A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula

Seth W. Ruffins* and Charles A. Ettensohn†

Department of Biological Sciences and Center for Light Microscope Imaging and Biotechnology, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA

*Present address: Developmental Biology Center, University of California, Irvine CA 92717, USA
†Author for correspondence

### SUMMARY

Previous lineage tracing experiments have shown that the vegetal blastomeres of cleavage stage embryos give rise to all the mesoderm and endoderm of the sea urchin larva. In these studies, vegetal blastomeres were labeled no later than the sixth cleavage division (60-64 cell stage). In an earlier study we showed that single cells in the vegetal plate of the blastula stage *Lytechinus variegatus* embryo could be labeled in situ with the fluorescent, lipophilic dye, DiI(C18), and that cells labeled in the central region of the vegetal plate of the mesenchyme blastula primarily gave rise to homogeneous clones consisting of a single secondary mesenchyme cell (SMC) type (Ruffins and Ettensohn [1993] *Dev. Biol.* 160, 285-288). Our clonal labeling showed that a detailed fate map could be generated using the DiI(C18) labeling technique. Such a fate map could provide information about the spatial relationships between the precursors of specific mesodermal and endodermal cell types and information concerning the movements of these cells during gastrulation and later embryogenesis. We have used this method to construct the first detailed fate map of the vegetal plate of the sea urchin embryo. Ours is a latitudinal map; mapping from the plate center, where the mesodermal precursors reside, through the region which contains the endodermal precursors and across the ectodermal boundary. We found that the precursors of certain SMC types are segregated in the mesenchyme blastula stage vegetal plate and that prospective germ layers reside within specific boundaries. To determine whether the vegetal plate is radially symmetrical with respect to mesodermal cell fates, single blastomeres of four cell stage embryos were injected with lysyl-rhodamine dextran (LRD). The resulting ectodermal labeling patterns were classified and correlated with the SMC types labeled. This analysis indicates that the dorsal and ventral blastomeres do not contribute equally to SMC derivatives in *L. variegatus*.

Key words: sea urchin, fate map, in situ cell marking, vegetal plate, gastrulation, cell fate specification

### INTRODUCTION

Fate maps provide essential information concerning the differentiation and movements of cells in the undisturbed embryo. Fate maps also make it possible to explore those factors that regulate the process of cell fate specification, including interactions among embryonic cells. Fate maps of the unperturbed sea urchin embryo have shown that animal and vegetal blastomeres give rise to predictable and different arrays of cell types, and that blastomere fates become progressively restricted as cleavages proceed. Fate maps of cleavage stage embryos have also been instrumental in analyzing cell interactions that take place between blastomeres during early development (e.g., Henry et al., 1989; Khaner and Wilt, 1991; Ransick and Davidson, 1993; for general review see Ettensohn et al., 1995). However, existing fate maps of the sea urchin embryo have been constructed by labeling blastomeres no later than the 60-64 cell stage (sixth cleavage).

The vegetal region of the embryo following the sixth cleavage division comprises four tiers of blastomeres (listed from animal to vegetal); the veg1 and veg2 tiers, each of which contains eight blastomeres, and the large and small micromeres. Following the ingestion of the large micromere descendants at the late blastula stage, the vegetal plate consists of cells derived from the veg1 and veg2 tiers, and the small micromeres. This region of the embryo is distinctive in that it gives rise to a highly diverse array of cell types, including cells of all three germ layers. In addition, the vegetal plate undergoes dramatic shape changes during gastrulation.

Several cell marking reagents and techniques have been used to map the descendants of the vegetal blastomeres. Höörstadius (1973) generated a fate map of the 32 vegetal blastomeres of *Paracentrotus lividus* using Nile Blue as a lineage tracer. This fate map showed that the veg1 tier gave rise to ectoderm on the anal surface of the pluteus larva, the veg2 tier gave rise to the non-skeletogenic mesodermal precursors (SMCs) and endoderm, and the micromeres gave rise only to the skeletogenic PMCs. Recently, however, Logan and McClay (1994),
using DiI as a lineage tracer, have shown that the *veg*1 blastomeres of *L. variegatus* contribute to the endoderm and occasionally give rise to SMCs. Young (1956), using neutral red, mapped the pigment cells to the *veg*1 blastomeres of *L. variegatus*. In a detailed study, Cameron et al. (1987) utilized lysine-fluorescein-dextran to construct a fate map of the eight-cell stage *Strongylocentrotus purpuratus* embryo. The resulting fate map has been used to show the relationship between cell lineage and the domains of expression of specific genes (Davidson, 1989). For example, the macromere descendants that constitute the vegetal plate territory express gene transcripts such as *S9* (Angerer and Angerer, personal communication), *LvN 1.2* (Wessel et al., 1989) and *Endo 16* (Ransick et al., 1993), which are specific to the vegetal plate, while the mesomere descendants express genes such as the *Spec1* and *Spec2* genes and *CyIIa*, which are specific to the aboral regions of the embryo (Davidson, 1989).

