Morphological differences in *Xenopus* embryonic mesodermal cells are specified as an early response to distinct threshold concentrations of activin

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SUMMARY

The involution of presumptive mesoderm that occurs during amphibian gastrulation is a complex process requiring the coordinated action of a diverse range of cells. We show that cells with distinct morphologies, resembling each of those normally found in the involuting tissue of the *Xenopus* embryo, are induced in dispersed animal pole cells by different doses of the potent mesoderm-inducing factor activin. Each cell type is induced within a restricted dose range of activin concentrations, the boundaries of which are well demarcated shortly after activin treatment. In contrast, *Brachyury* and *goosecoid*, two genes thought to pattern the presumptive mesoderm, and the gene encoding platelet-derived growth factor receptor α, which is expressed in the mesoderm of gastrula stage embryos, are induced by broad, overlapping ranges of high activin concentrations at such early times. Similarly, the response of the gene encoding platelet-derived growth factor A, which is expressed normally in ectoderm of gastrula stage embryos, diminishes gradually as the activin concentration increases. Dose windows for the expression of these four genes narrow and become distinct from one another in cell aggregates after several hours in culture, suggesting that activin prompts a dynamic program of gene expression in induced mesoderm.

Key words: mesoderm induction, gastrulation, cell morphology, activin
in dispersed cells from the animal pole which do not normally exhibit these properties.

In this paper, we explore the possibility that different concentrations of a single mesoderm-inducing factor can specify distinct cell behaviors. Recently, Green et al. (1992) demonstrated that animal pole cells can distinguish small differences in the concentration of a mesoderm-inducing factor, activin, such that the expression of several genes occurs within tightly restricted dose windows. Moreover, activin induction alters the shape of animal pole cells and elicits spreading and migration on a fibronectin substrate (Smith et al., 1990; Howard and Smith, 1993). We find that the three cell types normally present in the DMZ can be induced from dispersed animal pole cells within distinct, narrow dose ranges of activin. The boundaries between these dose ranges are well demarcated prior to those for the expression of the gsc and Brachury (Xbra) genes, which are thought to pattern the marginal zone along its dorsoventral axis (Cunliffe and Smith, 1992, 1994; Niehrs et al., 1993, 1994). We go on to show that induced cells alter their pattern of gene expression over time suggesting that different doses of activin prompt distinct temporal and dynamic programs of gene expression. For any given gene, this appears as a sharpening of the dose windows for expression over time after activin treatment. We discuss how cell programming influences morphogenetic movements and gene expression in induced mesoderm.

MATERIALS AND METHODS

Embryos

Xenopus embryos were obtained by artificial fertilization as described by Smith and Slack (1983). They were chemically dejellied using 2% cysteine hydrochloride (pH 7.8), washed and transferred into Petri dishes containing 0.1× Marc’s modified Ringer’s (MMR; Peng, 1991). The embryos were staged according to Nieuwkoop and Faber (1967).

Mesoderm-inducing factor

Recombinant human activin was obtained by collection of serum-free medium conditioned by Cos cells electroporated with a stable human βA activin construct (the generous gift of Sergei Sokol).

Embryo manipulations (as diagrammed in Fig. 1)

Cell spreading assays

Animal pole explants were made at the mid blastula stage (stages 8-9) using electrolytically sharpened tungsten needles. Twenty explants were made for each sample. They were then transferred to a Petri dish coated with a thin layer of agarose containing Ca²⁺- and Mg²⁺-free medium (CMFM; Sargent et al., 1986). The outer layer of cells, which is difficult to dissociate, was discarded and the inner layer was dispersed into a single-cell suspension (Smith et al., 1990). The cells were cultured in a two-fold dilution series of activin concentrations for 30 minutes, rinsed in CMFM and, at the onset of gastrulation (stage 10), plated onto dishes coated with fibronectin. The cells were cultured in 0.75× MMR. Cell morphology was scored between stages 10.5 and 11. Three fields of cells from each dish were selected at random and photographed using a Nikon FM2 camera attached to an Olympus inverted microscope equipped with differential interference contrast optics. The identity of each cell was later assessed from the contact sheets.

Aggregate assays

Activin-treated animal pole cells were treated as described above (Cell spreading assays) except that after incubation in activin, the cells were rinsed in CMFM and aggregated at stage 10 by transferring them into agarose-coated dishes containing 0.75× MMR. Aggregates were cultured until either stage 11 or 17 when they were prepared for RNase protection analysis (see below).

