Self-Organization of Vertebrate Mesoderm Based on Simple Boundary Conditions

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Embryonic development requires cell movements whose coordination is robust and reproducible. A dramatic example is the primary body axis of vertebrates: despite perturbation, cells in prospective axial tissue coordinate their movements to make an elongated body axis. The spatial cues coordinating these movements are not known. We show here that cells deprived of preexisting spatial cues by physical dissociation and reaggregation nonetheless organize themselves into an axis. Activin-induced cells that are reaggregated into a flat disc initially round up into a ball before elongating perpendicular to the disc. Manipulations of the geometry of the disc and immunofluorescence micrography reveal that the edge of the disc provides a circumferential alignment zone. This finding indicates that physical boundaries provide alignment cues and that circumferential “hoop stress” drives the axial extrusion in a manner resembling late-involuting mesoderm of Xenopus and archenteron elongation in other deuterostome species such as sea urchins. Thus, a population of cells finds its own midline based on the form of the population’s boundaries using an edge-aligning mechanism. This process provides a remarkably simple organizing principle that contributes to the reliability of embryonic development as a whole. Developmental Dynamics 231:576–581, 2004.

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INTRODUCTION

Embryonic development is evolved to be robust and reproducible (Kirschner and Gerhart, 1998). This finding is often expressed in terms of redundancy in regulatory networks, but it applies just as significantly to cell movements. A dramatic example is the primary body axis of chor-dates and vertebrates in particular: despite perturbation, cells in prospective axial tissue coordinate their movements to make an elongated body axis (Keller et al., 1985; Munro and Odell, 2002). Although the cell movements involved in this morphogenesis have been described in exquisite detail, the nature of the cues that coordinate these movements and provide the necessary robustness is not known.

The vertebrate embryo transforms itself during development from a one-layered structure into a three-layered one and from a round shape into an elongated one by means of the well-studied orchestration of cellular movements and behaviors collectively known as gastrulation. The best known of the cell behaviors is mediolateral intercalation in which mediolaterally elongated cells crawl between one another to drive narrowing and elongation of axial mesoderm (Keller, 2002; Wallingford et al., 2002). A fundamental but unanswered question is where the directional information that initiates and coordinates the orientation of these movements comes from. The initial randomly oriented protrusive activity of mesodermal cells first becomes oriented at the intersection of the future notochord-somite boundary (NSB) and the vegetal alignment zone (VAZ; Domingo and Keller, 1995; Lane and Keller, 1997). There is
no evidence for a midline signaling center that would orient and attract these movements (Keller et al., 2000). Despite the beauty of the observations and the models that describe this process, they do not adequately explain why notochordless ultraviolet (UV)-ventralized embryos can still undergo gastrulation (Gerhart and Keller, 1986), and, therefore, how cell intercalation becomes oriented in the absence of a NSB. Furthermore, the absence of evidence for a midline signaling center does not constitute evidence of its absence, and a formal test of this model has been lacking.

Many aspects of axial convergent extension, including migratory and protrusive cell behaviors characteristic of endogenous mesoderm, can be mimicked by growth factor treatment, especially activin treatment, of animal pole explants (animal caps; Symes and Smith, 1987). Elongation of animal cap explants has now become a commonplace assay for convergent extension, in part because the assay is relatively straightforward and provides great manipulative freedom (Green, 1999; Yao and Kessler, 2000). Convergent extension of animal caps also requires coordinated cell orientation.

Both mesodermal explants and animal cap explants contain potentially orienting information derived from the zygote. For mesodermal explants, cellular orientation clearly derives from that of the zygote, because all the relevant tissue arrangements are explanted intact. For ani-

Fig. 1. Aggregates of activin-treated cells elongate in vitro. a–f: Elongation is dose-dependent (treated with 0, 0.25, 0.5, 1.0, 2.0, and 4.0 units/ml activin, respectively). g,a–g': Elongation is evident at neurula stages (g, control embryo, stage 18) and is more pronounced by tail bud stage (aggregates a–f, control embryo, g', stage 24). h: Aggregates contain notochord (MZ15 antibody staining). Scale bars = 300 μm in a (applies in a–g'), 100 μm in h.

