

# Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species

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## SUMMARY

During early embryogenesis, the highly regulative sea urchin embryo relies extensively on cell-cell interactions for cellular specification. Here, the role of cellular interactions in the temporal and spatial expression of markers for oral and aboral ectoderm in *Strongylocentrotus purpuratus* and *Lytechinus pictus* was investigated. When pairs of mesomeres or animal caps, which are fated to give rise to ectoderm, were isolated and cultured they developed into ciliated embryoids that were morphologically polarized. In animal explants from *S. purpuratus*, the aboral ectoderm-specific *Spec1* gene was activated at the same time as in control embryos and at relatively high levels. The *Spec1* protein was restricted to the squamous epithelial cells in the embryoids suggesting that an oral-aboral axis formed and aboral ectoderm differentiation occurred correctly. However, the Ecto V protein, a marker for oral ectoderm differentiation, was detected throughout the embryoid and no stomodeum or ciliary band formed. These results indicated that animal explants from *S. purpuratus* were

autonomous in their ability to form an oral-aboral axis and to differentiate aboral ectoderm, but other aspects of ectoderm differentiation require interaction with vegetal blastomeres. In contrast to *S. purpuratus*, aboral ectoderm-specific genes were not expressed in animal explants from *L. pictus* even though the resulting embryoids were morphologically very similar to those of *S. purpuratus*. Recombination of the explants with vegetal blastomeres or exposure to the vegetalizing agent LiCl restored activity of aboral ectoderm-specific genes, suggesting the requirement of a vegetal induction for differentiation of aboral ectoderm cells. These results demonstrate that differences exist in aboral ectoderm differentiation between *S. purpuratus* and *L. pictus* and suggest that the formation of a cell type may occur by alternative mechanisms in two related species.

Key words: sea urchin development, *Spec* genes, ectoderm differentiation, animal cap

## INTRODUCTION

During early embryogenesis, a cleaving embryo produces blastomeres that adopt vastly different developmental fates. Single blastomeres or groups of blastomeres divide to produce clones of cells that constitute embryonic territories giving rise to specific tissues or organs in the embryo. How such territories are specified during development is a key question in embryology. In some species, all blastomeres are initially totipotent and their fates specified by cell-cell interactions while, in others, segregated cytoplasmic determinants restrict the developmental fates of blastomeres (Davidson, 1986; Slack, 1991). Even in these 'mosaic' embryos, however, some cell fates are specified by inductive events mediated through cell-cell interactions (Kimble, 1981; Sternberg and Horvitz, 1986; Priess and Thomson, 1987; Sternberg, 1988; Nishida and Satoh, 1989; Goldstein, 1992).

The highly regulative sea urchin embryo has been used for many investigations of the specification of cell fates. By the 60-cell stage the embryo is arranged into five distinct tiers

along the animal vegetal axis: An1, An2, Veg1, Veg2 and the micromeres. Pluteus larvae can form after the deletion of any tier and the isolated vegetal hemispheres produce their fated structures such as gut and skeleton, and can regulate to produce structures normally derived from blastomeres of the animal half (Horstadius, 1973). In contrast, isolated animal tiers of animal halves (animal caps) produce ciliated epithelial balls described as 'animalized' Dauerblastulae (Horstadius, 1973) or 'arrested in differentiation' (Cameron and Davidson, 1991). Horstadius found that normal development can be restored by recombination of animal and vegetal fragments, and proposed that territorial specification along the animal-vegetal axis depends on the interaction of two opposing morphogenetic gradients (Horstadius, 1973).

Fate mapping indicates that in *S. purpuratus*, founder cells for five distinctive spatial territories have been segregated by the 60-cell stage (Davidson, 1989; Cameron and Davidson, 1991). These territories are the oral and aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme derived from large micromeres and the small micromeres. The aboral ectoderm

territory gives rise to a single differentiated cell type of squamous epithelium characterized by a specialized pattern of gene expression initiated in the blastula (Cameron and Davidson, 1991). Oral ectoderm later differentiates into several cell types, including the ciliary band and stomodeum. Cells of the An1 and An2 tiers give rise to both oral and aboral ectoderm.

Horstadius' proposed morphogens have never been identified and his hypothesis has been reassessed in recent years (Wilt, 1987; Davidson, 1989; Slack, 1991). Davidson (1989) has proposed that the five spatial territories are specified as a result of localized determinants in the vegetal pole and inductive interactions. This view postulates that the large micromeres, containing inherited determinants, act as a vegetal signaling center and induce the Veg2 tier of cells above them, specifying the vegetal plate. These induced cells then induce the cells in the next tier, initiating an inductive cascade which spreads to the animal pole. Consistent with this view is the observation that ectopic micromeres can induce an archenteron and other vegetal plate structures to form in the animal hemisphere (Horstadius, 1973; Ransick and Davidson, 1993).

The advent of sensitive methods to detect molecular markers of embryonic territories has allowed an assessment of the role of cell-cell interactions in their specification. Cultures of cells from completely dissociated embryos express a variety of specific markers, suggesting an autonomy of specification. In particular, genes expressed only in aboral ectoderm are activated on time in a subset of cells, but their transcripts accumulate to a much lower level than in intact embryos (Hurley et al., 1989; Stevens et al., 1989, 1990). In this study, we make use of markers of differentiation of ectoderm cells to address the extent of differentiation of aboral and oral ectoderm in animal fragments cultured in the absence of vegetal cells. Unexpectedly, our results are different for *S. purpuratus* and *L. pictus* fragments, suggesting that different mechanisms result in differentiation of aboral ectoderm in these two sea urchins.

## MATERIALS AND METHODS

### Obtaining gametes and culturing embryos

*S. purpuratus* and *L. pictus* were obtained from Pacific Biomarine (Venice, CA) or Marinus (Long Beach, CA). Gametes were obtained by intraceolomic injection of 0.5 M KCl. Embryos were cultured with stirring in artificial sea water (ASW; pH 8.0) at 15°C.