Fibronectin-coated dishes

A 500 µl puddle of 10 µg/ml recombinant human fibronectin (Gibco) diluted in water was placed on a 35 mm Petri dish for 4 hours. The fibronectin was removed and the dish rinsed with phosphate-buffered saline (PBS). The non-specific binding sites were then blocked for 20 minutes with 0.1% bovine serum albumen (BSA) in PBS. The blocking solution was aspirated away before the cells were plated.

RNase protection analysis

Groups of 10 embryos or individual aggregates were frozen on dry ice in a minimum volume of 0.75× MMR and then stored at −80°C. Cells that had been cultured on fibronectin-coated dishes were lysed immediately after being photographed, at stage 11, in 1 ml of denaturing solution (Chomczynski and Sacchi, 1987), frozen and then stored at −80°C. RNA was prepared according to the method of Chomczynski and Sacchi (1987). RNase protection assays were carried out as described by Melton et al. (1984). The hybridization probes used were: PDGF-A (Mercola et al., 1988), protected fragment 230 bp; PDGFR-α (Jones et al., 1993), protected fragment 101 bp; Xbra, Xenopus Brachury gene homolog (Smith et al., 1991), protected fragment 214 bp; goosecoid organizer-specific homeobox gene probe consisting of a PstI fragment from the gscA cDNA clone (Cho et al., 1991) protected fragment 248 bp. The ubiquitously expressed gene elongation factor 1-α (EF1-α) was used as a loading control (Krieg et al., 1989). The probes were simultaneously hybridized to each sample. Autoradiography used Kodak X-Ormat film.

RESULTS

Three cell types can be distinguished in dispersed dorsal marginal zone (DMZ) explants

The diversity of dorsal marginal zone (DMZ) cells is evident when the inner, presumptive mesodermal cells are dissociated in medium lacking Ca²⁺ and Mg²⁺ (CMFM) at the onset of gastrulation (stage 10) and plated on a substratum of fibronectin. We can identify three populations of cells under these conditions: spread, motile cells (Fig. 2A,D; solid arrows), cells undergoing circus movements (see below; Fig. 2B,D; hollow arrows) and round cells (Fig. 2C,D; arrowheads).

Spread cells are flat, adherent and highly motile. They have a bipolar or multipolar morphology with prominent lamellipodia (Fig. 2A; large arrows) and fine filopodia (Fig. 2A; small arrow). The morphology of the spread cells resembles that of the migrating head mesoderm cells both in situ and when dispersed on a fibronectin substratum (Keller and Schoenwolf, 1977; Winklbauer et al., 1991). The spread cells also resemble presumptive notochord and somite cells as they appear in situ (Keller et al., 1989). When viewed in embryos by scanning electron microscopy (SEM), these cells appear bipolar with lamellipodia extending from their medial and lateral ends and filopodia extending from their anterior and posterior sides. When dissociated and plated on fibronectin, however, about half appear spread and elongated and the remainder undergo circus movements as discussed below (Winklbauer, 1990).

The term circus movement describes the formation of a bleb or blebs of cytoplasm at the cells’ periphery (Fig. 2B; hollow...
Mg\textsuperscript{2+}-free medium (CMFM). The outer pigmented layer of cells was in 0.75 × CMFM and either plated on fibronectin-coated dishes or aggregated of activin concentrations for 30 minutes. They were then washed in discarded. Cells were cultured dispersed in a two-fold dilution series of activin before being washed, aggregated and on fibronectin-coated dishes at the onset of gastrulation in sibling embryos (stage 10).

Fig. 1. A schematic representation of the induction protocol. Blastula stage (stages 8-9) animal pole explants were dispersed in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free medium (CMFM). The outer pigmented layer of cells was discarded. Cells were cultured dispersed in a two-fold dilution series of activin concentrations for 30 minutes. They were then washed in CMFM and either plated on fibronectin-coated dishes or aggregated in 0.75× MMR.

The round cells are attached to the fibronectin and occasionally have fine filopodia. While their counterpart in the embryo is not known they are probably ectodermal cells because they are the most abundant cell type in dissociated animal pole explants (see below).

Cells resembling each of the cell types in the DMZ can be induced by distinct activin concentrations

Animal pole tissue, which would normally form ectoderm, can be induced to form mesoderm by treatment with the potent mesoderm-inducing factor activin (for reviews see Sive, 1993; Slack, 1993). The response of the animal pole tissue depends on the concentration of activin used. High activin concentrations induce dorsoanterior mesoderm, whereas low activin concentrations induce more lateral and posterior mesoderm. Green et al. (1992) have shown that, when animal pole cells are dispersed in activin before being washed, aggregated and cultured, their response to activin changes sharply over narrow dose ranges. Thus, it has been suggested that mesodermal pattern may arise by differential responses to different concentrations of mesoderm-inducing factors (Green and Smith, 1990; Green et al., 1992). We chose, therefore, to examine whether graded doses of activin can induce cells with morphologies and behaviors like those present in the DMZ.

