Microtubule disruption reveals that Spemann’s organizer is subdivided into two domains by the vegetal alignment zone

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SUMMARY

Mediolateral cell intercalation is proposed to drive morpogenesis of the primary embryonic axis in Xenopus. Mediolateral intercalation begins in a group of cells called the vegetal alignment zone, a subpopulation of cells in Spemann’s organizer, and spreads through much of the marginal zone. To understand the functions of the vegetal alignment zone during gastrulation and axis formation, we have inhibited its formation by disrupting microtubules with nocodazole in early gastrula embryos. In such embryos, mediolateral intercalation, involution and convergent extension of the marginal zone do not occur. Although cell motility continues, and the anterior notochordal and somitic mesoderm differentiate in the pre-involution marginal zone, posterior notochordal and somitic mesoderm do not differentiate. In contrast, microtubule depolymerization in midgastrula embryos, after the vegetal alignment zone has formed, does not inhibit mediolateral cell intercalation, involution and convergent extension, or differentiation of posterior notochord and somites. We conclude that microtubules are required only for orienting and polarizing at stage 10† the first cells that undergo mediolateral intercalation and form the vegetal alignment zone, and not for subsequent morphogenesis. These results demonstrate that microtubules are required to form the vegetal alignment zone, and that both microtubules and the vegetal alignment zone play critical roles in the inductive and morphogenetic activities of Spemann’s organizer. In addition, our results suggest that Spemann’s organizer contains multiple organizers, which act in succession and change their location and function during gastrulation to generate the anterior/posterior axis in Xenopus.

Key words: gastrulation, microtubules, organizer, anterior/posterior axis, Xenopus

INTRODUCTION

Complex inductive interactions and morphogenetic movements establish the vertebrate embryonic axis. In the amphibian Xenopus laevis, inductive interactions occur in the blastula between the prospective endoderm and ectoderm in the vegetal and animal hemispheres, respectively, that induce the marginal zone, an equatorial belt of cells that forms mesoderm (Sudarwati and Nieuwkoop, 1971). The prevailing model for mesoderm induction in Xenopus proposes that the vegetal hemisphere emits two signals (reviewed in Slack, 1994). Ventrolateral vegetal cells induce the overlying cells in the ventrolateral marginal zone to differentiate into non-organizer mesoderm. Dorsal vegetal cells (the ’Nieuwkoop Center’) induce overlying cells in the dorsal marginal zone to form Spemann’s organizer, a region that induces a partial second embryonic axis when transplanted to the ventral side of a gastrula embryo (Spemann and Mangold, 1924). Functions attributed to Spemann’s organizer include: induction of the nervous system, dorsalization of the mesoderm in the lateral marginal zone, initiation and execution of convergent extension and self-differentiation into notochord, prechordal mesoderm and pharyngeal endoderm (Gerhart et al., 1991). Thus, Spemann’s organizer regulates both the induction and morphogenesis of axial structures in amphibians.

As the belt of morphologically homogeneous mesodermal cells in the marginal zone transforms itself into the distinctive structures of the vertebrate embryonic axis, the notochord and somites, the anterior/posterior axis emerges. This transformation begins with involution and convergent extension of the marginal zone. Most of the involution and all of the convergent extension of the mesoderm are proposed to be driven by cell rearrangement; initially cells in the marginal zone intercalate along the radial axis of the embryo; subsequently cells intercalate in the mediolateral axis, in a specific spatial and temporal progression that originates in Spemann’s organizer (Shih and Keller, 1992a). As a result of the pattern of intercalation, the involutioning marginal zone progressively rolls over, sweeping mesodermal tissues deep inside the embryo. Involuted mesodermal cells continue to intercalate mediolaterally, which results in mediolateral narrowing (convergence), and anterior/posterior lengthening (extension) of the axial tissues (convergent extension, Fig. 1A,B) as well as closing of the blastopore. Inductive interactions pattern the embryo concomitantly with morphogenesis, during the late blastula and early gastrula stages. As the mesodermal cells converge upon
the dorsal midline, cells assume specific fates based on their position within the mesodermal mantle (Domingo and Keller, 1995).

When the early gastrula stage dorsal marginal zone (which includes Spemann’s organizer) is explanted and cultured in vitro, the mesodermal cells execute their normal morphogenetic routines (Keller et al., 1991). At stage 10\textsuperscript{1}, a subpopulation of cells begins mediolateral intercalation behavior (see Definitions below; Shih and Keller, 1992b). The initiation of this motility behavior manifests itself in the formation of elongate, bipolar cells that can be distinguished visually from surrounding isodiametric cells. Shih and Keller (1992a) demonstrated that bipolar cell morphology arises initially as multipolar, isotropic filopodial and lamellipodial protrusions are restricted to the medial and lateral ends of mesodermal cells. They proposed, based on studies of ‘shaved explants’, that the cells exert traction upon one another using these bipolar protrusions, which results in mediolateral intercalation. The first cells to assume bipolar morphology associate in an arc which they named the vegetal alignment zone. This arc has its ends to either side of Spemann’s organizer, and traverses the organizer (see Fig. 1C,D). Mediolateral intercalation behavior spreads progressively from the vegetal alignment zone through the prospective axial and paraxial mesoderm in the marginal zone between stages 10\textsuperscript{1} and 13 (Shih and Keller, 1992a; Domingo and Keller, 1995). Because mediolateral intercalation behavior is the only motility behavior observed in explants of deep mesoderm that converge and extend, mediolateral intercalation is sufficient for convergent extension of the mesodermal tissues (Shih and Keller, 1992b).

The restriction of multipolar protrusions to generate bipolar cell morphology in *Xenopus* mesodermal cells appears morphologically similar to mesenchymal cell polarization in vitro; multipolar mesenchymal cells assume bipolar morphology by restricting protrusive activity to limited domains of the cell surface. In cultured fibroblasts, this process requires an intact microtubule system (Vasiliev et al., 1970). The formation of bipolar cells in the vegetal alignment zone from multipolar cells raises the possibility that microtubules are needed in the embryo to polarize cells and form the vegetal alignment zone. Two previous studies in *Xenopus laevis* examined the effects of microtubule depolymerization on gastrulation (Cooke, 1973; Nakatsuji, 1979). Results presented in those papers suggested that there was a critical time in the late blastula before which gastrulation was inhibited by microtubule disruption, and after which it was not (Fig. 1 in Cooke, 1973). We hypothesize that this critical time reflects vegetal alignment zone formation. To investigate the function of microtubules during vegetal alignment zone formation and mediolateral intercalation behavior, and the role of the vegetal alignment zone in gastrulation and axis formation, we have disrupted microtubules using nocodazole and assayed the effects on development in whole embryos and in dorsal marginal zone explants.