### Isolation of mesomere pairs and animal caps

Eggs were fertilized in the presence of 0.5 mM 3-amino-1,2,4 triazole (ATA) to prevent fertilization envelope (FE) hardening. Embryos were cultured until the equatorial third cleavage (8-cell stage) following which the FE was removed by passing the embryos through a Nitex filter. The embryos were then placed in hyaline extraction medium (HEM; 317 mM NaCl, 10 mM KCl, 50 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 300 mM glycine, 10 mM Tris, 2 mM ethylene glycol-bis(β-aminoethylether) N, N, N', N'-tetra acetic acid) (McClay, 1986) for 5 minutes, settled and resuspended in calcium-free sea water (CaFSW) (McClay, 1986). After a brief agitation for 1-2 minutes, the embryos were observed under a microscope for blastomere dissociation. To achieve complete dissociation of blastomeres, the embryos were gently passed through a 10 ml syringe (McClay, 1986). Dissociated blastomeres were placed on 1% noble agar-coated Petri dishes (Falcon®, Becton Dickinson) and observed under a stereomicroscope.

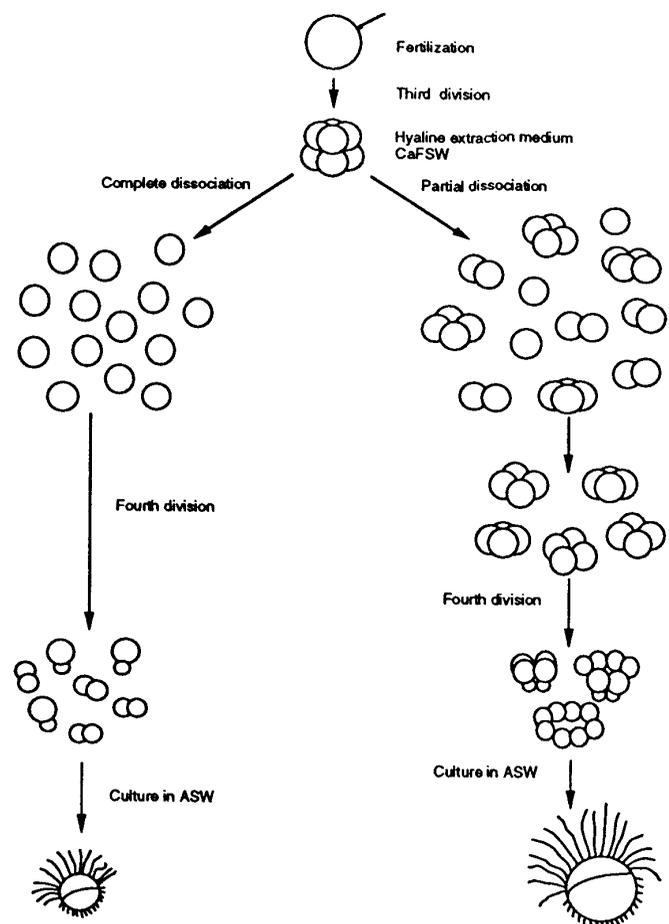
At the 4th cleavage, the four animal blastomeres divided equally to produce a pair of mesomeres, while the four vegetal blastomeres divided unequally to produce one large macromere and one small micromere.

The mesomere pairs were collected using a mouth pipette and cultured in ASW in the presence of 50 µg/ml Gentamycin (Sigma). This protocol is slightly modified from that used by Livingston and Wilt (1989) to isolate mesomeres from 16-cell embryos. Whereas these workers placed embryos in CaFSW at the 4-cell stage and dissociated the embryos at the 16-cell stage, we dissociated embryos at the 8-cell stage after loosening blastomeres with HEM and CaFSW. By doing this, we were able to separate animal and vegetal blastomeres soon after they originated at the equatorial third cleavage.

In order to collect animal caps, we examined partially dissociated cultures for blastomere quartets and, using a mouth pipette, placed these quartets in a Petri dish containing ASW at 4°C. After sufficient numbers had been collected, the quartets were observed under the microscope until the 4th cleavage. Quartets from the animal half produced eight equal mesomeres (animal caps), while the vegetal halves produced four large blastomeres (macromeres) and four small blastomeres (micromeres). The animal caps were collected with a mouth pipette and cultured in ASW in the presence of Gentamycin.

### Blastomere recombination

Partially dissociated embryos were sorted into animal and vegetal halves. Vegetal halves could be easily recognized by the four large macromeres and the four smaller micromeres. To facilitate embryo



**Fig. 1.** Isolation of mesomeres and animal caps from *S. purpuratus* embryos. Details of the protocol are discussed in Materials and Methods.

recombination, we used 72-well plates with a 20  $\mu$ l well volume (Nunc, Denmark). A layer of 1% agarose was placed at the bottom of each well and, after the agarose solidified, a small hole was made in the agarose in each well using a sharp needle. An animal half and a vegetal half were placed in a hole in the agarose and, under a Nikon SMZ-U stereo microscope, they were brought into contact using a mouth pipette and a glass needle made from a drawn out capillary tube. After a few hours, the halves fused and developed into normal appearing embryos.

#### LiCl treatment of animal caps

Animal caps were treated with LiCl essentially as described by Livingston and Wilt (1989). Isolated animal caps were placed in 25 mM LiCl in ASW and cultured for 12-15 hours. Following this incubation, the embryoids were transferred and cultured in fresh ASW.

#### RNA isolation

RNA was extracted from embryos and embryoids as described by Zhao et al. (1990). Briefly, embryos and embryoids were collected using a mouth pipette, pelleted and washed in 0.5 M NaCl-50 mM EDTA-10 mM Tris-HCl (pH 7.4). Embryos and embryoids were then solubilized in 200  $\mu$ l of 0.1 M NaCl-1 mM EDTA-10 mM EGTA-10 mM Tris-HCl-0.5% SDS (pH 7.4). After 10  $\mu$ g yeast tRNA was added as carrier, the lysate was extracted with 400  $\mu$ l phenol-chloroform (1:1) and then precipitated from 0.5 M ammonium acetate and 2.5 volumes of ethanol. Usually 100-200 embryoids derived from mesomere pairs and 25-50 embryoids derived from animal caps were used for each treatment.

