

The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo

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SUMMARY

During sea urchin development, a tier-to-tier progression of cell signaling events is thought to segregate the early blastomeres to five different cell lineages by the 60-cell stage (E. H. Davidson, 1989, *Development* 105, 421-445). For example, the sixth equatorial cleavage produces two tiers of sister cells called 'veg1' and 'veg2,' which were projected by early studies to be allocated to the ectoderm and endoderm, respectively. Recent in vitro studies have proposed that the segregation of veg1 and veg2 cells to distinct fates involves signaling between the veg1 and veg2 tiers (O. Khaner and F. Wilt, 1991, *Development* 112, 881-890). However, fate-mapping studies on 60-cell stage embryos have not been performed with modern lineage tracers, and cell interactions between veg1 and veg2 cells have not been shown in vivo. Therefore, as an initial step towards examining how archenteron precursors are specified, a clonal analysis of veg1 and veg2 cells was performed using the lipophilic dye, DiI(C16), in the sea-urchin species, *Lytechinus variegatus*. Both veg1 and veg2 descendants form archenteron tissues, revealing that the ectoderm and endoderm are not segregated at the sixth

cleavage. Also, this division does not demarcate cell type boundaries within the endoderm, because both veg1 and veg2 descendants make an overlapping range of endodermal cell types. The allocation of veg1 cells to ectoderm and endoderm during cleavage is variable, as revealed by both the failure of veg1 descendants labeled at the eighth equatorial division to segregate predictably to either tissue and the large differences in the numbers of veg1 descendants that contribute to the ectoderm. Furthermore, DiI-labeled mesomeres of 32-cell stage embryos also contribute to the endoderm at a low frequency. These results show that the prospective archenteron is produced by a larger population of cleavage-stage blastomeres than believed previously. The segregation of veg1 cells to the ectoderm and endoderm occurs relatively late during development and is unpredictable, indicating that later cell position is more important than the early cleavage pattern in determining ectodermal and archenteron cell fates.

Key words: sea urchin, cell lineage, specification, cleavage, blastomere, *Lytechinus variegatus*.

INTRODUCTION

Early sea urchin development is characterized by both a stereotyped pattern of cleavage divisions and striking regulative abilities. The invariant cleavages are thought to accompany the predictable segregation of early blastomeres to five different cell lineages, or territories, which include the oral and aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme and the small micromeres (Cameron and Davidson, 1991; Davidson, 1989). The ability of the sea urchin to regulate in response to microsurgical perturbations reveals the importance of cellular interactions in establishing cell identities within this embryo (Hörstadius, 1973). To explain how blastomeres are reproducibly allocated to specific lineages in an embryo dependent on cell signaling, a current model of sea urchin development proposes the following: (1) a signaling center forms at the vegetal pole of 16-cell stage embryos; (2) inductive signals emanate from this signaling center and propagate from tier-to-tier to allocate cells to distinct lineages by the 60-cell stage; and (3) because the early cleavages are stereotyped and because the cleavage boundaries are sites of

inductive interactions, the five different cell lineages are the outcome of the cell divisions that create both the signaling cells and the recipients of those cues (Davidson, 1989).

Several studies provide support for this model. At the 16-cell stage, the skeletogenic mesenchyme territory is established when the micromeres form at the vegetal pole of the egg (Fig. 1A). The micromeres are autonomously specified to make only skeletogenic primary mesenchyme cells (PMCs) (Okazaki, 1975; Kitajima and Okazaki, 1980) and are thought to induce the overlying macromeres to form archenteron cell types (Hörstadius, 1939; Ransick and Davidson, 1993, 1995). Consistent with this idea, embryos are impaired in their ability to gastrulate on a normal time schedule following deletion of the micromeres, and ectopic placement of micromeres at the animal pole induces a secondary archenteron. Therefore, micromere-derived cues may play an important role in the specification of archenteron tissues in the normal embryo, and the cleavage plane between the micromeres and macromeres appears to be a site of cell-cell signaling.

Subsequently, interactions between macromere descendants are thought to segregate the endoderm from the ectoderm. At

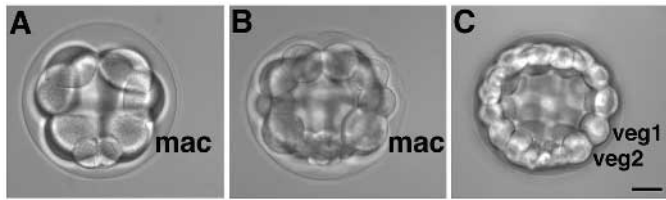


Fig. 1. Side views of normal embryos during early cleavage. Embryos are oriented with the animal pole up and vegetal pole down. (A) 16-cell stage embryo; (B) 32-cell stage embryo; (C) 60-cell stage embryo. Early blastomeres are identifiable by their location and size within the embryo. The macromeres (mac) overlie the vegetal micromeres, and lie beneath the mesomeres. At the 60-cell stage, the macromeres cleave into the veg1 and veg2 tiers which contain eight cells each (C). Scale bar, 20 μ m.

the sixth cleavage, the macromeres cleave equatorially into two tiers called 'veg1' and 'veg2' (Fig. 1B,C). In vitro cell aggregation experiments have shown that both veg1 and veg2 cells possess the ability to make endoderm (Khaner and Wilt, 1991), but earlier fate-mapping studies projected that only the veg2 tier makes the archenteron in the intact embryo (Hörstadius, 1973; Cameron and Davidson, 1991). One interpretation of these results is that disaggregation reveals an underlying competence of veg1 cells to make endoderm, and in normal embryos where spatial relationships between veg1 and veg2 are not disrupted, the inherent endoderm-forming ability of veg1 is prevented by suppressive signals from veg2 (Khaner and Wilt, 1991). Thus, the segregation of ectoderm from the prospective archenteron may be regulated by signaling across the sixth equatorial cleavage plane that separates the veg1 and veg2 tiers.

The cleavage boundary between the veg1 tier and the mesomeres may also be an important interface for cell-cell signaling because mesomeres appear to possess an inherent capacity to form endoderm that must be suppressed in the normal embryo. For example, mesomeres differentiate into endoderm if cultured as isolated pairs or if obtained from sub-equatorially cleaving embryos (Henry et al., 1989). As the normal fate of mesomeres is to form ectoderm (Cameron and Davidson, 1991), this suggests that signaling between the mesomeres or from the veg1 tier prevents mesomeres from assuming an endodermal fate. These data are consistent with the hypothesis that the segregation of blastomeres to distinct lineages is regulated by a tier-to-tier progression of intercellular signaling events. This hypothesis places great emphasis on the stereotyped lineage relationships that result from the invariant cleavages, because they put blastomeres in unique positions along the animal-vegetal axis, thereby determining the signals they will receive and/or send to specify cells to distinct fates.

