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The Cellular Basis of Sea Urchin Gastrulation

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I. Introduction—General Approaches to Gastrulation

In his introduction to the inaugural issue of *Archiv für Entwicklungsmechanik der Organismen*, Wilhelm Roux discussed at length the significance of the term "Entwicklungsmechanik," a term he had specifically coined to refer to the new

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science of experimental embryology that had emerged in the latter part of the 19th century (Roux, 1895). The German term was intended to be flexible enough to encompass both the *mechanics* (i.e., the physical forces and underlying physical/chemical processes) and the *mechanisms* of embryonic development (e.g., cell–cell interactions, cell movements, etc.). Nowhere was the confluence of mechanics and mechanism more apparent than during gastrulation. Because gastrulation involves the wholesale reorganization of the embryo to produce a recognizable body plan, it requires consideration of both the *forces* underlying cellular movements and the *patterning* processes that result in the establishment of localized information to which moving cells respond.

A. The Tissue Is the Basic Mechanical Unit of Gastrulation

Although it is clear that any thorough understanding of gastrulation will require an understanding of molecules that mediate cell movement and cell differentiation, the fundamental unit of analysis in gastrulation is the tissue. Cell sheets, streams of cells, and individual or groups of migratory mesenchymal cells constitute the germ layers that must be correctly positioned during gastrulation, so although it is certainly appropriate to discuss the "molecular basis of gastrulation," the role(s) of various molecules is probably best thought of in terms of how they affect the behaviors of cells and tissues. In this sense, understanding the cellular basis of gastrulation is fundamental to our ultimate understanding of the role that various molecules play in regulating cell behaviors: it provides the context within which specific molecular interactions can be analyzed and interpreted. In the case of systems allowing robust developmental genetic studies, the characterization of these behaviors is crucial for establishing the wild-type phenotype as a baseline to which mutant phenotypes are compared.

As D'Arcy Thompson pointed out in his *On Growth and Form*, the organism is in many ways a "diagram of forces" at work within it (Thompson, 1927). Thus, clarifying the nature and magnitude of forces that act within the embryo to shape it will be part of a comprehensive analysis of gastrulation. Mechanical properties are composite characteristics of tissues that reflect forces actively generated within the cells of the tissue, those generated outside the tissue, and the passive mechanical properties of the tissue (viscoelasticity, plasticity, etc.). Thus, a complete understanding of tissue mechanics will require an examination of the cytoskeleton, intercellular adhesion, and the interaction of cells with the extracellular matrix. These interactions contribute to the composite mechanical properties of the embryo (for a foray into this sort of analysis, see Koehl, 1990).

B. Patterning Events Regulate Gastrulation

Because cells migrate and tissue sheets deform in response to incipient axial information within the embryo, an understanding of the mechanisms by which

spatial differences arise in the embryo is important for understanding gastrulation (see Davidson, 1993, for further discussion on this point). First, the boundaries at which different cells engage in different movements or the expression of different cell adhesion molecules or receptors for extracellular matrix are extremely sharp, in some cases on the order of a single cell diameter (for examples, see Leptin and Gruenwald, 1990; Keller *et al.*, 1992; Shih and Keller, 1992a,b). Understanding how such sharp differences arise ontogenetically serves as an important backdrop for understanding the movements of gastrulation themselves. Second, sharp tissue boundaries are established via local cell–cell interactions (for examples, see Hörstadius, 1973; Ransick and Davidson, 1993; Keller *et al.*, 1992). Third, these interactions are progressively refined over time (for examples, see reviews by Sive, 1993; Kimelman *et al.*, 1992; Davidson, 1993). Fourth, these interactions lead to patterning of both regulatory molecules, such as transcription factors, and morphogenetic programs, such as invagination, convergent extension, and involution. Finally, although the definitive body plan arises as a result of the morphogenetic movements of gastrulation, it is clear that substantial axial polarity exists prior to the movements of gastrulation. Although systems such as *Drosophila* and *Xenopus* are the best studied in this sense (e.g., St. Johnston and Nüsslein-Volhard, 1992; Leptin *et al.*, 1992; Keller and Winklbauer, 1992; Slack *et al.*, 1992; Kimelman and Moon, 1992; Sive, 1993), a thorough understanding of gastrulation in other organisms will necessitate a similar analysis.

II. The Sea Urchin as a Model for Studying Gastrulation

A. The Need for an Up-to-Date Review of Sea Urchin Gastrulation

The sea urchin embryo has been an historically important system for investigating the cellular basis of gastrulation. Sea urchin embryos can be obtained in large numbers, they develop synchronously, they are optically transparent, and their organization is relatively simple. For these reasons, the sea urchin embryo was one of the experimental systems of choice for studying the range of fates of early blastomeres and the ways that cell–cell interactions can modulate the development of individual cells. The pioneering studies of Driesch (1892) on regulation of development and Hörstadius on cell–cell interactions and the existence of graded properties within the unfertilized egg and the early embryo are well known (reviewed in Hörstadius, 1939, 1973). In addition, the sea urchin system was one of the first in which time-lapse cinèmicroscopy was exploited extensively. The classic studies of Gustafson and colleagues characterized many of the basic behaviors exhibited by cells in the gastrula (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960; Gustafson and Wolpert, 1961), and for many years these studies served as seminal examples of a system in which gastrulation was thought to be largely understood at the cellular level. Perhaps as

important as the descriptive studies themselves were two influential reviews written by Gustafson and Wolpert (1963a, 1967). Because of their comprehensive scope, as well as their attempts to employ physical models to account for the observed behaviors of gastrulation, these reviews became influential and served as the nucleus around which many textbook discussions of gastrulation were constructed.

B. Toward a Mechanistic Understanding of Sea Urchin Gastrulation

The simplicity of organization of the sea urchin embryo makes it an appealing model system for undertaking a cellular analysis of gastrulation. Because of its optical clarity, it is possible to observe morphogenesis *in vivo*, and modern microscopy can be used to examine the dynamics of gastrulation in living embryos. Furthermore, unlike amphibian embryos, in which deep, nonepithelial cells behave as partially integrated mechanical units during gastrulation, there are only two basic sorts of cells in the sea urchin embryo: epithelial cells and mesenchyme. This simplification means that one need only consider tissues for which a considerable amount of cell biological information already exists; thus, it ought to be possible to gain insights into the cell biological basis of the morphogenetic movements that occur during gastrulation.

The reviews of Gustafson and Wolpert sought to understand sea urchin gastrulation in terms of a set of unifying hypotheses concerning cell adhesion and cell motility, with a heavy emphasis on physical processes involved in morphogenetic movements. At the time, their fundamental conviction was that

> We are, however, still ignorant about the final steps in the causal chain between the genes and the shapes they control. . . In order to bridge the gap, it seems both logical and necessary to reduce the complex processes at the organ level to activities of the individual cells, that is, to activities that may be more meaningful for the biochemist than concepts such as gastrulation, mesenchymal patterns and coeloms. . . When we look for such activities, we must realize that any change in shape of a system, either physical or biological, requires forces to bring it about. . . Our purpose is therefore to reduce the complex morphogenetic events of organ rudiments and of the whole embryo into a question about the cellular forces involved. (Gustafson and Wolpert, 1967)

Although our knowledge of the molecular basis of developmental processes has advanced considerably in the past 25 years, our understanding of the links between molecules, cell behavior, and forces in the embryo remains incomplete at best, and so the task of understanding how individual cell behaviors produce the morphogenetic movements of gastrulation is far from over. There are several additional reasons for an up-to-date review of sea urchin gastrulation. First, these reviews are now 30 years old, and while much of the data produced by Gustafson

and colleagues remained unembellished for nearly two decades, more recent experiments have considerably clarified the cellular mechanisms of sea urchin gastrulation. Second, extensive lineage studies by Cameron and colleagues have extended the classic studies of Hörstadius to provide a more detailed picture of the establishment of tissue territories prior to gastrulation (Cameron and Davidson, 1991; Cameron *et al.*, 1991). Third, although direct genetic manipulation of the sea urchin is laborious (but see Hinegardner, 1975; Leahy *et al.*, 1994), this system has a robust molecular biology, and progress has been made regarding characterization of molecules that may be involved in gastrulation. Finally, the devotion of several recent international meetings to the subject of gastrulation (Stern *et al.*, 1992; Keller *et al.*, 1992) attests to its current relevance, and so a comprehensive review of sea urchin gastrulation within this modern context seems desirable.

The following sections examine the cellular basis of the morphogenetic movements of sea urchin gastrulation and the patterning events that organize them. Although an historical development of each of these aspects of gastrulation might be desirable, for clarity the discussion that follows is arranged topically. For an excellent recent review of sea urchin morphogenesis not restricted to gastrulation, see Ettensohn and Ingersoll (1992). For reviews of specific aspects of sea urchin morphogenesis the reader is referred to articles by Ettensohn (1992), McClay *et al.* (1992), and Hardin (1990, 1994, 1995). Earlier general reviews include Gustafson and Wolpert (1963a, 1967), Okazaki (1975), Spiegel and Spiegel (1986), and Solursh (1986).

III. An Overview of Pregastrula and Gastrula Morphogenesis

A. Founder Lineages That Give Rise to Territories in the Gastrula Are Established during Cleavage

Scrutiny of the fate map of the sea urchin embryo yields insights into the overall patterns of morphogenetic movements during gastrulation and hints at detailed mechanisms by which the embryo is transformed during gastrulation. This is particularly true in the sea urchin embryo, because spatial relationships between lineages are so reliable in this organism. When the isolecithal sea urchin egg is fertilized, it undergoes two meridional holoblastic cleavages to produce a four-celled zygote. At the third division, the four cells divide equatorially to produce two tiers of four cells each: a quarter of animal pole blastomeres and four vegetal pole blastomeres. At the fourth cleavage, the four animal pole cells divide meridionally to produce eight *mesomeres*; the four vegetal pole blastomeres undergo an unequal equatorial cleavage to produce four large *macromeres* and four *miromeres*, which lie at the extreme vegetal pole of the embryo. At the next division, the mesomeres divide equatorially to produce two tiers of cells (an_1 and an_2 in Hörstadius' terminology; see below). The micromeres divide once to produce four small micromeres, at the vegetal pole of the embryo, and four

larger micromeres. By the 64-cell stage,[1] the macromeres have divided first meridionally and then equatorially to produce two tiers of cells: the veg_1 cells, lying adjacent to the animal tiers of blastomeres, and the veg_2 cells, which lie just animal to the larger micromeres. Lineage studies by Hörstadius and Cameron and colleagues (reviewed in Hörstadius, 1939, 1973; Cameron and Davidson, 1991) indicate that by this time many of the founder lineages which give rise to the major tissue territories of the later embryo are established. Hörstadius ound that the tiers of the 32- and 64-cell embryo give rise to distinct tissues: an_1 and an_2 progeny give rise to animal pole and equatorial ectoderm, respectively, and veg_1 progeny give rise to vegetal ectoderm. veg_2 progeny produce tissues contributing to all three germ layers: anal ectoderm, endoderm, and mesoderm. Hörstadius also showed that the micromeres of the 16-cell embryo give rise to primary mesenchyme cells (PMCs), which produce the larval skeleton. More recent studies have shown that the larger progeny of the micromeres give rise to the PMCs; the smaller progeny contribute to the coelomic pouches (Endo, 1966; Pehrson and Cohen, 1986; Tanaka and Dan, 1990).

Recently, these lineage studies have been extended by Cameron and colleagues both to refine the existing lineage data and to study tissues along the dorsoventral (oral–aboral) axis. The result is an extensive lineage of each cell of the 16-cell embryo, with additional refinements to selected lineages and/or tissues (Cameron and Davidson, 1991; Cameron *et al.*, 1991; see Fig. 1). Several salient features of this analysis are germane to the discussion of gastrulation that follows. First, lineage founder cells for most tissues are identifiable by the 64-cell stage, with some notable exceptions. Second, dorsoventral differences in ectodermal lineages can be inferred at the 16-cell stage. In some species, these differences can be inferred as early as the 2-cell stage based on the relation between the plane of first cleavage and the subsequent dorsoventral axis (Cameron *et al.*, 1989; Henry *et al.*, 1992; reviewed by Jeffery, 1992). These lineage boundaries can be demonstrated well before any morphological differences appear in the embryo, and spatially restricted patterns of gene expression correspond to these lineage boundaries. One major exception to this pattern of lineage restriction is the ciliated band, a structure which forms at the boundary between dorsal (aboral) and ventral (oral) ectoderm. Founder cells from both dorsal and ventral lineages contribute progeny to this structure (Cameron *et al.*, 1993). Third, in the pregastrula, ectodermal founder cells lie in the animal two-thirds of the embryo. During gastrulation, however, the boundaries of ectoderm distort, so that by the end of gastrulation only ectodermal tissue lies on the surface. Thus it is only macromere-derived material at the vegetal pole that participates in archenteron formation; the fate map rules out any large-scale, long-distance involution of cells into the interior. In fact, only veg_2 cells give rise to the archenteron in species that have been thoroughly examined (Hörstadius, 1973). The formation

[1] In *S. purpuratus* there are actually 60 cells at this stage, due to the different cleavage schedule of the micromeres; for simplicity, this stage will be referred to as the '64-cell stage' here. See Cameron and Davidson (1991) for further details.

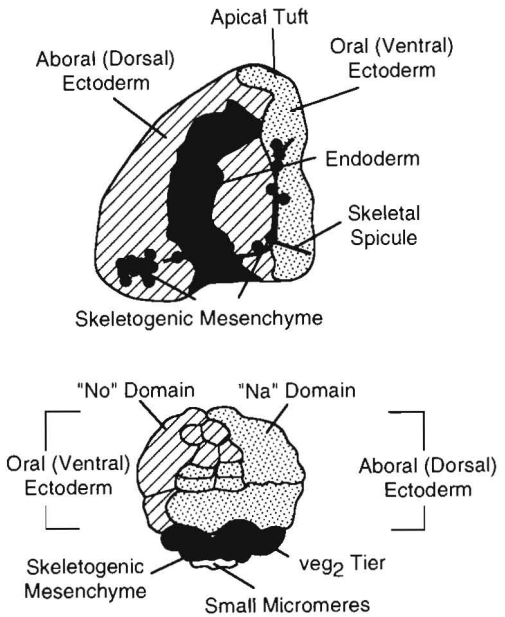


Fig. 1 Tissue territories of the gastrula are established during early cleavage stages (adapted from Cameron and Davidson, 1991). Lineages are derived from the work of Hörstadius (see Hörstadius, 1973) and Cameron and colleagues (Cameron and Davidson, 1991; Cameron *et al.*, 1991).

and elongation of this structure must be accounted for solely based on changes in the position and shape of the veg_2 progeny (however, for an abstract of a recent study suggesting that both veg_1 and veg_2 progeny contribute to the vegetal plate in *Lytechinus variegatus*, see Logan and McClay, 1994).