#### Preparation of probes and RNase protection assays

cDNA clones in the plasmid Bluescript II (Stratagene, La Jolla, CA) representing the 400 bp *Eco*RI fragment from the coding region of *Spec1* (Hardin et al., 1988), the 440 bp *Eco*RI fragment from the coding region of *LpS1- $\beta$*  (Xiang et al., 1988; referred to as *LpS1* in the remainder of the text) and the 448 bp fragment from the 3' untranslated region of *LpC2* (Fang and Brandhorst, unpublished observa-

tions) were linearized with *Bam*HI, *Nsi*I and *Hinc*II, respectively. The 3' overhang created by *Nsi*I in the *LpS1* plasmid was filled in using Klenow DNA polymerase. As an internal control for RNA loading the *Hind*III-linearized pT7 RNA 18S plasmid (Ambion, Austin, TX) was used. The linearized plasmids were used as templates for synthesizing T7 polymerase-directed [<sup>32</sup>P]UTP labeled (DuPont, Boston, MA) anti-sense RNA using the Maxiscript II kit from Ambion (Austin, TX). Ribonuclease protection assays were done using the RPA II kit from Ambion (Austin, TX) following the manufacturer's instructions.

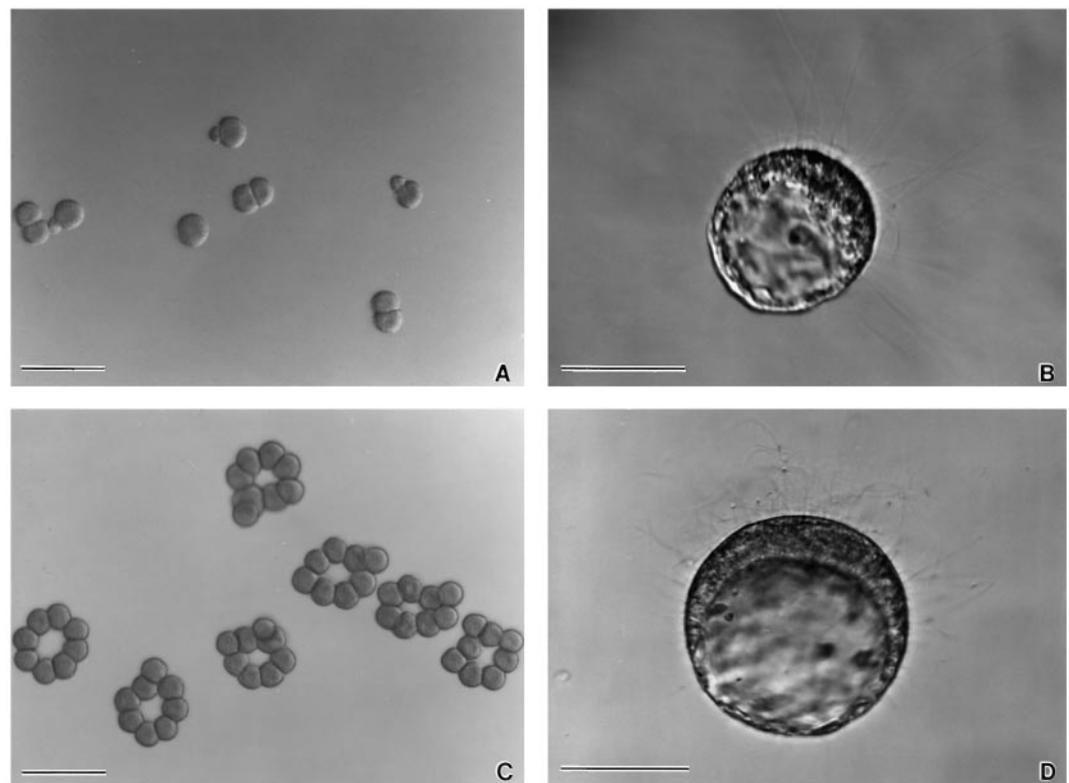
#### Preparation of fusion proteins and antibodies

The 400 bp *Eco*RI fragment of the *Spec1* coding region (Hardin et al., 1988) was fused in frame downstream from the *malE* gene in the pMALc2<sup>tm</sup> vector (New England Biolabs), which encodes the *Escherichia coli* maltose-binding protein (MBP). The MBP-*Spec1* fusion protein was produced in *E. coli*, affinity purified using an amylose resin column and used for immunization of rabbits to generate antibodies. Before use, the serum was preabsorbed with an acetone powder extracted from early blastula stage *S. purpuratus* embryos (Harlow and Lane, 1988).

#### Western analysis

Pluteus stage *S. purpuratus* embryos were solubilized in 62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol (BME) and 0.001% bromophenol blue and electrophoresed on 12% SDS polyacrylamide gels (Laemmli, 1970). After transferring to nitrocellulose (Towbin et al., 1979), the blots were blocked in 3 mg/ml BSA/0.2% Tween-20 in 10 mM phosphate-buffered saline (PBS) (pH 7.4). Blots were incubated with the preabsorbed antiserum or the preimmune serum at a 1:1000 dilution for 3 hours. After washing, the blots were incubated with a horseradish peroxidase conjugated anti-rabbit goat secondary antibody (Cappel) for 2 hours. The blots were developed using chloronaphthol as a substrate (Harlow and Lane, 1988).

**Fig. 2.** Morphology of mesomeres and mesomere derived embryoids. (A) Blastomeres from embryos dissociated at the third cleavage division that have undergone the fourth cleavage. (B) An embryoid derived from a mesomere pair after 3 days of culture. (C) Mesomeres from an intact animal cap. These blastomeres have retained their cell contacts through the dissociation and isolation protocol. (D) An embryoid derived from all mesomeres of animal caps after 3 days of culture. (All bars=50  $\mu$ m.)



### Indirect immunofluorescence

Indirect immunofluorescence was conducted using the preabsorbed Spec1 antibodies and the Ecto V monoclonal antibody (a gift from Dr David McClay, Duke University). Embryos were fixed in 4% paraformaldehyde in PBS for staining with the Spec1 antibody and in methanol for staining with the Ecto V antibody. Indirect immunofluorescence was done essentially as described in Ettensohn and McClay (1988) using PBS instead of ASW as buffer. The primary antibodies were followed by fluorescein-conjugated secondary antibodies (affinity-purified rabbit IgG; Cappel) for the Spec1 antibody, or rhodamine-conjugated secondary antibodies (goat anti-mouse IgG, IgA, IgM; Cappel) for the Ecto V antibody. The embryos were observed on a Nikon Diaphot-TMD inverted microscope equipped with differential interference contrast (DIC) and epifluorescence optics.