**Fig. 1.** The marginal zone in *Xenopus*. Convergence of the marginal zone in the mediolateral direction after stage 10\textsuperscript{1} produces extension, or lengthening of the dorsal mesodermal tissues in the anterior/posterior direction. (A,B) A simplified stage 10 fate map is shown on the embryo on the left, with the head mesoderm (blue), the prospective axial tissue (notochord; anterior is red, posterior is orange) and paraxial mesodermal tissue (somites in brown; anterior is heavily stippled, posterior is lightly stippled) shown centered on the dorsal midline (dashed line). Mediolateral intercalation results in notochordal and somitic tissue involution, which inverts the anterior/posterior directional polarity of the tissues with respect to the animal/vegetal axis of the embryo. Mediolateral intercalation also causes convergence upon the dorsal midline (B; stage 23) and extension of the anterior/posterior axis. (C) Dorsovegetal view of a stage 10\textsuperscript{1} embryo, showing the relationship between the vegetal alignment zone, the prospective anterior notochord and somites, and the prospective posterior notochord and somites. A→P indicates the orientation of the anterior/posterior axis in the notochordal and somitic fields. The gastrulation movements bring these essentially perpendicular axes into parallel alignment. In this view, the epithelium is removed, revealing the deep mesodermal component of the marginal zone. Head mesoderm cells have moved beneath the prospective notochord. The vegetal alignment zone (black cells) begins and ends to either side of the dorsal marginal zone, and traverses the prospective anterior notochordal and somitic tissues. (D) In a sagittal section, the arc (black) is present in the pre-involutive mesoderm, beneath the epithelium of the organizer. The head mesoderm is migrating on the deep surface of the prospective notochord. an, anterior; A, animal; Bl, blastocoel; DL, dorsal lip of blastopore; LI, limit of involution; p, posterior; V, vegetal; VAZ, vegetal alignment zone; VL, ventral lip of blastopore.

**MATERIALS AND METHODS**

**Embryo culture and explant isolation**

Embryos obtained by standard methods were dejellied in 2% cysteine HCl, pH 8.0, and maintained in one-third strength Modified Barth’s Solution (MBS) or 1/10 MMR (Kay and Peng, 1991). Regularly cleaving embryos were selected at the 4- to 8-cell stage. Stages 10 through 10\textsuperscript{1} were determined by the criteria of Keller (1991). Imme-
diately prior to surgery, the embryos were transferred to modified Danilchik’s solution containing 0.1% bovine serum albumin (DFA+BSA; Sater et al., 1993) and the vitelline membranes removed manually with forceps.

**Explant and isolates**
Dorsal marginal zones were surgically prepared as explants (meaning they were cultured for extended periods under coverslip bridges, Figs 4 and 6), or as isolates (meaning they were gently flattened beneath a coverslip bridge for 3-5 minutes before fixation, Fig. 2A-C). Dorsal open-faced explants, freed of head mesoderm and bottle cells, were prepared as described by Keller (1991). Dorsal lip isolates retain head mesoderm, bottle cells and archenteron roof, along with involuted and non-involuting dorsal mesoderm.

**Microtubule fixation and immunofluorescence**
Specimens were fixed using formaldehyde and glutaraldehyde (0.5%), immunostained and mounted using the procedures of Gard (1991). Microtubules were immunostained with a mouse monoclonal antibody to tubulin (Tub 2.1, Sigma), and visualized with a Texas Red-conjugated donkey anti-mouse IgG (Jackson Immunolabs). The specimens were observed with a 10x0.5 NA fluorescence objective on a Bio Rad MRC 600 confocal scanning laser microscope.

**Nocodazole and taxol treatment**
Taxol and nocodazole (Calbiochem) stocks were prepared in tissue culture grade DMSO (Sigma) at 10 mg/ml and 5 mg/ml, respectively, and stored at -20°C. Both drugs cross the vitelline envelope, and nocodazole is more effective at 21°C than 16°C. To determine how long microtubules persisted in embryos treated with nocodazole, at various timepoints, embryos in 15 μg/ml nocodazole were fixed and immunostained for microtubules. Within 15 minutes, microtubules in the deep cells of the marginal zone, endoderm and animal cap were disrupted; sparse microtubules persisted only in the apical region of ectodermal cells.

Whole embryos or explants were cultured continuously at 21-23°C in DFA+BSA containing 15 μg/ml nocodazole, 20 μg/ml taxol or 3 μl/ml DMSO (at 7 embryos/ml), until control embryos reached approximately stage 23-25. Vitelline envelopes were removed manually, and embryos were either dissected and scored for archenterons or fixed for 3 hours in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), post-fixed in 100% methanol and scored after clearing in benzyl benzoate:benzyl alcohol (2:1; BB/BA). Explants were transferred immediately upon isolation to fresh DFA+BSA containing either 15 μg/ml nocodazole, 20 μg/ml taxol or DMSO and restrained by a coverslip bridge held in place by silicone grease. The explants were cultured at 21-25°C until control embryos reached stage 18-20, when the explants were inspected for notochord/somite boundary formation and scored for convergent extension.

We verified that the effects of nocodazole on morphogenesis were reversible in whole embryos by washing out the drug after 1-7 hours of treatment. Following two gentle washes with DFA+BSA within 1 hour of drug removal, the embryos were cultured in 1/10 MMR until control embryos reached stage 23-27, fixed and immunostained for differentiated tissues as described below.