B. Tissue Architecture in the Blastula

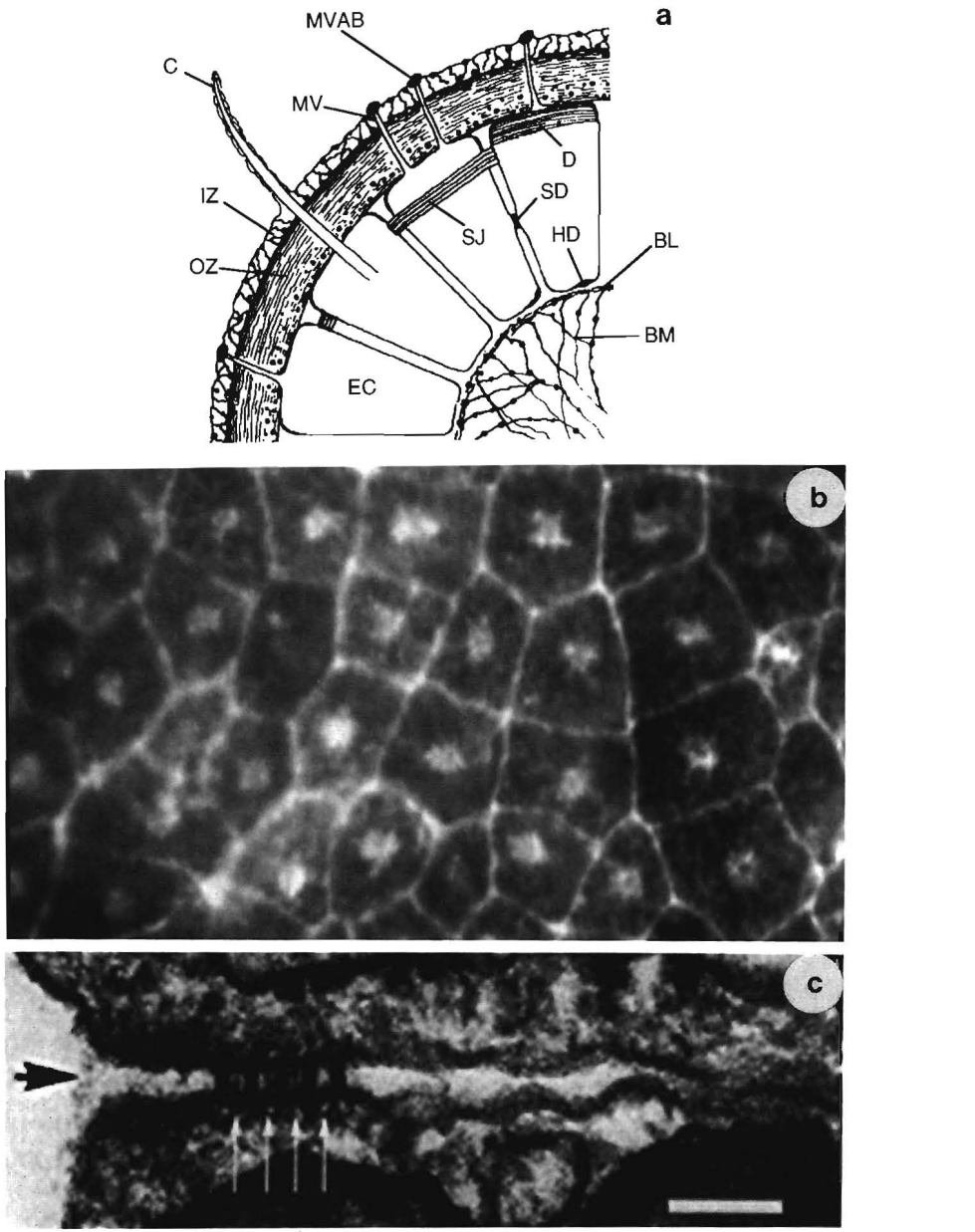
Regionalization of the blastula involves the segregation of its epithelial cells into several distinct regions with different appearance and differing patterns of gene expression. In addition, the blastula stage marks the time at which a bona fide epithelium appears. The early cleavage divisions lead to the formation of a hollow cavity, the blastocoel, within the embryo. As development proceeds, the cells surrounding the blastocoel acquire well-defined apical–basal polarity, including an apical Golgi apparatus, numerous apical microvilli, and a single apical cilium (Wolpert and Mercer, 1963; Tilney and Gibbins, 1969a,b; Anstrom, 1992a,b; reviewed in Okazaki, 1975; Spiegel and Spiegel, 1986; Ettensohn and Ingersoll, 1992). Certain aspects of this polarization are already noticeable at the 16-cell stage and do not require cell–cell contact for maintenance

(Schroeder, 1988). The blastular wall has a relatively simple structure and is fairly typical of invertebrate epithelia. The cells are joined at their apical (outer) surfaces by junctional complexes, including zonulae adherens and septate junctions, as well as spot desomosomes (Wolpert and Mercer, 1963; Spiegel and Howard, 1983; Ettensohn, 1985; see Fig. 2). The formation of junctional complexes coincides with a decrease in permeability of the blastula wall to small molecules such as sucrose (Moore, 1940) and with an apparent increase in adhesion between cells.

Under the light microscope, the *hyaline layer* can be seen at the apical surfaces of the cells in the blastula. Electron microscopy suggests that there are several layers of extracellular matrix associated with the apical surfaces of the epithelium, arranged in concentric shells around the embryo (Spiegel and Howard, 1983). There is biochemical, immunocytochemical, and structural evidence (see below) for an inner *apical lamina* more tightly associated with the apical plasma membrane, and an outer layer associated with the tips of microvilli. Depending upon the fixation protocol, a multiplicity of layers has been identified; in some case, immunogold labeling has identified certain molecules as being closer to or further away from the apical plasma membrane (Coffman and McClay, 1990). Structures termed *microvillus-associated bodies* are associated with the tips of microvilli and appear to be sites of attachment of microvilli to the hyaline layer (Spiegel and Howard, 1983; Fig. 2). For a discussion of the role of the hyaline layer and cell–cell adhesion in blastocoel formation, see the reviews by Dan (1960), Gustafson and Wolpert (1963a,b, 1967), Dan and Inaba (1968), Ettensohn and Ingersoll (1992), and McClay *et al.*, (1992). The cells of the blastula secrete a thin basal lamina at their basal surfaces; this layer increases in thickness over most of the embryo as development proceeds (Wolpert and Mercer, 1963; Okazaki and Nijima, 1964; Galileo and Morrill, 1985; Amemiya, 1989). In addition, the blastocoel becomes filled with a progressively more substantial blastocoel matrix (reviewed by Solursh, 1986; see also Burke and Tamboline, 1990; Ingersoll and Ettensohn, 1994).

The epithelial cells of the hatched blastula are not identical in appearance. Three distinctive regions of epithelium are identifiable at this time: (1) the *apical*, or *animal plate*, a thickened region at the extreme animal pole of the embryo whose cells have unusually long cilia; (2) the *vegetal plate*, a thickening at the extreme vegetal pole of the embryo, from which the endoderm and mesoderm are derived; and (3) the *lateral ectoderm*, which displays optical patterns in the microscope reminiscent of a Japanese fan (Okazaki *et al.*, 1962; see Fig. 5).

Fig. 2 Structure of the epithelium of the blastula. (a) Schematic diagram of the ultrastructure of the epithelium (adapted from Spiegel *et al.*, 1989). BM, blastocoel matrix; BL, basal lamina; HD, hemidesmosome; SD, spot desmosome; SJ, septate junction; EC, epithelial cell; D, belt desmosome (zonula adherens); MV, microvillus; MVAB, microvillus-associated body; C, cilium; IZ, inner zone of hyaline layer; OZ, outer zone of hyaline layer. (b) Rhodamine phalloidin staining of ectoderm in a *L. pictus* gastrula. Note that concentration of actin at junctional regions and around the basal body/ciliar rootlet (central spots within each cell). (c) Junctional structure in gastrulae of *Strongylocentrotus drobachiensis*. Large arrow, zonula adherens; small arrows, septate junction.


a
MVAB
C
MV
D
SD
IZ
SJ
HD
BL
OZ
BM
EC
b
c

C. The Onset of Gastrulation

As cell division continues, the embryo develops a hollow cavity, the *blastocoel*, and eventually the cells of the embryo organize into an epithelium, forming a blastula. Midway through the blastula stage the embryo secretes a hatching enzyme and the embryo hatches to become a free-swimming blastula. Ultimately, the epithelium at the vegetal pole flattens and thickens to form the *vegetal plate* (Fig. 3). All of the endodermal and mesodermal cells of the embryo derive from the vegetal plate, beginning with the *primary mesenchyme cells* (PMCs). These cells (typically 32 or 64, depending on how many rounds of cell division occur following the 16 cell stage), are derived from the large micromeres. They detach from the vegetal plate epithelium, round up, and ingress into the blastocoel. After a short pause, they migrate within the blastocoel using short, seemingly stiff filopodia (for reviews of PMC morphology during migration, see reviews by Gustafson and Wolpert, 1963a, 1967; Solursh, 1986). After migration, many PMCs form two patterned clusters in the ventolateral regions of the embryo; other PMCs form a ring connecting these clusters in the lateral ectoderm (see Ettensohn, 1992, for a further discussion of the details of this ring structure). Ultimately, the PMCs secrete spicules, which contain calcium and magnesium carbonate in an organic matrix (reviewed by Decker and Lennarz, 1988; Wilt and Benson, 1988; Benson and Wilt, 1992). The skeleton is secreted in a precise pattern, in part presaged by the position of PMCs at the gastrula stage.

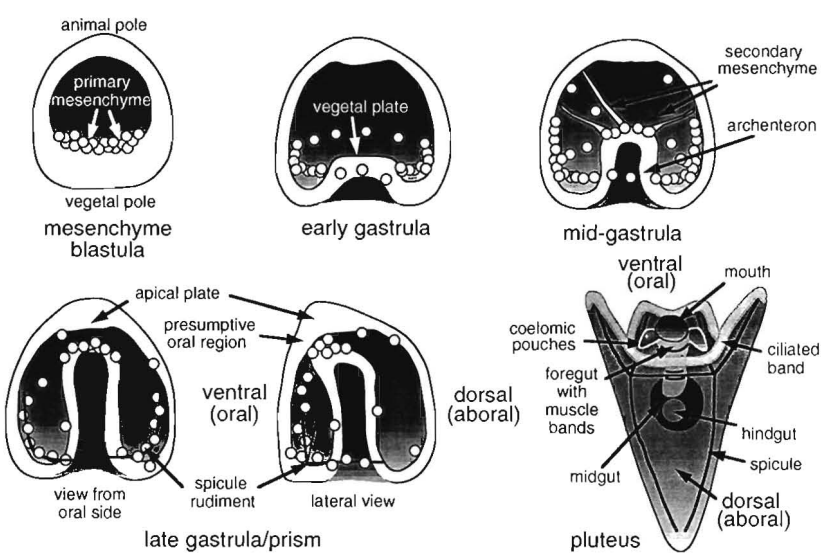


Fig. 3 A schematic overview of sea urchin gastrulation (adapted from Hardin, 1994. with permission).

Following ingression of the PMCs, three other vegetal plate derivatives begin their morphogenetic movements; the timing of onset of these activities appears to vary among different species. The most dramatic of these movements involves the vegetal plate epithelium, which invaginates to form the *archenteron*. Time-lapse studies by Gustafson and colleagues indicate that the process of invagination can be separated into two distinct phases with distinctly different rates (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960; see Fig. 10). The first, *primary invagination*, transforms the flat vegetal plate first into a shallow invagination and then into a short tube with flat walls and a flat top. During the second phase, *secondary invagination*, the archenteron elongates across the blastocoel to the animal pole. At about the time secondary invagination ensues, a second population of cells, *secondary mesenchyme cells*, engages in morphogenesis (Gustafson and Kinnander, 1956; Dan and Okazaki, 1956). These cells, located at the tip of the archenteron, become protrusively active, sending long filopodia into the blastocoel. Eventually, the tip of the archenteron reaches the ectoderm near the animal pole and attaches to a specific region of the ectoderm, marking the end of secondary invagination (Kinnander and Gustafson, 1960; Hardin and McClay, 1990; see Fig. 11).

A third population of migratory cells that become active at this time is the presumptive *pigment cells* (chromogenic mesenchyme.) One population of pigment cells ingresses from the vegetal plate or from the tip of the forming archenteron early in invagination; these cells migrate within the blastocoel, disperse, and subsequently invade the ectoderm, where they differentiate to produce the pigment echinochrome (Gibson and Burke, 1985; see Fig. 27). A second population of pigment cells ingresses from the tip of the archenteron relatively late in gasrulation in normal embryos (Ettensohn and McClay, 1986; Ruffins and Ettensohn, 1993).

After the major movements of gastrulation are completed, the various embryonic tissues undergo differentiation and the definitive larval axes become established. The ventral (oral) ectoderm flattens and thickens, giving the embryo a prism shape. At the same time, the spicules produced by the PMCs continue to grow. Eventually the larva develops four arms: two oral arms and two postoral arms (for nomenclature relating to specific, identified rods, see Hörstadius, 1973; Ettensohn, 1992). The oral arms flank the mouth, which results from the fusion of the tip of the archenteron with the ectoderm (Gustafson and Kinnander, 1960; Gustafson and Wolpert, 1963b). The archenteron also differentiates, subdividing to produce a foregut, midgut, and hindgut. Esophageal muscles, derived from cells at the tip of the archenteron, encircle the foregut and undergo peristaltic contractions (Gustafson and Wolpert, 1963b; Ishimoda-Takagi *et al.*, 1984; Burke and Alvarez, 1988; Wessel *et al.*, 1990). Coelomic pouches bud off of the archenteron; these vesicles subsequently expand, with the left one typically forming the internal structures of the juvenile urchin at metamorphosis (Czihak, 1962; reviewed in Czihak, 1971).