Various experimental strategies have been used to define the fates of the vegetal-most cells of the cleavage stage embryo – the micromeres and their descendants. Urben et al. (1988) injected micromeres of *L. pictus* with Lucifer Yellow and confirmed that the PMC precursors reside in the central region of the vegetal plate prior to ingress. Persson and Cohen (1985) exploited the cleavage stage-specific isof orm of histone H1 to demonstrate that the small micromere descendants contribute to the coelomic pouches, a finding that has been confirmed by labeling these cells with a thymidine analog, 5-bromodeoxyuridine (Tanaka and Dan, 1990). By integrating RITC-labeled micromeres into unlabeled host embryos of the ‘primitive’ sea urchin, *Eucidarlis tribulodes*, Wray and McClay (1988) were able to show that even though this species lacks an early-ingressing, skeletogenic mesenchyme, the micromeres of the 16 cell stage embryo are the precursors to the skeletogenic mesenchyme that forms later in gastrulation.

During gastrulation, vegetal plate epithelium undergoes striking shape changes and movements and gives rise to several cell types. As the PMCs ingress, the SMC precursors, which encircle the PMCs, shift to occupy the space vacated by the ingressing cells. These movements displace the SMC precursors toward the center of the vegetal plate which, during gastrulation, becomes the tip of the archenteron. The SMCs give rise to four non-skeletogenic mesodermal cell types; pigment cells, blastocoelar muscle, circumsophageal muscle and most of the cells that make up the coelomic pouches (Cameron et al., 1991; Ruffins and Etteno, 1993). At the mesenchyme blastula stage, endodermal precursors ring the SMC precursors. Archenteron formation begins as a shallow dimple in the vegetal plate epithelium which eventually buckles inwards to form the blastopore and the short archenteron rudiment. After primary invagination, the archenteron elongates approximately two-thirds of the distance across the blastocoel by convergent extension, a process by which the cells intercalate, resulting in a lengthening and narrowing of the archenteron (Etteno, 1985b; Hardin and Cheng, 1986; Hardin, 1989). Once the archenteron has fully elongated, it partitions into the foregut (esophagus), midgut (stomach) and hindgut, each separated by a sphincter. The three gut segments are distinct with respect to morphology and patterns of gene expression. Eventually, only the midgut and hindgut express *Endo 1* (Wessel and McClay, 1985) while *Endo 16* (Ransick et al., 1993) and alkaline phosphatase are expressed solely in the midgut of the pluteus larva.

In a previous study, we reported that greater than 80% of the SMC precursors in the mesenchyme blastula stage vegetal plate give rise to clones composed of a single cell type (Ruffins and Etteno, 1993). Unexpectedly, the percentage of precursors that gave rise to pigment cells and blastocoelar cells differed considerably from the observed distribution of SMC derivatives in the pluteus larva. In that study, we were careful only to label the central region of the vegetal plate, which could have excluded populations of peri-centrally located SMC precursors. To explore the possibility that SMC precursors are differentially distributed throughout the mesenchyme blastula vegetal plate and to provide a more detailed fate map of this region of the embryo, we have proceeded to clonally map the vegetal region to 45° from the plate center within 15° intervals (Fig. 2A) and non-clonally in the region between 45°-60°. The resulting map represents the latitudinal distribution of precursors in the vegetal hemisphere and maps across mesodermal, endodermal and ectodermal boundaries. The latitudinal map, however, cannot be used to determine whether precursors may be radially asymmetrically distributed in the vegetal plate around the animal/vegetal axis (dorsal/ventral or left/right polarity). To test this possibility, we injected lysyl-rhodamine dextran (LRD) into single blastomeres of the four cell stage embryo and correlated the resulting ectoderm labeling patterns with the labeled SMC derivatives in the pluteus larva.