**Immunohistochemistry**
Specimens fixed in MEMFA were stained with Tor 70 (Kushner, 1984), which stains the notochord, and 12-101 (Kintner and Brockes, 1984), which stains the somites, following published protocols (Bolce et al., 1992). Immunostaining utilized a horseradish peroxidase-conjugated, goat anti-mouse IgG secondary antibody (Jackson Immunolabs), with diaminobenzidine tetrahydrochloride (Polysciences), with or without NiCl₂, as the substrate. Some specimens were embedded

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Fig. 2. A subpopulation of cells in the dorsal lip/organizer which become bipolar in the mediolateral axis during mid-gastrulation. Cell outlines are revealed by immunostaining with the anti-integrin antibody, SC8. In all panels, the animal pole is up and the dorsal lip is down (AA’). The epithelium of the organizer is composed of isodiametric, polygonal cells at stage 10⁺ (shown) and 10⁻ (not shown). (B,B’) Cells in the first deep cell layer beneath the organizer epithelium at stage 10⁺ are likewise polygonal, and essentially isodiametric. These cells are undergoing radial intercalation (in and out of the focal plane). (C,C’) Some cells in the first deep cell layer beneath the organizer epithelium at stage 10⁺ are bipolar and elongated (solid circles) in the mediolateral axis of the embryo. Cells in the first deep cell layer situated more anteriorly in the organizer show no mediolateral elongation at stage 10⁻ (stars), while cells vegetal of alignment zone are also not mediolaterally elongated (squares). Scale bar, 25 μm.
in paraffin following standard procedures and sections of 10-15 μm were cut. Sections were cleared and mounted in BB/BA.

For observation of cell shapes in nocodazole-treated and control dorsal lips, pigmented embryos at stage 10+ or 10 were treated with carrier or nocodazole until stage 12. Whole embryos were fixed overnight in Dent’s fixative (80% methanol/20% DMSO, Dent et al., 1989), and stored in 100% methanol. Samples were serially rehydrated in PBS, blocked and immunostained with the 8C8 monoclonal antibody (to β1-integrin) ascites diluted 1:2000 (gift of Dr D. DeSimone), and visualized with HRP-goat anti-mouse IgG (Jackson Immunolabs). The specimens were partially bleached in 70%methanol/30%H2O2, dehydrated in methanol and cleared in BB/BA. Dorsal lips were dissected from cleared embryos before viewing (Fig. 3D-F).

Grafting of labeled cells

Newly fertilized embryos were dejellied, transferred to 6% Ficoll in 1x MBS and injected by air pressure with approximately 15 nl of rhodamine-dextran amine (RDA, 33 mg/ml in H2O; Molecular Probes) within 30 minutes of fertilization. The embryos were transferred to 1/3 MBS + 6% Ficoll and cultured to early gastrula (stage 10-14). Labeled embryos were transferred to DMSO+BSA and vitelline envelopes removed with forceps. The suprablastoporal epithelium was peeled back, a small clump of cells was removed from the presumptive notochord region and transplanted to the deep surface of the presumptive notochord region of an unlabeled dorsal open-face explant, isolated from either a stage 10+ or stage 10 embryo. Explants were screened at the outset and only explants receiving a single, contiguous clump of labeled cells were retained. The open-face explants were subsequently cultured in DMSO+BSA containing either DMSO (control) or nocodazole (15 μg/ml) until control embryos reached stage 20. Cell motility and behaviors were filmed in some explants using a Hamamatsu C2400-08 STF camera, a Nikon Diaphot epifluorescence microscope and Image One image processor, as previously described (Domingo and Keller, 1995). Other explants were fixed at the end of the culture period in MEMFA, post-fixed in methanol, cleared and mounted in BB/BA, observed in a fluorescence microscope and photographed using Kodak TMax 100 print film.

DEFINITIONS

Convergent extension morphogenesis

Convergent extension is the narrowing and lengthening of a tissue, without reference to particular cell behaviors. In Xenopus, mesodermal convergent extension is the outcome of cell intercalation in the mediolateral axis of the embryo. Mediolateral intercalation results in cells accumulating at the dorsal midline, called convergence. As more cells arrive at or near the midline, the midline extends to accommodate the increasing number of cells. (We emphasize that, in Xenopus, convergence is not the result of directed cell migration towards the dorsal midline. Other instances of convergent extension may result from different morphogenetic processes.)

Mediolateral intercalation

This is the rearrangement of mesodermal cells within a cell layer. Exerting traction on mediadly and laterally situated neighbors, cells pull themselves between these neighbors.

Mediolateral intercalation behavior

Mediolateral intercalation behavior is the set of stereotyped cell behaviors that, in a population of cells, drives mediolateral cell intercalation of Xenopus mesoderm (see Fig. 3 in Keller et al., 1992). These behaviors include: (1) initially, randomly multipolar cells restrict their protractive activity to the medial and lateral (relative to the embryo) ends of the cells; (2) bipolar cells apply protractive forces to neighboring cells, aligning themselves and eventually their neighbors into arcs spanning the dorsal midline; (3) both protractive activity and the proposed cell traction are roughly balanced at the two ends of a bipolar cell; (4) once a cell contacts the notochord/somite boundary (defined below), the area of the cell contacting the boundary ceases protractive activity.

Vegetal alignment zone

This is an arc comprising the first mesodermal cells undergoing mediolateral intercalation behavior. The arc forms during stage 10+ in the region above the dorsal lip of the blastopore. The ends of the arc are in the lateral marginal zone, and the arc traverses Spemann’s organizer through the region of the prospective anterior axial and paraxial mesoderm (see Fig. 1C).

Notochord/somite boundary

A visible boundary forms between the notochordal and somitic tissues beginning at stage 11+. By stage 12+, laminin is present in the extracellular matrix that separates the notochord from somitic tissues (Fey and Hausen, 1990), and a post-translationally modified tubulin that marks stable microtubules is found under the plasma membrane of notochordal cells at the boundary (M. C. Lane and R. Keller, manuscript in preparation).

RESULTS

The vegetal alignment zone is present in the preinvolution mesoderm of the intact Spemann’s organizer at stage 10+. The vegetal alignment zone was observed originally in cultured, shaved explants of the dorsal marginal zone, but shaved explants differ in two important aspects from intact dorsal marginal zones. First, all but one of the deep layers of the mesoderm was removed (Shih and Keller, 1992b), thereby preventing radial cell intercalation, which normally occurs at this time. Second, although cells underwent mediolateral intercalation behavior, actual cell rearrangement was thwarted intentionally by applying pressure on the explant with a coverslip bridge. Thus, the formation of the vegetal alignment zone could be an artifact of the culture conditions.