As our main interest lies in how blastomeres are specified to form archenteron cell types, we have re-examined the allocation of the veg2 tier to the prospective archenteron and the segregation of the ectoderm from the endoderm at the 60-cell stage. We began these studies because signaling between veg1 and veg2 cells has been inferred on the basis of cell aggregation experiments, but interactions between veg1 and veg2 cells have not been documented in undissociated embryos. In addition, cell-marking studies on veg1 and veg2 cells have not

been repeated since the 1930s and modern lineage tracers now provide more powerful tools than were available to early investigators. Therefore, we performed a clonal analysis of veg1 and veg2 cells of *Lytechinus variegatus* embryos using the lipophilic dye, DiI(C16). Unexpectedly, individually labeled veg1 cells as well as veg2 cells form tissues of the archenteron, demonstrating that the sixth cleavage does not segregate the ectoderm from the endoderm and eliminating the possibility that signaling between veg1 and veg2 cells at this cleavage allocates them to separate lineages. In addition, some of the same endodermal cell types made by veg1 progeny are also made by veg2 descendants, showing that the sixth equatorial division does not predict tissue boundaries within the gut. The ectoderm and endoderm are not segregated as a consequence of a specific, later division because the eighth cleavage also fails to separate the ectoderm from the endoderm, and the contribution of veg1 progeny to the ectoderm is highly variable. Finally, mesomere descendants form endoderm in normal embryos, although at low frequency. These data show that allocation of veg1 and veg2 descendants to the ectoderm and endoderm occurs after the early cleavage stages and is not correlated with cleavage boundaries, implying a significant role for later, position-dependent cell-cell signaling between macromere descendants in establishing the ectoderm-endoderm boundary and cell identities within the archenteron.

MATERIALS AND METHODS

Embryo handling and culture

Lytechinus variegatus were obtained from Sue Decker Services (Hollywood, FL) or from Duke Marine Laboratory (Beaufort, NC). Gametes were collected by intracoelomic injection of 0.5 M KCl. Embryos were fertilized with a dilute sperm suspension in 10 mM p-aminobenzoic acid (Sigma), washed 3 \times in artificial sea water (ASW), and cultured in glass bowls at 20–24°C until the desired stage was reached. To remove fertilization envelopes with minimal disruption of cleavage planes, small numbers of embryos were placed on glass slides. The fertilization envelopes stuck to the glass and the embryos were collected by releasing them from their fertilization envelopes with a gentle stream of sea water issuing from a mouth pipette. The embryos were subsequently placed in microinjection chambers as described by Etensohn and McClay (1988). The chambers were assembled with 100 μ m thick double stick tape (3M Company) to avoid disruption of cleavage orientations by compression. All labeled embryos were cultured individually in ASW in 96-well microtitre plates. Embryos were scored at the 48-hour pluteus stage.

Embryo labeling

Double-labeling of embryos with DiI(C16) and DiO(C16) (Molecular Probes) was performed by pressing dye-coated needles against the desired cell, as described previously (Ruffins and Etensohn, 1993). With this method, dye transfer of both dyes occurred in 3–5 minutes. Veg1 and veg2 cells were identified by immobilizing an embryo in a manipulation chamber at the 32-cell stage and by watching the macromeres until the sixth cleavage produced the veg1 and veg2 tiers. A single veg2 cell was labeled first with DiO, followed by a quick confirmation by fluorescence microscopy to insure transfer of the dye. The overlying veg1 cell was identified by reconfirming the fluorescence in the sister veg2 blastomere below. The veg1 cell was labeled, success of dye transfer was verified, and the embryo was cultured individually.

For all other experiments, embryos were labeled iontophoretically

with DiI (5 mg/ml in EtOH) or, in a few cases, with rhodaminated dextran (50 mg/ml in water, 10,000 M_r , Molecular Probes), with a 20-30 second pulse for DiI or a 15 second pulse for dextran (Birgbauer and Fraser, 1994). In about 10% of the labeled cases, large fluorescent punctate spots were observed inside the blastocoel at the pluteus stage, which were interpreted to indicate dead cells. All embryos containing fluorescent debris were omitted from further analysis.

Embryos labeled at the 32- or 60-cell stages were placed individually in microinjection chambers immediately before iontophoresis to minimize the length of time in confinement. To examine whether cell fates of veg1 or veg2 progeny are influenced by their radial position around the animal-vegetal axis, single veg1 or veg2 cells were DiI-labeled and the positions and cell fates of their descendants were documented at the pluteus stage. The location of each cell in relation to the oral-aboral axes or left-right axes could not be identified at the time of labeling. However, because there is little lateral movement of labeled veg1 and veg2 descendants within the archenteron, the initial position of labeling could be inferred based on the final location of labeled cells within the gut. SMCs such as the blastocoelar and pigment cells disperse throughout the embryo, but because veg2 descendants always form some foregut tissues, a position of origin could be assigned to these mesenchymal cells. Where multiple veg1 cells were labeled within a single tier, embryos were examined from the vegetal pole prior to labeling to confirm that there were eight veg1 and veg2 cells in each tier. The desired cells were labeled, followed by a quick confirmation that the appropriate blastomeres were marked before overnight culture.

For 8th cleavage labeling experiments, embryos were placed in chambers at the 60-cell stage. Embryos were observed continuously with a camera connected to a monitor, and their cell outlines were drawn directly on the monitor screen to follow the blastomeres as the embryos divided. At the eighth cleavage, the orientation of the division plane through the veg1-derived blastomere was documented. Where the division was equatorial, the more animal veg1 descendant was labeled with DiI.

Imaging

All DiI-labeled embryos were examined live using a cooled CCD camera (either Photometrics or Princeton Instruments) mounted on a Zeiss Axiovert 135 TV microscope or Leica DMRB microscope. Images were captured using PMIS or Metamorph image processing software. Color images were photographed using Ektachrome 400 slide film with embryos mounted on a Nikon Diaphot DMT microscope. Embryos were immobilized on poly-L-lysine (0.1% w/v, Sigma)-coated slides under glass coverslips separated by clay feet.

To follow veg1 descendants during gastrulation, specimens were placed in modified glass slides in which a circular hole had been cut in the center. As images were captured on an inverted microscope, a small chamber that would hold the embryo was created by affixing a poly-L-lysine-coated coverslip with vacuum grease to the lower surface of the slide. A smaller coverslip fragment separated by clay feet was placed on top of this larger coverslip to create a glass sandwich that could hold the specimen. The chamber was filled with sea water and an embryo was inserted and oriented under the small coverslip fragment with a mouth pipette. Care was taken not to compress the embryo in order to minimize the possibility of affecting gastrulation movements, so some shifting of the embryo was observed during the experiment. The entire chamber was then flooded with ASW, and another coverslip was placed on the top surface of the slide with vacuum grease to prevent evaporation of sea water from the chamber. The sea water was changed periodically during the course of the experiment without disturbing the embryo. Specimens mounted in these modified glass chambers were healthy for over 5 hours and gastrulated on a similar time-schedule as controls that had been cultured in glass bowls throughout development.

RESULTS

Both veg1 and veg2 descendants contribute to the archenteron

The veg1 and veg2 blastomeres are easily identified at the 60-cell stage as two tiers of eight cells each, which overlie the micromeres in the vegetal half of the embryo (Fig. 1C). Earlier studies suggested that the segregation of the ectoderm from the endoderm accompanies the formation of the veg1 and veg2 tiers (Cameron and Davidson, 1991; Hörstadius, 1973). To examine the cell fates of veg1 and veg2 blastomeres in *L. variegatus*, single veg1 or veg2 cells were labeled with the lipophilic dye, DiI(C16), and the embryos were examined at the pluteus stage.

