites and defective/missing caudal structures. Reinterpreting these mutants in light of the revised frog fate map suggests that the problem in these mutant embryos is depletion of the reservoir of quiescent cells that express Myo D early but normally do not differentiate immediately as somitic muscle. These cells should have slowly entered the somites to construct the long array of normal somites, but instead, these cells entered the somite file almost concomitantly, building massive rostral somites early in development, and leaving too few prospective somite cells to make the trunk and tail.

This developmental strategy of the frog embryo stands in stark contrast to the strategy used by avians and mammals. In chick and mouse, extensive growth accompanies all stages of development, including gastrulation and neurulation. In a mouse or chick embryo, the cells that form somite 15 probably are not present until shortly before somite 15 is constructed—the rostral–caudal axis is most likely generated as a small population of “stem” cells instructs or influences newly born cells that arise during growth of the embryo. Therefore, neither the chick nor the mouse requires a widespread system of repression by BMPs to maintain a large population of pre-existing cells in a quiescent reservoir.

**Conclusion**

As we learn more about frog embryos, our working models must change. Spemann believed that the area of the embryo with the organizer at early gastrula stage was determined, while the opposite side of the embryo was indifferent or naive. Most contemporary authors believe that the side of the embryo with the organizer is specified and fated as dorsal and the opposite side is specified and fated as ventral. Our results suggest a third view—that the side of the early gastrula containing the organizer is rostral–anterior,
and it is in an advanced state of differentiation compared to the opposite side of the embryo, which is caudal/posterior tissue. The presence of restricted expression domains on the caudal side (e.g., Xbra, menf, MyoD) indicates the region is not indifferent/naive, and the expression of dorsal-type markers indicates the region is not specified as “ventral”. The caudal side is undifferentiated in early gastrula, but both the animal and vegetal regions of the caudal marginal zone are somewhere in the middle of differentiation pathway—the animal region is within the muscle pathway, while the vegetal region is within the lateral plate pathway (e.g., blood, vasculature). Our data indicate that in the case of the caudal, dorsal mesoderm (i.e., the prospective muscle), BMPs actively suppress the differentiation and morphogenesis of the tissue. BMP antagonists from the organizer recruit suppressed cells into the morphogenetic behaviors that construct the vertebrate embryonic axis (i.e., mediolateral intercalation behavior) in an orderly spatial and temporal progression.

The revised fate map, combined with an understanding of the morphogenetic movements underlying gastrulation, provides different, and in most cases, much simpler explanations for many classical experiments in amphibian embryology, not just H. Mangold’s grafting experiment. For example, Spemann noted that an early lip graft (arrowhead in Fig. 8C, early gastrula stage) yielded duplicated heads, while a late lip graft (arrowhead in Fig. 8E, late gastrula) yielded duplicated trunk/tail structures (discussed in Saxén and Toivonen, 1962 and Spemann, 1938). The old fate maps (e.g., Fig. 1A) offer a complex and convoluted interpretation of the result: the early lip graft, from the “dorsal marginal zone”, induces a rostral structure, the head, while the late lip graft, which is composed of cells originally situated in both the dorsal and ventral marginal zones, induces a caudal structure, the trunk and tail. The result is confusing for two reasons. First, the dorsal marginal zone/early lip gives a rostral duplication, not a strictly dorsal duplication. Second, both duplications contain anatomically dorsal and ventral tissues. In contrast, the new map (Fig. 1B) reveals a simpler interpretation. The early lip graft from the rostral marginal zone, which normally forms head structures, self-differentiates as a second head, while the late lip graft from the caudal marginal zone, which normally forms trunk structures, self-differentiates as a second set of trunk/tail structures. Thus, the results of the grafts are neither surprising nor confusing. Each graft self-differentiates as it would in the intact embryo, as rostral, dorsal and rostral, ventral structures (i.e., a head) in the case of the early lip and as caudal, dorsal and caudal, ventral structures (i.e., a trunk/tail) in the case of the late lip. Some host cells are recruited by the grafts, but the major phenomenon underlying the partial secondary axes observed following grafting is self-differentiation. This view becomes obvious only when the revised fate map is used to interpret experimental results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.08.012.

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