To determine whether the vegetal alignment zone forms in embryos, we examined cell shapes in dorsal lip isolates from stage 10 and stage 10+ embryos. To visualize cell shapes, we immunostained with 8C8, an antibody to β1-integrin that primarily stains cell surfaces in early Xenopus embryos (Gawantka et al., 1992). In stage 10+ dorsal lip isolates, we see only isodiametric cells in the epithelial and deep layers of the marginal zone (Fig. 2A,B). In stage 10+ dorsal lip isolates, most cells in the mesodermal region of Spemann’s organizer are isodiametric, but some bipolar cells elongated in the mediolateral axis of the embryo can be distinguished (solid circles, Fig. 2C'). Scanning at high magnification in three dimensions throughout the dorsal marginal zone, we find that bipolar cells associate with one another in an arc above the dorsal lip in the first (and occasionally second) deep cell layer beneath the suprablastoporal epithelium. A portion of a vegetal alignment zone is shown in Fig. 2C,C'. When first detected, the arc is subtle but is observed reproducibly; it is about 1-2 cells in animal-vegetal extent, and at its highest point, which should coincide with the dorsal midline, the arc is approximately 5-8 cells above the dorsal lip. Cells higher in the marginal zone.
retain a cobblestone appearance and are not mediolaterally elongated (stars, Fig. 2C). Cells immediately below the arc are not mediolaterally elongated (squares, Fig. 2C), nor are cells located deeper radially in the marginal zone or in the overlying epithelium (not shown). Thus, the vegetal alignment zone forms in preinvoluted mesoderm. The approximate position of the vegetal alignment zone is schematically represented in Fig. 1C,D. The arc traverses Spemann’s organizer in the middle of the notochord territory and more laterally it crosses through the somitic territories.

We examined the microtubule arrays in bipolar cells of the vegetal alignment zone to see if they differed from arrays in surrounding cells. The vegetal alignment zone is present in the preinvoluted mesoderm of the dorsal marginal zone, confirming our observations based on integrin staining, and cells in the arc can be distinguished from their isodiometric neighbors by their bipolar shape (not shown). At stage 10\textsuperscript{\textcircled{i}}, we observe nothing distinct about the microtubule arrays in bipolar cells. In all deep cells of Spemann’s organizer, microtubules course throughout the cytoplasm, wending between yolk platelets and along the plasma membrane. The microtubules in mediolaterally intercalating cells will be dramatically reorganized in the next few hours (M. C. Lane and R. Keller, manuscript in preparation), but at the onset of gastrulation, there are no obvious differences between microtubule arrays in bipolar and isodiometric cells.

Our observations demonstrate that the vegetal alignment zone is first present in the embryo in pre-involution mesoderm and is not an artifact of shaved explant culture conditions.

Gastrulation of intact embryos and formation of the vegetal alignment zone are inhibited by nocodazole exposure prior to stage 10\textsuperscript{\textcircled{i}}

To determine whether microtubules are involved in restricting protrusive activity and generating bipolar cells, intact embryos were treated with nocodazole or taxol (reviewed in Hamel, 1989). Nocodazole depolymerizes dynamically unstable microtubules, while taxol both enhances tubulin assembly and stabilizes microtubules against some depolymerizing conditions (e.g. cold, Ca\textsuperscript{2+} concentration, tubulin monomer dilution). We cultured intact embryos in nocodazole, taxol or carrier DMSO from stages 10\textsuperscript{+}, 10\textsuperscript{S} or 11, and examined data not shown), demonstrating that bottle cell morphogenesis does not depend on the presence or stabilization of microtubules from stage 10 through 10\textsuperscript{\textcircled{i}}, when all of the bottle cells have formed.

Continuous treatment of embryos with taxol had no pronounced effect on any of the morphogenetic events associated with gastrulation and primary axis formation, although in embryos treated prior to stage 10\textsuperscript{\textcircled{i}}, blastopore closure lagged slightly. At all stages tested, during continuous exposure to taxol, somites failed to segment and take on their characteristic chevron shape during tailbud stages. We confirmed by immunostaining that taxol entered cells and caused the formation of extensive arrays of straight, bundled microtubules, but did not inhibit cell division (data not shown, but see treated explant, Fig. 4B).

The effects of microtubule depolymerization on development varied with the stage at which the embryos were initially exposed to the drug, as reported by Cooke (1973). Exposing embryos to nocodazole after stage 10\textsuperscript{\textcircled{i}} did not inhibit blastopore closure (Fig. 3B), nor archenteron formation (94\% at stage 10\textsuperscript{\textcircled{i}}; 100\% at stage 11; Table 1; Fig. 5E below). These embryos formed neural plates but not neural tubes. However, nocodazole exposure before stage 10\textsuperscript{\textcircled{i}} inhibited involution and convergent extension. Embryos cultured continuously in nocodazole from stages 10\textsuperscript{+} or 10\textsuperscript{S} do not close the blastopore (Fig. 3C), form archenterons (Fig. 5E below) or internalize the marginal zone (0\% formed archenterons when treated at stages 10\textsuperscript{+} and 10\textsuperscript{S}; Table 1 and Fig. 5E below). By morphological criteria, no neural plate develops in these embryos.

We examined embryos treated with nocodazole for microtubule persistence. No microtubules remain in the mesoderm or in the endoderm after exposure to nocodazole, precluding cell division and indicating that the conditions employed effectively eliminated microtubules (see explant, Fig. 4C below; data for whole embryos not shown).