IV. Morphogenetic Movements during Sea Urchin Gastrulation

A. Ectoderm

1. Epiboly Prior to Gastrulation

Involution, i.e., the inward rolling of a sheet of cells, has been postulated as a mechanism contributing to the invagination of the vegetal plate for over a century (reviewed in Hörstadius, 1935, 1973). Expansion of a tissue sheet toward the site of an invagination was suggested by His (1874) as a mechanism by which an invagination could be initiated and was the favored mechanism by which primary invagination was thought to occur by many early investigators. Early fate mapping studies carried out by von Ubisch using pieces of agar impregnated with vital dye seemed to confirm this result; indeed, von Ubisch suggested that as much as half of the epithelium of the blastula moved into the interior during gastrulation (reviewed in Hörstadius, 1973). As mentioned earlier, more careful studies by Hörstadius showed that this was not the case; by staining single veg_2 blastomeres, he was able to show that widespread involution does not occur, but that the archenteron is formed only from veg_2 progeny that are in the vegetal plate (Fig. 4). However, Hörstadius' studies did not rule out a role for involution; they simply limited its scope. In fact, several different marking experiments indicate that demonstrable involution occurs prior to and during primary invagination. Ettensohn performed careful volumetric measurements on serially reconstructed *L. pictus* gastrulae, and he could show that there was a reproducible, though limited, increase in the number of cells within the invagination during its initiation. More recent studies by Burke and colleagues (Burke *et al.*, 1991) have refined these studies. By injecting horseradish peroxidase into single *Strongylocentrotus purpuratus* 16-cell stage blastomeres, and by applying local spots of Nile blue to lateral ectoderm cells, they demonstrated that there is a net movement of ectodermal cells toward the vegetal plate just prior to and during the onset of primary invagination. At the same time, the animal ectodermal cap expands vegetally. Sectors of ectoderm labeled by injecting mesomeres with horseradish peroxidase narrow in the circumferential dimension, while extending along the animal–vegetal axis (Burke *et al.*, 1991; Fig. 5). It is not currently known by what mechanism this overall shape change occurs.

As gastrulation progresses, it is clear that the tissue that remains on the outside of the gastrula must undergo marked changes in overall shape. First, the presumptive ventral (oral) ectoderm must flatten and converge toward the ventral side (Fig. 5). At the same time, the dorsal (aboral) ectoderm must flatten and expand to form a large portion of the outer covering of the larva. How such movements occur is unknown, although there is at least one cell adhesion or extracellular matrix molecule expressed differentially in the two major ectodermal territories (Coffman and McClay, 1990; see below).

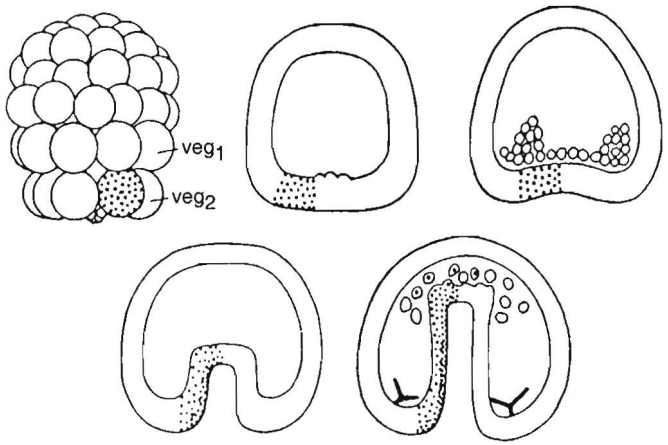


Fig. 4 Involution during gastrulation is measured by marking of veg_2 cells with Nile blue (from Hörstadius, 1936).

B. Primary Mesenchyme

1. Primary Mesenchyme Cells Can Undergo Morphogenesis Autonomously

Primary mesenchyme cells are perhaps the best-characterized cell population in the sea urchin embryo. One reason for this is that PMCs can be isolated and cultured *in vitro* (Okazaki, 1975), thus permitting many straightforward pharmacological experiments, as well as *in vitro* adhesion studies. When micromeres from 16-cell embryos are isolated based on their small size and cultured in the presence of horse serum *in vitro*, they appear to undergo many of the changes in morphology, motility, and gene expression exhibited by PMCs *in vivo*, including the production of spicules (Okazaki, 1975; Harkey and Whiteley, 1980; reviewed by Wilt, 1987; Decker and Lennarz, 1988; Ettensohn and Ingersoll, 1992; Benson and Wilt, 1992). Such *in vitro* methods also demonstrate that micromeres are probably committed to the PMC pathway of differentiation as soon as they are born and that the developmental processes required for differentiation as PMCs are autonomous within this lineage. Transplantation studies and chimeric embryos further support this view (see below). Although PMCs appear to differentiate autonomously from the larger descendants of the micromeres, they are nevertheless profoundly influenced by their environment, particularly with regard to their migration and patterning (see below).

2. Ingression of Primary Mesenchyme

a. Morphological Changes at Ingression. Prior to their ingression, PMCs are integrated into the vegetal plate epithelium. From the outside of the vegetal

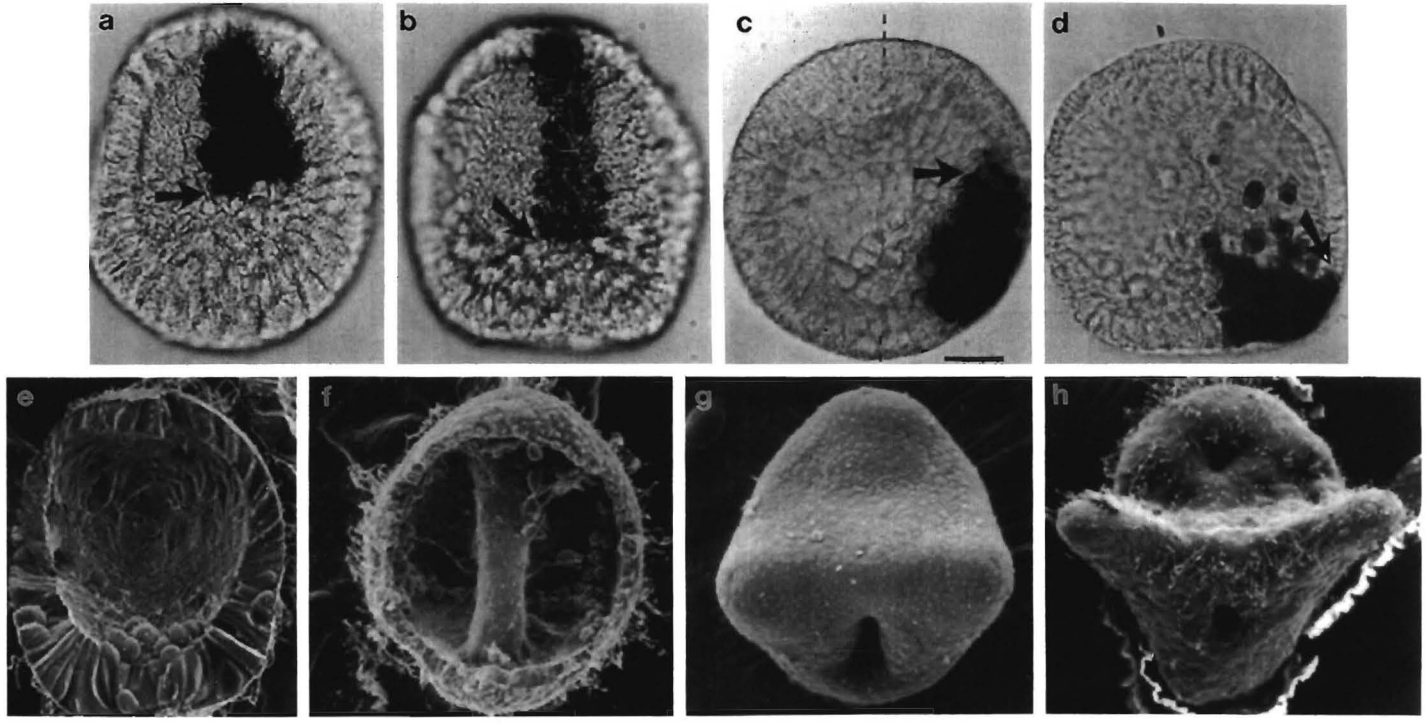


Fig. 5 Ectodermal morphogenesis prior to, during, and after gastrulation. (a) Clone of cells marked by injection of a mesomere with horseradish peroxidase (HRP) in *S. purpuratus*. (b) As gastrulation begins, the clone elongates. (c) HRP-marked clone derived from a macromere at the mesenchyme blastula stage. (d) Similar clone in an early gastrula. Arrows indicate the mesomere/macromere clonal boundary. (a–d) From Burke *et al.* (1990), with permission. (e) Scanning electron micrograph of a *L. variegatus* mesenchyme blastula, showing the fan-shaped "Okazaki pattern" in the lateral ectoderm. (f–h) Successive stages of postgastrula bending, viewed from the oral (ventral) side. (e–h) Courtesy of J. Morrill.

plate, a transient depression can be seen to form as PMC ingression begins (Katow and Solursh, 1980; Solursh, 1986; Amemiya, 1989; Fig. 6). A central cluster of nonciliated cells at the center of the plate does not ingress; these cells are presumably descended from the small micromere daughters (Katow and Solursh, 1980; Amemiya, 1989). A number of morphological changes accompany ingression; these have been studied at the light microscope level by Gustafson and colleagues (1960) and at the ultrastructural level by Gibbins *et al.* (1969), Katow and Solursh (1980; reviewed in Solursh, 1986), and Anstrom and Raff (1988). The onset of ingression is marked by waves of pulsaltile activity at the bulbous, basal ends of the ingressing cells as they protrude into the blastocoel (Gustafson and Kinnander, 1956).

Changes in ultrastructure coincide with the onset of the pulsatile behavior. Ingressing cells elongate, and eventually their apical ends decrease in diameter until a stalk of apical surface remains. The stalk contains numerous oriented microtubules (Gibbins *et al.*, 1969; Katow and Solursh, 1980; Anstrom, 1989; Fig. 6). Initially, the apical surface maintains its attachment to the hyaline layer/apical lamina; as the cells begin to ingress, they display elongated microvilli which maintain attachment to the hyaline layer. Eventually this attachment is lost, as are desmosomal attachments to neighboring cells (Katow and Solursh, 1980; Solursh, 1986; Amemiya, 1989). Changes in the extracellular matrix are coincident with ingression of PMCs. A wrinkle or blister in the hyaline layer

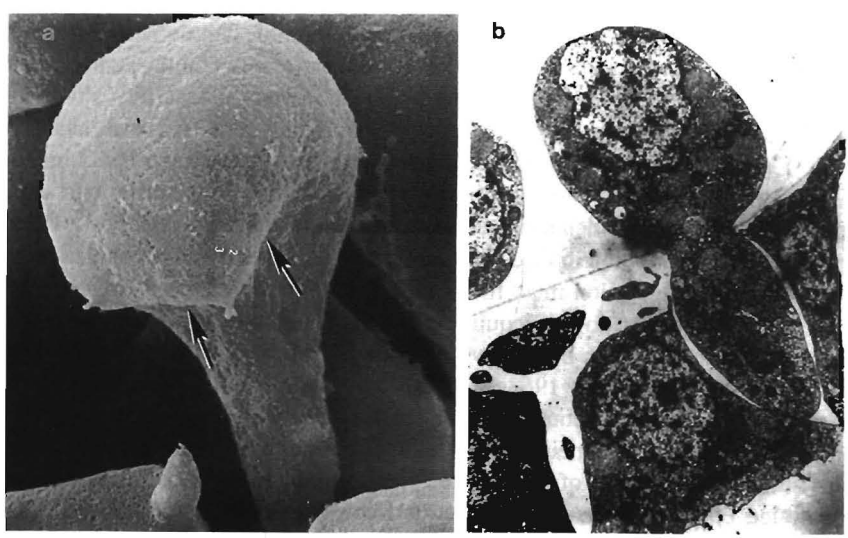


Fig. 6 Ingression of primary mesenchyme cells. (a) Scanning electron micrograph of PMC ingression in *L. pictus* (from Katow and Solursh, 1979, with permission). (b) Transmission electron micrograph of ingression in *S. purpuratus*. Note the basal lamina surrounding the site of ingression (courtesy of J. Morrill).

appears at the site of ingression (Dan, 1960; Dan and Inaba, 1968), presumably created by the release of PMCs from the hyaline layer. At the time that the basal ends of presumptive PMCs begin to protrude into the blastocoel, the basal lamina breaks down over the surface of the vegetal plate (Katow and Solursh, 1980; Wessel *et al.*, 1984; McCarthy and Burger, 1987).

The internal structure of PMCs changes markedly as they undergo the epithelial–mesenchymal transition at ingression. The Golgi apparatus and microtubule organizing center shift from an apical position to the apical side of the nucleus, and microtubules become oriented in the stalk region parallel to the apical–basal axis of the cell (Anstrom and Raff, 1988). Oriented microtubules appear in cells adjacent to bottle-shaped cells; it has been suggested that the oriented microtubules reflect lateral compressive forces experienced by PMCs as they ingress (Solursh, 1986; see below). Detailed observations of the actin cytoskeleton of PMCs have also been made recently; the apical actin meshwork characteristic of epithelial cells is lost as PMCs ingress, while the remaining cells in the vegetal plate retain an actin-rich apex (Anstrom, 1992).

3. Mechanisms of Ingression

Given the ease with which PMC ingression can be observed, surprisingly little is known about its cellular mechanisms. However, several pharmacological experiments rule out certain components of the intracellular machinery as being crucial for this process. Although Gibbins *et al.* (1969), Katow and Solursh (1980), and Anstrom (1989) have documented changes in the distribution of microtubules during ingression, treatment of embryos with microtubule depolymerizing agents indicates that microtubules are not necessary for ingression (Tilney and Gibbins, 1969a; Anstrom, 1989). In an attempt to test models of motility involving insertion of membrane at the leading edge of crawling cells, Anstrom and Raff (1988) showed that embryos treated with monensin still undergo PMC ingression. Ingression in this case occurs despite a blockage of secretion and other membrane trafficking, including secretion of the glycoprotein msp130, a PMC-specific cell surface protein (see below). These experiments at least indicate that mechanisms of ingression must focus on the actin cytoskeleton and changes in the PMC surface that do not require secretion immediately prior to ingression. However, little more can be said with confidence about how ingression occurs.

Gustafson and Wolpert (1963a,b, 1967) postulated that changes in the adhesive properties of PMCs could account for their ingression. They suggested that as PMCs lose affinity for neighboring cells in the vegetal plate, they would round up and be squeezed out of the vegetal plate by lateral pressure exerted by the remaining epithelial cells. Although Gustafson and Wolpert assumed that presumptive PMCs should round up as they ingress, ultrastructural data do not support this view. Rather, PMCs elongate prior to ingression; it is only as the last vestige of attachment to the apical lamina/hyaline layer is lost that the cells round up. The rapid detachment seems to be a viscoelastic process; cells can on occa-

sion be observed to "snap" away from the apical surface, in some cases leaving a remnant of their apical surface behind.