**MATERIALS AND METHODS**

**Embryo culture**

Adult *Lytechinus variegatus* were purchased from a commercial supplier (Susan Decker, Hollywood, FL). Animals were induced to shed gametes by intracoelomic injection of 0.5 M KCl. Eggs were washed 2-4 times in Instant Ocean (IO; Aquarium Systems, Inc.) and fertilized with a dilute sperm suspension. Embryos were cultured in IO in glass bowls until the mesenchyme blastula stage. The rate of development was regulated by maintaining cultures between 17.5° and 25°C in temperature-controlled water baths.

**DiI(C18) labeling**

DiI(C18) is a brightly fluorescent, lipophilic dye that intercalates into the lipid bilayer of the cell membrane. We coated glass needles with DiI(C18) and used them to bring the solid dye crystals into direct contact with cells in specific regions of the vegetal plate. Mesenchyme blastula stage embryos were labeled during the interval between the start of PMC migration and the beginning of invagination; i.e., between the mid and late mesenchyme blastula stages (an interval of 60-90 minutes). PMC migration was complete in all cases prior to cell labeling. Embryos were immobilized in Kiehart micropinjection chambers as described by Etteno and McClay (1988) and labeled with DiI(C18) as described by Ruffins and Etteno (1993). An ocular micrometer with angle markings was used to determine the position being labeled on the embryo. Briefly, the image of the embryo was centered in the cross hairs of the micrometer and the micrometer was aligned so that the 0° line bisected the vegetal plate. A DiI(C18)-coated needle was pressed against the embryo for 8-12 minutes in a selected position as described by Ruffins and Etteno (1993). Single cells were labeled at 15°-60° from the vegetal plate center within regions defined by 15° of arc (see Fig. 2A). Embryos were inspected by fluorescence microscopy immediately after labeling to assess the success of the procedure. Those embryos that
had more than one cell labeled (except those labeled between 45°-60°) or no cells were labeled were discarded. Those embryos that had only a single vegetal plate cell labeled were allowed to develop to the pluteus stage (24-48 hours postlabeling) to ensure that all SMC derivatives had differentiated. Up to six embryos were successfully labeled per trial.

**Scoring DiI(C18)-labeled vegetal plate derivatives**

Live pluteus larvae were collected and scored for labeled derivatives as described by Ettensohn and Ruffins (1993). When possible, the number of labeled cells in each embryo was counted.

**Deciliation and mass DiI(C18) labeling**

Embryos were deciliated by hypotonic shock as described by Merlino et al. (1978) and Gong and Brandhorst (1987). Briefly, 0.12 ml of 4.45 M NaCl was added to a 1 ml suspension of embryos and incubated for 1.5 minutes. After the incubation, 1.2 ml of ‘special sea water’ was added to the embryos and they were left to incubate for 2-4 minutes. ‘Special sea water’ contains 11 mM KCl, 29 mM MgCl2, 15.5 mM MgSO4, 11.7 mM CaCl2 and 2.6 mM NaHCO3, pH 8.0. The embryos were gently pelleted in a clinical centrifuge and transferred into 1:1000 DiI(C18) stock (stock solution=25 mg/ml DiI(C18) in 95% ethanol) and gently agitated on a rocking platform for 15 minutes. The embryos were washed 4-5 times with ASW and scored for DiI(C18) labeling. Control embryos were labeled with DiI(C18) in parallel.

**Lysyl-rhodamine dextran (LRD) injections**

Two-cell stage embryos were collected shortly before the second cleavage (90-120 minutes postfertilization) and placed in calcium- and magnesium-free artificial sea water (CMF-ASW) for 4-6 minutes to soften the hyaline layer. The embryos were transferred to a depression slide where they were selected for injection. Selected embryos were collected by mouth pipetting through a finely drawn Pasteur pipette and transferred into a microinjection boat containing 1:1 CMF-ASW:ASW. Briefly, the microinjection boat consisted of an aluminum slide (75×25×5 mm) with a machined 46×18 mm window opening which could accommodate a 22×50 mm cover glass. The boat was hallowed so that its walls and floor were 1 mm in thickness. The center of the cover glass was coated with 1 mg/ml poly-lysine solution in deionized H2O (dH2O), dried and washed by dipping 5-10 times in double dH2O. Once dried, the cover glass was sealed to the boat using high vacuum grease (Dow Corning Corporation, Midland, MI). Fila-