## RESULTS

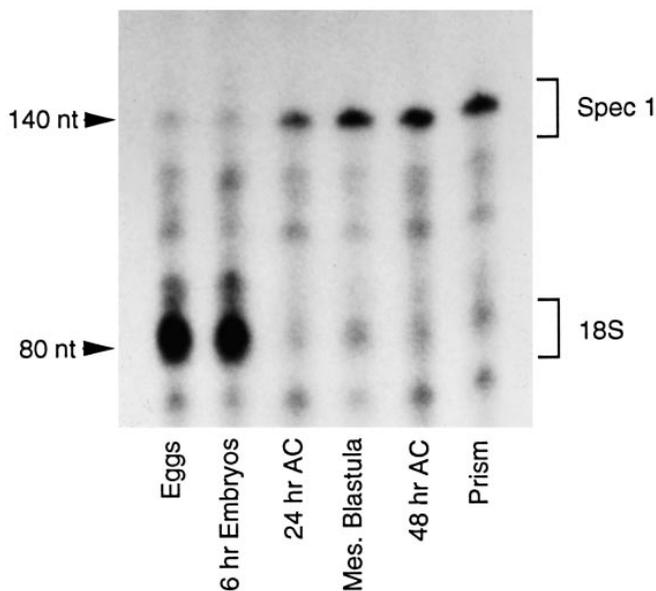
### Isolation of mesomere pairs and animal caps

Livingston and Wilt (1989) described a useful method for isolating large numbers of mesomere pairs from sea urchin embryos. In the current study, we modified this method slightly by using HEM to remove the hyaline layer and dissociating embryos earlier (Fig. 1). This minimized contact time between blastomeres from the animal and vegetal halves. After 48 hours in culture, a majority of the mesomere pairs isolated using this method produced polarized, ciliated, swimming embryoids (Fig. 2A,B). Survival of these embryoids was generally greater than 90%. We were also able to collect large numbers of animal caps (>100) from both *S. purpuratus* and *L. pictus* embryos by partially dissociating embryos at the 8-cell stage, collecting quartets and screening the quartets at the fourth division for equal sized mesomeres (Figs 1, 2C). As with the

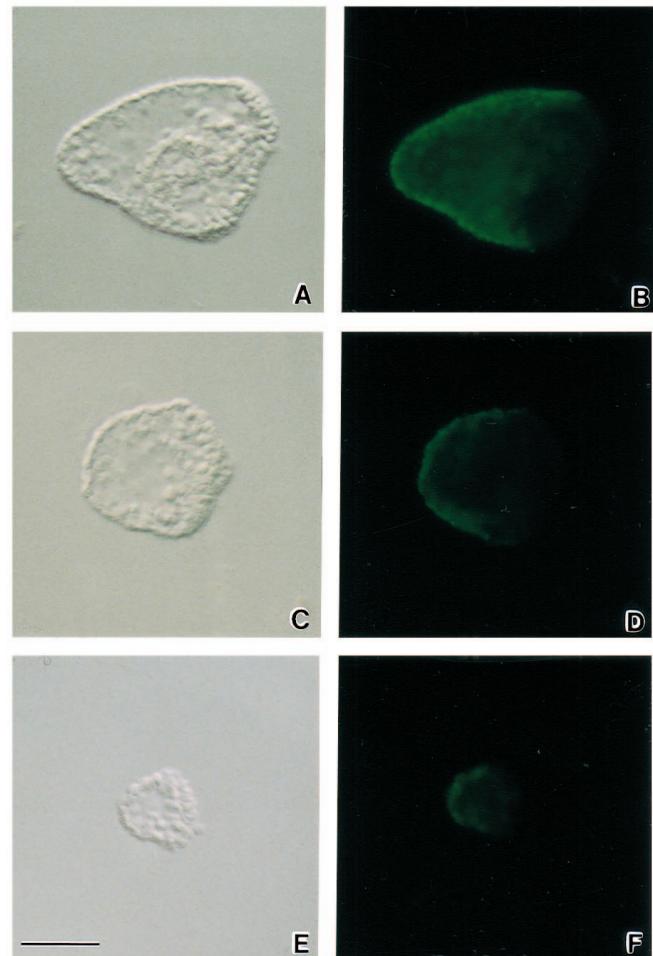
cultured mesomere pairs, these animal caps gave rise to polarized, ciliated, swimming embryoids (Fig. 2D). Embryoids derived from either mesomere pairs or animal caps had a thickened epithelium on one side and a squamous epithelium on the other indicating a morphological polarity of ectoderm similar to intact embryos (Fig. 2B,D). These embryoids also had a polarized distribution of 'stereocilia'. We rarely saw signs of gut or spicules in these cultures even after prolonged incubations and we never saw evidence of a stomodeum or ciliary band. The few cases where vegetal structures appeared may be explained by a subequatorial third cleavage (Livingston and Wilt, 1989; Henry et al., 1989). Thus, culturing animal caps and mesomere pairs from either *S. purpuratus* and *L. pictus* led to the classic ciliated epithelial balls (Horstadius' Dauerblastulae) that have been reported over the years by many investigators.