We examined cell shapes in nocodazole-treated embryos, as revealed by 8\textsuperscript{C8} immunostaining. In control embryos at stage 12\textsuperscript{\textcircled{i}}, mediolateral intercalation behavior has spread through much of the involuted marginal zone. The cells of the involuted marginal zone are bipolar (arrowheads, Fig. 3D) and the notochord/somite boundary is prominent (arrow). Involuting cells in embryos treated after stage 10\textsuperscript{\textcircled{i}} are mediolaterally

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Numbers stated are embryos, which were scored at control stage 23-25 by dissection or observation of fixed, cleared embryos. The presence of a shallow indentation (Nieuwkoop and Faber, 1967) as a result of radial intercalation, bottle cell formation and head mesoderm migration was scored as (lacking in archenteron). True involution of the marginal zone results in a much longer impoertting easily distinguished from the shallow, broad indentation present in stage 10\textsuperscript{\textcircled{i}} embryos.
Nocodazole prevents convergent extension and notochord/somite boundary formation in dorsal open-faced explants

To determine whether an intact microtubule cytoskeleton is required specifically for convergent extension, we used dorsal open-faced explants, which contain mesoderm and endodermal epithelium but not bottle cells or head mesoderm, two cell types undergoing morphogenetic activity in the dorsal marginal zone of the early gastrula. Dorsal open-faced explants were made at stage 10, and incubated immediately and continuously, in nocodazole or taxol, until stage 20. Explants treated with carrier (DMSO) elongate as expected (100%, Table 2, Fig. 4A, and see Fig. 6B) and form notochord/somite boundaries; explants treated with taxol undergo significant convergent extension (Fig. 4B). The notochord/somite boundaries reveal a slight reduction in convergent-extension, which is apparent in the increased breadth and shortened length of the notochord. Nocodazole-treated explants rarely extend (12% extend, Table 2, Fig. 4C, and see Fig. 6C), and those that do are consistently much shorter than controls. Notochord/somite boundaries do not form in the nocodazole-treated explants. Convergent extension is not affected and notochord/somite boundaries form in explants made at stage 10 and cultured in nocodazole (74% converge and extend; Table 2, and not shown, but see Fig. 6D). These results indicate that microtubules are required in the cells of the dorsal marginal zone at stage 10 for convergent extension of the dorsal axial mesoderm after stage 10.

Nocodazole blocks differentiation of posterior axial and paraxial mesoderm

A simple explanation for the inhibition of morphogenesis caused by nocodazole exposure of stage 10 embryos is that all developmental processes are inhibited. We determined whether histodifferentiation of tissues occurs in nocodazole-treated embryos by immunostaining with antibodies to notochordal and somitic markers at the equivalent of stage 22-25. The epitopes recognized by these antibodies are first detectable in late neurulae (approximately stage 17-19), so these antigens are not present in the embryos when they are initially exposed to nocodazole. We find that both antibodies recognize coherent blocks of tissue in control and nocodazole-treated embryos (Fig. 5), indicating that differentiation occurs in the presence of nocodazole, and development in general is not blocked by drug treatment.

There is however a significant difference in the amount of dorsal mesodermal tissue in the embryos treated at different stages. While there is an anteriorly/posteriorly complete notochord and two somite files in control embryos (Fig. 5A), embryos exposed to nocodazole before stage 10 have a very small, button-like notochord approximately five cells in height,
with small blocks of somitic tissue to either side (Fig. 5B), located directly above the dorsal lip. At stage 10, Spemann's organizer extends approximately 15 cells animalward from the dorsal lip (excluding the bottle cells, R.K., unpublished observations); thus, not all of the prospective notochord field within the nucodazole-treated organizer forms notochord. Likewise, cells in the lateral marginal zone, which normally form somitic tissue, do not do so. From these results we conclude that not all of the tissue fated to form axial and paraxial mesoderm actually differentiates into notochord and somites in embryos treated with nucodazole before stage 10]. This implies that the inductive interactions required for formation of the entire axis are interrupted as a consequence of microtubule depolymerization.

In contrast, extensive axes and archenterons form in embryos exposed to nucodazole at or after stage 10] (Fig. 5C,D). This indicates that more axial mesoderm differentiates in embryos treated with nucodazole at stage 10]. Treatment at this stage usually results in two types of embryos: a less advanced type (Fig. 5C), with a protruding yolk plug, and a type that resembles the embryos treated with nucodazole at stage 11 (Fig. 5D); in these latter embryos, the blastopore is completely closed. Neither type of embryo forms a neural tube from the neural plate (see Fig. 5E,F below). Otherwise, these embryos appear to reach the stage of development when the tailbud assumes dorsal mesoderm production.

We determined the location of the notochordal and somitic tissues in the marginal zone by sectioning immunostained embryos. Prior to involution, the tissues destined to form notochordal and somitic tissues lie immediately beneath the single-layered suprablastoporal epithelium. Intercalation results in involution of the marginal zone and post-involution mesoderm lies beneath the two cell-layered neural plate. 12-101- and Tor 70-positive mesoderm cells lie just below the superficial layer, indicating the mesoderm did not involute. In addition, sectioning demonstrates that no archenteron is present in the embryos treated with nucodazole prior to stage 10], whereas archenterons are present and the notochord and somites occupy post-involution positions in embryos treated after stage 10] (compare Fig. 5E and F; see Table 1]. These results indicate that differentiation can occur when dorsal mesoderm fails to involute into the interior of the embryo.

**The effect of nucodazole on the gastrulation movements and posterior mesoderm differentiation are reversible**

After 1-7 hours of exposure beginning at stage 10], nucodazole was washed away and the embryos cultured until controls reached stage 23-27. Microtubules return (not shown), the embryos gastrulate and form somite files, a midline notochord and an archenteron (Table 1 and Fig. 5G,H below), indicating that nucodazole treatment is reversible. There were no apparent differences between embryos rescued after 1-7 hours of nucodazole exposure from controls.

**Individual cell motility is not inhibited by nucodazole treatment**

Differentiation of notochordal and somitic tissues in pre-involution positions raises the possibility that nucodazole-treated embryos fail to gastrulate because cells are not motile. We tested this hypothesis by grafting isochronically single clumps of labeled cells into unlabeled, dorsal open-faced explants (Fig. 6A) at stages 10] or 10], in the presence or absence of nucodazole. In some cases we video-recorded these explants to determine whether cells were motile, and in others we examined the explants at control stage 20 for dispersal of the labeled cells as a measure of their motility.