Both veg1 and veg2 cells of normal embryos contribute to the archenteron (Table 1, Figs 2-6). The presence of veg1 descendants in gut tissues was not due to toxic effects of DiI, because veg1 cells labeled iontophoretically with rhodaminated dextran also contributed progeny to the archenteron ($n=5$, data not shown). In addition, both heavily and lightly DiI-labeled veg1 blastomeres formed archenteron tissues, eliminating the possibility that veg1 cell fate could be affected by dye concentration.

As confirmation that the presence of labeled veg1 cells in the archenteron was not due to incorrect identification of blastomeres, pairs of sister veg1 and veg2 cells were labeled with different dyes. A single veg1 cell was labeled with DiI(C16), and the veg2 sister blastomere below it was labeled with DiO(C16) (Fig. 2A). In all cases ($n=5$), descendants of both blastomeres formed archenteron tissues (Fig. 2B,C). Veg1 descendants (Fig. 2C, red) occupied regions toward the base of the archenteron, whereas veg2 descendants (Fig. 2C, green) lay in more distal regions, near the archenteron tip.

To document when veg1 and veg2 progeny enter the archenteron, single veg1 ($n=9$) and veg2 ($n=8$) cells were labeled and their descendants were followed during gastrulation. Veg2 descendants exclusively formed the buckled vegetal plate

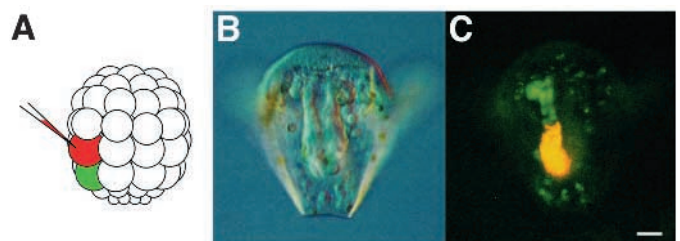


Fig. 2. Both veg1 and veg2 cells are precursors of the archenteron. (A) A diagram illustrating double-labeling of the veg1 and veg2 blastomeres. A single veg2 blastomere was labeled with DiO(C16), shown in green, and the sister veg1 cell above it was labeled with DiI(C16), shown in red. (B) A bright-field image of a double-labeled embryo at the early pluteus stage, showing the archenteron. (C) The same embryo demonstrating that both veg1 and veg2 descendants have contributed to the archenteron. In this embryo, a small amount of DiO was allowed to transfer to the veg1 cell by labeling the sister veg2 blastomere prior to completion of cleavage by the macromere. When subsequently labeling the veg1 cell with DiI, the presence of DiO in both veg1 and veg2 cells permitted unambiguous identification of veg1/veg2 sister-cell pairs. The yellow color of veg1 descendants results from photographing DiI and DiO together. The dispersed green cells are SMCs. Scale bar, 20 μm .

Table 1. Frequency with which single labeled veg1 and veg2 descendants form archenteron-derived tissues in individual embryos

	Veg2			Veg1		
	Total % †	Oral %*	Aboral %*	Total % †	Oral %*	Aboral %*
Pigment cells	51	0	49	2	0	4
Blastocoelar cells	62	100	33	0	0	0
Coelomic Sac cells	40	30	42	0	0	0
Foregut (and Muscle)	100	100	100	35	21	48
Midgut	60	60	48	100	100	100
Hindgut	0	0	0	100	100	100
Anal Ectoderm	0	0	0	91	95	89
Number of cases scored (<i>n</i>)	43	10	33	46	19	27

†For each embryo, the presence or absence of fluorescent archenteron cell types derived from single-labeled veg1 or veg2 cells was scored. The above values do not reflect the number of fluorescent cells in each of the tissues. Values are expressed as a percentage of the number of cases scored.

*Labeled veg1 or veg2 descendants were classified as either 'oral' or 'aboral' depending on their position within the pluteus. The frequency with which they contributed to various tissues is represented as a percentage of cases scored in either the oral or aboral sectors.

during early archenteron formation (data not shown). Veg1 progeny remained outside the archenteron until the mid- to late-gastrula stages (Fig. 3A-C), after which they involuted over the blastopore lip, rearranging into a long thin array of cells (Fig. 3D-F). In a few cases, the column of veg1 descendants was interrupted by non-fluorescent cells. Whether the unlabeled cells originated from a neighboring veg1 or veg2 clone was not determined. Cell movements of labeled veg1 descendants continued at least through the late gastrula to early prism stages, but the increasing curvature of the gut made filming difficult, so embryos were not followed after this time.

These results demonstrate that the equatorial cleavage that produces the veg1 and veg2 tiers does not demarcate a precise segregation of cell fates to the ectoderm and the endoderm, as previously predicted. Rather, the sixth cleavage provides two populations of presumptive archenteron cells that enter the gut

at distinct times during gastrulation. Veg2 descendants initiate archenteron invagination; veg1 descendants subsequently contribute to the archenteron after the mid- to late-gastrula stage.

Veg1 and veg2 descendants differentiate into an overlapping range of cell types within the archenteron

Cell types formed by the archenteron include the fore-, mid- and hind-gut as well as the secondary mesenchyme cells (SMCs). SMCs differentiate into four different subpopulations: pigment cells, blastocoelar cells, circumesophageal muscle cells and coelomic sac cells. Earlier cell aggregation

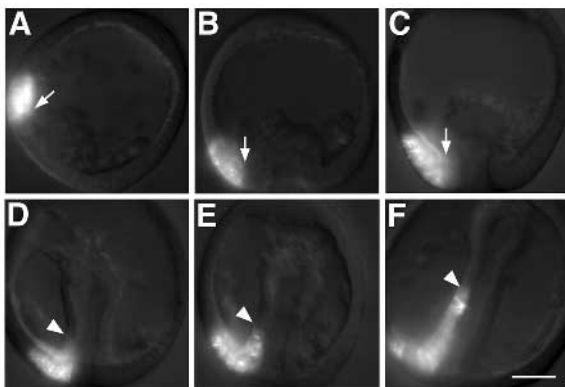


Fig. 3. Cell movements of DiI-labeled veg1 descendants during gastrulation. All panels are side views of the same embryo at different times. (A) Embryo prior to primary invagination of the vegetal plate. Veg1 descendants are a coherent patch at the vegetal plate periphery (arrow). (B,C) Embryos during primary invagination. Veg1 descendants do not participate in the initial buckling of the vegetal plate and lie outside of the archenteron. Veg1 descendants remain separate from the veg2 descendants, as shown by the arrow, which marks the location of the veg1/veg2 boundary. (D-F) Embryos during the mid- to late-gastrula stages. Veg1 descendants involute over the blastopore lip after the mid-gastrula stage. Arrowhead marks the advancing front of labeled veg1 descendants. Scale bar, 20 μ m.