Evidence supporting lateral pressure as a mechanism aiding ingression of PMCs has been provided by exerting extrinsic pressure on embryos with a microneedle; this results in accelerated extrusion of PMCs from the vegetal plate (Solursh, 1986). As mentioned above, oriented microtubules in the stalk region of PMCs have been suggested to be due to lateral pressure exerted on PMCs (Solursh, 1986). Depressions can be seen on PMCs using scanning electron microscopy, suggesting that these cells experience lateral pressure (Katow and Solursh, 1980; see Fig. 6). What might generate such lateral compressive forces? Experiments by Ettensohn (1984) indicate that such forces must be localized in the vicinity of the vegetal plate, since PMC ingression occurs when vegetal plates are isolated prior to ingression. One possible source of such lateral pressure could be the convergence of material toward the center of the vegetal plate prior to its invagination that was observed by Burke *et al.* (1991; for more on the role of such convergence during invagination of the archenteron, see below). If vegetal plate thickening is simply the beginning of a process which is continued through the end of primary invagination, then this might be a means by which such lateral squeezing could be produced. Alternatively, Anstrom (1992) has suggested that apical constriction of PMCs may be required for the extrusion of mesenchyme into the blastocoel. If ingression is blocked with papaverine, the basal ends of PMCs protrude into the blastocoel, but apical narrowing of the cells is prevented (Anstrom, 1992). However, such suggestions are conjectural, and the cellular mechanisms by which mesenchyme ingression occurs remain surprisingly unclear.

4. Migration of Primary Mesenchyme Cells

a. The Motile Repertoire of PMCs. Once PMCs ingress into the blastocoel, they undergo extensive migratory movements, eventually adopting a characteristic ring-like pattern in the lateral ectoderm. In particular, two clusters of PMCs form in the ventrolateral ectoderm, and these clusters give rise to the spicule rudiments of the larva. PMCs migrate via thin filopodia approximately 0.5 μm in diameter and as long as 40 μm (Gustafson and Kinnander, 1956; Katow and Solursh, 1981; Karp and Solursh, 1985). In some cases, filopodia are unbranched, straight protrusions, but in other cases multiple filopodia may be extended by a single PMC, and individual filpodia can be branched (Gustafson and Kinnander, 1956; Karp and Solursh, 1985). The filopodia of PMCs do not appear to terminate within the blastocoel, but only appear to make stable contacts with the ectoderm and/or its associated basal lamina (Gustafson, 1963; Amemiya, 1989). The filopodia are actin-rich structures (Gibbins *et al.*, 1969; Katow and Solursh, 1981) and are thought to mediate motility via the production of contractile tension (reviewed by Trinkaus, 1984). When filopodia of PMCs detach, they can bend near the base, giving the impression that they behave me-

chanically as "stiff bristles" (Gustafson and Kinnander, 1956; Karp and Solursh, 1985; reviewed by Gustafson and Wolpert, 1963a, 1967; Solursh, 1986).

Given the basic motile repertoire of PMCs and the intricate pattern that they adopt after migration, Gustafson and Wolpert proposed that PMC migration and patterning could be accounted for largely by "random exploration" of PMCs within the blastocoel, combined with trapping or "selective fixation" (Weiss, 1947). The directionality of PMC migration would then be controlled by the relative stability of attachments that the PMCs make within the embryo, and PMCs would accumulate at sites where the density of such attachments was highest. Gustafson (1963) reported that PMCs appeared to prefer attachment at the junctions between epithelial cells; in compressed embryos observed with DIC microscopy, PMCs attached preferentially at clefts between epithelial cells. Gustafson interpreted this behavior as reflecting greater surface area for attachment in these regions, based both on clefts between epithelial cells and on the overall curvature of the epithelium at these sites (Gustafson, 1963). More recently, Amemiya examined the attachments of PMCs to the substratum via scanning electron microscopy and could find no evidence for attachment of filopodia to clefts between cells; they were observed to make attachments only to fibrillar material in the basal lamina (Amemiya, 1989). However, based on immunofluorescence data obtained using confocal microscopy on uncompressed embryos, the analysis of Gustafson may be correct for at least some species: embryos stained with PMC-specific monoclonal antibodies appear to insert filopodia between ectoderm cells in some cases (C. Ettensohn, personal communication; J. Hardin, unpublished observations).

More recently, it has been suggested by Solursh and colleagues (Karp and Solursh, 1985; Solursh and lane, 1988; see also Solursh, 1986) that the predominant means by which filopodia find their way may not be via adhesive sampling of the substratum. Instead, they suggest that filopodia may be sensory structures, sampling the local molecular environment and transducing signals to the rest of the cell. They base their arguments on the behavior of PMCs *in vitro*: when PMCs contact an aggregate of embryo-derived extracellular matrix, they appear to make stable attachments to the aggregate and preferentially shorten the attached filopodium (Solursh and Lane, 1988). In addition, Solursh and colleagues argue that the surface area at the tip of a filopodium is simply too small to provide adequate attachment sites for a purely adhesive process. It is not clear whether such "sensory" behavior is operating in this system. Miller *et al.* (1995) have made a similar suggestion (see Note added in proof). Nerve growth cones are apparently capable of integrating ionic signals from multiple filopodia (Kater and Mills, 1991; Davenport *et al.*, 1993), so it is certainly possible that such signal transduction is a component of the directed migratory response of PMCs (and SMCs as well; see below). On the other hand, it has been pointed out that due to the high curvature at its surface, a filopodium is ideally suited to overcome the activation energy barrier required for attachment to a topographically complex substratum (Pethica, 1961; see the extensive discussion in Trinkaus, 1984).

5. Molecular Requirements for PMC Migration

A number of chemical and pharmacological treatments have been used to disrupt PMC migration. These include sodium selenate (Sugiyama, 1972), tunicamycin (Schneider *et al.*, 1978; Heifetz and Lennarz, 1979; Akasaka *et al.*, 1980), arylxylosides (Kinoshita and Saiga, 1979; Akasaka *et al.*, 1980; Solursh *et al.*, 1986; Lane and Solursh, 1988), and sulfate deprivation (Herbst, 1904; Immers and Runnström, 1965; Karp and Solursh, 1974; Akasaka *et al.*, 1980). A common theme among these various treatments is their perturbation of glycoprotein or proteoglycan synthesis or processing. In the case of sulfate deprivation and xyloside treatment, the effects are largely reversible: when the agents are removed from the seawater, development resumes (Karp and Solursh, 1974; Solursh *et al.*, 1986). This suggests that these agents exert widespread epigenetic effects on the onset of gastrulation; while they also block migration of PMCs, this probably results from indirect effects upon entry into gastrulation rather than effects on migratory events specific to PMCs (see below).

The chemical treatments do not resolve whether the defects in treated embryos lie with the embryonic environment or the PMCs. However, culturing PCMs *in vitro* under defined conditions indicates that many of the defects lie with the PMCs themselves (Venkatasubramanian and Solursh, 1984). PMCs from embryos treated with xylosides or reared in sulfate-free seawater cannot migrate *in vitro*. The inability of such PMCs to migrate can be overcome at least in part by supplementing them with a urea extract of cell surface components derived from normal PMCs (Lane and Solursh, 1988). Reciprocal transplantation of PMCs between tunicamycin-treated and normal embryos indicates that blocking glysolylation produces defects in both the PMCs and their environment (E. Ingersoll and C. Ettensohn, personal communication). Thus, although there is presumptive evidence indicating a role for cell surface proteoglycans in PMC migration, the precise identity of such components awaits further study.

PMCs express other cell surface proteins just prior to their ingression. Several of these appear to be involved in spiculogenesis, based on their appearance in larval and adult skeletal tissues and a number of other criteria. These include SM50, a 50-kDa protein isolated from *S. purpuratus* (Benson *et al.*, 1987; Sucov *et al.*, 1987), and msp130, a PMC-specific glycoprotein which is apparently diagnostic for spiculogenic cells in echinoids (Leaf *et al.*, 1987; Anstrom *et al.*, 1987; Farach *et al.*, 1987; Wray and McClay, 1988). A third protein originally identified via monoclonal antibodies and termed "Meso1" identifies a separate 380-kDa cell surface protein expressed by PMCs, based on electrophoretic mobility and specificity of expression (Wessel and McClay, 1985). There is some evidence that antibodies recognizing Meso1 may also recognize a panmesenchymal epitope recognized by another monoclonal antibody, Sp12 (Wray and McClay, 1988; Tamboline and Burke, 1989). Finally, PMCs possess a high density of wheat germ agglutinin (WGA) binding sites on their surfaces (Spiegel and Burger, 1982; DeSimone and Spiegel, 1986); the proteins to which the lectin

binds have not been characterized. Although there are clearly numerous proteins expressed uniquely on the surfaces of PMCs, few have been shown to play a significant role in the ingression or migration of PMCs. Suppression of secretion of msp130 by monensin treatment does not prevent ingression of PMCs, although migration does not occur normally (Anstrom and Raff, 1988); whether the latter effects are due to the specific prevention of deposition of msp130 or to other more general defects is not known. Injection of WGA into the blastocoel can inhibit migration of PMCs (C. Ettensohn, personal communication); what specific role(s) WGA-binding moities may play in PMC migration is not known.

6. Control of PMC Migration and Patterning

Specific experiments have been performed that shed light on the sorts of cues PMCs use to find their way within the blastocoel. These experiments indicate that PMCs respond to multiple sites within the blastocoel; in addition, their competence to respond to such signals lasts for considerable periods of time relative to their normal schedule of migration. Most of these experiments have involved heterotopic or heterchronic transplantation or displacement of PMCs. Okazaki *et al.* (1962) observed that PMCs localize at sites in the ectoderm where the epithelial cells are thickened, producing a fan-like optical effect (see Fig. 5). When this belt of cells is shifted along the animal–vegetal axis by classic "vegetalizing" agents such as lithium chloride, PMCs localize to the shifted ectoderm (Wolpert and Gustafson, 1961c). These experiments suggest that regionally specific information that specifies the pattern of PMCs is expressed within the ectoderm; whether the thickened epithelial cells are a correlative marker for the actual guidance information or represent an epithelial response to molecular or adhesive specializations of the ectoderm in this region is not known. Gustafson (1963, 1964) suggested that the localization of PMCs to the fan-like arrays was due to mainly physical factors; he proposed that the curvature of the ectoderm in this region would present a greater density of adhesive sites for attachment of filopodia.

Additional evidence for guidance cues within the embryo is provided by cell displacement and transplantation experiments. When PMCs are displaced from the vegetal pole by centrifugation, the displaced cells migrate back to the vegetal pole region (Okazaki *et al.*, 1962; Fig. 7). When rhodamine-labeled PMCs are injected into the animal pole region of a recipient mesenchyme blastula, the injected cells migrate to the vegetal plate region. The ability of PMCs to respond to the vegetal plate region extends over a considerable period of time, since PMCs transplanted from older donor embryos into younger hosts localize to the vegetal pole as well. Furthermore, even though they are competent to ingress and begin formation of the skeleton (as do their siblings in control embryos), older transplanted PMCs remain at the vegetal plate until the host PMCs ingress. The donor PMCs then join the host PMCs to produce a normal skeleton (Ettensohn

and McClay, 1986; Fig. 7). The nature of the vegetal plate signals is uncertain. However, transplanted cells appear to migrate with little persistence when initially transplanted; after this initial phase, they appear to migrate directionally toward the vegetal plate. The distance involved appears to preclude simple "trapping" of randomly extended filopodia, since the diameter of the embryo is roughly 120 μm and the maximum length of a protrusion is ~40 μm (Gustafson and Wolpert, 1963a,b; C. Ettensohn, personal communication). Similar behavior is observed in vegetalized embryos, in which PMCs migrate toward the animal pole after an initial period of relatively nondirectional migration (Wolpert and Gustafson, 1961c; Fig. 7).

Further evidence for distributed signals with highest strength at the vegetal plate comes from transplantations into microsurgically isolated animal halves; PMCs localize to the vegetal end of such isolates, even though a vegetal plate is

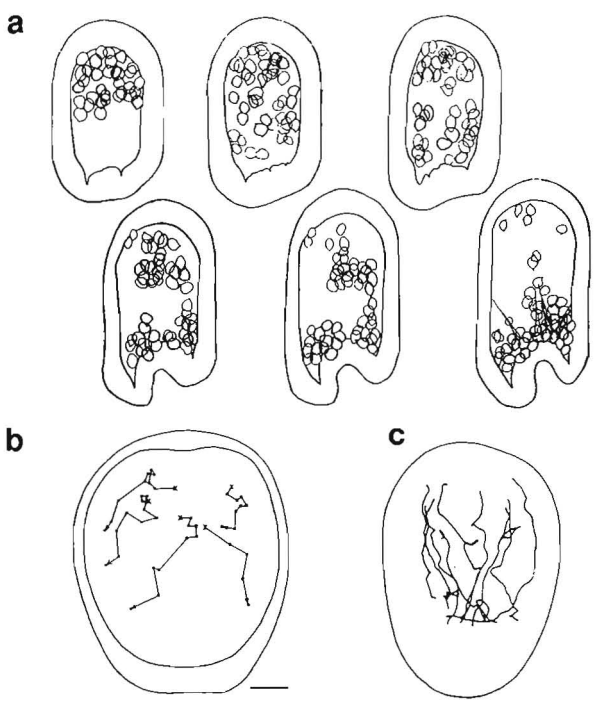


Fig. 7 Directed migration of primary mesenchyme cells. (a) Displacement of PMCs by centrifugation. Displaced PMCs move back to the vegetal regions of the embryo. Tracings were made at 30 min. intervals (from Okazaki *et al.*, 1962, with permission). (b) PMCs injected into the animal pole of a *L. variegatus* embryo migrate toward the vegetal plate (from Ettensohn, 1992, with permission). (c) PMCs migrate toward the animal pole in a *Psammechinus miliaris* embryo vegetalized with LiCl (from Wolpert and Gustafson, 1960, with permission).

not present, suggesting that PMCs respond to directional cues of a spatially distributed nature (McClay *et al.*, 1992; Malinda and Ettensohn, 1994). Although it is probably easiest to envision PMCs responding to a haptotactic gradient of a substrate-bound signal, short-range diffusible cues have not thus far been ruled out. Perfusion experiments that should shed light on this question are currently underway (K. Malinda and C. Ettensohn, pers. commun.).