**Table 2. Effect of microtubule perturbation on convergent extension morphogenesis in dorsal open face explants**

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Using low-light-level videomicroscopy, we find that, in all situations, grafted cells are actively motile and disperse as they incorporate into the explant. In the control explants made at stages 10^9 and 10^1, labeled cells intercalate with host cells and assume notochordal and somitic morphologies (Fig. 6B), and the explants undergo convergent extension. In explants made at stage 10^9 and cultured in nocodazole, labeled cells disperse, but the host explants do not undergo convergent extension, nor do the notochord/somite boundaries form. Video recordings demonstrate that cells remain motile for at least 7 hours in nocodazole (Fig. 7). However, no axis is constructed, and the cells do not assume notochordal or somitic morphologies (Fig. 6C). In explants made at stage 10^9 and treated with nocodazole, an axis forms, and grafted cells disperse and join with host cells in notochordal (arrow, Fig. 6D) and somitic tissues. Clearly, cells are not paralyzed by nocodazole treatment at gastrula stages.

DISCUSSION

The vegetal alignment zone forms in the preinvolution mesoderm of the dorsal marginal zone at stage 10

The original observation of the vegetal alignment zone in shived, dorsal marginal zone explants led Keller et al. (1992) to suggest that the vegetal alignment zone formed in postinvolution mesoderm. In the proposed model for gastrulation, mediolateral cell intercalation commenced only after the first mesoderm cells rolled over the dorsal lip into the interior of the embryo (as a result of radial intercalation, bottle cell formation and head mesoderm migration). Our observations indicate that mediolateral intercalation behavior and vegetal alignment zone formation commence in preinvolution mesoderm.

Convergent extension requires microtubules before, but not after, stage 10

Exposing embryos at an early stage to nocodazole severely affected morphogenesis. Whole embryos cultured continuously in nocodazole prior to stage 10^9 failed to form a vegetal alignment zone and gastrulate. Embryos treated at stage 10^9 formed mediolaterally elongated cells, gastrulated and formed an axis, in agreement with the results of Cooke (1973) and Nakatsuji (1979). Exposing open-faced explants to nocodazole before stage 10^9 prevented convergent extension, while exposing explants to nocodazole after stage 10^9 did not prevent convergent extension. We conclude that convergent extension requires an intact microtubule array in the embryo only until stage 10^9, coincident with the time at which the vegetal alignment zone forms. After stage 10^9, bipolar cell formation and convergent extension proceed in the absence of microtubules until the tailbud assumes axis production.

Although our data confirm and extend the results of Cooke, we differ in our determination of the stage at which axial morphogenesis becomes insensitive to microtubule depolymerization. While Cooke reported that the critical change in sensitivity occurred between stages 8 and 9 (see Fig. 1, Cooke, 1973), we found that the critical time is between stages 10^9 and 10^1. Since our treatment resulted in the same morphological series observed by Cooke, we interpret the difference in timing to reflect a difference in efficacy of the treatments used in the two studies. In our hands, nocodazole at 15 μg/ml disrupts the microtubules throughout the mesoderm and endoderm in intact embryos in 15 minutes. There was no analysis of the time course of action of the 0.5 μg/ml colcemid treatment used in the earlier study, but it is unlikely that such a low concentration of colcemid acted as rapidly as the relatively high concentration of nocodazole we employed. This may underlie the observed timing difference between the two studies.

Nocodazole inhibits tissue-level morphogenesis, but not cell motility

The experiments grafting clumps of fluorescently labeled cells demonstrate that embryonic cells are motile in the presence of nocodazole. In general, cultured cells do not require microtubules to migrate randomly; they require microtubules to polarize protrusive activity and undergo directed motility (Vasliev et al., 1970; Gail and Boone, 1971). It is not surprising then that labeled cells grafted onto a stage 10^9 dorsal open face explant and treated with nocodazole disperse in the explant, indicating the cells are motile, while the explant itself does not undergo morphogenesis, which apparently requires polarization of a population of cells, i.e. the vegetal alignment zone, to organize tissue movements. No vegetal alignment zone is observed in the organizer region when embryos are treated with nocodazole before stage 10^9. These results indicate that, although the cells remain motile, they do not restrict protrusive activity to their medial and lateral ends. Cell intercalation occurs, but it is not directed in the mediolateral axis. Thus, convergence at the dorsal midline does not occur, and the anterior/posterior axis does not extend.

Immunostaining of nocodazole-treated explants for microtubules confirmed that the nocodazole effectively disrupted all of the microtubules in the mesoderm. This leads us to conclude that once the vegetal alignment zone has formed, the disruption of microtubules has little consequence for the elongation and bipolar protrusive activity of the axial and paraxial mesoderm cells that lie posterior to the vegetal alignment zone. Apparently, though posterior cells lack microtubules, they can read cues from the cells that polarized in the first arcs of the vegetal alignment zone, and adopt mediolateral intercalation behavior. A possible explanation for this phenomenon is that the bipolar cells in the vegetal alignment zone organize fibrillar extracellular matrix in their local environment. Adjacent cells sense the organized matrix, adopt a bipolar morphology and subsequently organize their local matrix, passing the information on to their posterior neighbors.

The specificity of nocodazole treatment

Experiments based on an inhibitor are limited by the specificity of the inhibitor. We chose nocodazole for our studies because, in comparison with other microtubule-depolymerizing drugs, nocodazole has no known side effects and is the most readily reversed. However, one must keep in mind that, first, nocodazole may affect cellular structures other than microtubules, and second, depolymerization of microtubules may affect more processes than cell polarization. We have addressed some of these concerns in experiments not included in this report.

Much more is known about the action of nocodazole in cultured cells than in embryos. We have repeated some of the
experiments performed on cell lines with Xenopus embryos, and found no aberrant results, suggesting that nocodazole’s effects in embryos are similar to its effects in single cells. We have reduced the temperature (to 13-16°C) during nocodazole exposure, which normally reduces the effectiveness of nocodazole in disassembling microtubules. We found that embryos retained numerous microtubules, gastrulated, and formed dorsal, axial structures. We cultured embryos at various concentrations of nocodazole and found that lower concentrations, which did not block morphogenesis, did not disrupt all of the microtubules. We also co-cultured with taxol and nocodazole, which in rare instances has been reported to protect microtubules from depolymerization. We did not find this result using Xenopus embryos; the microtubules were disassembled and the embryos did not gastrulate. Finally, we examined a panel of marginal zone-specific marker genes by in situ hybridization and found that these genes were turned on in their normal distributions in nocodazole-treated embryos. These results suggest that nocodazole acts in embryos as it does in cultured cells and that the principal target of the reagent is the microtubules.