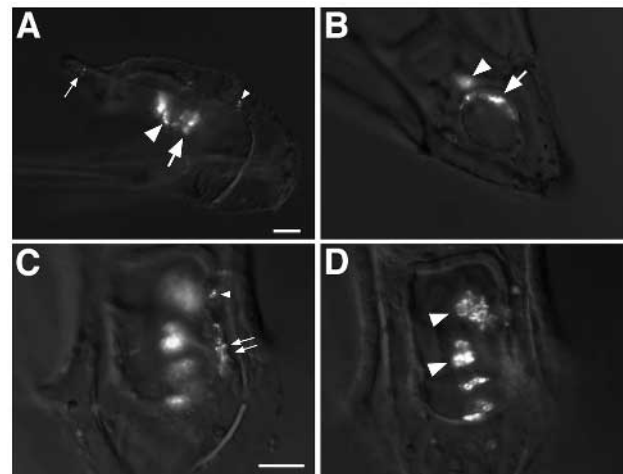


Fig. 4. Descendants of DiI-labeled veg2 cells at the pluteus stage. (A) Side view of a pluteus showing veg2 descendants on the oral surface of the gut. Labeled cells occupy the foregut and midgut as well as pigment and blastocoelar cells. (B) Pluteus showing labeled veg2 cells in the foregut and midgut. (C) A higher magnification view of a pluteus examined through the oral hood possessing fluorescent blastocoelar cells and cells in the coelomic sacs. (D) A different focal plane of the same embryo as in C, showing labeled veg2 descendants in the foregut. Although the clone of veg2 progeny is discontinuous along the length of the gut, there is little lateral dispersion of cells. Large arrowhead, foregut; large arrow, midgut; small arrow, pigment cells; small arrowhead, blastocoelar cells; double arrows, coelomic sac cells. Scale bars, 20 μ m.

experiments showed that both veg1 and veg2 cells could make endoderm in vitro but differed in their ability to make foregut cell types (Khaner and Wilt, 1991). In addition, our observation demonstrating a temporally distinct entry of DiI-labeled veg1 and veg2 descendants into the archenteron during gastrulation suggested that perhaps the sixth equatorial cleavage would predict a segregation of endodermal cell fates within the archenteron. To test this hypothesis, single veg2 or veg1 blastomeres were labeled and the cell types formed by their descendants at the pluteus stage were documented.

Veg2 descendants

Veg2 descendants form secondary mesenchyme, foregut and midgut, but do not make hindgut (Table 1, Fig. 4A-D). The labeled secondary mesenchyme population includes widely dispersed pigment and blastocoelar cells, coelomic sac cells and circumesophageal muscle (Table 1, Fig. 4A,C). Because circumesophageal muscle cells are difficult to distinguish reliably from other labeled veg2 descendants in the foregut, they were scored together with 'foregut' in this study. Within the endoderm, veg2 descendants sometimes form patches that are discontinuous along the length of the gut (Fig. 4D), suggesting that cell rearrangements during gastrulation disrupt the continuity of labeled cells within this tissue. However, fluorescent cells are generally confined to a single vertical area within the archenteron and are not displaced laterally over long distances.

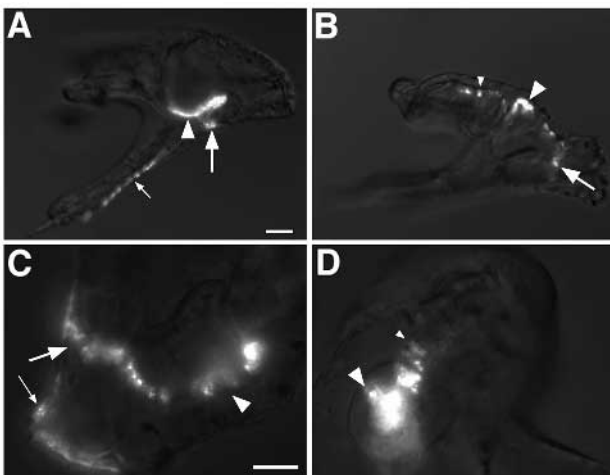


Fig. 5. Descendants of DiI-labeled veg1 cells at the pluteus stage. (A) Side view of a pluteus larva showing fluorescent cells in the ectoderm, hindgut and midgut. Veg1 progeny lie on the oral surface of the gut. (B) Side view of a pluteus larva showing labeled veg1 progeny in the hindgut, midgut and foregut. Veg1 descendants lie on the aboral surface of the gut. (C) Side view of a pluteus larva shown at higher magnification. Veg1 descendants occupy the ectoderm near the apex of the pluteus as well as the hindgut and midgut. (D) Higher magnification of a pluteus viewed through the oral hood, containing labeled cells in the foregut and midgut. The clone of veg1 descendants is interrupted by unlabeled cells, indicating that cell movements during gastrulation can disrupt the continuity of cells derived from a single blastomere. Small arrow, ectoderm; large arrow, hindgut; large arrowhead, midgut; small arrowhead, foregut. Scale bars, 20 μ m.

Veg1 descendants

Veg1 descendants contribute to all tissues of the endoderm (Table 1, Fig. 5A-D). Descendants of labeled veg1 cells always occupy the midgut and hindgut (Fig. 5A,B,D) and sometimes contribute to foregut tissues (Fig. 5B,D). Veg1 descendants undergo cell rearrangements during gastrulation, as seen by the transformation of veg1 clones that begin as a small coherent patch into a narrow column of cells by the end of archenteron formation (Fig. 3). Veg1 descendants only rarely form secondary mesenchyme cell types (Table 1). Specifically, veg1 descendants formed pigment cells in only one specimen and were not found to differentiate into any other SMC subtypes.

These results demonstrate that although veg1 and veg2 descendants differ in that veg1 progeny make ectoderm whereas veg2 progeny make SMCs, there is a high degree of overlap and variability in the range of endodermal cell types formed by veg1 and veg2 descendants. Therefore, the equatorial division that produces the veg1 and veg2 tiers does not delineate endodermal cell type boundaries within the gut.

Fate choices within the endoderm are variable and are not dictated by radial position around the animal-vegetal axis

DiI-labeling of single veg1 cells within a given embryo reveals the range of tissue types that the descendants of an individual blastomere can form. However, cell-marking studies of this type cannot distinguish whether each veg1 cell assumes a unique repertoire of cell fates that is different from its other veg1 neighbors within the embryo, or whether the fates exhibited by a given veg1 cell are identical for all of the neighboring veg1 blastomeres. For example, veg1 progeny make foregut at a 35% frequency, but this number could represent either (1) foregut formation by all veg1 blastomeres in 35% of embryos or (2) foregut formation by only a subset of veg1 progeny within a given embryo, which is revealed 35% of the time. To distinguish between these two possibilities, the entire veg1 tier was DiI-labeled and examined at the pluteus stage ($n=9$). The distribution of labeled veg1 cells is variable between embryos (Fig. 6), indicating that the range of cell fates exhibited by veg1 cells is not highly stereospecific.