### Spec1 expression in *S. purpuratus* animal caps and mesomere pairs

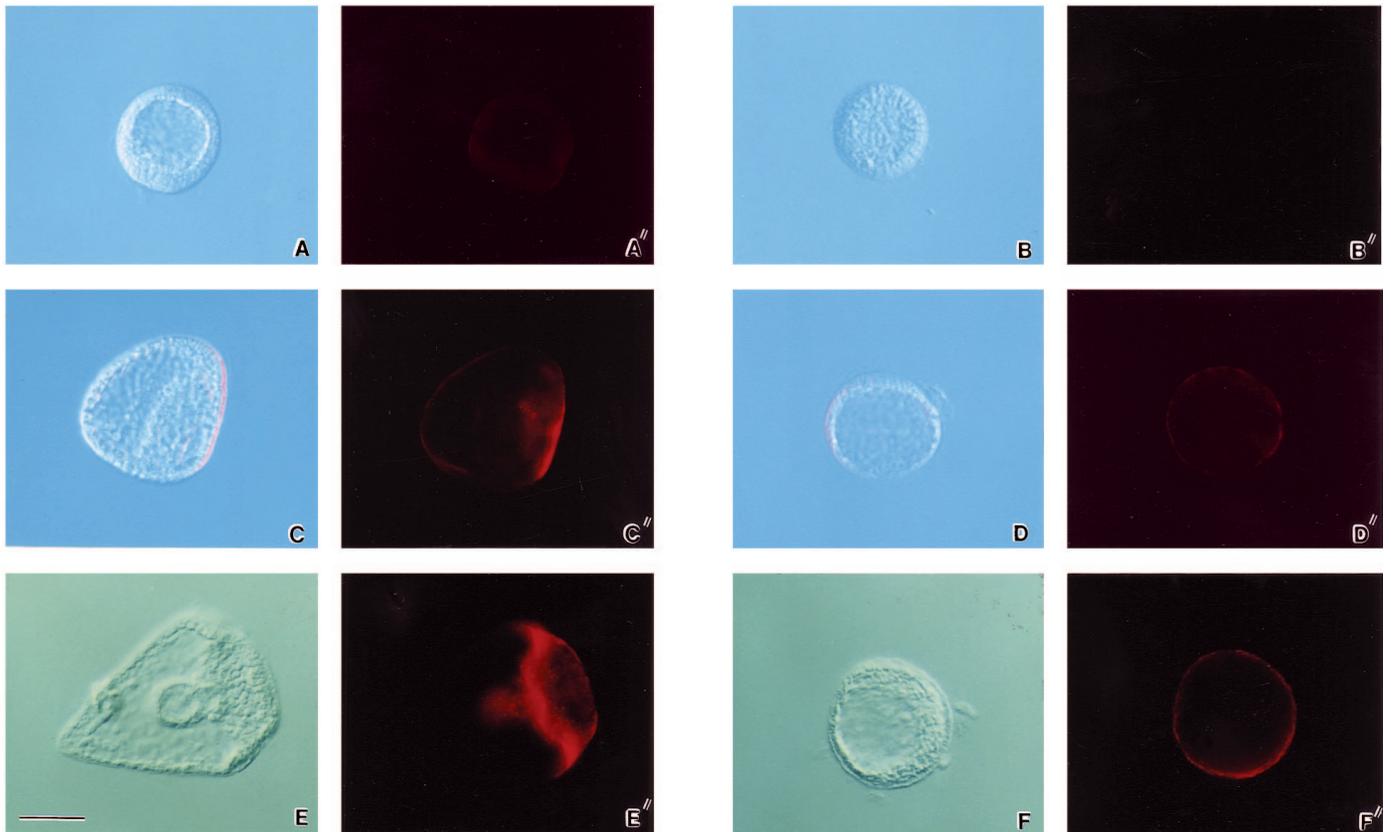
RNase protection analysis of the animal explants derived



**Fig. 3.** *Spec1* expression in *S. purpuratus* animal caps and embryos. RNA isolated from eggs, embryos and animal caps was assayed for *Spec1* expression using RNase protection. RNase digestion produced the expected 140 nt *Spec1* fragment and the 80 nt 18S ribosomal RNA fragment used as an internal control. Eggs and 6 hour lanes are overloaded with respect to the other lanes.



**Fig. 4.** Localization of *Spec1* in *S. purpuratus* embryos and embryoids. *Spec1* protein was localized using indirect immunofluorescence on paraformaldehyde fixed embryos. (A,C,E) Differential interference contrast images of a 3 day pluteus larva and identically staged animal caps and mesomere embryoids, respectively. (B,D,F) The corresponding epifluorescence images. (Bar=50  $\mu$ m.)



**Fig. 5.** Localization of Ecto V in *S. purpuratus* embryos and animal caps. Ecto V was localized using indirect immunofluorescence on methanol-fixed embryos and animal caps. (A-F) Differential interference contrast images; (A'-F') the respective immunofluorescence images. (A,C,E) Embryos at 24, 48 and 72 hours, respectively; (B,D,F) animal caps at the corresponding time points. (Bar=50  $\mu$ m.)

from mesomere pairs or animal caps showed that they expressed the aboral ectoderm-specific *Spec1* gene (Lynn et al., 1983) with the correct temporal pattern and at levels comparable to control embryos (Fig. 3). Variability in the amount of *Spec1* transcript levels in animal caps made from different cultures ranged from 70% to 140% of control embryos, but overall *Spec1* levels were much higher than those reported when *S. purpuratus* embryos were dissociated and cultured as individual cells (Hurley et al., 1989; Stevens et al., 1989). These results indicated that full aboral ectoderm differentiation, as monitored by *Spec1* gene expression, occurred autonomously in mesomeres in the absence of more vegetal blastomeres.

We asked if *Spec1* expression was localized to a particular region of the embryoids. A polyclonal antibody generated in rabbits against a MBP-*Spec1* fusion protein showed a strong reactivity to the *Spec1* protein on Western blots (not shown). When pluteus stage embryos were stained with the anti-*Spec1* serum, the *Spec1* protein was localized to the aboral ectoderm cells as had been previously demonstrated (Carpenter et al., 1984; Fig. 4 A,B). When the antibodies were used to detect *Spec1* protein in cultured animal caps or mesomere pairs, we observed a highly localized signal in the squamous epithelium on one side of the embryoids (Fig. 4 C-F). These results provided evidence that cultured mesomere pairs and animal caps were polarized along the oral-aboral axis and that aboral ectoderm cells had differentiated. Our results argue for a large

component of autonomy in the differentiation of the aboral ectoderm cell type in *S. purpuratus*. It cannot be ruled out, however, that vegetal interactions take place earlier, that is, between animal and vegetal quartets of the 8-cell-stage embryo, since animal halves were isolated after the third cleavage.