In addition to cues directing cells to the vegetal plate and perhaps to the ventrolateral ectoderm, it is clear that additional pattern regulation occurs during construction of the larval skeleton. The cellular mechanisms of skeletogenesis have been extensively reviewed elsewhere (Okazaki 1975; Decker and Lennarz, 1988; Wilt, 1987; Wilt and Benson, 1988; Ettensohn, 1992; Ettensohn and Ingersoll, 1992; Benson and Wilt, 1992). The details of this process are outside the scope of this review; interested readers can consult these references for further information on this process. Here we will focus on the patterning that guides individual PMCs to the correct sites as a prerequisite for construction of a bona fide skeleton. Single micromeres added ectopically to host embryos produce PMCs that ingress on schedule and incorporate into the host skeleton normally (Wray and McClay, 1988), demonstrating that there is nothing about the site at which PMCs ingress that is important for pattern formation. When supernumerary PMCs are transplanted into host embryos, the pattern of the skeleton that results is indistinguishable from the normal pattern, even though as many as two to three times the normal number of PMCs participate in skeleton production (Ettensohn, 1990; Fig. 8). There seems to be no preference of the additional PMCs for any part of the patterned skeletal array over another, suggesting that site selection by PMCs for different regions of the forming skeleton is not based on prior "addressing" of PMCs to specific sites (e.g., the ventrolateral ectoderm or intervening ring regions; Ettensohn, 1990). In half- and quarter-sized dwarf larvae, the pattern produced by PMCs is proportionately correct (Driesch, 1892, 1900a,b; Takahashi and Okazaki, 1979), again indicating that regulation of the size of the skeleton occurs. Transplantation of PMCs into dwarf larvae indicates that the ectoderm of the dwarf larva governs the size of the skeleton, since PMCs from full-size donor embryos form a skeleton with an appropriately reduced size when transplanted into a dwarf host (Armstrong and McClay, 1994; Fig. 8). Ettensohn and Malinda (1993) have shown that size regulation may involve "plug" PMCs, i.e., aggregates of PMCs located at the tips of the growing arms of the larva; when the number of such cells is artificially increased by transplantation or reduced by photoablation, arm growth is enhanced or retarded accordingly (Ettensohn and Malinda, 1993). Taken together, results from these numerous studies indicate that the embryo restricts the number of spicules, the sites at which they form, and the timing of their formation.

Recent experiments involving embryos radialized with nickel chloride indicate that much of this control resides with the ectoderm. Radialized embryos produce

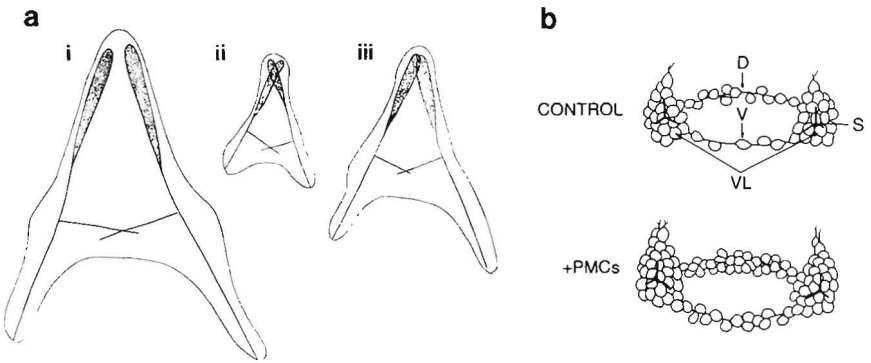


Fig. 8 Regulation of overall pattern of primary mesenchyme cells. (a) Skeletal size regulation in normal, $\frac{1}{2}$, $\frac{1}{4}$ dwarf larvae of *Echinus* (from Driesch, 1900a). (b) Size regulation in a normal embryo which has received additional PMCs via transplantation (adapted from Ettensohn, 1992, with permission).

the normal number of PMCs, yet can produce as many as a dozen small triradiate skeletal elements (Hardin *et al.*, 1992; Fig. 9). Based on the alterations in ectodermal gene expression induced by nickel treatment, much of the aboral (dorsal) ectoderm is converted to oral (ventral) ectoderm. The PMCs localize at the border of the remaining aboral ectodermal tissue with the ciliated band/ventral ectoderm (Hardin *et al.*, 1992). Armstrong *et al.* (1993) used the perturbation of skeletal pattern to test directly the role of the ectoderm in patterning by performing reciprocal transplants between normal and nickel-treated embryos. Normal PMCs transplanted into radialized hosts from which all PMCs had been removed produce a radialized skeleton; conversely, PMCs from radialized embryos transplanted into normal embryos after removal of their own PMCs result in a normal, bilateral skeleton (Armstrong *et al.*, 1993; Fig. 9).

While these experiments demonstrate a major role for the ectoderm in mesenchymal patterning, many unanswered questions remain. First, the molecular nature of the interaction between ectoderm and PMCs is unknown. Recently, it has been demonstrated that PMCs do show regional differences in their patterns of gene expression, particularly with respect to the cell surface protein msp130 (Harkey *et al.*, 1992) and the spicule matrix protein SM30 (K. Guss and C. Ettensohn, pers. commun.). These results suggest that the ventrolateral ectoderm may be inducing such differences via local cell–cell signaling, since there is a noticeably higher concentration of mRNAs derived from both of these genes in ventrolateral cluster PMCs. Additional experiments must be performed to establish this with certainty; the molecular nature of such cues remains obscure. Second, although the ectoderm profoundly influences PMC patterning, the local details of skeletal pattern are due to autonomous processes among PMCs. Thus,

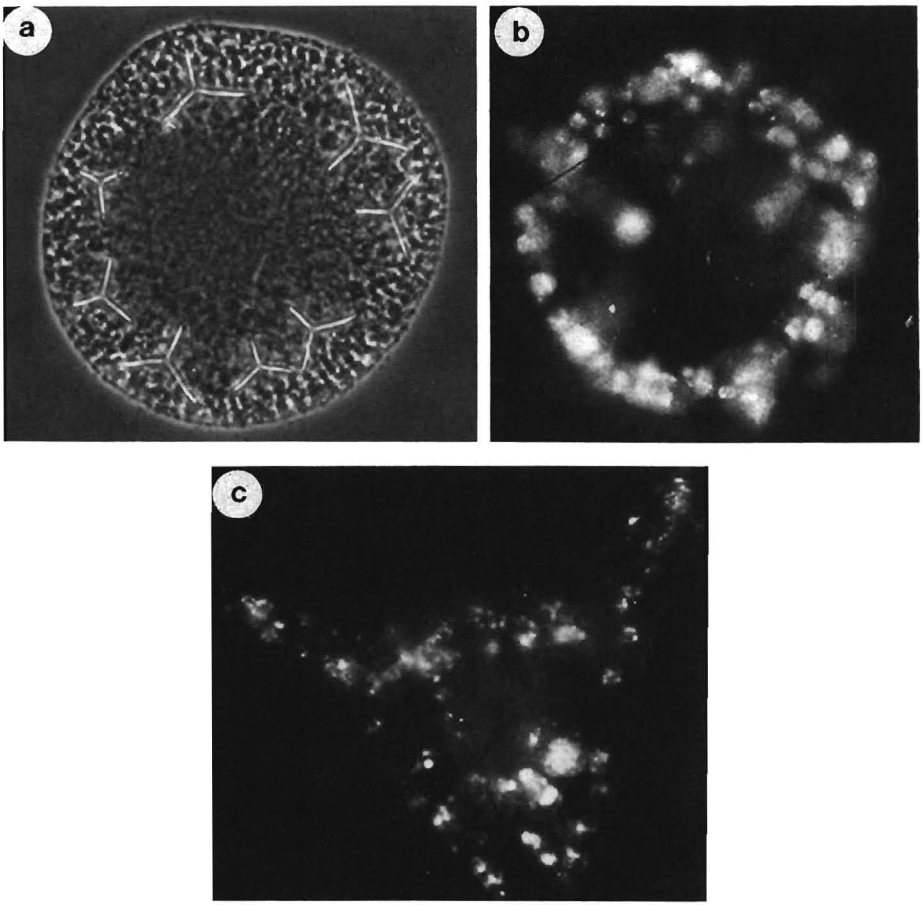


Fig. 9 Regulation of PMC patterning of ectoderm. (a) Embryo radialized with $NiCl_2$, compressed and viewed from the vegetal pole. Note the multiple triradiate spicule elements. (b) Radialized embryo whose PMCs were removed and replaced with PMCs from a normal embryo. (c) Normal embryo whose PMCs were removed and replaced with PMCs from a radialized embryo. (b and c) Adapted from Armstrong *et al.*, 1993, with permission.

when PMCs from one species are transplanted into another, the overall shape of the skeleton produced is appropriate to the host embryo (Armstrong and McClay, 1994). However, the fenestration pattern at the local level is determined by the species from which the PMCs are derived (Armstrong and McClay, 1994). How and when PMCs use autonomous (or at least PMC-endogenous) patterning mechanisms vs ectodermally provided signals remains unknown.

C. Endoderm

1. Initial Invagination of the Archenteron

a. The Process of Primary Invagination. The studies of Gustafson and colleagues revealed that the invagination of the archenteron occurs in two distinct phases displaying different characteristic rates and qualitatively different cell behaviors (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960; reviewed in Gustafson and Wolpert, 1967; Fig. 10). During the first phase, *primary invagination*, the vegetal plate invaginates to form a shallow invagination, which ultimately produces a flat-topped archenteron with straight sides (Fig. 11). Following a noticeable pause (see Fig. 10), the archenteron resumes elongation during *secondary invagination*, which is characterized by the onset of protrusive activity by secondary mesenchyme cells (Fig. 11). Time-lapse studies by Gustafson and colleagues indicate that a marked changed in the behavior of cells in the vegetal plate occurs at the time of primary invagination. The basal surfaces of the cells appear to "boil," displaying pulsatile bleb-like extensions that are dynamically extended and retracted; in some cases, small vesicles of

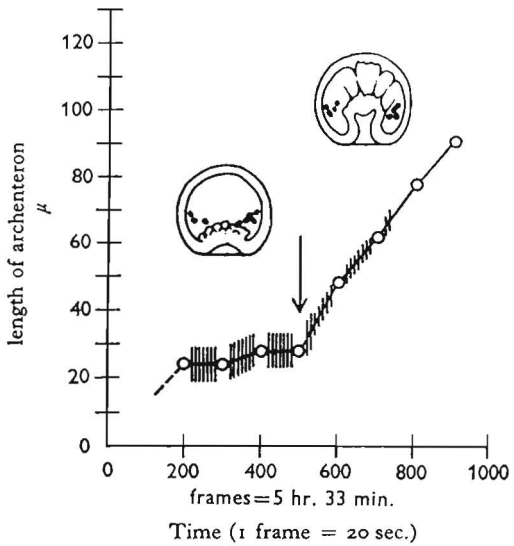


Fig. 10 Invagination occurs in two distinct phases with characteristic rates. Plot of archenteron length vs time in *P. miliaris*. The vertical bars represent qualitative extent of protrusive activity. During primary invagination (portion of the graph preceding the arrow), protrusive activity takes the form of cortical blebbing; during secondary invagination, protrusive activity refers to protrusive activity of secondary mesenchyme cells (from Gustafson and Wolpert, 1967, with permission).

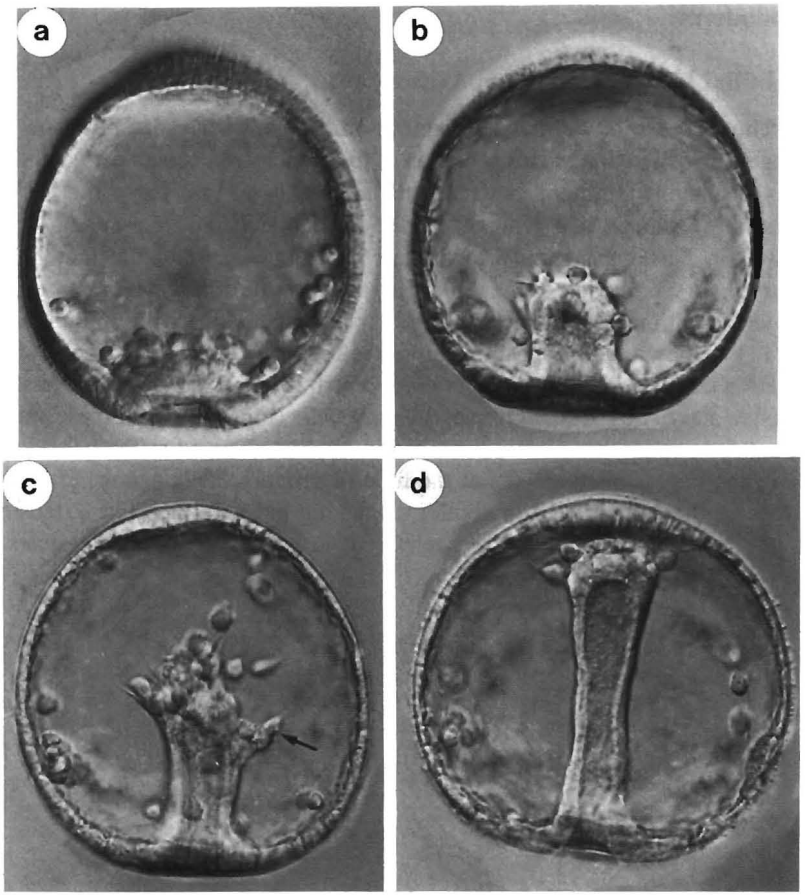


Fig. 11 Invagination of the archenteron in *L. pictus*. (a) Early primary invagination. (b) End of primary invagination. (c) Secondary invagination is underway; secondary mesenchyme cells are protrusively active (arrow). (d) End of secondary invagination. The archenteron makes contact with a specific region near the animal pole. Adapted from Hardin and Cheng (1986), with permission.

basal cytoplasm appear to be liberated (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960). These extensions are small (~0.1–0.5 μm) and are restricted to the vegetal plate. The significance of this behavior is not known; it may be that such behavior results from the dissolution of the basal lamina in this region, or may reflect a direct alteration in the cortical cytoskeleton of the presumptive endoderm. Studies of the rate of primary invagination indicate that it is roughly constant throughout; in addition, one "side" (in profile; in reality a radial sector of the vegetal plate) of the invagination often bends inward first,

followed by the other (Dan and Okazaki, 1956; Ettensohn, 1984). Thus, there is some temporal inhomogeneity with respect to the initiation of invagination throughout the plate. In addition to changes in cell motility, the cells of the vegetal plate undergo marked changes in shape. Ultrastructural and histological studies have documented these changes rather extensively (Ettensohn, 1984, 1985; Galileo and Morrill, 1985; Morrill and Santos, 1985; Hardin, 1989; Burke *et al.*, 1991; Fig. 12). By the time primary invagination is over, the archenteron is cylindrical in shape, with straight sides and a flat top.

b. Primary Invagination: A Regionally Autonomous Process. Classic experiments by Moore and Burt (1939) and more recent experiments by Ettensohn (1984) have demonstrated conclusively that the forces required for primary invagination are generated in the immediate vicinity of the vegetal plate. Moore and Burt (1939) isolated vegetal hemispheres at the gastrula stage and found that they underwent invagination. Vegetal plates in which a radial cut was made did not appear to invaginate. Ettensohn extended these experiments by isolating vegetal plates several hours before they would normally invaginate and then immobilizing them to prevent wound healing (Ettensohn, 1984). In this case, PMCs ingress from the isolated plate, and thereafter the plate invaginates on schedule. These experiments demonstrate that the vegetal plate is autonomous with respect to (1) the *forces* responsible for its invagination and (2) the *signals* required to initiate an invagination. The radial incision experiments further suggest that forces must be integrated circumferentially within the plate.