Fig. 2. a: Frames of a time-lapse video recording showing side and top views of the same elongating reaggregate at successive times shown. Arrows (center) show upward elongation (black arrow) and direction of toppling (white arrow). b: The shape of the initial disc of cells determines subsequent elongation: a disc of reaggregating cells with a "peninsula" (arrowed, left) rounds up but then elongates both on its main axis and on a branch at the site of the "peninsula" (arrowheads and three-headed arrows, right). Scale bar = 250 μm in a,b.

Fig. 3. Notching of the aggregate disc periphery inhibits elongation. a,b: Smaller excised piece and larger remaining aggregate (a) show much less coherent elongation that uncut induced aggregate (b), although a small elongated peninsula is sometimes seen (compare with Fig. 2b). c: Control uninduced aggregate.
mal cap explants, it has been shown that they are highly asymmetrical with respect to inducibility of dorsal–anterior- and ventral–posterior-derived halves (Sokol and Melton, 1991) and, thus, clearly contain potentially orienting information. It is plausible, therefore, that coherent convergent extension requires the orienting influence of something laid down earlier in development. The alternative hypothesis is that coherent convergent extension is generated autonomously and autocatalytically with minimal polarity information from prior events.

RESULTS

We examined the gastrulation behaviors of inner layer animal cap cells that had been dissociated and reaggregated to abolish preexisting polarity information. Unlike intact mesodermal explants and explanted animal caps, such cells contain no global intrinsic orientation deriving from the zygote. They are, thus, a good test of the autonomy of the cell orientation process. In previous work, we dissociated cells from animal cap explants by incubation in calcium-free medium and then treated them with the protein growth factor activin to make multiple mesodermal tissue types (Green et al., 1990). At the right dose, aggregates consist almost entirely of notochord, but surprisingly failed to elongate (Green et al., 1992). We had thought that this finding was because they lacked directional cues. However, in previous work, rapid reaggregation was achieved by low-speed centrifugation of cell suspensions (Green et al., 1990). This centrifugation step abolishes elongation ability (J.B.A. Green and A. De Jesus-Escobar, data not shown), perhaps because hydrostatic pressure during centrifugation disrupts microtubules that are essential for orientation of intercalation behavior (Lane and Keller, 1997). Convergent extension was observed when, instead of centrifuging the cells, they were allowed to reaggregate spontaneously by pipetting into a pile in medium containing calcium ions. Uninduced cells simply rounded up into a ball, but cells treated with 0.5–2 units/ml activin initially rounded up into a ball then reorganized themselves into an elongated shape (Fig. 1a). Thus, a population of cells deprived of all preexisting directional cues from the egg or zygote was able to reconstitute directional cues to organize a coherent axis. When allowed to differentiate, such axes contained notochord arranged cylindrically around the aggregate’s long axis (Fig. 1b).

To understand the mechanism of elongation, we made time-lapse video recordings (see Supplementary Material, which can be found at http://www.interscience.wiley.com/jpages/1058-8388/suppmat). In the initial stages, cells rapidly aggregated into a flat disc or pancake shape (Fig. 2a). This finding presumably represents simple maximization of cell–cell contact dominated by the initial condition that the cells are sitting on a flat surface. With time, these disc aggregates “rounded up” into a spherical shape, regardless of activin induction and with little influence of the initial disc shape, whether circular, ellipsoid, or irregular. This spherical shape would be predicted by a simple maximization of cell–cell contact. However, after this stage, control and activin-induced aggregates immediately diverged. Control aggregates remained spherical, whereas aggregates of induced cells elongated upward, perpendicular to the plastic or agarose-coated surface of the dish. Eventually, the now-elongated cell mass would topple over but continue elongating (Fig. 2a). The elongation and its upward orientation were highly reliable, occurring in 100% of cases.

Simple consideration of the asymmetries of the aggregate led to the conclusion that cells at the periphery of the disc stage are likely to experience the most asymmetrical environment and might, perhaps, provide the leading cue for the coordination of subsequent convergent extension. By altering the geometry of the cell aggregates at the disc stage, we were able to determine its significance for subsequent cell movements. First, we allowed the disc to form and then cut a notch or “cake slice” from it. This strategy dramatically inhibited convergent extension (Fig. 3), suggesting that the disc periphery is indeed an important component of the elongation mechanism. We also made video recordings of aggregates with small peninsulas (Fig. 2b and Supplementary Material). Like the other aggregates, these rounded up into spheres, with no obvious appendage at the site of the preceding peninsula. However, upon elongation, a secondary axis appeared at the site of the peninsula (Fig. 2b), showing the significance of the disc stage periphery and its significance at the disc rather than the subsequent ball stage.