We find that populations of cells enriched for particular DMZ cell morphologies can be made by induction of dispersed animal pole cells in different activin concentrations when they are plated onto fibronectin-coated dishes at stage 10 (Fig. 1). High concentrations of activin (20 units/ml) induce spread, motile, bipolar and multipolar cells with lamellipodia and filopodia (Fig. 2E). Intermediate activin concentrations (2 units/ml) induce cells that exhibit circus movements (Fig. 2F). Low activin concentrations (0.2 units/ml) or no activin give rise to mostly round cells (Fig. 2G).

The results of a typical two-fold dilution series of activin concentrations (of a total of 6 experiments) are shown in Fig. 3. We find distinct boundaries between the activin levels that induce each cell population (Fig. 3A-C). At high activin concentrations spread cells are most abundant (Fig. 3A). As the concentration of activin is decreased, the proportion of spread cells also decreases (Fig. 3A) whereas the proportion of circus movement cells increases (Fig. 3B). At the lowest doses of activin, the proportion of circus movement cells is small and round cells predominate (Fig. 3C). These data suggest that a gradient of activin-like activity in the embryo could account for the morphology and behavior of cell types normally found in the DMZ.

Gene expression patterns of cells cultured on fibronectin

We have examined whether the distinct cell populations induced by graded activin doses correlate with the expression of three genes, *Xenopus Brachyury* (*Xbra*), *goosecoid* (*gsc*) and platelet-derived growth factor receptor \(\alpha\) (*PDGFR-\(\alpha\)*), which are expressed normally by marginal zone tissue as well as a fourth, platelet-derived growth factor (*PDGF-A*), which is expressed by ectoderm. In the mouse, the *Brachyury* gene is required for mesoderm formation (Chesley, 1935; Grüneberg, 1958; Herrmann et al., 1990). Loss of its function leads to a disturbance of the primitive streak so that insufficient mesoderm is generated during gastrulation (Chesley, 1935; Grüneberg, 1958). In gastrula stage *Xenopus* embryos, the entire marginal zone expresses *Xbra* mRNA (Smith et al., 1991). By mid gastrula stages, however, *Xbra* expression is confined to the notochord and a ring around the yolk plug (Smith et al., 1991). *gsc*, a homeobox gene, is expressed specifically in the dorsal blastopore lip of the *Xenopus* gastrula (Blumberg et al., 1991; Niehrs et al., 1994). Microinjection of *gsc* mRNA into either dorsal or ventral blastomeres causes cells that inherit this factor to move towards the anterior end of the embryo, thus implicating *gsc* in the control of gastrulation movements (Niehrs et al., 1993). Both *gsc* and *Xbra* mRNA are induced as an immediate early response to mesoderm-inducing factors (Cho et al., 1991; Smith et al., 1991). Zygotic transcripts for *PDGFA* and *PDGFR-\(\alpha\)* are expressed in the ectoderm and presumptive mesoderm, respectively, and appear at stage 10, coincident with the onset of gastrulation (Jones et al., 1993; Lap Ho and MM, unpublished observations). Mesoderm induction by either activin or vegetal cells suppresses *PDGFA* mRNA in cultured animal pole explants while inducing *PDGFR-\(\alpha\)* mRNA (Jones et al., 1993). Following the assessment of cell morphology, at the early to mid gastrula stage (stage 10.5-11), cells cultured on fibronectin-coated dishes were lysed in denaturing solution and processed for RNase protection analysis. Results from typical protection assays (of a total of 4 experiments) are shown in Fig. 4. We find that the expression of mRNA for gsc, *Xbra* and *PDGFR-\(\alpha\)* is almost coincident at the high dose range (Fig. 4). *PDGFA* mRNA expression is detected at all doses, however, its expression is strongest at low activin concentrations, decreasing in level as gsc, *Xbra* and *PDGFR-\(\alpha\)* increases.
These data suggest that there is no direct correlation between the expression patterns of gsc, Xbra and PDGFR-α and the induction of one particular cell type. However, these three genes are expressed predominantly by populations enriched for spread and circus movement cells whereas PDGF-A is expressed mostly in populations enriched for round cells.