Other studies place boundaries on the sorts of models that can be proposed to account for primary invagination. First, inhibition of DNA synthesis and subsequent mitosis using aphidicolin do not prevent invagination in *L. pictus* (indeed, secondary invagination can take place as well in this species in the presence of the drug; Stephens *et al.*, 1986). These results indicate that in the case of *L. pictus*, oriented cell divisions resulting in "mitotic pressure" are not crucial for invagination (for a discussion of the merits of mitotic pressure as a general morphogenetic mechanism, see Ettensohn, 1985; Fristrom, 1988). However, it should be noted that there may be species-specific differences in the relative importance of mitosis at this time, since cells in the vicinity of the vegetal plate are mitotically active in *L. variegatus*, and blocking DNA synthesis blocks invagination in this species (Nislow and Morrill, 1988). Second, disruption of cytoplasmic microtubules does not prevent primary invagination (Tilney and Gibbins, 1969; Hardin, 1987); thus, any motility or cell shape changes that occur during invagination are presumably either actin-mediated or are due to forces exerted extrinsically on the vegetal plate. Even given these limitations on plausible models of invagination and the seeming simplicity of this process, primary invagination remains poorly understood. Recently, however, several novel proposals have been made regarding the cellular mechanisms of primary invagination.

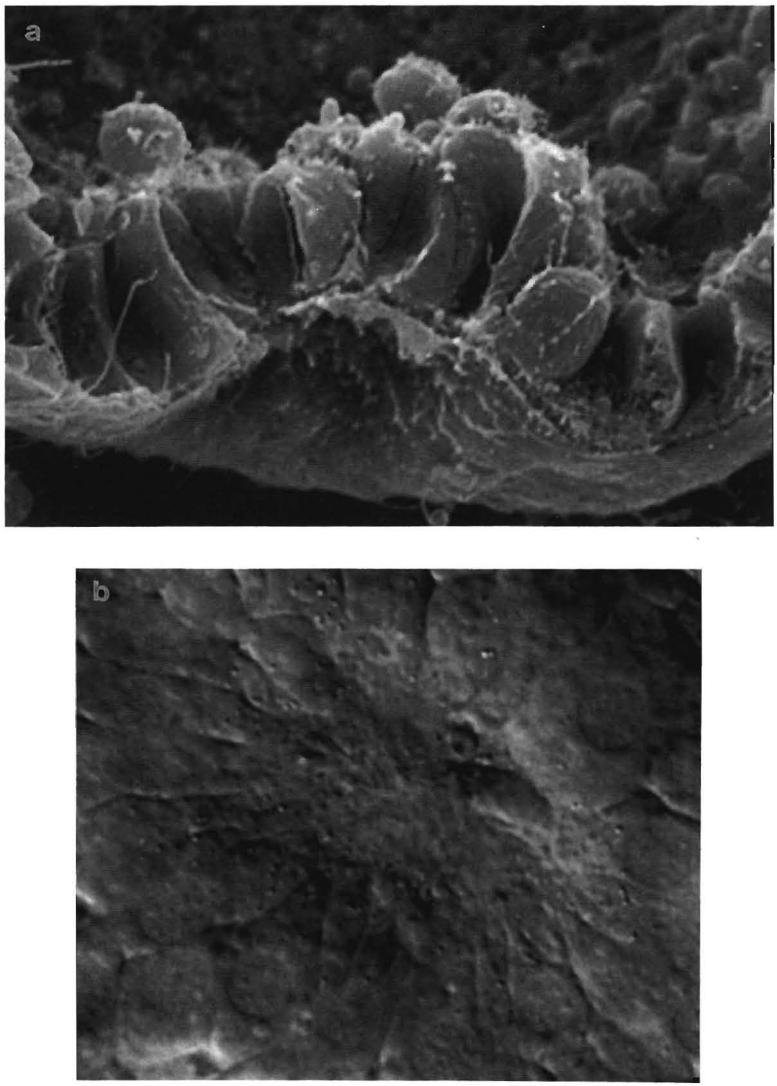


Fig. 12 Primary invagination of the vegetal plate. (a) Scanning electron micrograph of a *L. pictus* archenteron. Note the conspicuous indentation and the apical stretching of nearby cells (J. Hardin, unpublished). (b) A vegetal pole view of a *L. pictus* vegetal plate at the onset of invagination. Note the radial orientation of cells just outside the center of the vegetal plate (J. Hardin, unpublished).

c. Proposed Mechanisms of Primary Invagination

Mechanical models of invagination. One of the earliest paradigms for the application of the mechanical notions inherent in the new *Entwicklungsmechanik*

was gastrulation in "simple" systems such as echinoderms and *Amphioxus*, in which gastrulation is typified by the invagination of a simple epithelium. Mechanical modeling of invagination was performed by Rhumbler in 1902 using metal rods and elastic bands; later, differential swelling models were constructed by Bütschli (using paper and gelatin; Bütschli, 1915) and Spek (using gelatin and agarose; Spek, 1917, 1919). The latter attempted to simulate the properties of the cytoplasm as an osmotically active gel. Spek was able to demonstrate that differential swelling of a three-dimensional lamellar gel could cause both invaginations and evaginations. Somewhat later, Lewis (1947) used brass plates and elastic bands to simulate apical constriction in a three-dimensional model. While crude, such models were quite useful for clarifying rather vague notions about how mechanics specifically affect morphogenesis. As we shall see, these models have been given new relevance by recent hypotheses concerning primary invagination.

In addition to such early attempts at modeling, actual mechanical measurements have been attempted on sea urchin embryos. Moore (1941) exploited the development of a permeability barrier to sugar molecules at the mid-blastula stage to swell embryos using isotonic sucrose solutions; by calculating the osmotic pressure required to do so, he could estimate the mechanical work required to produce an invagination. Moore found that the central region of the invaginated archenteron persists following swelling, but the periphery of the vegetal plate everts. This led Moore to postulate that the vegetal plate is actually composed of two zones: an actively invaginating central region and a passively translocated periphery (Moore, 1941). Gustafson and Wolpert (1963a) used a cell elastimeter originally developed by Mitchison and Swann (1954) to measure the stiffness of the gastrula epithelium. While such measurements were certainly rough at best, they do provide upper bounds on the forces required to produce an invagination. Gustafson and Wolpert used these calculations to suggest that filopodial traction could provide a sufficient mechanical explanation for archenteron elongation (see below).

More recently, mathematical models derived from continuum mechanics have been used to simulate various aspects of gastrulation, including primary invagination. Such models are valuable in that they suggest experiments that can be performed on real embryos to elucidate the actual mechanisms of primary invagination. Odell *et al.* (1981) postulated that stretch-activated contraction of apical microfilament bundles could produce an invagination in two-dimensional models. One consequence of their model was extensive involution, something that does not occur in *L. pictus* (Ettensohn, 1984; Hardin, 1989). Computer-generated, three-dimensional continuum mechanical models have also been constructed to model primary invagination (J. Hardin and L. Cheng, unpublished; Davidson *et al.*, 1995). and exogastrulation (Zinemanas and Nir, 1992; J. Hardin and L. Cheng, unpublished observations). Based on their results, Zinemanas and Nir (1992) suggest that there may be mechanical anisotropies within the vegetal plate that account for some of its behaviors.

Hypotheses regarding cellular mechanisms of primary invagination. Gustafson and Wolpert proposed that *changes in cell adhesion* within the vegetal plate could drive invagination. In this model, increased lateral cell–cell adhesion would first result in the formation of a placode (the vegetal plate itself) and, as the cells attempt to "zip up" further, an invagination. This model would require a progressive increase in height of the cells of the vegetal plate as invagination proceeds. However, careful measurements on sectioned material by Ettensohn (1984) and several scanning electron microscopy studies (Morrill and Santos, 1985; Hardin, 1989) indicate that this sort of systematic cell shape change does not occur.

Recently, several competing (but by no means mutually exclusive) hypotheses concerning primary invagination have emerged (summarized in Fig. 13). Burke *et al.* (1991) demonstrated that extensive vegetalward movement of material lateral to the vegetal plate occurs just prior to primary invagination (see above). Because these "apical tractoring" movements are roughly coincident with primary invagination, it has been suggested by Burke and colleagues that such movements could generate lateral compressive forces that cause the vegetal plate to buckle inward. Presumptive evidence in favor of this model comes from antibody blocking studies; when embryos are reared in the presence of antibodies against fibropellins (glycoproteins of the apical lamina, and hence in immediate contact with the apical surfaces of cells of the embryonic epithelium; see below) primary invagination is blocked (see Fig. 28). At the same time, convergence of material toward the vegetal pole is halted, based on movement of Nile blue spots (Burke *et al.*, 1991). Although these experiments are open to other interpretations, they

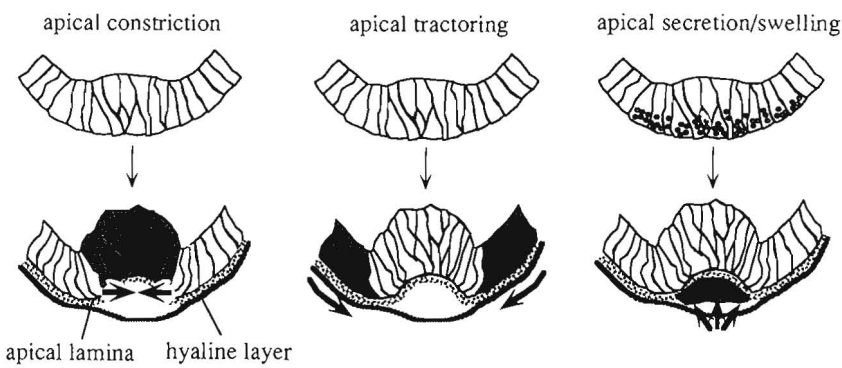


Fig. 13 Models of primary invagination. In the *apical constriction* model, active constriction of some cell apices in the center of the vegetal plate generates stretching of nearby cells. Because the stretching is not uniform with respect to the apical basal axis of the epithelium, shearing occurs lateral to the invagination. In the *apical tractoring* model, active crawling of lateral cells generates compressive force within the vegetal plate, causing it to buckle. In the *apical secretion/swelling* model, local secretion of proteoglycan and its subsequent swelling generate radially directed compressive force in the vegetal plate, causing it to buckle. From Hardin (1994), with permission.

suggest that cells lateral to the vegetal plate may actively converge toward it, using the apical lamina as a substrate for movement. Burke and colleagues have offered the following supporting morphological evidence for this model: (1) the apices of lateral cells are sheared with respect to their basal surfaces, suggesting that they are migrating; and (2) apical extensions on lateral cells are visible during this time under the scanning electron microscope. Burke *et al.* (1991) suggest that these extensions are active, protrusive appendages that "tractor" along the apical lamina (Burke *et al.*, 1991).

The apical tractoring model of primary invagination is appealing in several respects. First, it integrates involution with primary invagination. The tight temporal correlation between these events would then have a direct causal explanation. Second, although not specifically mentioned in their model, the ectodermal spreading that occurs concomitant with primary invagination would presumably result from pulling by the tractoring cells, much as a knit cap is pulled down over a person's head. Third, a compressive buckling model makes the phenomenon of exogastrulation easier to understand. A disc experiencing compressive force at its margin can buckle inward or outward, depending on slight changes in the initial conditions or the presence or absence of factors that would restrain buckling in one direction or the other. One could imagine that in exogastrulation the shape of the vegetal region or the properties of its extracellular matrix are altered somehow. Since so many factors can result in exogastrulation (see below), the notion that buckling is rather like a "gun" that can be "fired" in one of two directions, with the direction being influenced by many factors, is parsimonious. However, this argument works both ways: because it would be so easy to exogastrulate if this model were correct, there must presumably be some facet of the process that guarantees reliability. One possibility is that in the normal embryo the hyaline layer restrains the vegetal plate, preventing it from buckling outward.

Despite its appeal, there is no unambiguous support for this model, and it presents some challenges with respect to our current understanding of the properties of epithelia. First, apical shearing of cells lateral to the vegetal plate could simply reflect an asymmetric distribution of forces within the vegetal plate (e.g., apical constriction) that is transmitted to the lateral cells. Shearing would then be an effect, rather than cause, of invagination. Second, given the structural properties of the apical surfaces of epithelial cells, there is little precedent for such motility at this surface. While it must be true that cells modulate their contact with the apical lamina and hyaline layer as gastrulation proceeds, "crawling," i.e., the production of actin-rich protrusions capable of shortening to produce force, is not a property associated with the apical surfaces of epithelia. It should be pointed out, however, that the apices of rearranging enveloping layer cells during epiboly in the teleost *Fundulus* display "flower-like" surface activity as they rearrange (Keller and Trinkaus, 1987). In this case, apical extensions are present during dynamic changes in epithelial structure, so such activity in the sea urchin ectoderm cannot be ruled out. The available evidence suggests that the

basolateral surfaces of epithelial cells could produce such protrusions (see below), but then the *basal* lamina would be the substratum for migration, and shearing in the *opposite* direction from that observed in *S. purpuratus* would be required. Such a proposal, called the "cortical tractor" model, has been put forward to account for neurulation in urodeles (Jacobson *et al.*, 1986). Finally, antibody treatments that disrupt the apical lamina do not adjudicate between various specific mechanisms, since they presumably disrupt attachment to the molecule of interest globally. Thus, blocking of vegetal convergence in this manner may simply reflect a general requirement (structural or otherwise) for the apical lamina in primary invagination. Perturbation of a specific region of the ectoderm would have to be performed, or a restricted pattern of receptors for apical lamina proteins would have to be found, to add weight to this argument.

Another recent model to account for primary invagination, put forward by Lane *et al.* (1993), involves the *localized secretion of proteoglycans* at the vegetal plate. After their release, these materials are thought to swell, producing a local plug of material that forces the vegetal plate inward. Several pieces of evidence have been put forward in support of this model. First, material that shares epitopes with vertebrate chondroitin sulfate proteoglycans is released at the time of primary invagination by cells of the vegetal plate (Lane *et al.*, 1993). The immunoreactive material is present in vegetal plate cells prior to this time as small granules. Second, when mesenchyme blastulae are treated with calcium ionophore, they undergo precocious primary invagination, which is accompanied by precocious release of the proteoglycan. Third, when all secretion is blocked with monensin, precocious primary invagination is blocked, as is the release of this material. Finally, precocious invagination can still be induced in the presence of anti-mitotic doses of cytochalasin, which would presumably interfere with any constriction of microfilaments. Given these results, Lane *et al.* (1993) propose that the vegetal plate behaves like a bimetallic strip; the rapid swelling of the proteoglycan, combined with the relative inflexibility of the surrounding hyaline layer, results in bending, in a manner reminiscent of the suggestions made by Bütschli and Spek 90 years ago.