#### Expression of Ecto V in *S. purpuratus* animal caps

Since the thickened epithelium opposite the squamous epithelium expressing *Spec1* resembled oral ectoderm, we asked if these cells were differentiated oral ectoderm cells. The monoclonal antibody Ecto V recognizes an antigen that is initially seen at low levels on the surface of fertilized eggs and early embryos. Zygotic synthesis of the Ecto V antigen subsequently increases several fold and becomes restricted to the oral ectoderm and foregut by the pluteus stage (Coffman and McClay, 1990). By 24 hours maternal Ecto V antigen had almost disappeared from blastomere surfaces with little or no expression seen on the blastula stage embryos or embryoids derived from animal caps (Fig. 5A,A',B,B'). However, by 48 hours (approximately gastrula) intense staining with Ecto V was seen in the oral ectoderm of control embryos, while all cells of the animal caps expressed the antigen (Fig. 5C,C',D,D'). This indicated that while Ecto V was clearly restricted to the oral ectoderm by the mid-gastrula stage embryos, all cells in the animal caps actively expressed the antigen. Animal caps incubated for 72 hours showed an

increased intensity of Ecto V staining throughout the embryoid surface and showed no apparent restricted expression pattern (Fig. 5F,F'), while intact controls showed an intense staining of oral ectoderm and foregut (Fig. 5E,E'). We conclude that, while certain features of ectoderm differentiation such as correct temporal and spatial activation of the aboral ectoderm-specific *Spe1* gene occurred in these animal explants, other features, such as restricted expression of Ecto V and formation of the ciliary band and stomodeum did not occur.

### ***LpS1* expression in *L. pictus* animal caps and mesomere pairs**

To determine if these patterns of ectoderm differentiation were a general feature of sea urchins, we performed similar experiments with *L. pictus*. When animal caps and mesomere pairs from *L. pictus* were cultured, they formed polarized embryoids similar to those of *S. purpuratus* (Figs 6A,8A) Unexpectedly, however, neither the embryoids from mesomere pairs (Fig. 6B) nor the embryoids from animal caps (Fig. 6C) produced detectable *LpS1* mRNA, a marker for aboral ectoderm differentiation (Xiang et al., 1988). That the animal caps had the

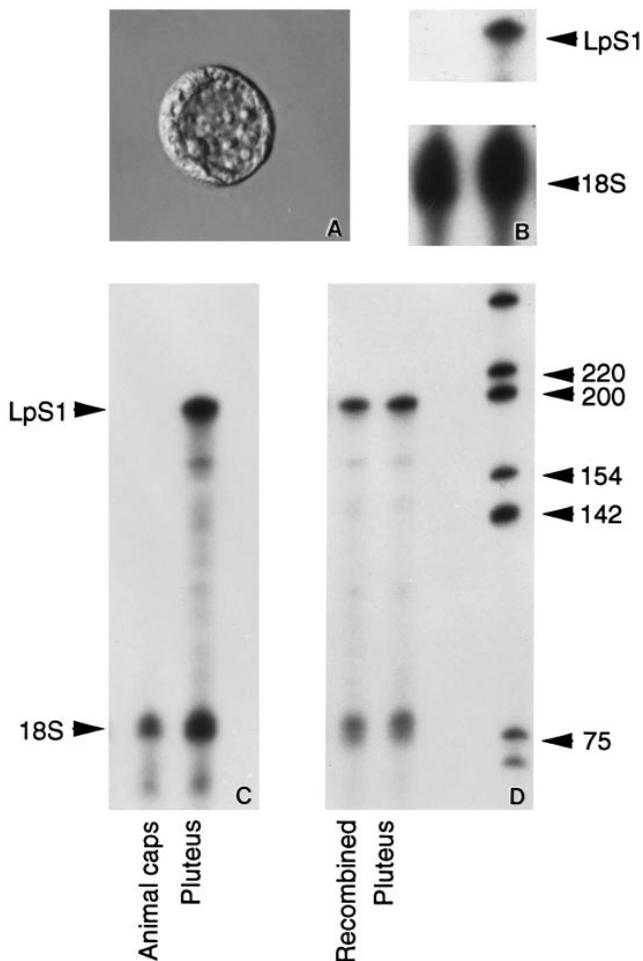
ability to express *LpS1* gene was demonstrated by recombination with vegetal blastomeres. These explants formed normal embryos and readily accumulated *LpS1* transcripts (Fig. 6D). These results indicated that in *L. pictus* *LpS1* gene expression was non-autonomous and that activation of the gene required a vegetal influence. Messenger RNA of another aboral ectoderm marker, *LpC2* actin, was not detected in animal caps embryoids (Fig. 7). Together, these results suggest that aboral ectoderm differentiation did not occur in isolated *L. pictus* animal halves and that vegetal blastomeres were required for the differentiation of this tissue.

### **Ecto V expression in *L. pictus* animal caps**

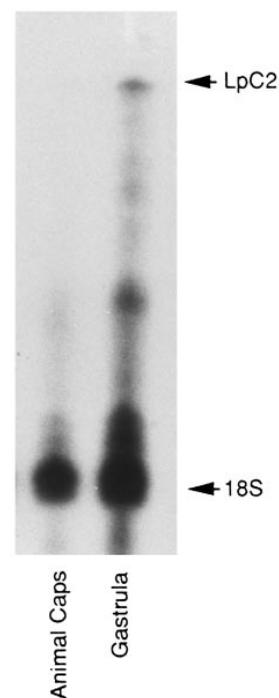
Staining of *L. pictus* embryos and animal caps with Ecto V at 24 and 48 hours showed the same pattern of staining as seen with *S. purpuratus*; i.e. very little staining was seen at 24 hours in animal caps and blastula stage embryos, while localization of Ecto V was seen in the oral ectoderm in mid-gastrula stage embryos and global staining was seen in the animal caps (not shown). In a previous study, Livingston and Wilt (1990) found that embryoids derived from *L. pictus* mesomeres did not show localized expression of Ecto V even after a prolonged incubation period.