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**RESULTS**

**Estimation of numbers of SMC precursors**

To estimate the number and density of cells in the vegetal plate, confocal optical sections of the vegetal region of mesenchyme blastula stage embryos that had been double-labeled with PI and mAb 6a9 were collected along the animal/vegetal axis and projected into a single image plane (Fig. 1). A radial arrangement of 6a9-labeled PMCs was used as an indication that embryos were optically sectioned along the animal/vegetal axis. The vegetal pole was considered to be the point in the image at which perpendicular diameters intersected. Projected angles (Fig. 2A), were traced over the image, and the nuclei
and PMCs were counted in each region. The apparent crowding of nuclei in the peripheral regions due to the curvature of the vegetal plate was compensated for by transforming the angular measurements into linear measurements \((\sin\theta) r = l(\theta)\) in Fig 2A. The nuclei in the region between 45° and 60° could not be counted directly due to crowding in the projected images. The number of cells in this region was estimated by assuming that cell density is constant over the entire surface of the embryo (see below) and calculating the surface area of the arc between 45° and 60° as if it were a cylinder with a diameter of \(2\sin(52.5°) r\) and a height of 15 mm. We checked the validity of our assumptions by applying the cylinder surface area calculations for the region between 15° and 30° using a diameter of \(2\sin(22.5°) r\) and found a discrepancy of only 0.7% compared to the actual measurements of this region. To determine the number of nuclei in the vegetal plate epithelium, the number of PMCs was subtracted from the number of nuclei counted. Cell density was estimated by calculating the area of each region and dividing by the number of nuclei. The cell density in the vegetal plate is approximately 3 cells/100 \(\mu\)m² in the three central regions labeled \((n=7\) embryos) (Table 1). The number of SMC precursors of various classes in each region was estimated by multiplying the total number of ciliated cells per region by the cell type labeling frequency for each cell type (see below and Table 2).

### Table 1. Numbers and densities of cells in the DiI(C18) labeled regions

<table>
<thead>
<tr>
<th>Region of embryo</th>
<th>0-15</th>
<th>15-30</th>
<th>30-45</th>
<th>45-60°*</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclei/region (±s.d.)</td>
<td>24.57 (3.11)</td>
<td>62.43 (14.71)</td>
<td>84.86 (11.27)</td>
<td>135</td>
</tr>
<tr>
<td>density [nuclei/100 (\mu)m²] (±s.d.)</td>
<td>3.39 (0.95)</td>
<td>3.02 (0.72)</td>
<td>3.06 (0.69)</td>
<td>3.16</td>
</tr>
</tbody>
</table>

(* ) denotes that the number of cells in this region was extrapolated from data acquired from the three central regions. Because it was not possible to count the nuclei in the 45°-60° region in the 2-D projections (Fig. 1), the number of cells was estimated by assuming that the cell density for this region was equal to the average of the three central regions (see Results).
SMCs (Fig. 3B-D). The labeling frequency for any given cell type ($f_{\text{type}}$) within a specific region of the vegetal plate ($\Theta$) was calculated by dividing the number of larvae with at least one labeled cell of that type ($n_{\text{type labeled}}$) by the total number of embryos labeled in that region of the vegetal plate ($\text{Total}$) (i.e. $f_{\text{type}} \Theta = \frac{n_{\text{type labeled}}}{\text{Total}}$).

(i) SMC precursor distribution in the vegetal plate
All of the SMC precursors lie in the region between 0° and 30° of the vegetal pole (Fig. 4). The coelomic pouch cell precursors are labeled with the same frequency in the 0°-15° and 15°-30° regions, suggesting that they are approximately evenly distributed. Blastocoelar cells are labeled about 1.4 times as frequently in the 0°-15° region as in the 15°-30° region. Pigment and muscle cells are labeled twice as frequently in the 15°-30° region as they are in the 0°-15° region. The difference in the frequencies of SMC labeling suggest that SMC precursors are partially segregated in the vegetal plate of the mesenchyme blastula stage (Fig. 4B). Although the SMC precursors appear to be differentially distributed in the vegetal plate, all classes of SMC precursors are present in both the 0°-15° and 15°-30° regions.