Several lines of evidence suggest that the formation of different endodermal cell types does not correlate with the radial position of veg1 blastomeres around the animal-vegetal axis. First, the distribution of fluorescent tissues that results from labeling all veg1 cells within an embryo is variable. As shown in Fig. 6A, fluorescent veg1 progeny lie within the foregut on the oral surface of the gut, whereas in another embryo labeled in the same way, fluorescent veg1 descendants occupy the foregut on the aboral side (Fig. 6B). Second, a correlation between cell fate and radial position around the animal-vegetal axis should reveal an identifiable pattern between the range of cell types made by single blastomeres within the gut and the final location of fluorescent cells with respect to the left-right or oral-aboral axes. Single veg1 or veg2 cells were marked and the identities and location of the labeled progeny were documented. It was difficult to match the resulting labeled cell types with a specific position in the embryo because the cleavages that produce the veg1 and veg2 blastomeres do not lie in a rigid relationship with respect to the

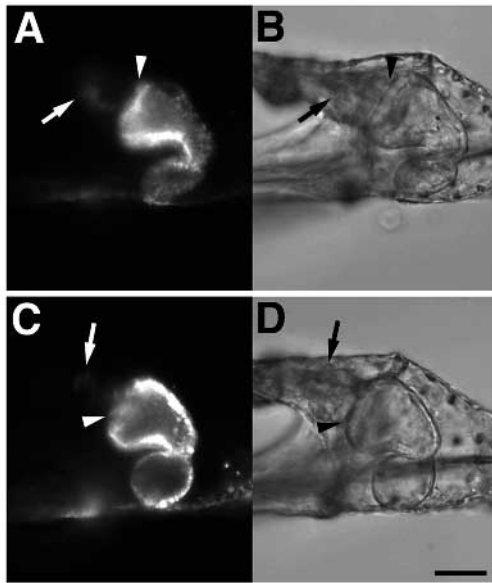


Fig. 6. The distribution of veg1 descendants varies in the gut tissues of different embryos. Shown are two embryos resulting from DiI-labeling of the entire veg1 tier. The plutei contain veg1 descendants that occupy the foregut, midgut, hindgut and ectoderm, but some veg1 cells lie either on the oral (A,B, arrows) or on the aboral surfaces (C,D, arrows) of the foregut, while other veg1 descendants only occupy up to the midgut (A-D, arrowheads). Scale bar, 20 μ m.

oral-aboral and left-right axes (data not shown). Descendants of veg1 and veg2 cells spanned the axial midline in some cases and roughly abutted the midline in others. To simplify the analysis, the resulting labeling patterns were classified as either 'oral' or 'aboral', depending on the general location of the clones within the plutei. All staining on the lateral sides of the archenteron were included in the 'aboral' category, and for veg1 cells, the location of labeled progeny within the ectoderm was used as an additional indicator of position within the archenteron. As shown in Table 1, no predictable pattern of endodermal cell types formed by veg1 and veg2 descendants could be identified on the basis of their radial position around the animal-vegetal axis. Therefore, cell fates within the endoderm do not correlate with early blastomere position in the cleavage stage embryo, but rather appear to be influenced by cell position later in development.

Labeled veg2 progeny exhibited distinct differences in the differentiation of some SMC cell types, depending on their oral or aboral location within the embryo (Table 1). Veg2 progeny on the oral side of the archenteron made blastocoelar cells ($n=10/10$) and never made pigment cells ($n=0/10$). Pigment cells were formed by aboral veg2 descendants ($n=16/33$), although some of these cells also made blastocoelar cells ($n=11/33$). A caveat of the experiment is that because DiI becomes faint with time, embryos were scored at 48 hours instead of at 60 hours when all pigment cells have differentiated (Ettensohn and Ruffins, 1993). Where both pigment and blastocoelar cells were seen, some of the blastocoelar cells could have been undifferentiated pigment cells. This oral-aboral bias in SMC fates has been observed previously by Ruffins and Ettensohn (1996) and our results are in agreement with their findings.

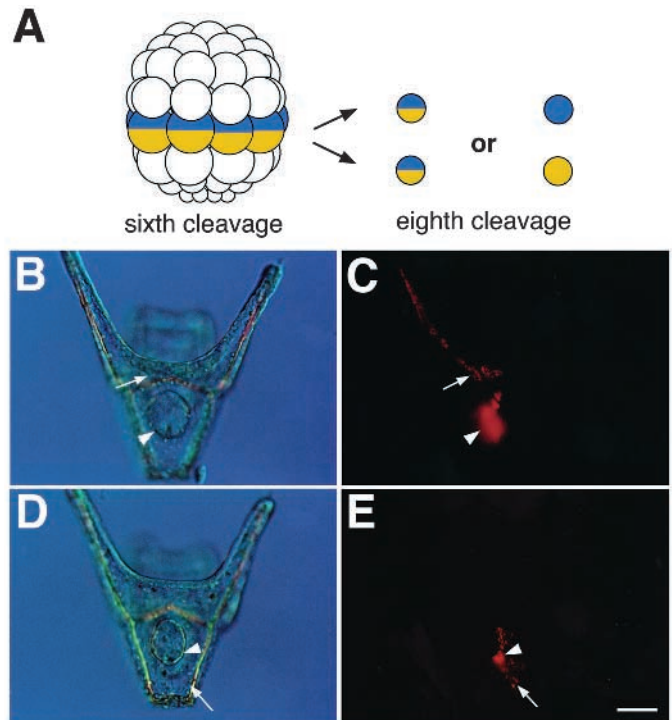


Fig. 7. The segregation of the prospective ectoderm from the endoderm does not occur at the eighth cleavage. (A) A diagram of two possible outcomes of a labeling experiment performed at the eighth cleavage. Colors represent the tissue types that a single labeled blastomere can form (blue, ectoderm; yellow, endoderm). Labeling of veg1 descendants at the eighth cleavage could give progeny that will each form both ectoderm and endoderm, showing that the segregation of ectoderm from endoderm does not occur at this stage. Alternatively, each veg1 descendant could form ectoderm or endoderm but not both tissues, indicating that the allocation of these cells to different prospective fates has occurred. (B-E) Two examples of plutei resulting from DiI-labeling of a single veg1 descendant at the eighth cleavage. Only the more animally located veg1-derived cell was labeled, with the prediction that all of its progeny would form ectoderm if the segregation of ectoderm from endoderm occurs at this time. Resulting plutei contain labeled cells that span both the ectoderm and endoderm, showing that the eighth cleavage does not demarcate the ectoderm-endoderm boundary. In these two cases, some veg1 descendants lie in the ectoderm (arrow), and other veg1 progeny are found in the hindgut (arrowhead). (B,D, bright-field; C,E, fluorescence) Scale bar, 40 μ m.

The ectoderm/endoderm germ layer boundary is not dictated by cell lineage

Veg1 descendants labeled at the eighth cleavage

Descendants of veg1 cells labeled at the 60-cell stage form ectoderm in addition to endoderm (Table 1, Fig. 5A,C). The segregation of these two germ layers therefore is not correlated with the sixth equatorial cleavage division and must occur later in development. In the sea urchin embryo, the early divisions alternate between an equatorial and a meridional orientation (reviewed in Czihak, 1975; Summers et al., 1991, 1993). If ectoderm and endoderm are separated by an equatorial cleavage, the eighth cleavage would be the next possible division when cells could be allocated to different lineages. The more animally located veg1-derived blastomere was DiI-

labeled at the eighth cleavage, with the prediction that all descendants would contribute to the ectoderm if a segregation in germ layer were to occur at this stage (Fig. 7A). In nine out of 13 cases, veg1 descendants cleaved equatorially at the eighth division; the remaining embryos cleaved meridionally or at other angles. Of the nine equatorially cleaving embryos, the labeled descendants of five cases (56%) formed both ectoderm and endoderm (Fig. 7B-E). Although labeled descendants within the endoderm occupied only the hindgut in most cases, one embryo contained fluorescent veg1 progeny in both midgut and hindgut tissues, suggesting that veg1 descendants born at the eighth cleavage are not necessarily restricted to the hindgut at this time. The remaining four cases contributed only to the ectoderm. All embryos in which veg1 cells cleaved non-equatorially produced descendants that segregated to both the ectoderm and the endoderm. Therefore, the allocation of cells to the prospective ectoderm or endoderm does not occur predictably during the eighth cleavage. As cleavage orientations become increasingly variable over time, the segregation of veg1 progeny to either germ layer is not correlated with specific cleavage boundaries of the early embryo.