Determining whether or not this model is correct awaits further experiments. Unfortunately, the drug treatments used in this study are global and affect any process requiring elevation of cytoplasmic calcium or secretion, so the process responsible for the observed effects on invagination cannot be determined. Second, assays were not performed to assess the extent to which the actomyosin cytoskeleton was disrupted in these experiments; prolonged cytochalasin treatment results in the dissolution of the embryo into single cells (Ettensohn, 1984; Katow, 1989), so treatments could be applied for only short time periods. Third, since the actual invagination produced is not hemispherical, but cylindrical, the resistance to deformation of the archenteron upon swelling of the proteoglycan must be quite anisotropic to yield the observed shape. It is not known whether such anisotropy exists. Finally, this model would require the hyaline layer to

restrain the ejection of the proteoglycan; currently, nothing is known about how the hyaline layer interacts structurally or biochemically with the released material, although there is a noticeable wrinkling of clear material at the open end of the invagination (Dan and Inaba, 1968).

Another model that has found its way into prominent undergraduate cell biology texts (e.g., Alberts *et al.*, 1994) involves the *constriction of apical microfilaments*. There have been several variations of this model proposed (reviewed in Ettensohn, 1985), including stretch-activated firing of microfilaments (Odell *et al.*, 1981; see above). The apical constriction of the cells produced by microfilament contraction would result in local bending of the sheet, and since the epithelium is polarized, the bending in this case would always be directed inward. What evidence supports this model of primary invagination? First, it is clear that an apically directed, local bending force applied to the vegetal plate will produce an invagination. Mechanical simulations suggest that such a bending moment, if applied long enough, will produce an invagination with straight sides and a flat top (J. Hardin and L. Cheng, unpublished observations). Second, the cells of the vegetal plate epithelium have typical circumferential actin bundles at their apical surfaces, based on ultrastructural studies and confocal microscopy (Ettensohn, 1984; Anstrom, 1992b; J. Hardin, unpublished observations). In addition, flask-shaped cells are seen in the vegetal plate at the time when it invaginates. If such cells are a cause of invagination rather than an effect, then neighboring, unconstricted cells would be expected to be stretched at their apical surfaces, and this is in fact what is observed (Hardin, 1989; Fig. 12).

Conclusive evidence is lacking for this model as well. First, all epithelia have apical microfilaments; their presence says nothing about their contractility. In some epithelia, apical constriction is certainly possible (Owaribe *et al.*, 1981; Brady and Hilfer, 1982; Hardin and Keller, 1988; see reviews by Ettensohn, 1985; Hilfer and Searles, 1986; Fristrom, 1988); whether or not contraction occurs in the vegetal plate is unclear. Second, the results of prolonged cytochalasin treatment mean that this drug cannot be used in an interpretable fashion (see above). Third, if active contraction of these cells is occurring, then one might expect that vegetal plate fragments would constrict autonomously. In one clear case of active apical constriction, bottle cell formation in *Xenopus*, such autonomous contraction occurs (Hardin and Keller, 1988). Thus far, such autonomous constriction has not been demonstrated in the vegetal plate. Finally, this model does not account well for exogastrulation, if one assumes that exogastrulation results from a process very similar to the normal situation, since this would require a reallocation of contractile elements to the basal, rather than apical, side of the cells.

Davidson *et al.* (1995) have recently performed mechanical simulations using three-dimensional finite element analysis to test the role of the hyaline layer, apical lamina, secreted proteoglycan, and the vegetal plate epithelium during primary invagination. The vegetal plate's behavior could be modeled to simulate

various postulated mechanisms of primary invagination, including (i) apical constriction of cells at its center, (ii) constriction of a ring of material at the periphery of the plate, (iii) lateral compressive forces acting at its edge, or (iv) passive indentation following swelling of secreted proteoglycan. Although the models do not rule out any particular hypothesis, they do suggest valid parameter ranges within which such models reasonably reproduce the observed behavior of the vegetal plate. In particular, for apical constriction or a ring constriction to produce an invagination in the models, the elastic modulii of the apical lamina and hyaline layer must be relatively low. In contrast, for proteoglycan swelling to produce an invagination in the models, these modulii are required to be rather high. Apical tractoring requires a stiff apical lamina but permits a range of values for the stiffness of the hyaline layer (Davidson *et al.*, 1995). Unfortunately, no data are currently available regarding the actual physical properties of these extracellular matrix layers.

None of the models proposed above mutually excludes the others, and it is quite possible that multiple mechanisms contribute to the initial invagination of the archenteron. Nothing is currently known about what, if any, motility cells employ in the vegetal plate as it invaginates. A detailed, dynamic cellular analysis of this process is sorely needed. In addition, confirmation of one or more of these models must await new, more specific experiments.

2. Elongation of the Archenteron

a. Cell Rearrangement Occurs during Elongation. The second phase of invagination requires a dramatic elongation of the archenteron. In comparison with primary invagination, less attention was focused on secondary invagination by early workers. Morgan (1985a,b) claimed that the internuclear distance between cells in the vegetal plate increased following its invagination, suggesting to him that the cells might be swelling, a suggestion reiterated by Spek (1917). As mentioned above, fate mapping and volumetric measurements indicate that some involution of material occurs during primary invagination (Ettensohn, 1984; Burke *et al.*, 1991). In contrast, similar measurements indicate that there is no additional involution during secondary invagination in *L. pictus* (Hardin, 1989). In addition, secondary invagination does not require mitosis in *L. pictus* (Stephens *et al.*, 1986), and cell counts indicate that the archenteron possesses the same number of cells at the beginning and end of secondary invagination (Hardin, 1989). Thus, the elongation of the archenteron must occur via changes within the cohort of cells it comprises.

The fate-mapping studies of the 64-cell embryo performed by Hörstadius (1935) provide clues as to possible mechanisms by which elongation occurs. Hörstadius found that veg_2 cells give rise to sectors of labeled cells in the archenteron; when examined early in gastrulation and then at the end of gastrulation, these sectors appear to become longer and narrower (Hörstadius, 1935;

Fig. 14). Such behavior is reminiscent of the ways in which labeled regions of the amphibian embryo deform during gastrulation, based on studies by Vogt and Keller (reviewed in Keller, 1986). In the amphibian, such "convergent extension" occurs via the rearrangement of cells in both the radial and mediolateral dimensions. Thus, cells from fluorescently labeled patches of tissue intercalate with unlabeled neighboring cells; as many cells change position in the *Xenopus* marginal zone, it elongates dramatically during involution (reviewed in Keller and Winklbauer, 1992; Keller *et al.*, 1992). Several studies indicate that cell rearrangement accounts for much of the elongation of the sea urchin archenteron as well.

Cell rearrangement was initially deduced by Ettensohn (1985), who observed that the number of cells in cross sections of archenterons decreases as gastrulation proceeds (Fig. 15a). The only way that this could be explained was by the rearrangement of the cells in the wall of the archenteron. Rearrangement occurs to differing extents in different species and correlates well with the total elongation required to complete gastrulation (Hardin and Cheng, 1986). More recent studies have employed labeled clones of cells introduced into the archenteron to show that, as in the case of amphibian embryos, neighboring cells intercalate between one another (Cameron *et al.*, 1987; Wray, 1987; Hardin, 1989). These studies also showed that the rearrangements are quite local; initially contiguous cells are only separated from one another by one to two cell diameters by the end of gastrulation (Hardin, 1989; Figs. 15c–15f). Because such rearrangement occurs simultaneously among many cells, however, the net effect is a dramatic reshaping of the tissue, such that initially contiguous sectors of the archenteron elongate into narrower strips as gastrulation proceeds. This is no longer surprising, since in many instances cell rearrangement has been shown to be a powerful means by which to reorganize tissues (Schoenwolf and Alvarez, 1992).

Remarkably, ultrastructural studies indicate that the archenteron maintains its integrity as an epithelium as cell rearrangement occurs; adherens and septate

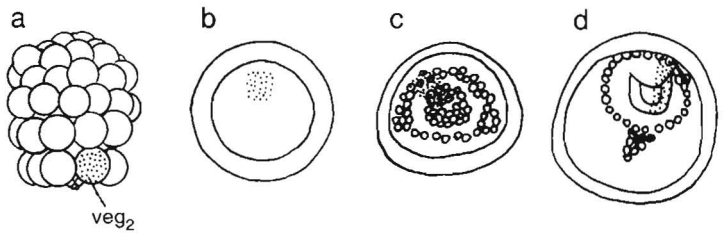


Fig. 14 Overall movements during invagination of the archenteron, based on the fate maps of Hörstadius (1936). Labeled veg_2 clones form rectangular patches near the vegetal pole in hatched blastulae (b), which subsequently elongate by the late gastrula stage (d). (b–d) Animal pole views. Adapted from Hörstadius (1936).

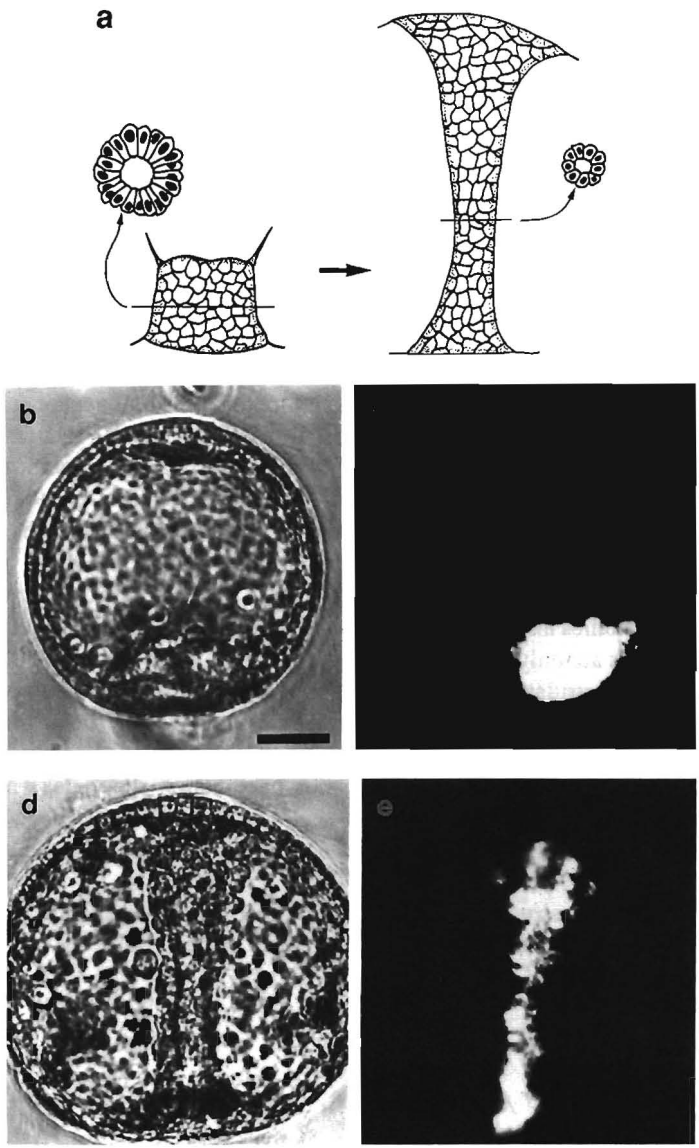


Fig. 15 Evidence for cell rearrangement within the archenteron. (a) Summary of experiments by Ettensohn (1985) and Hardin and Cheng (1986). The number of cells within a given cross section of the archenteron decreases as gastrulation proceeds. (b–e) Rhodamine-labeled clones of cells incorporated into the vegetal plate undergo elongation and intercalation with neighboring unlabeled clones (adapted from Hardin, 1989, with permission). Bar = 20 μm.

junctions are maintained throughout the rearrangement process (Spiegel and Howard, 1983; Ettensohn, 1985). Other epithelia known to undergo rearrangement exhibit a similar integrity; an unanswered question in all systems where epithelial rearrangement has been documented concerns how modulation of junctional components occurs as cells change position (Keller, 1987; Keller and Hardin, 1987; Fristrom, 1988; Schoenwolf and Alvarez, 1992).

In addition to cell rearrangement, cell shape changes occur in the archenteron. The wall of the archenteron thins during secondary invagination (Dan and Okazaki, 1956; Ettensohn, 1985; Hardin and Cheng, 1986; Hardin, 1989). Extensive morphometric analysis indicates that cells flatten in the radial dimension and spread in the other dimensions to accomplish this (Hardin, 1989): the inner and outer surface areas of the archenteron increase roughly twofold as the archenteron elongates in *L. pictus* (Hardin, 1989). These shape changes contribute significantly to the increase in length of the archenteron (Hardin, 1989). Flattening appears to occur to a greater or lesser extent in most, if not all, species of sea urchin (Dan and Inaba, 1968; Hardin and Cheng, 1986), but the contribution probably varies depending on the species (for example, considerably less flattening occurs in *L variegatus* than *L. pictus;* Morrill and Santos, 1985). Epithelial cell rearrangement is also accompanied by extensive flattening of the cells as they rearrange in other systems (examples include the superficial layer of *Xenopus* gastrulae, Keller, 1978; teleost epiboly, Keller and Trinkaus, 1987).

The realization that cell rearrangement occurs during sea urchin gastrulation says little about the forces that produce the rearrangement. There are two ways in which the archenteron could elongate: (1) via intrinsic, or *active* forces, i.e., the cells that rearrange are also the cells that produce the forces required for rearrangement; or (2) via externally applied forces, i.e., *passive* rearrangement. Several investigators have made the argument that the observed cell-shape changes within the epithelium should be different in these two cases (Keller and Hardin, 1987; Fristrom, 1988; Irvine and Wieschaus, 1994). Passively rearranging cells would be expected to elongate along the axis of extension of the tissue as they respond to the forces exerted on them, whereas actively rearranging cells would not show such elongation, or may even elongate perpendicular to the axis of extension as they intercalate. An example of the latter is the marginal zone of the *Xenopus* gastrula, where cells are markedly elongated along the mediolateral axis as the entire tissue converges and extends along the anteroposterior axis (Shih and Keller, 1992a,b). Likewise, rearranging epithelial cells in the *Drosophila* germ band do not show elongation (Irvine and Wieschaus, 1994). Based on the accumulated evidence, a combination of both active and passive cell rearrangement seems to account for elongation of the archenteron of the sea urchin embryo.

b. Archenteron Elongation Involves Autonomous Extension and SMC-Dependent Elongation. A striking feature of the latter part of gastrulation is the protrusive activity of secondary mesenchyme cells (SMCs) at the tip of the

archenteron. Forty years ago, Gustafson and co-workers (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960; reviewed by Gustafson and Wolpert, 1963a, 1967) and Dan and Okazaki (1956) independently proposed that tension exerted by these cells could suffice to explain the elongation of the archenteron. A number of observations indicate that filopodia extended by SMCs can exert appreciable tension. When the filopodia of SMCs attach to the ectoderm, they pull out what Gustafson and colleagues termed *cones of attachment*, distensions of the ectoderm and/or basal lamina where they attach (Gustafson, 1963; Fig. 16). Whole regions of the ectoderm deform in regions where SMCs attach in *Pseudocentrotus depressus*, apparently due to their concerted mechanical effects (Okazaki, 1956). In this species, the entire embryo visibly shortens along the animal–vegetal axis; this shape change was ascribed by Okazaki to the SMCs (Okazaki, 1956; Fig. 16). When individual filopodia detach from the ectoderm, the archenteron and the site of filopodial attachment often visibly recoil, indicating that individual protrusions generate substantial force (Gustafson and Kinnander, 1956; Dan and Okazaki, 1956). In sand dollar gastrulae, SMCs appear to rip out of the tip of the archenteron, or in some cases even appear to tear the distal portion of the archenteron away from the base (Dan and Okazaki, 1956), again suggesting that the combined mechanical effects of these cells can be substantial.