(ii) Endoderm precursor distribution
Our fate map extends to 60° from the vegetal plate center; through the prospective endoderm and into the
Table 2. Calculated number of SMC precursors in the vegetal plate

<table>
<thead>
<tr>
<th>Region of embryo</th>
<th>Cell type</th>
<th>No. of precursor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-15</td>
<td>pigment</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>blasto.</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>muscle</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>coelom</td>
<td>1.8</td>
</tr>
<tr>
<td>15-30</td>
<td>pigment</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>blasto.</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>muscle</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>coelom</td>
<td>12.4</td>
</tr>
</tbody>
</table>

The calculations assume that cilia are necessary for DiI(C18) uptake.

prospective ectoderm (Fig. 5A). We found that when a single endodermal precursor was labeled at the mesenchyme blastula stage, the cell gave rise to a small patch of labeled cells in the gut of the larva (Fig. 3A). Some foregut labeling was seen in the 15°-30° region, but most prospective foregut cells were located in the 30°-45° region and no foregut cells were labeled outside this region. The prospective midgut lay primarily in the 30°-45° region but there was a small contribution from the region between 15° and 30°. No midgut labeling was seen from the region between 45° and 60°. Hindgut and ectoderm labeling was found exclusively in embryos labeled between 45° and 60° (Fig. 5A,B). Clonally labeled precursors did not give rise to labeled derivatives in more than one gut segment. The region between 45° and 60° was not necessarily clonally labeled so it is not known whether endoderm and ectoderm are derived from a common precursor in the mesenchyme blastula. No clones labeled in the region between 0° and 30° gave rise to both endoderm and mesoderm, indicating that the cell division that segregates endodermal lineages from mesodermal lineages occurs at a time before the mesenchyme blastula stage.

The 0°-15° region is made up of a total of about 25 cells, eight of which are small micromere descendants and 17 cells which are SMC precursors. We have calculated that there are a total of about 66 macromere-derived SMC precursors, all found in the region from 0°-30°. Approximately 12 cells (20%) of the 62 cells in the 15°-30° region and all 85 cells in the 30°-45° region are endodermal precursors (see Table 1). Assuming that there is no preference for endoderm or ectoderm labeling, our calculations show that there are approximately 135 cells in the 45°-60° region of which 58 cells are presumptive endoderm and 77 cells are ectoderm. By adding the number of endodermal precursors in these various regions, we calculate that in the mesenchyme blastula there are a total of approximately 155 endodermal precursors, all of which reside in an arc spanning from 15° to 60° from the vegetal pole.

Cilia and DiI(C18) labeling

In the pluteus larva, pigment cells comprise almost 50% of the SMC derivatives (Ettensohn and Ruffins, 1993) but are labeled in only 13% and 27% of pluteus larvae in the 0°-15° and 15°-30° regions, respectively. The reason for the under-representation of labeled pigment cells may be the inaccessibility of dye to the pigment cell precursors. Pigment cells are the first SMCs to ingress and may resorb their cilia prior to ingestion as do presumptive PMCs (Katow and Solursh, 1980). The ciliary membrane is a good candidate for the site of DiI(C18) uptake because it appears to be the only apical membrane not enveloped within the hyaline layer.

To test whether cilia are necessary for the uptake of DiI(C18), mesenchyme blastula stage embryos were deciliated by hypertonic shock as described by Merlino et al. (1978) and Gong and Brandhorst (1987). After the hypertonic shock treatment, embryos were examined with DIC optics and were found to be denuded of most of their cilia as compared to control embryos. The control and deciliated embryos were then incubated in ASW containing a suspension of DiI(C18) for 15 minutes washed in ASW and scored to determine the percentage of embryos with any cells labeled. Approximately 50% of control embryos had cells labeled (n=119) while only 5% of the deciliated embryos had cells labeled (n=187). This suggests that DiI(C18) is taken up through the cilia.

Distribution of cilia

Because the cilia appear to be the site of DiI(C18) uptake, we determined the distribution of cilia by surveying the epithelium of the mesenchyme blastula stage embryo by SEM. We predicted that in all regions we would observe at least some cells lacking cilia due to resorption during mitosis (J. Morrill, personal communication). We also predicted that fewer cells in the vegetal region would have cilia, compared to the more animal regions, because mitotic activity is higher in this region (Agrell, 1953; Nislow and Morrill, 1988). Cells undergoing mitosis can be identified by SEM as unciliated pairs (Fig 6A). Our survey shows that most of the epithelial cells of the mesenchyme blastula stage embryo have a single cilium but the epithelial cells making up the central region of the vegetal plate have short or missing cilia (Fig 6B). Approximately 17.3 cells (n=6, s.d.=8.6) are lacking cilia in the vegetal region, encompassing, but not limited to, the region between 0° and 15°. The non-ciliated cells represent about 77% of the cells residing in the region between 0° and 15°. The findings of Agrell (1953) and Nislow and Morrill (1988) in conjunction with our survey suggest that although mitotic activity is highest in the vegetal region, it cannot account for all the missing cilia.