The number of veg1 descendants allocated to the ectoderm is variable

As another test to confirm that the segregation of the ectoderm from the endoderm is not dictated by the early cleavage pattern, multiple veg1 cells were labeled and their descendants were counted in the ectoderm. If there were a predictable allocation of veg1 cells to the ectoderm, the number of ectodermal veg1 descendants should be similar, either between all labeled veg1 blastomeres within the same embryo, or between labeled blastomeres occupying similar positions in different embryos.

Four out of eight veg1 cells were labeled at the 60-cell stage by marking every other veg1 blastomere within the embryo ($n=18$; Fig. 8A). The distribution of labeled cells within the ectoderm was recorded, and the number of veg1 descendants was counted in each clone. All embryos were scored at the pluteus stage, at 48 hours post-fertilization. Because our labeling method did not always mark the four blastomeres with the same intensity, the descendants of each clone could be distinguished in the same specimen. In addition, the DiI-labeling was stable enough throughout the duration of the experiment

so that cells could be identified by the fluorescent, punctate cytoplasmic staining surrounding the nucleus which remained dark. Four clonal patches of ectoderm were seen in every embryo. In addition, similar patterns of distribution in the four patches could be identified between different embryos. Although marking four out of eight veg1 cells predicts that only two patterns of labeling should be produced, at least six distinct patterns of ectodermal staining were documented (Fig. 8B). This indicates that the early cleavages are not stereotyped with respect to the embryonic axes in *Lytechinus variegatus*.

Embryos that displayed similar distributions of labeled veg1 descendants were grouped together and compared further. Fig. 8C shows a diagram of one type of pattern observed. The variability in the numbers of labeled ectodermal cells affects the size and shape of the fluorescent ectodermal patches. Although clones generally lie in similar positions, the number of veg1-derived cells that occupy the ectoderm differs from embryo to embryo as well as from clone to clone within the same embryo.

When labeled veg1 progeny were counted in the ectoderm and endoderm, no strict relationships between the number of cells allocated to each tissue were identifiable ($n=43$). Although there are roughly twice as many veg1 descendants in the endoderm as in the ectoderm (mean number of endodermal cells= 30.7 ± 9.4 ; mean number of ectodermal cells= 13.8 ± 11.8), there is a large degree of variability in the number of endodermal and ectodermal veg1 descendants derived from a single blastomere. A small number of veg1 descendants in the endoderm does not always predict a large number of ectodermal cells, or vice versa. Separating the labeled clones into 'oral,' 'lateral' or 'aboral' categories, based on their position, also did not reveal any easily identifiable patterns to the cell numbers allocated to the ectoderm or endoderm. Therefore, the segregation of veg1 cell fates to the ectoderm must be determined independently for the descendants of each veg1 cell.

The sum of ectodermal and endodermal progeny derived from a single veg1 clone was also variable between different clonal patches of veg1 descendants. As *Lytechinus variegatus* embryos undergo cell divisions within the archenteron during gastrulation (C. Y. Logan, unpublished observations; Nislow and Morrill, 1988), this variability may be explained by differences in the patterns of cell divisions within and between the ectoderm and endoderm.

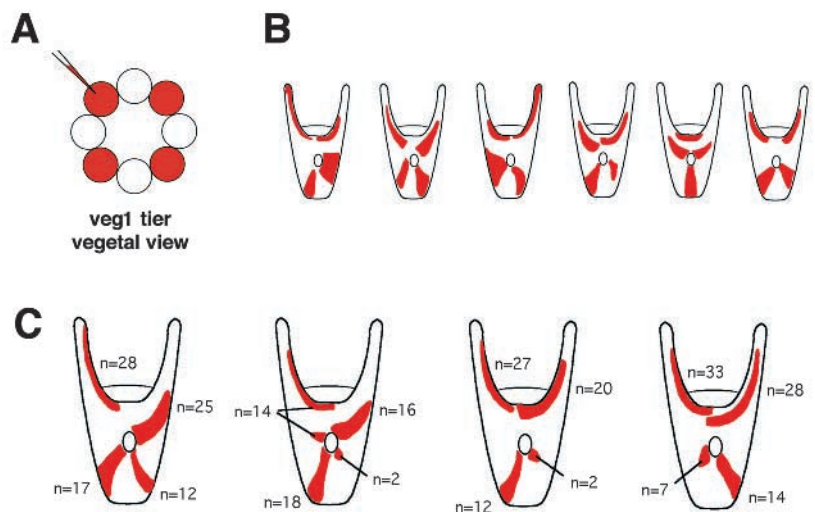
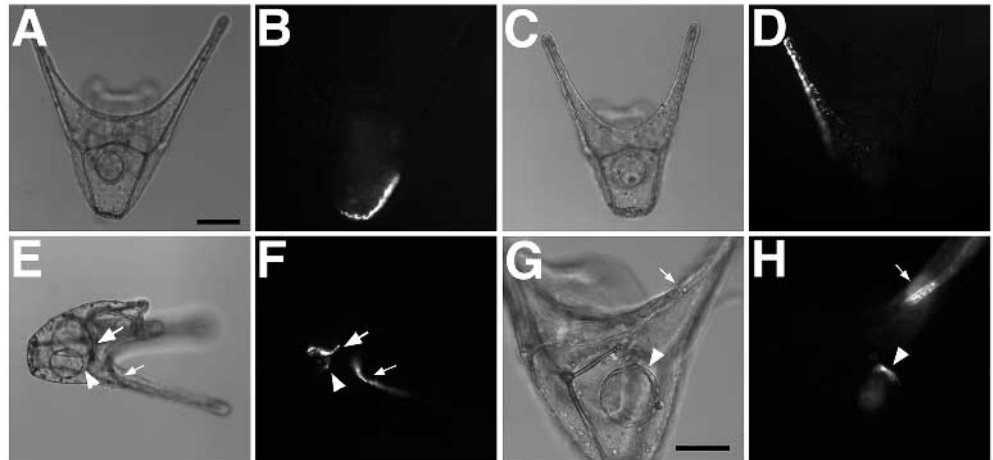


Fig. 8. Descendants of veg1 in the same embryo contribute different numbers of progeny to the ectoderm. (A) DiI-labeling of alternating veg1 blastomeres results in four out of eight fluorescently labeled cells within the tier. (B) Six different labeling patterns obtained show that there is variability in the positions and sizes of labeled veg1-derived patches within the ectoderm. (C) A diagram showing four embryos with similar labeling patterns and the number of veg1-derived cells in each fluorescent ectodermal patch. Veg1 descendants within the ectoderm contain different numbers of labeled progeny, both within the same embryo and in different embryos, even when occupying similar positions. Note that n values here represent the number of DiI-labeled cells within the ectoderm and not the number of cases labeled.

Fig. 9. Mesomeres can contribute descendants to the endoderm at low frequency. Four plutei resulting from DiI-labeling of single mesomeres overlaying the macromeres at the 32-cell stage. In most cases, mesomeres form only ectoderm (A-D). However, at low frequency, mesomeres can also contribute descendants to the hindgut (arrowhead, E-H), midgut (large arrow, E,F) as well as the ectoderm (small arrow in E-H). (A,C,E,G, bright-field; B,D,F,H, fluorescence). Scale bars, 50 μm (A-F); 40 μm (G,H).