### **Lithium-induced activation of aboral ectoderm gene expression in *L. pictus* animal caps and mesomere pairs**

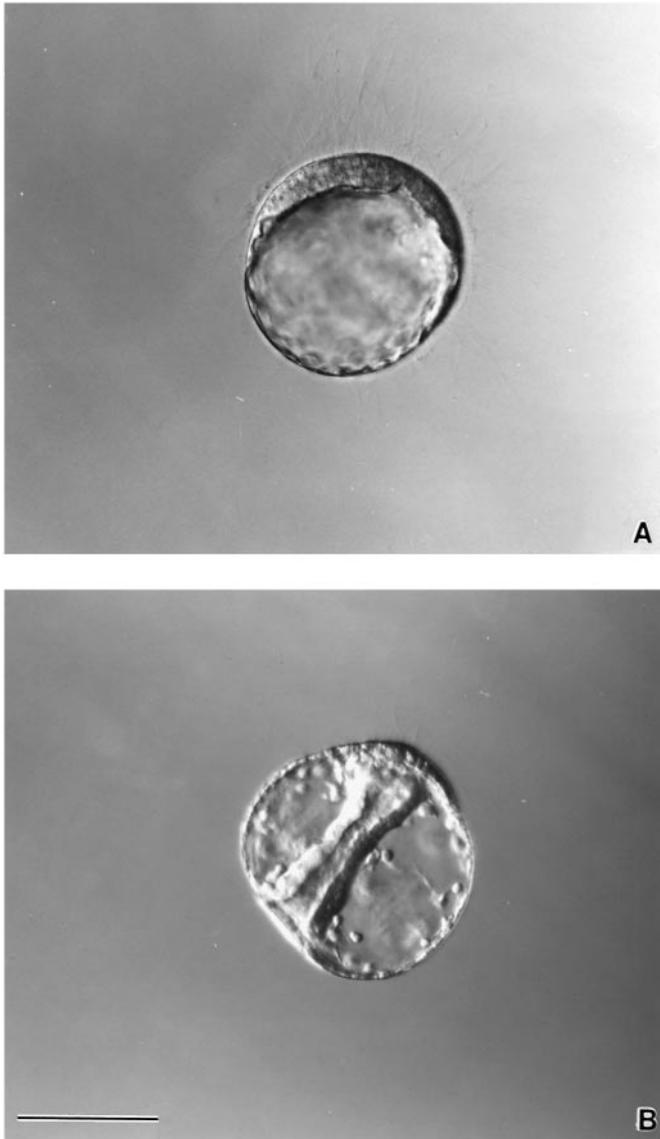
Previous workers have shown that addition of LiCl to isolated animal caps or mesomeres can evoke the expression of vegetal markers in these cells normally fated to form ectoderm (Livingston and Wilt, 1989, 1990; Horstadius, 1975). In the case of animal caps, exposure to LiCl transforms them into



**Fig. 6.** *LpS1* expression in *Lytechinus pictus* embryos and embryoids. RNA isolated from embryos, embryoids from mesomere pairs and animal caps was assayed for *LpS1* using RNase protection. (A) A 3 day embryoid derived from an *L. pictus* mesomere pair. Recombined indicates separated animal and vegetal halves that were recombined following the fourth cleavage.



**Fig. 7.** *LpC2* actin expression in *L. pictus* animal caps. RNA isolated from embryos and animal caps and assayed for *LpC2* actin expression using RNase protection.

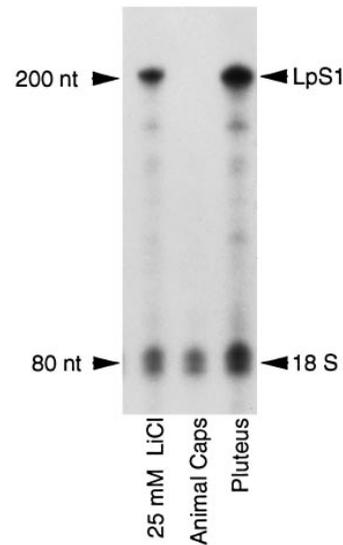


**Fig. 8.** Effect of LiCl on *L. pictus* animal caps. (A) An *L. pictus* embryoid derived from an animal cap. (B) An embryoid derived from an *L. pictus* animal cap treated with 25 mM LiCl. (Bar=50  $\mu$ m.)

what appear to be normal plutei (Von Ubisch, 1929). When we treated *L. pictus* animal caps with LiCl they formed gastrula-like embryos at 48 hours (Fig. 8B). RNase protection analysis showed that LiCl-treated animal caps readily accumulated *LpS1* mRNA to levels approaching intact embryos (Fig. 9). Similar results were obtained for *LpC2* actin mRNA (data not shown). These results indicate that the differentiation of aboral ectoderm in animal caps depends on the presence of cells normally derived from the vegetal half, but was formed by animal cells in the presence of LiCl.

## DISCUSSION

Availability of molecular probes for differentiated oral and aboral ectoderm and development of techniques to selectively isolate mesomeres and animal caps has allowed a reassessment



**Fig. 9.** LiCl rescue of *LpS1* expression in *L. pictus* animal caps. Pluteus stage *L. pictus* embryos along with identically staged animal caps and animal caps treated with 25 mM LiCl were assayed for *LpS1* expression using RNase protection.

of autonomy of determination of the developmental fate of mesomeres. Our results with two sea urchin species showed significant differences in the dependence of aboral ectoderm differentiation on cellular interactions with vegetal cells. These studies also showed, however, that many aspects of animal blastomere development appeared similar in these species and required interactions with vegetal blastomeres.

## Aboral ectoderm differentiation

Our results with *S. purpuratus* animal caps and mesomere pairs argue that, in this species, the aboral ectoderm lineages derived from the mesomeres are specified without a requirement for interaction with vegetal blastomeres, at least subsequent to the third cleavage. Other workers have found that *Spec1* gene expression is activated at the appropriate time in completely dissociated cultures, but expression is severely attenuated (Hurley et al., 1989; Stevens et al., 1989). The increased level of expression that we observed in the embryoids may be due to a community effect. Khaner and Wilt (1990) showed that large aggregates of mesomeres survive better than smaller aggregates or individual cells. Consequently, single blastomeres may accumulate less mRNA because of their decreased viability. Alternatively, lateral cell interactions may be required for completion of aboral ectoderm differentiation and full expression of the *Spec1* marker gene.