Radial asymmetry

The fate map generated by DiI(C18) labeling shows the distribution of SMC precursors as a function of their latitudinal position along the animal/vegetal axis. However, this map is based on a single coordinate system which ignores the radial position in which a precursor resides. To examine the radial distribution of SMC precursors around the animal/vegetal axis, single blastomeres of four cell stage embryos were injected with LRD. Injected embryos were allowed to develop to the pluteus stage and then scored for SMC derivatives in relation to the resulting ectodermal labeling pattern. Six major classes of ectoderm labeling patterns were observed; ventral left, ventral center, ventral right, dorsal left, dorsal center, dorsal right (VL, VC, VR, DL, DC, DR, respectively; Fig. 7). Larvae that had any oral ectoderm labeled were considered ventrally labeled, whereas all others were classified as dorsally labeled. There seemed to be no preferential pattern of ectoderm labeling. Dorsal/ventral ectodermal labeling was distributed almost equally (47%:53% respectively) while the left/right patterns were observed in 28% and 36% of the cases, respec-
At the mesenchyme blastula stage, one-quarter of the embryo was labeled, spanning a region from the animal pole to the vegetal plate. Clones from the labeled blastomere formed a contiguous patch of labeled cells but the lineage boundaries were frequently irregular with occasional fingers of labeled clones protruding into unlabeled territories (Fig. 8).

All labeled blastomeres gave rise to SMCs. Muscle cells were not scored because they could not be distinguished from the LRD-labeled foregut. In 22% of the embryos, the coelomic pouches were not easily identifiable and were not scored (ND in Table 3). In the cases in which coelomic pouches could be identified, at least one pouch was labeled. Surprisingly, only the left coelom was labeled in larvae with the DC ectodermal labeling pattern.

The blastomeres that gave rise to the VC and DC labeling patterns did not contribute to all SMC derivatives equally. All of the larvae with the VC labeling pattern had labeled blastocoelar cells but only 50% had any labeled pigment cells. Conversely, all blastomeres that showed a DC labeling pattern gave rise to labeled pigment cells but 40% did not give rise to labeled blastocoelar cells. All larvae with the VC labeling pattern had pigment cells and all larvae with the DC labeling pattern had blastocoelar cells, demonstrating that these cell types differentiate in all larvae. All of the laterally labeled larvae (DL, DR, VL, VR) contributed to all three SMC types scored (Table 3). These results suggest that the precursors of blastocoelar cells and pigment cells have an asymmetric distribution along the dorsal/ventral axis of the embryo.

DISCUSSION

The fate map presented here was generated using DiI(C18) as a lineage tracer. The advantage of this method is that it can be used to label single cells in situ, even at late developmental stages (Ruffles and Ettensohn, 1993; Bossing and Technau, 1994). Using this technique we labeled cells at the mesenchyme blastula stage, later than any previous studies, and have produced the most detailed fate map of the vegetal region to date.

In this study, we have attempted to describe the latitudinal and radial distribution of SMC precursors as well as the boundaries between the precursors of mesoderm, endoderm and ectoderm. We found that all the mesodermal precursors reside within the central 30° region of the vegetal plate. Mesodermal precursors appear to be segregated, to a limited extent, in that blastocoelar cells are labeled more frequently in the 0°-15° region than in the 15°-30° region while muscle cells and pigment cells are labeled twice as frequently in the 15°-30° region as in the 0°-15° region. We have estimated that the vegetal plate contains approximately 74 mesodermal precursors (66 macromere descendants and eight small micromere descendants) and 155 endodermal precursors—a total of some 230 cells that will be internalized during gastrulation. Endodermal precursors are broadly distributed in the vegetal plate in a zone that extends from 15°-60°. However, the precursors of each gut segment (foregut, midgut and hindgut) appear to occupy very narrow ring-like regions in the vegetal plate, each only one to two cells wide. Most of the presumptive foregut and midgut is located in the 30°-45° region and all of the presumptive hindgut is in the 45°-60° region. Ectodermal precursors were labeled only in the 45°-60° region.