Mesomeres can form gut tissues in normal embryos

Some *veg1* descendants do not contribute any progeny to the ectoderm (Table 1), indicating that in some embryos, the ectoderm-endoderm boundary must lie at the cleavage plane between the *veg1* tier and the mesomeres or within the mesomeres. The normal fate of mesomeres is to form ectoderm (Cameron and Davidson, 1991; Cameron et al., 1987), but several studies have shown experimentally that mesomeres can make endodermal tissues (Henry et al., 1989; Khaner and Wilt, 1990). Because the allocation of cells to either ectoderm or endoderm correlates more strongly with the position of cells within the embryo rather than with the pattern of early cleavages, we hypothesized that mesomeres might also form endoderm in normal embryos. As a test, single mesomeres directly overlying the macromeres at the 32-cell stage were DiI-labeled and examined at the pluteus stage.

In agreement with previous fate-mapping studies (Cameron and Davidson, 1991; Cameron et al., 1987; Hörstadius, 1973), mesomere descendants contributed to only the ectoderm in 43 out of 51 embryos labeled (Fig. 9A-D). However, in eight cases (16%), mesomeres formed both ectoderm and endoderm (Fig. 9E-H). Some specimens possessed endodermal mesomere descendants that were confined to the hindgut (Fig. 9G,H), but other embryos displayed mesomere descendants in both midgut and hindgut regions (Fig. 9E,F). Although the labeled mesomere descendants in the two embryos shown in Fig. 10E-H lie towards the oral side of the embryo, as seen by fluorescent cells in the ciliary band, a mesomere in any radial position around the animal-vegetal axis could contribute progeny to the endoderm. The fluorescence in the endoderm was not due to accidental delivery of dye to *veg1* cells, since cells were marked prior to the macromere cleavage that forms the *veg1* and *veg2* tiers. In addition, labeled cells in the endoderm were not a consequence of mis-labeled macromeres, since this would have resulted in fluorescent SMCs and large sectors of labeled archenteron tissues, a pattern that was not observed. These results confirm that establishment of the ectoderm-endoderm boundary is not correlated with specific early cleavage events and that the presumptive endoderm of normal embryos includes more blastomeres than was thought previously.

Curiously, in a separate batch of embryos not included in the analysis described above, we noticed that the majority of 32-

cell stage macromeres were unusually small. In this batch, the third cleavage plane had apparently been more subequatorial than normally seen, extending the mesomeres into a more vegetal region of the embryo than usual. When mesomeres of these embryos were labeled with DiI, 8/12 cases contributed labeled cells to the endoderm.

DISCUSSION

Early blastomeres in the sea urchin have been shown by a variety of experimental means to possess a much greater potency than is exhibited in the normal embryo (for examples, see Hörstadius, 1939; Henry et al., 1989; Khaner and Wilt, 1991). However, previous fate maps of normal 60-cell stage embryos have suggested a tight correlation between the pattern of early cleavages and the segregation of cell fates to distinct lineages (Cameron and Davidson, 1991). In this study, we demonstrate a much less rigid relationship between cleavage boundaries and cell-fate boundaries for the macromere descendants. We show that (1) prospective endoderm consists of a larger population of early blastomeres than was previously thought; (2) ectoderm and endoderm are not segregated by the early cleavage divisions; and (3) establishment of ectoderm and different endodermal cell identities is correlated with the later position of blastomeres within the embryo rather than with the early cleavage pattern. As illustrated in Fig. 10B, which shows a revised fate map of the 60-cell stage sea urchin embryo, both *veg1* and *veg2* cells contribute progeny to the endoderm, some *veg2* descendants make SMCs, and a subset of *veg1* progeny form anal ectoderm. Mesomeres also contribute to the gut in some embryos, but we have chosen not to represent this in Fig. 10B, as mesomeres form gut tissues in only a minority of labeled cases.

Precursors of the archenteron include both *veg1* and *veg2* descendants

The descendants of both *veg1* and *veg2* cells form archenteron tissues (Figs 2-6, Table 1), showing that the prospective endoderm population occupies much of the vegetal half of the embryo. This finding was initially surprising because earlier studies had suggested that *veg1* and *veg2* progeny contribute to distinct tissues, and that the archenteron was derived from

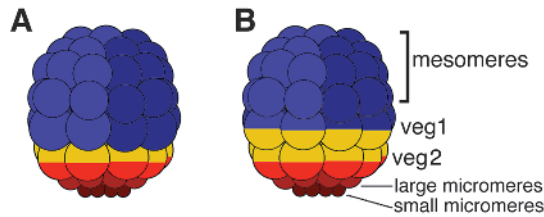


Fig. 10. Old and revised fate maps of the 60-cell stage embryo. The lineages have been color-coded: brown, small micromeres; dark red, PMCs; light red, SMCs; yellow, endoderm; blue, oral and aboral ectoderm. (A) The old fate map of the 60-cell stage sea urchin embryo shows that the segregation of the ectoderm and endoderm accompanies the cleavage that produces the veg1 and veg2 tiers. Precursors of the archenteron derive from only the veg2 layer. (B) The revised fate map shows that the allocation of fates to distinct lineages has not been completed by the 60-cell stage, as both veg1 and veg2 cells form precursors of the archenteron. Although a subset of mesomere descendants form endoderm in some embryos, this has not been included in the figure because endoderm formation by mesomeres occurs in only 16% of labeled cases.

only the veg2 tier (Hörstadius, 1939). It is unclear why there is a discrepancy between our results and those reported earlier. One explanation is that veg1 and veg2 fates are affected by our labeling technique, by the confinement of the embryos in manipulation chambers, or by toxic effects of the dye. However, identical results were obtained with both DiI and rhodaminated dextran, and when cells were DiI-labeled using either coated needles or iontophoresis. In addition, compression of embryos and disruption of cleavage planes were minimized by a variety of methods, prior to and during labeling (see Materials and Methods). An alternative explanation is that our results are specific to *L. variegatus* and that separation of the prospective ectoderm from endoderm occurs differently in other species. However, the separation of the ectoderm from endoderm also fails to occur at the sixth equatorial cleavage in *S. purpuratus* (Ransick and Davidson, 1995), indicating that allocation of veg1 and veg2 descendants occurs after the 60-cell stage for at least two different sea urchin species.

The results of the cell-marking studies also reveal that allocation of veg1 and veg2 cells to specific endodermal compartments occurs after the 60-cell stage because the sixth cleavage does not correlate with any endodermal cell type boundaries within the gut. In addition, the observations that the distribution of veg1 progeny differs between embryos, and that the range of endodermal cell types made by veg1 cells is not related to their radial position around the animal-vegetal axis, indicate that specification of endodermal cell fates may be largely dictated by later cell position. In agreement with this, previous studies have shown that cells within the archenteron are plastic throughout gastrulation (McClay and Logan, 1996). The ability of archenteron tissues to regulate in response to a variety of microsurgical perturbations has been interpreted to suggest that cells continuously evaluate their position throughout archenteron elongation, presumably through cell-cell signaling. Cells appear to be specified during gastrulation, as indicated by expression of region-specific markers within the gut, but the commitment of cells to specific endodermal fates is determined according to the final position of cells after all gastrulation movements have ceased.