In addition, we find that the dose ranges for expression of mRNAs for Xbra, gsc, PDGFR-α and PDGF-A are broad and that the levels of expression change gradually as a function of activin dose (Fig. 4). This contrasts with the narrow, sharp boundaries of gene expression observed in response to threshold activin concentrations in aggregates of induced cells cultured until the equivalent of the late neurula stage (stage 17;
To understand how sharp threshold concentrations of activin might arise, we have investigated how aggregation and length of time in culture influence gene expression. Time in culture alters the expression pattern of molecular markers in aggregates. Animal pole cells were dispersed at stage 9 and treated with a two-fold dilution series of recombinant activin conditioned medium. The cells were rinsed in CMFM and, at stage 10, were plated onto fibronectin coated dishes. Cell morphology was assessed between stages 10.5-11. Approximately 150 cells were scored for each data point. (A) High activin concentrations induce spread cells (black bars); (B) intermediate activin concentrations induce circus movement cells (shaded bars); (C) and low or zero activin concentrations give round cells (hatched bars).

Fig. 3. Different activin concentrations induce cells exhibiting distinct morphologies. Animal pole cells were dispersed at stage 9 and treated with a two-fold dilution series of recombinant activin conditioned medium. The cells were rinsed in CMFM and, at stage 10, were plated onto fibronectin coated dishes. Cell morphology was assessed between stages 10.5-11. Approximately 150 cells were scored for each data point. (A) High activin concentrations induce spread cells (black bars); (B) intermediate activin concentrations induce circus movement cells (shaded bars); (C) and low or zero activin concentrations give round cells (hatched bars).

Fig. 4. gsc, Xbra, PDGFR-α and PDGF-A genes are expressed by induced animal pole cells plated on a fibronectin substratum in response to broad, overlapping ranges of activin concentrations. RNA extracted from cells that had been cultured on fibronectin-coated dishes until sibling embryos had reached stage 11 was analyzed by RNase protection analysis. The sibling control lane corresponds to RNA isolated from 10 whole stage 11 sibling embryos. The expression of gsc and Xbra genes are examined in one experiment (upper gel) and that of PDGF-A and PDGFR-α genes in another (lower gel). The gene EF1-α was used as a loading control.

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Time in culture alters the expression pattern of molecular markers in aggregates

Animal pole cells were dispersed in activin before being rinsed and aggregated at stage 10. Aggregates were then cultured until stage 11, the time at which the cells cultured on fibronectin-coated dishes were analyzed, or until stage 17. Results of typical experiments (of a total of 5 experiments at each stage) are shown in Fig. 5. At stage 11 (Fig. 5; upper gel), the general pattern of gene expression is similar in aggregates to that of the cells cultured on fibronectin-coated dishes. gsc, Xbra and PDGFR-α are expressed coincidentally at the high concentration range of activin. Xbra and PDGFR-α mRNAs are only expressed at high activin doses. In contrast, while gsc mRNA is strongly induced by high activin doses, a low level is present at all activin concentrations and in the absence of activin. PDGF-A is expressed at low activin concentrations, decreasing as the levels of Xbra, gsc and PDGFR-α increase. In contrast, at stage 17 (Fig. 5; lower gel) gsc and PDGFR-α are expressed only at high activin concentrations. As the concentration of activin is decreased the expression of gsc and PDGFR-α diminishes sharply and Xbra expression is induced. Xbra is not expressed at low activin concentrations. PDGF-A is expressed biphasey at both the high and low concentration ranges. Thus, we find that the boundaries of gene expression in aggregates are both changed and sharpened with increasing time in culture. In a separate series of experiments, the induction of PDGF-A and PDGFR-α was examined in aggregates that had been cultured until sibling control embryos reached the tailbud stage (stage 24). In these experiments, the boundaries of gene expression are more narrow and further sharpened than those observed at stage 17 (data not shown). This result indicates that aggregates of induced cells alter their pattern of gene expression over time. For a given gene, this appears as a restriction and sharpening of the dosage window of expression the longer the aggregates are cultured.

DISCUSSION

We have used the mesoderm-inducing factor, activin, to induce cell types that mimic those normally found in the marginal
zone during gastrulation. We find that animal pole cells respond to various concentrations of activin by exhibiting a particular morphology according to the dose used. In contrast to the induction of cell morphology, the initial expression patterns of \textit{gsc}, \textit{Xbra}, PDGFR-\(\alpha\) and PDGF-A genes are not narrowly restricted by activin dose. Narrow and sharp dose windows for expression of these molecular markers do appear, however, in aggregates of induced cells cultured for several hours after activin treatment. Below we discuss how the distinct cell types induced in vitro correlate with marginal zone cells in an intact embryo and present a model to account for the dynamic changes in expression of molecular markers that occur with time after activin treatment.