We examined cell shape in aggregates by fixing and immunofluorescently staining for β-catenin protein to reveal cell outlines. Confocal sections showed that control aggregates had small lumens and contained cells with no signs of elongation (Fig. 4). Elongating aggregates, by contrast, contained many elongated cells arranged circumferentially around the geometrical center of the long axes of the aggregates (Fig. 4). The elongating cells were all aligned underneath a layer or two of nonelongated cells. The arrangement of cells was very reminiscent of that in posterior late-involved mesoderm of normal tadpoles (Keller et al., 1989; Hausen and Riebesell, 1991).

DISCUSSION

Our results prove that vertebrate axial cells—which may or may not be a homogeneous population—do not need global spatial cues from the egg or sperm to organize an axis. Instead, a population of such cells regenerates an axis based on the form of the population’s boundaries. This finding strengthens the hypothesis proposed by Keller and colleagues, that the shape of dorsal axial mesoderm is determined by tissue boundaries and the center of mass, not by some preexisting midline signal (Keller et al., 2000). Although sophisticated cell intercalation and bipolarity behaviors underlie this mechanism, it nonetheless provides a remarkably simple organizing principle whereby these behaviors are coordinated macroscopically.

Our data also suggest a mecha-
nism whereby the global orientation of convergent extension can be triggered with minimal (indeed, inevitable) spatial cues (Fig. 5). We observed that the superficial cells in the aggregates are not elongated and do not appear to adhere well to one another (Fig. 4). Therefore, if cells have a tendency to bipolarity and exert tensile force along their bipolar axis, this force will be random in the middle of an aggregate (Fig. 5a) but must be circumferential near its edge, where cells lack outward tension-generating neighbors (Fig. 5b). If there is positive feedback between tensile force and bipolarity, as demonstrated, for example, in bipolar fibroblasts cultured in collagen (Stopak and Harris, 1982), cells at the periphery will increasingly tend to align their long axes with each other circumferentially. Coherent alignment in response to an edge is observed in other systems (Elsdale and Wasoff, 1976; Bard, 1990). Circumferentially aligned cells will initially link up to create short arcs of aligned cells, eventually joining into hoops of tension running in a great circle within the aggregate (Fig. 5c). As these hoops contract they will extrude deep cells into an axis as circumferential intercalation shrinks the perimeter. Thus, a local cell behavior, namely tension-stimulated tensile bipolarity, would propagate by local mechanical interactions to achieve a macroscopic organization and to orient tissue shape change on a large scale.

Importantly, this mechanism would also apply at any boundary between a bipolar cell population and another for which its adhesiveness or tensile resistance is weak. In the aggregate, this could arise at the surface layer because superficial cells are a different cell type (due to signals within the aggregate) and/or because they are physically poorly anchored. In the embryo, the edge alignment mechanism would apply to the boundary between prospective endoderm and mesoderm—in other words, at the VAZ. Selective adhesion between germ layer cells is a well-established cell sorting behavior (Townes and Holtfreter, 1955) and is particularly evident between marginal zone and vegetal cells of Xenopus (Turner et al., 1989). The localization of the Xenopus VAZ has not been established precisely with respect to cell fate, but it is certainly in the vicinity of the junction between marginal chordamesodermal cells and more vegetal endodermal or mesendodermal cells.

An alternative a priori model of aggregate elongation could have included radial contraction mediated by radially elongated cells. Radial elongation does occur in vivo: it accompanies radial intercalation movements that cause spreading and thinning (i.e., epiboly) in the embryonic ectoderm (Keller, 1980; Marsden and DeSimone, 2001) and may play a role in axial extension after neural tube closure (Davidson and Keller, 1999). We did not see any indications of radial elongation, ruling out this mode of tissue extension.