Although veg1 and veg2 descendants are similar in their ability to form endodermal tissues, it is worth mentioning that these two cell types are distinct in their contributions to the SMCs and ectoderm. Whether this reflects real differences in the ability of these cell types to differentiate into mesoderm or ectoderm, or whether the descendants of these cells are simply not in the appropriate location within the embryo to form these other tissue types, is unclear at this time. Further experimental studies will be required to address both how veg1 and veg2 acquire the ability to form endoderm, and how cell fate differences between veg1 and veg2 descendants arise during development.

Cell movements during gastrulation

The observation that involution of cells over the blastopore lip occurs surprisingly late during archenteron elongation extends our understanding of gastrulation movements in the sea urchin (Fig. 3). Previous studies suggested that the cells peripheral to the vegetal plate might contribute a few cells to the archenteron, but the late wave of veg1 cell involution has not been reported previously (Ettensohn, 1984; Ruffins and Ettensohn, 1996). Descriptions of gastrulation have outlined archenteron formation as occurring in three steps, with (1) primary invagination that involves an initial buckling of the vegetal plate (Burke et al., 1991; Ettensohn, 1984; Lane et al., 1993; Nakajima and Burke, 1996); (2) an elongation phase that is accompanied by cellular rearrangements (Ettensohn, 1985; Hardin, 1989; Hardin and Cheng, 1986); and (3) a third phase that utilizes SMC recognition of the roofplate target to pull the archenteron to its final length (Hardin and McClay, 1990). The movement of a large quantity of veg1-derived tissue into the archenteron late in gastrulation (Figs 3 and 6) reveals a fourth phase to gastrulation movements in the sea urchin that contributes much of the presumptive midgut and all of the hindgut tissues to the developing larval endoderm.

Specification of the ectoderm-endoderm boundary

The results of the lineage analysis indicate that allocation of cells to the ectoderm or endoderm does not occur at either the sixth or eighth cleavages and instead appears to be refined on the basis of the later position of cells within the embryo. Two observations support this conclusion. First, when veg1 progeny were labeled at the eighth cleavage, we observed that although many embryos at this division cleave equatorially, many other embryos divide at variable orientations, suggesting that the allocation of veg1 progeny to ectoderm and endoderm is not rigidly correlated with cleavage pattern. Such unpredictability in the cleavage orientations of early blastomeres has also been reported by Summers et al. (1991). In addition, as shown by marking multiple veg1 cells within the same embryo, the number of veg1 progeny allocated to the ectoderm is variable and therefore must be determined by the location of individual cells within the embryo later in development (Fig. 7C).

Descendants of labeled mesomeres form endoderm in normal embryos at a low frequency, showing that the ability to segregate progeny to both ectoderm and endoderm is not restricted to the veg1 population. The significance of the contribution of mesomeres to the endoderm in 16% of labeled cases is unclear at this time. Our labeling technique does not distinguish between whether a single mesomere contributes to endoderm in every embryo or whether mesomeres simply form

endoderm at low frequency. If one out of eight mesomeres were to contribute to the endoderm in all embryos, one might find mesomeres in the endoderm 16% of the time, but this seems unlikely, given that endodermal mesomere descendants are not always found in the same position between embryos. The fact that mesomeres form endoderm at all is consistent with the hypothesis that the ability to contribute to the gut is mediated by both the initial position of the blastomere along the animal-vegetal axis and probably later cell signaling that refines the final fates of cells near the ectoderm-endoderm junction.

Implications of the cell lineage analysis

The results of the clonal analysis presented here suggest that the model of early cell fate specification in the sea urchin involving a tier-to-tier signaling cascade to segregate cell fates during the cleavage stages requires some modification. First, the sea urchin does not display cell fate restriction during early cleavage, but instead exhibits a mixture of both determinate and indeterminate cleavages. For some cell populations, such as the skeletogenic mesenchyme and small micromeres territories, cleavage boundaries also demarcate lineage boundaries. Within the macromere descendants, however, early cleavage boundaries and tissue boundaries are not highly correlated, suggesting that cell fates are refined according to later cell position rather by the cleavage process. This feature makes the sea urchin embryo in some ways much like many other deuterostome embryos in which tissue boundaries do not become clearly established until fairly late in development (for examples see Dale and Slack, 1987; Kimmel et al. 1990). Second, our results do not support the hypothesis that signaling between the veg1 and veg2 tiers segregates cells to the ectoderm and endoderm. The 'vegetal plate territory' has been thought to originate from only the veg2 tier and to constitute the entire presumptive gut (Davidson, 1993). In contrast, the results presented here demonstrate that the archenteron includes both veg1 and veg2 descendants and is not segregated from the ectoderm until much later in development. Cleavage boundaries certainly may play some role in normal development because the 'vegetal plate,' which has been described as the thickened region that undergoes primary invagination (Ransick and Davidson, 1995), is segregated at the sixth equatorial cleavage from the rest of the embryo (Fig. 3). However, if cell-cell signaling between veg1 and veg2 cells does exist, its function does not appear to be related to cell-fate segregation during early cleavage.

Clonal analyses do not yield strong insights into the mechanisms that drive cells along distinct pathways of differentiation, and cannot distinguish between autonomous or conditional modes in cell-fate specification. Because veg1 blastomeres labeled during cleavage are variable in their contribution to the ectoderm and endoderm, our results can be best explained by cell-signaling events that allow cells to make fate choices on an individual basis, based on their later position. Signaling during fairly late stages has been demonstrated within the archenteron, and appears to play an important role in assigning identities appropriate to the location of cells within the gut (McClay and Logan, 1996). Thus, it is also possible that cell interactions during later development play a role in the position-dependent segregation of cells to distinct germ layers.

However, autonomous mechanisms may also contribute to

endodermal specification. First, Henry et al. (1989) have reported that animal caps obtained from subequatorially cleaving embryos can form endoderm. In addition, we have observed that mesomeres of intact, subequatorially cleaving embryos frequently contribute progeny to the gut. These observations suggest that the competence to form endoderm may be broadly distributed very early in development and can be localized to mesomeres in some embryos, depending on the early cleavage pattern. Second, it has been shown recently that nuclear beta-catenin protein becomes localized to the nuclei of veg2 blastomeres in 60- and 120-cell stage embryos, and that its nuclear translocation is independent of early micromere signals (J. R. Miller, C. Y. Logan and D. R. McClay, unpublished observations). The function of nuclear beta-catenin in early sea urchin development is unclear. However, used simply as a marker, the distribution of beta-catenin in veg2 nuclei indicates that veg1 and veg2 blastomeres are inherently different from the time that they are born. Finally, a recent report has demonstrated that the expression of hatching enzyme in *Paracentrotus lividus* embryos is an autonomous property of the animal regions of the embryo and is not regulated by micromere signaling (Ghiglione et al., 1996).

Taken together, these data suggest that the sea urchin embryo may be organized in broad domains along the animal-vegetal axis prior to any cell interactions that occur during early cleavage, and the function of later cell signaling may be to refine tissue boundaries. Further experiments will be required to directly examine the relative contributions of cell-autonomous and conditional mechanisms in cell-fate specification of veg1 and veg2 descendants. The revised fate map presented here provides a starting point for experimental studies that examine how cell types within the archenteron and the ectoderm-endoderm boundary are established during early development.

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