While our results demonstrate that nocodazole prevents the formation of bipolar cells and the vegetal alignment zone, microtubule disruption should have other effects on the embryo. In particular, secretion of many of the putative inducing factors identified in Xenopus may be reduced by treatments that depolymerize microtubules. Secretion is not completely blocked, since the Tor 70 epitope is synthesized and transported to the extracellular compartment in the presence of nocodazole. We are presently testing whether exogenous factors such as noggin, activin or FGF can rescue either differentiation or morphogenesis in nocodazole-treated embryos.

A second potential target of nocodazole could be intermediate filaments. Microtubules and intermediate filaments are physically connected and microtubule depolymerization can cause intermediate filaments to collapse to a perinuclear distribution in cultured cells. Two groups have shown that perturbation of intermediate filaments (using either antisense oligos or microinjected antibodies) in early Xenopus development causes gastrulation defects (Torpey et al., 1992; Klymkowsky et al., 1992). Both groups suggested that disrupting the intermediate filaments would affect the biomechanical properties of cells, and that this might underlie the observed defects. Although it would be informative to determine whether any part of the organizer and marginal zone differentiates in such embryos, there is no data that suggests that intermediate filaments are involved in the establishment of cell polarity, and we have shown that nocodazole prevents cell bipolarization in the embryo; thus, we believe that the microtubules themselves are the important cellular target of nocodazole in our studies.

Disruption of the microtubules reveals two domains of both notochordal and somitic tissues in the marginal zone.

Our results demonstrate that the differentiation of both notochordal and somitic tissues in the head and trunk regions of the embryo are differentially sensitive to microtubule disruption before stage 10]. According to the fate map (Keller, 1975), the small notochord and somites observed in embryos exposed to nocodazole prior to stage 10] (Fig. 5B) are the most anterior notochordal and somitic tissues present in the embryo. Clearly, not all of the cells in the embryo fated to form notochord differentiate as notochord under these circumstances, suggesting that within Spemann’s organizer, two domains comprise the notochord field. One domain, comprising the anterior notochord, differentiates independently of microtubules and the vegetal alignment zone. The second group of cells requires an intact microtubule array until stage 10] in order to undertake mediolateral intercalation behavior and differentiate into posterior notochord. A connection between notochord differentiation and mediolateral intercalation behavior was observed previously by Domingo and Keller (1995). The observation of two domains supports the finding of Stewart (1990) that the notochord field in the upper dorsal marginal zone cannot autonomously differentiate into notochord in the late blastula (stage 9).

The fated somitic territory also exists as two domains, which extend around the marginal zone towards the ventral side. Only the anterior somitic tissue that lies within or near the organizer proper differentiates into somitic tissue in stage 10]-treated embryos. Cells lying in the lateral marginal zone, which are fated to form posterior somites, do not differentiate as somitic tissue when the microtubules are disrupted prior to stage 10]. Thus, both the notochord and the somites are constructed from two domains that differ in the ability to differentiate autonomously in the early gastrula embryo. The anterior domains of both tissues do not require intact microtubule arrays after stage 10], while induction of the posterior notochordal and somitic domains requires that microtubules be present until stage 10].

A short notochord/somite boundary is visible in the nocodazole-treated embryos immunostained with Tor 70. We do not know if this boundary forms in the manner observed in the shaved explant. The first indication of the notochord/somite boundary in culture is a small cleft located within the vegetal alignment zone at stage 11] (Fig. 4, Shih and Keller, 1992b). The boundary then spreads primarily towards the posterior, but also towards the anterior (towards the dorsal lip in the explant, but away from the dorsal lip in the intact embryo because of involution). Based on the stage 10 fate map, the position of the vegetal alignment zone, and the size of the notochord ‘button’ in nocodazole-blocked embryos, we suspect that the small notochord and somites in the nocodazole-treated embryos are the cells located between the vegetal alignment zone and the dorsal lip in Spemann’s organizer (Fig. 1C). If this is the case, then anterior axial and paraxial mesoderm form independently of the vegetal alignment zone while the differentiation of posterior axial and paraxial mesoderm is apparently dependent on another event, which we propose is formation of the vegetal alignment zone. This is presently under investigation.

Several lines of evidence suggest that the anterior and posterior notochord and somites in Xenopus are inherently different. The anterior and posterior notochord may form by different morphogenetic mechanisms (Novoselov, 1995). The most anterior five or six somites are derived entirely from the two dorsoanterior blastomeres at the 4-cell stage; more posterior somites have complex origins from both the dorsoanterior and posterior blastomeres of the 4-cell embryo (Cooke and Webber, 1985; ‘dorsoanterior’ and ‘posterior’ as used by these authors are currently referred to as ‘dorsal’ and ‘ventral’ respectively; we employ Cooke and Webber’s termi-
technology as it reflects more accurately the fate of the blastomeres at the 4-cell stage). The anteriormost five somites in *Xenopus* degenerate regardless of their location in the embryo, while posterior somites do not (Chung et al., 1989). Recently, Smith and his colleagues (Conlon et al., 1996) have shown that posterior notochord and somite formation are inhibited by a fusion protein in which the transcriptional activation domain of the *Xenopus brachury* protein was replaced by a repressor domain from the *Drosophila engrailed* protein. Anterior mesoderm was largely unaffected in these embryos. Such observations indicate that anterior and posterior mesodermal structures in *Xenopus* may differ more than their gross morphology suggests. Our observation that microtubule disruption

![Fig. 5. Nocodazole-blocked embryos express notochordal and somitic differentiation markers. Anterior is to the left in A,C and D. Animal is up in B.](image)