The finding that some populations of SMC precursors are segregated in the vegetal plate, in conjunction with our earlier finding that by the mesenchyme blastula stage about 80% of SMC precursors give rise to a single cell type, suggests that cell fate restrictive events have taken place. Clearly, however, throughout gastrulation cell-cell interactions are required for the regulation of cell fates (see review by Ettensohn et al., 1995). Venuti et al. (1993) showed that removing the ectoderm before the mid-gastrula stage prevented the activation of muscle-specific genes. Experiments in which PMCs are ablated at progressively later times have shown that SMCs retain skeletogenic potential until at least the mid-late gastrula stage (Ettensohn, 1990). Logan and McClay (1994) have demonstrated that cells in the vicinity of the blastopore at the gastrula stage are capable of replacing the gut and at least some of the mesodermal derivatives upon removal of the archenteron. This suggests that these vegetal cells have the capacity to give rise to a range of mesodermal and endodermal cell types that clearly exceeds their prospective fate, but are prevented from doing so by signals originating from the archenteron. The experiments described above demonstrate that some cells of the vegetal plate remain labile and competent to receive instructive signals during gastrulation.

An unexpected result of our LRD lineage tracing experiments was the observation that the vegetal plate is radially asymmetric with respect to mesodermal fates. We have found that a larva with a DC ectodermal labeling pattern is 40% less likely to have blastocoelar cells labeled than a larva with any other ectodermal labeling pattern. Likewise, a larva with a VC pattern is only half as likely to have pigment cells labeled as any other ectodermal labeling pattern. Moreover, when
pigment cells were labeled in embryos with the VC pattern, the number of labeled pigment cells was reduced. This result strongly suggests that the vegetal plate has dorsal/ventral polarity. Consistent with this is the finding that, in *S. purpuratus*, the cytoplasmic actin isoform CyIIa is specifically restricted in its expression to the ventral region of the vegetal plate at the mesenchyme blastula stage (Miller and Ettensohn, unpublished data).

An explanation for the variability among embryos that we observed with respect to the dorsal and ventral labeling patterns may be that cells from one lineage mix with those of a neighboring lineage during early development. At the mesenchyme blastula stage, clones from a labeled blastomere can sometimes extend several cell diameters into a neighboring lineage’s territory (Fig. 8). Logan and McClay (1994) recently reported that, in *L. variegatus*, the veg1 tier of blastomeres contributes to both ectoderm and endoderm. Surprisingly, the veg1 tier also occasionally contributes to a small population of SMCs, which our observations show arise from the central-most region of the vegetal plate. This supports the idea that some mixing of cells takes place within the vegetal epithelium prior to gastrulation, and suggests that cells that cross into neighboring environments might respond to the instructive signals specific to that locale and adopt fates appropriate to that region.

Cameron et al. (1991) showed that, in *S. purpuratus*, each macromere contributes to all SMC types, though not equally. The VAM (aboral) macromere contributes either to the left or right coelomic pouch while the VOM (oral) macromere contributes to both the left and right pouches. The lateral macromeres, VLM (lateral left and right), contribute to the left or right coelomic pouch, depending on which macromere is labeled. In our study, a surprising finding was that the blastomeres that gave rise to the DC pattern only contributed to the left coelomic pouch while no other ectoderm labeling pattern showed preferential labeling of either coelomic pouch.

Our blastomere labelings suggest that the first cleavage plane does not predictably divide the *L. variegatus* embryo...
symmetrically along the left/right axis, as reported by McCain and McClay (1994). Although we labeled at the four cell rather than the two cell stage, our labeling patterns suggest that the first cleavage plane can lie at virtually any position with respect to the left/right axis. The explanation for this discrepancy is unclear, although the labeling techniques used were different.

Pigment cells make up approximately 50% of the SMCs in the pluteus larva but are labeled with a frequency of 13% and 27% in the central two regions of the vegetal plate, where the SMC precursors reside. It appears unlikely that the explanation for this discrepancy is that cells of the pigment cell lineage undergo a greater number of rounds of cell division during later development than do other SMC types, as typically only two pigment cells are derived from a single, labeled vegetal plate precursor (Ruffins and Ettensohn, 1993). Instead, we suspect that some pigment cell precursors cannot be labeled by the Dil(C18) method described here, possibly because they lack cilia. Katow and Solursh (1980) showed that PMCs lose their cilia prior to ingression. Pigment cells are the first class of SMC to ingress (Gustafson and Wolpert, 1967; Gibson and Burke, 1985; Ettensohn and McClay, 1988), even preceding the PMCs in some species, and probably lose their cilia in the process. We found that deciliated mesenchyme blastula stage embryos were labeled with Dil(C18) ten times less frequently than control embryos, suggesting that cells are labeled through their cilia. We then looked at the distribution of cilia in the mesenchyme blastula stage embryo and found that virtually all cells had a single, long cilium except those in the central region of the vegetal plate.