The results with *L. pictus* animal isolates are in striking contrast to those with *S. purpuratus*. Animal caps and mesomere pairs from this species did not activate the *LpS1* gene. Recombination with vegetal blastomeres or treatment with lithium chloride, which redirects some mesomeres to vegetal fates, led to the expression of *LpS1* at levels comparable to control embryos. At a minimum, these results point out a need for vegetal cell interactions in the expression of *LpS1*. Because *LpS1* is closely linked to aboral ectoderm differentiation, the results also imply that this cell type cannot form in the absence of vegetal cells or agents that permit formation of

vegetal structures. The inability of animal caps and mesomere pairs to activate an unrelated aboral ectoderm-specific gene encoding *LpC2* actin suggests that the entire program of aboral ectoderm differentiation was inhibited by the removal of vegetal blastomeres.

A possible explanation for the difference seen in the two species may be a difference in timing of vegetal signaling events. In this view, in both species a vegetal induction is necessary for specifying the aboral ectoderm territory but, in *S. purpuratus*, this signal may be transmitted prior to the third cleavage or just after the third cleavage. Thus, in this species, when animal isolates are prepared, the mechanisms that specify the aboral ectoderm lineage have already been activated. This would preclude a role for the large micromeres as part of a signaling cascade in this process as these cells do not segregate until the fourth cleavage. In *L. pictus* the inductive signal may be transmitted later in development and may require a signaling cascade originating from the large micromeres as envisioned by Davidson (1989). As a result, in this species, animal blastomere isolates prepared at the third cleavage are deprived of any inductive signal.

A subpopulation of cultured cells from dissociated *L. pictus* embryos express *LpS1* at a low level in a fashion similar to what is observed for *Spec1* in dissociated embryos of *S. purpuratus* (Stevens et al., 1990). In our studies, we never observed *LpS1* or *LpC2* expression in embryoids derived from mesomere pairs or animal caps. Since investigations of dissociated embryos did not identify which cells were expressing *LpS1* or *Spec* genes, it is possible that cells from macromere but not mesomere lineages were expressing this gene, or that signals were transmitted from vegetal cells co-cultured with the animal cells. An alternative and intriguing possibility is that, in *L. pictus*, cell-cell interactions among animal blastomeres suppress aboral ectoderm gene expression. In this view, vegetal blastomeres would be required to relieve the suppressive effect of the mesomere interaction.

### Oral ectoderm differentiation

During normal embryogenesis the Ecto V antigen localizes to the oral ectoderm by the prism stage and is thus a good marker for this territory (Coffman and McClay, 1990). Livingston and Wilt (1990) demonstrated that Ecto V does not localize in embryoids derived from mesomeres of 16-cell stage *L. pictus* embryos and we confirmed these results with embryoids derived from animal caps. In this study, however, we found that the Ecto V expression seen in the embryoids from both species is due to active synthesis of the antigen with the same temporal pattern as in intact embryos. Livingston and Wilt (1990) observed that treatment of isolated pairs of mesomeres with lithium chloride resulted in some localization of Ecto V expression often away from an invagination induced by this treatment. However, recombination of mesomeres with micromeres or macromeres did not result in a proper localization of the Ecto V antigen (Livingston and Wilt, 1990; Khaner and Wilt, 1991). To restore the normal pattern of Ecto V expression, animal fragments must be treated with lithium chloride and placed in contact with large micromeres (Livingston and Wilt, 1990). Thus, it appears that Ecto V localization requires multiple signals during normal embryogenesis. It is likely that, in both *S. purpuratus* and *L. pictus*, differentiation of the oral ectoderm as assessed by Ecto V

localization requires signals from the vegetal blastomeres. It is tempting to speculate that specification of oral and aboral ectoderm territories may involve a positive signal for activating aboral ectoderm differentiation and a negative signal for suppressing oral ectoderm differentiation in prospective aboral ectoderm. In this view, an oral ectoderm fate would be a default state for mesomeres. This model would also require differential signaling from vegetal blastomeres. The observation that *Spec1* expression is localized and not global in *S. purpuratus* embryoids supports this idea.

### Generation of the oral-aboral axis

Localization of the *Spec1* protein to the squamous epithelium suggests that the morphological axis in the animal caps corresponds to the oral-aboral axis. It is also possible, however, that this axis corresponds to the animal-vegetal axis. In this view, the extended cilia seen on the thickened epithelium would correspond to the apical tuft seen in the animal pole of early gastrula stage embryos. If this were the case, we would expect that *Spec1* expression would cross the morphologically distinct boundaries of animal caps in at least 50% of the embryoids since two blastomeres in the animal hemisphere of 8-cell embryos are specified to form both oral and aboral ectoderm (Cameron et al., 1990). However, we have never observed this result in our experiments. Our results are most consistent with the polarization seen in the animal caps as corresponding to the oral-aboral axis, but further study is necessary to rule out other possibilities. Embryoids from *L. pictus* also formed the morphological polarization seen in *S. purpuratus* embryoids, but aboral ectoderm differentiation clearly did not occur. This morphological polarity may also reflect the specified oral-aboral axis in *L. pictus* animal caps.

### Other aspects of ectoderm differentiation

Our results also show that other aspects of ectoderm differentiation are not autonomous events. We never observed a ciliary band or stomodeum in the animal caps made from either species. A recent study by Cameron et al. (1993) indicates that the ciliary band is formed as a result of an interaction at the boundary of oral and aboral ectoderm cells. The lack of the ciliary band in the embryoids suggests that either oral or aboral ectoderm or both are not capable of promoting the required inductive interaction in the animal-half embryoids in the absence of a vegetal contribution. Horstadius (1939, 1973) found that if the *Veg1* tier is incubated with animal caps, the embryoids form a stomodeum and ciliary band, indicating an inductive role for these vegetal cells. Moreover, the positioning of the stomodeum is influenced by an interaction between the oral ectoderm and the elongating archenteron (Hardin and McClay, 1990).

It has been reported that mesomere pairs or reaggregates cultured for over 60 hours display a remarkable developmental potency, substantially greater than that of isolated animal caps, forming endodermal and mesenchymal derivatives (Henry et al., 1989; Khaner and Wilt, 1990). We have rarely observed such differentiation in our experiments, but have not normally cultured embryoids for as long. We have concentrated on assessing whether the timing and the extent of ectoderm differentiation is normal. Our results indicate that generation of the oral-aboral axis and differentiation of the ectodermal territories requires signaling from the vegetal cells.

Future studies will focus on identification of the molecules involved in these early specification events during sea urchin embryogenesis.

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