(A) Dorso-vegetal view of a control embryo, immunostained with the Tor 70 and 12-101 antibodies at stage 23. (B) Dorsal view of an embryo treated with nocodazole from stage 10s, at stage 23. A button-like notochord (n) with small blocks of somitic tissue (s) on either side sits above the dorsal lip of the blastopore (arrows). (C) Dorsal view of an embryo treated with nocodazole from stage 10s, at stage 23. More notochordal and somitic tissue is present and arranged as an axis. Yolk endoderm (y) protrudes from the blastopore. (D) Lateral view of an embryo treated with nocodazole at stage 11. The dorsal mesoderm consists of an extended notochord with two paraxial somitic files. The archenteron (ar) is present ventral to the axial mesoderm. (E) A transverse section through the axial mesoderm of an embryo similar to the one shown in (B). No archenteron is present and notochordal and somitic tissues are in their pre-involution positions. (F) A transverse section through an embryo similar to the one shown in C. Notochordal and somitic tissue are situated in their post-involution positions and an archenteron is present. (G,H) Gastrulation is rescued when nocodazole is withdrawn. (G) Lateral view of an embryo treated with nocodazole for 2 hours beginning at stage 10s, and subsequently rescued. After removal of the drug, morphogenesis resumes. The notochord, somites and archenteron are visible. (H) A cross-section of an embryo similar to the one shown in G. The dorsal mesodermal structures are in their normal post-involution positions, and an archenteron is present. vp, vegetal pole; an, animal pole.

![Fig. 6. Nocodazole does not inhibit cell motility. Rhodamine-dextran-labeled cells were grafted to explants of the dorsal marginal zone, and the explants treated with DMSO or nocodazole.](image)

(A) A clump of labeled notochordal cells grafted into the notochordal region, after 15 minutes. (B) At stage 20 in a control explant constructed at stage 10s, the clump of cells is dispersed in an explant that converged and extended. Labeled cells joined with host cells in forming notochordal and somitic tissue. (C) By stage 20 in a nocodazole-treated explant constructed at stage 10s, the labeled cells are dispersed, demonstrating that cells are motile. The explant did not converge and extend, and cells did not assume notochordal nor somitic morphologies (arrow). (D) By stage 20 in a nocodazole-treated explant constructed at stage 10s, labeled cells have assumed notochordal morphology (arrow) in an explant that underwent convergent extension. Dashed lines indicate the edges of explants. Scale bar, 35 μm.
of nocodazole exposure. A third cell has surfaced (large open arrow). Every cell, including those in clumps, undergoes extensive shape changes between 5 and 7 hours after nocodazole treatment was initiated. (A) Two groups are visible. (B) Approximately 1 hour later, the clumps have changed slightly, and two cells (marked with arrows) have moved into view from deeper in the explant. (C) Approximately 30 minutes later, one cell (straight arrow) has migrated laterally and another cell (small curved arrow) has migrated posteriorly in the explant. A third cell has surfaced (large open arrow). Every cell, including those in clumps, undergoes extensive shape changes between 5 and 7 hours of nocodazole exposure.

The two-domain structure of Spemann’s organizer

Spemann’s organizer is defined empirically as the tissue in the amphibian dorsal marginal zone that induces a second embryonic axis when transplanted to the ventral marginal zone of an early gastrula stage embryo. The experiments of Nieuwkoop, Gerhart and their colleagues have led to the proposal that multiple, distinct organizers acting in a temporal and spatial succession are involved in anterior/posterior axis specification in Xenopus (Gerhart et al., 1991). The ‘multiple organizer’ hypothesis is summarized below and in Fig. 8A.

While the ventrolateral vegetal blastomeres induce mesoderm in the ventrolateral marginal zone (Nieuwkoop, 1969a), the dorsal vegetal cells, or Nieuwkoop Center, induce the overlying marginal zone to form the late blastula organizer (LBO). The late blastula organizer, located in the vegetal half of Spemann’s organizer at stage 9, includes head mesoderm, some prospective notochordal and somitic tissue in the deep layers, and pharyngeal endoderm in the epithelial layer (Stewart, 1990; Stewart and Gerhart, 1991). The late blastula organizer induces the gastrula organizer (GO), or ‘notochord territory’, immediately above it in the animal half of Spemann’s organizer sometime after stage 9 (Stewart, 1990; Gerhart et al., 1991). The gastrula organizer differentiates as notochord, executes convergent extension, and dorsalinizes the lateral marginal zone. Our results provide further insight into the structure and function of these organizers, and the time at which the gastrula organizer exerts its influence.

Our data support the conclusion of Stewart (1990) that Spemann’s organizer consists of two domains, which differ in their ability to differentiate autonomously into notochordal tissue. (Stewart, 1990; Gerhart et al., 1991). In addition, our results (Fig. 5B) provide a graphic demonstration of the late blastula organizer proposed by Stewart and Gerhart, and reveal that the animal half of Spemann’s organizer (the gastrula organizer) cannot become autonomous for notochord differentiation before stage 10. Results presented here indicate that the vegetal alignment zone is not required to form the most anterior notochordal and somitic tissue, but is required to organize or induce the posterior notochordal field, to generate posterior somites in the lateral marginal zone and to execute convergent extension.

These observations lead us to make four proposals. First, we propose that the late blastula organizer includes the head mesoderm cells, the pharyngeal endoderm cells and the most anterior notochordal and somitic cells that will lie vegetal of the vegetal alignment zone at stage 10 (Fig. 8B). Given this composition, the late blastula organizer may be perceived as the anterior, or head, organizer. Second, we propose that the anterior notochord and somites differentiate independently of the formation of the vegetal alignment zone. Third, because the vegetal alignment zone is required to execute convergent extension, to organize the posterior notochordal field, and to generate the posterior somitic tissue in the lateral marginal zone, we propose that the vegetal alignment zone is the gastrula organizer, or a critical component of this organizer. These functions define the gastrula organizer as the trunk organizer. Finally, we propose that the late blastula organizer operates until stage 10, when the gastrula organizer exerts its influence. This result in Xenopus agrees with the findings in Cynops that the notochord is induced some time after the early gastrula stage (Kaneda, 1980, 1981).

Our understanding of the molecular nature of the signals involved in the activities of Spemann’s organizer grows increasingly complex. We envisage that cells throughout the marginal zone receive and integrate multiple signals, and subsequently initiate responses. For a subpopulation of cells in the dorsal marginal zone, this response is to undertake mediolateral intercalation behavior, which begins internalization of the mesoderm in the marginal zone and leads ultimately to the generation of the dorsal, axial structures in Xenopus.
REFERENCES


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