Based upon these observations, our fate map assumes that cilia are necessary for the uptake of Dil(C18). This is relevant only with respect to cell fates in the central-most (0°-15°)
region of the vegetal plate, but implies that the map shown in Fig. 4 represents the fates of the approximately eight ciliated vegetal plate cells within this region. Of the approximately seventeen non-ciliated cells in this central region, which we assume cannot be labeled by the DII method, eight are likely to be the small micromere descendants as they reside in the center of the vegetal plate (Pehrson and Cohen, 1985; Urben et al., 1988) and are within the field of non-ciliated cells. This is reflected in the fate map shown in Fig. 4, which assumes that the small micromere progeny at the vegetal pole (colored blue) make up part of the non-ciliated cell population. If the approximately nine remaining non-ciliated cells are pigment cell precursors, then the 0°-15° region contains a total of some ten pigment cell precursors; almost 60% of the macromere descendants residing within this region. Although we have not demonstrated directly that these non-ciliated cells are pigment cell precursors, this would bring our fate map into close agreement with our SMC counts (Ettensohn and Ruffins, 1993). We should note, however, that it remains possible that all cells in the central region of the vegetal plate, not just those with cilia, can be labeled with DI. If true, then the orange and blue cells shown in the fate map shown in Fig. 4 would need to be re-colored as specific cell types in the same proportions as the other cells in this region, which were assigned fates based on our labeling frequencies for the various cell types. In this case, the cause for the discrepancy between our low pigment cell labeling frequency and the high proportion of SMCs in the larva that are pigment cells would remain unclear.

Our fate map bears on the issue of cell movements during gastrulation. During the earliest stage of invagination, we measured the angle of the initial invagination at the blastopore lip and at the maximum curve of the folding ectoderm sheet (Fig. 2B). When the average angles of these two landmarks (−15° for the lip and −20° for the ectoderm fold; n=22) are superimposed on the fate map (two tone grey circles in Figs 4 and 5), a majority of the endoderm precursors fall outside the presumptive blastopore. Several studies of sea urchin gastrulation show that there is some involution of cells over the blastopore lip during primary invagination (Ettensohn, 1984a,b; Hardin, 1989; Burke et al., 1991) and Kuraishi and Osanai (1992) provide evidence for involution of endoderm blastopore lip and at the maximum curve of the folding ectoderm sheet (Asterina pectinifera). Wray and Raff (1991) show that considerable numbers of cells involute over the blastopore lip during gastrulation in H. erythrogramma. The fate map presented here provides evidence that, in L. variegatus, the initial invagination of the archenteron involves only the prospective mesoderm, and that essentially all of the prospective endodermal cells are recruited into the archenteron after the start of invagination. As evident from Fig. 5, this would require a movement of a ring of cells about four cell diameters wide over the blastopore lip. A possibility that we are exploring is that some shifting of vegetal plate cells toward the vegetal pole occurs during the mesenchyme blastula stage, immediately before the start of invagination. Such a movement might shift at least some presumptive endodermal cells into the prospective invaginating region. During later gastrulation, when cell rearrangements occur that elongate the archenteron, neighboring cells remain within one cell diameter of each other (Hardin, 1989). The discrete, concentric arrangement of presumptive foregut, midgut and hindgut cells that we see in the fate map supports the view that extensive mixing of archenteron cells does not occur during gastrulation.

Our previous study showed that a single vegetal plate cell labeled at the hatched blastula stage was more than twice as likely to give rise to heterogeneous clones as a vegetal plate cell labeled at the mesenchyme blastula stage (Ruffins and Ettensohn, 1993). This suggests that developmental decisions regarding the specification of SMC precursors are being made during the interval between the hatched blastula and mesenchyme blastula stages. The remarkable regulative behavior of the embryo, however, demonstrates that at least some populations of vegetal plate-derived cells retain considerable flexibility with respect to their fate well past the start of gastrulation. It seems likely that cell-cell signaling events both within the epithelium of the archenteron (Logan and McClay, 1994) and between the archenteron and other cell populations in the embryo (Ettensohn, 1990) serve to progressively restrict the fates of these populations of vegetal plate cells during gastrulation.

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