The cellular morphology of the aggregates and the timing of elongation resemble that of late involuting mesoderm in Xenopus (see, for example, plate 22A of Hausen and Riebesell (1991)). It is also reminiscent of the radially symmetrical gastrulation of other species such as sea urchin, in which circumferential intercalation extends all the way around

**Fig. 4.** Cells within aggregate axis are circumferentially elongated. a–c: A montage of confocal sections collected through a bisected aggregate of deep ectoderm cells form cavities (a) and exhibit isodiametric cell shapes near the cavity (b) and far from the cavity (c). d: In contrast, a montage of confocal sections collected transverse to the axis of an activin-induced aggregate produces circumferentially elongated cells. e,f: The outer-most cell layer is not aligned, but alignment is strong two to three cells from the surface. Scale bars = 200 μm in a, 50 μm in b (applies to b,c,e,f), 120 μm in d.
The cellular morphology of the aggregates is indistinguishable from that of the central notochord region of the embryo undergoing dorsal convergent extension. In the latter, cells are generally bipolar and intercalate along their axis to shorten the tissue dimension in that direction. Only the notochord–somite boundary interrupts the propagation of arcs of alignment by anchoring cells at their lateral ends, in effect, limiting contraction to a segment of a hoop. More posteriorly, these arcs extend all the way around the blastopore. It therefore seems likely that hoop-stress-driven axial extrusion differs from dorsal convergent extension only in its circumferential extent.

Reaggregating activin-induced cells provides an accessible model system in which controlled geometry may enhance our ability to dissect the cellular and subcellular mechanisms of gastrulation morphogenesis. For example, this system could be used to analyze the gradual propagation of cellular alignment (which is seen in vivo) and to distinguish mechanical force from a graded external cue as the driver. The absence of a relatively impervious epithelial layer may also facilitate experiments with soluble factors added to the medium. Adding inhibitors, such as those for gap junctions, mitosis, transcription, and translation, at different times could be used to establish whether, when, and how particular programs of cell behavior are initiated. The morphogenetic effects of target protein depletion by antisense morpholino oligonucleotides of relevant proteins can also be analyzed in great detail. Likely interesting proteins would be those involved in planar cell polarity (PCP), because not only are several PCP-associated proteins implicated in convergent extension, but also the locally autocatalytic aspects of this model are reminiscent of a PCP propagation model recently proposed in flies (Tree et al., 2002). In that model, as in ours, relatively simple and subtle global cues are reinforced at the cellular level to align cell orientation. This type of mechanism for global and local polarization is evidently extraordinarily robust and resistant to radical perturbation.

EXPERIMENTAL PROCEDURES

Xenopus embryos and cells were obtained their animal caps excised in calcium- and magnesium-free medium as previously described (Green and Smith, 1990; Green et al., 1992). Xenopus activin B was made in baculovirus (materials were the gift of Dr. Doug Melton, Harvard University). For time-lapse recording, cells were pipetted next to a small 45 degree mirror (Edmund Optics, Inc., Barrington, NJ) mounted at approximately 60 degrees to the horizontal by use of a piece of modeling clay inside the Petri dish (Supplementary Fig. S1). Activin or control medium was used to fill the dish to the brim and a lid placed on top to prevent evaporation and rippling of the liquid surface. Images were captured using a Leica MZ stereo zoom microscope, a Q-Imaging Retiga digital CCD camera and Openlab (Improvision) software.

For fluorescent immunostaining of notochord, aggregates were fixed in buffered formaldehyde (MEMFA), embedded in acrylamide, frozen, cryostat-sectioned, and stained as previously described (Green et al., 1992). For immunostaining of β-catenin, aggregates were fixed in ice-cold Dent’s (80% methanol 20% dimethyl sulfoxide; Dent et al., 1989) and rehydrated and incubated overnight with rabbit polyclonal against β-catenin (Sigma; C-2206). After incubation with a rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) and washing, whole aggregates were cut with a scalpel transverse to the direction of elongation and the fragments dehydrated in ethanol and mounted in clearing medium (2:1 benzyl benzoate: benzyl alcohol). Cleared samples were mounted and sealed in shallow wells formed by nylon washers (Small Parts, Inc.; Miami Lakes, FL).
glued in place by finger nail polish (Sally Hanson “Hard as Nails,” clear; Farmingdale, NY). Serial optical sections were collected by using either a ×20 (dry lens; n.a. 0.70) or a ×60 (oil immersion; n.a. 1.40) and a confocal laser scan head (Nikon PCM2000; Melville, NY) mounted on an inverse compound microscope (Nikon). The eight-bit image stacks were transferred to a computer and analyzed with image processing software (ImageJ; Wayne Rasband NIH). Single image projections were created using the “brightest-point” algorithm.

NOTE ADDED IN PROOF

While this article was in press, Winklbauer and colleagues published data consistent with our findings, showing that an activin-inducible cell-type boundary corresponding to the VAZ aligns convergent cell movements (Ninomiya et al. (2004) Nature 430:364–367).

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