**DMZ cell types are mimicked by activin induction of animal pole cells**

When cells of the DMZ are dissociated and plated onto fibronectin-coated dishes, we can identify three morphologically distinct cell types. Enriched populations of similar cell types are induced by treating animal pole cells with different activin concentrations. At high activin concentrations spread, motile cells are induced. The spread cell morphology resembles two populations in the embryo, as seen in scanning electron micrographs; the migrating head mesoderm cells and the intercalating presumptive notochord and somite cells. We cannot distinguish whether the in vivo counterpart of the spread cells seen in our assay are head or axial mesoderm cells. However, these cell populations have different characteristics in vivo. During gastrulation the head mesoderm cells migrate over oriented fibronectin-rich fibrils that line the blastocoel wall and roof (Nakatsuji and Johnson, 1983; Lee et al., 1984; Nakatsuji et al., 1985). The migration of head mesoderm cells is impaired by injection of antibodies to fibronectin or \(\beta_1\) integrin into the blastocoel of gastrulating \textit{Xenopus} (Howard et al., 1992; Smith and Howard, 1992) as well as other amphibian (Boucaut et al., 1984, 1985; Darribère et al., 1990) embryos. In contrast, the behavior of presumptive notochord and somite cells is not affected (Smith et al., 1990). Furthermore, when dispersed on fibronectin-coated dishes, head mesoderm comprises only spread cells, whereas axial mesoderm comprises both spread and circus movement cells (Winklbauer, 1990). Thus, it seems likely that head and, possibly, axial mesoderm are the in vivo counterparts of the spread cells induced in our experiments.

Intermediate activin concentrations induce cells to undergo circus movements. This cell behavior is not seen in the intact embryo. However, four lines of evidence suggest that they may
represent a subset of the intercalating presumptive notochord and somite cells. First, about half of the axial mesoderm cells undergo circus movements when plated on fibronectin-coated dishes (Winklbauer, 1990). Second, the activin concentrations that give rise to circus movement cells induce narrowing and lengthening of cell aggregates characteristic of the convergent extension of the axial mesoderm (not shown). Third, such aggregates go on to differentiate as notochord and muscle (K. S. unpublished observations). Fourth, when ventral marginal zone (VMZ) explants are plated on fibronectin-coated dishes, there are few circus movement cells (less than 6% at stage 10.5, compared to 30% in the DMZ, K. S. unpublished observations) and the VMZ undergoes little convergent extension during gastrulation (for review see Keller, 1986; Keller and Winklbauer, 1992). Thus, it seems likely that the circus movement cells require cell-cell contact or different matrix components to behave as they would in vivo.

In the absence of activin or at low concentrations, round, adherent cells predominate. These cells probably represent ectodermal cells because dissociated animal pole cells are mostly round in appearance (Fig. 2G).

Most work to date has examined how activin patterns the marginal zone along its dorsoventral axis (for reviews see Sive, 1993; Smith, J. C. et al., 1993; Whitman and Melton, 1989). However, our studies indicate a role for activin within one region of the marginal zone, the DMZ. We find that different activin concentrations induced cells with morphologies that are normally present within the DMZ. This result was somewhat unexpected but is consistent with one view of mesoderm induction which postulates a localized DMZ signal propagated as a gradient (for review see Smith, J. C. et al., 1993). Activin is able to mimic this DMZ signal in vitro (for reviews see Sive, 1993; Slack, 1993; Smith, J. C. et al., 1993). Our results are consistent with this model such that a high activin concentration would be closest to the dorsal lip and would therefore, induce migratory head mesoderm cells, the first cells to involute during gastrulation. As the gradient trails off towards the animal pole, lower doses might induce the intercalating presumptive notochord and somite cells. Finally, at low activin concentrations or in the absence of activin, the morphology of the ectodermal cells of the animal pole would remain unchanged. One paradox, however, is that VMZ cells, dispersed on fibronectin-coated dishes, contain numerous spread, elongated cells (Gerhart et al., 1984; Howard and Smith, 1993; Niehrs et al., 1993; K. S. unpublished observations) which resemble those induced at the highest activin concentrations in our experiments. It seems likely, therefore, that additional factors induce spread, but few circus movement, cells on the ventral side of the embryo in vivo. Alternatively, ventrally localized factors might modulate the response of the dorsal inducer. The appearance of different cell types might be important in the temporal control of gastrulation. Gastrulation movements begin on the dorsal side of the embryo and spread bilaterally towards the ventral side. The timing of these movements may depend on when the different cell types are induced (in particular the presence of circus movement cells might be crucial for convergent extension during gastrulation). We are currently examining how the appearance of different cell types is spatially regulated and how the presence of different cell types might influence the timing of gastrulation movements.