In addition to the apparent force-producing abilities of these cells, their protrusive activity can be correlated with extension of the gut rudiment, based on a number of observations in normal and experimentally perturbed embryos. There is a temporal correlation between continued elongation of the archenteron and the onset of filopodial activity by SMCs in most species (Gustafson and Kinnander, 1956; Kinnander and Gustafson, 1960). Moreover, the activities of individual SMCs can be correlated with changes in the rate of extension of the archenteron in *P. milaris*, based on "case histories" of individual embryos (Kinnander and Gustafson, 1960; see Fig. 10). General treatments that result in poor attachment of SMCs often result in impaired elongation of the archenteron as well. These include osmotic swelling of the blastocoel (Dan and Okazaki, 1956), treatment of embryos with seawater containing low concentrations of calcium (Okazaki, 1956), incubation of embryos in pancreatin (Dan and Okazaki, 1956), injection of proteases such as pronase or collagenase into the blastocoel (Spiegel and Burger, 1982), injection of concanavalin A into the blastocoel (Spiegel and Burger, 1982), and treatment of embryos with lithium chloride, which results in partial or complete exogastrulation, in which the archenteron evaginates, rather than invaginates (Wolpert and Gustafson, 1961). Taken together, these results provided evidence for an important role for SMCs in elongating the archenteron.

The "filopodial traction" model remained the most appealing explanation for archenteron elongation for many years. However, much of the experimental evidence supporting it was open to other interpretations. Trinkaus pointed out 30 years ago that this model has difficulty explaining several aspects of SMC behavior (Trinkaus, 1965). Based on his preliminary observations in *L. variegatus*,

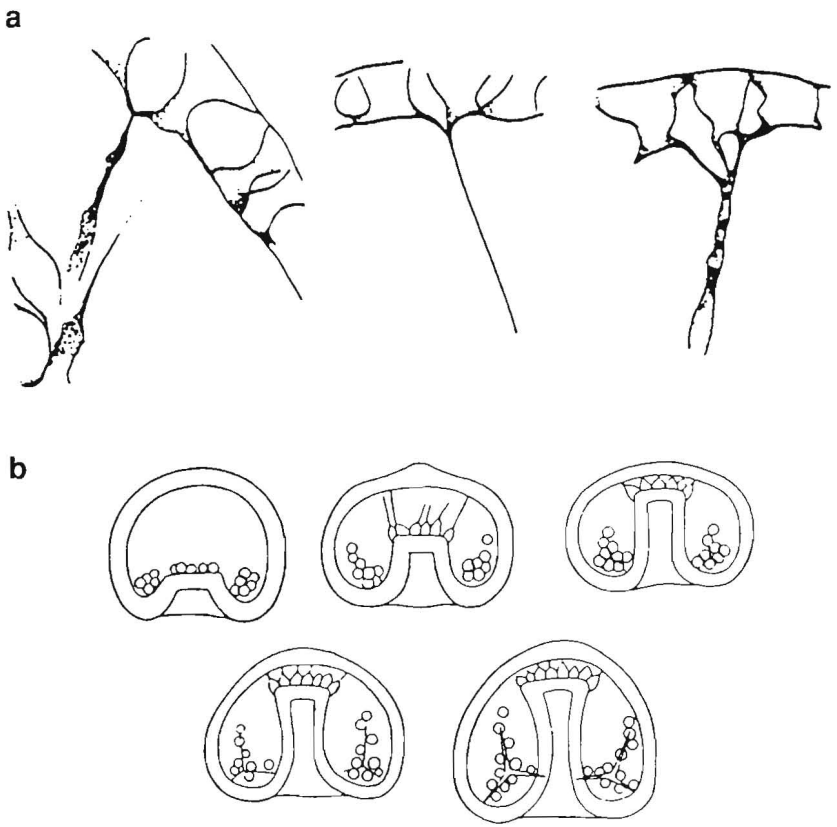


Fig. 16 Evidence for tension generated by filopodia during gastrulation. (a) Examples of "cones of attachment" produced by secondary mesenchyme cells as they attach to the ectoderm/basal lamina (from Gustafson, 1963 with permission). (b) Overall flattening of the embryo along the animal–vegetal axis during gastrulation in *Pseudocentrotus depressus* (from Okazaki, 1956, with permission).

Trinkaus reported that SMCs only appear to make successful attachments to the lateral ectoderm (also see Fig. 20); the force component generated by these cells along the animal–vegetal axis would be necessarily low at this time, yet the archenteron elongates at a roughly uniform rate throughout secondary invagination. In addition, in this species the total number of protrusions appears to be much lower at early stages of elongation, rather than later (Trinkaus, 1965; reviewed in Trinkaus, 1984; McClay *et al.*, 1991). Likewise, most of the experimental treatments used to disrupt attachment of SMCs are far from specific, such as low calcium, general proteases, or lectins, so the results of such experiments cannot be unambiguously ascribed to their effects on SMCs. Lithium chloride probably affects differentiation of cells in the embryo via the inositol triphosphate second messenger pathway; when high doses are used, it is quite clear that

wholesale shifts in differentiation occur; in particular, an overcommitment of cells to endoderm occurs (reviewed by Livingston and Wilt, 1990). Thus, it is difficult to interpret these affects solely based on alterations of SMC behavior.

More recent observations and experiments have clarified the cellular mechanisms of archenteron elongation. On the whole, these studies do support a role for SMCs, especially late in secondary invagination. However, they also indicate that active extension of the archenteron occurs early in secondary invagination. The result of the combination of these two processes is the complete elongation of the archenteron. Evidence for active extension of the archenteron comes from several observations and experiments. First, in at least two species, *L. variegatus* and *Eucidaris tribuloides*, filopodia only make lateral attachments at the onset of secondary invagination, confirming the impressions of Trinkaus (Schroeder, 1981; Hardin, 1989; Hardin and McClay, 1990; see Fig. 20a). It is only as the archenteron reaches roughly two-thirds of its final length that most filopodia contact the animal hemisphere in *L. variegatus* (Hardin and McClay, 1990). In *Eucidaris*, filopodia attach laterally throughout gastrulation, making it unlikely that they produce significant tension along the axis of elongation of the archenteron (Schroeder, 1981; Hardin, 1989). Second, substantial extension of the archenteron can occur in exogastrulae produced by several treatments, including lowered calcium (Dan and Okazaki, 1956) and low doses of lithium chloride (Hardin and Cheng, 1986; Hardin, 1988; see below, Fig. 24). In this case, SMCs remain at the tip of the archenteron and cannot exert tension on the archenteron, since there is no substratum available to them. Nevertheless, the archenterons of exogastrulae can elongate to two-thirds the length of normal gut rudiments (Hardin and Cheng, 1986; Hardin, 1988), and their walls thin as they do so (Dan and Okazaki, 1956; Hardin and Cheng, 1986).

The most convincing evidence that filopodial traction is not required during the early phase of elongation comes from laser ablation experiments (Hardin, 1988; Fig. 17). When all SMCs are ablated near the onset of archenteron elongation, the archenteron continues to elongate at a rate comparable to that in control embryos, but asymptotically slows and finally stops at the two-thirds gastrula stage (Hardin, 1988; Fig. 17). If all SMCs are ablated after the two-thirds gastrula stage elongation ceases, but if a few intact SMCs are allowed to remain, elongation continues at a slower rate directly related to the number of intact cells (Hardin, 1988; Fig. 17). The laser ablation results indicate that prior to the two-thirds gastrula stage the archenteron is capable of autonomous extension. However, autonomous extension is insufficient to completely elongate the archenteron; SMCs are required late in gastrulation for its successful completion. Observations of many different species suggest that archenteron elongation involves a combination of both active extension and continued elongation in response to filopodial traction, and that the mixture of these two processes varies depending on the distance the tip of the archenteron must travel (Hardin and Cheng, 1986).

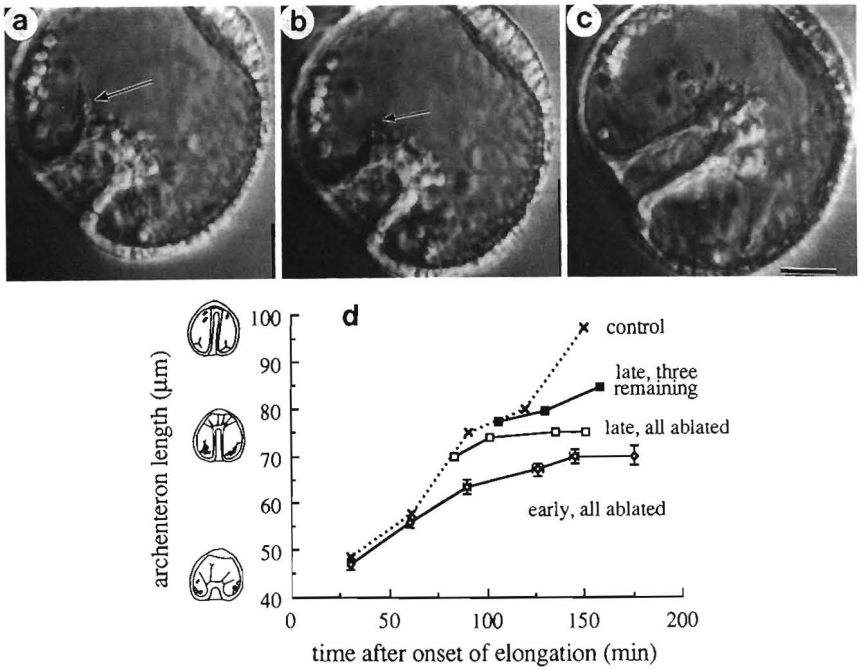


Fig. 17 Laser ablation of SMCs in *L. pictus*. (a) Midgastrula just prior to ablation (arrow denotes an active SMC). (b) Same embryo after ablation of all SMCs (the same SMC has now retracted its protrusion). (c) Two hours later, the archenteron has elongated, but has ceased elongation at two-thirds of its normal, final length. (d) Rate of archenteron elongation in normal and laser-irradiated embryos in which all or most of the SMCs were ablated. From Hardin (1988), with permission.

c. Mechanisms of Active Cell Rearrangement Early in Secondary Invagination. Cell rearrangement can be directly observed in the cidaroid urchin, *Eucidaris tribuloides*. Filopodia extended by SMCs are few in number well into gastrulation in this species (Schroeder, 1981); additionally, they *never* acquire an upward orientation (Hardin, 1989). Thus, gastrulation in *Eucidaris* provides a "natural experiment" by which to examine the relative mechanical importance of filopodia to archenteron elongation. The increase in length of the archenteron during gastrulation in *Eucidaris* corresponds very closely to the extent of rearrangement that has occurred. Indeed, as gastrulation proceeds in *Eucidaris*, the cells of the archenteron as a whole become more elongated in a direction *perpendicular* to its axis of extension, in marked contrast to the situation in *L. pictus* (Hardin, 1989; Fig. 18). As the cells of the archenteron rearrange, they undergo vigorous motile activity. Cortical bleb-like protrusions are continually sent out from the basal surfaces of endoderm cells; they rotate part of the way around the basal periphery of the cell and then disappear, with a lifetime of approximately

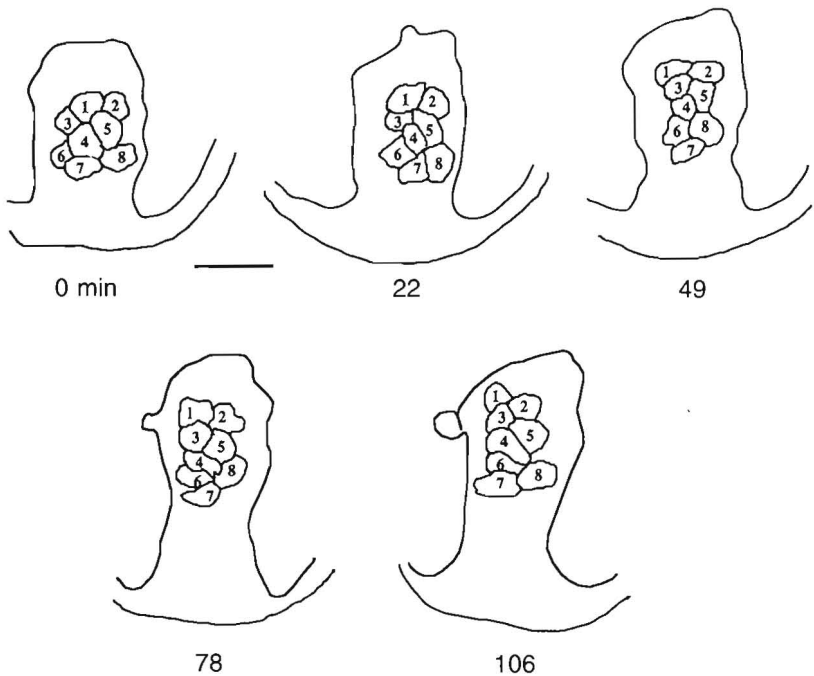


Fig. 18 Motility during active extension of the archenteron in *Eucidaris tribuloides*, followed via time-lapse videomicroscopy. An array of eight cells was followed as cells changed position to narrow and lengthen the array (from Hardin, 1989, with permission).

30 sec. When the blebbing behavior of an individual cell known to be undergoing intercalation is followed, its basal surface appears to continually "squeeze" up and down and back and forth with no apparent temporal periodicity (Hardin, 1989). However, given the optical limitations of these experiments, the presence of any fine protrusions could not be resolved.

In euechinoids, where cell rearrangement cannot be easily observed with DIC microscopy, little is known about the dynamics of rearrangement. However, scanning electron microscopy indicates that striking, stage-specific changes in the shape and protrusive activity of the basal surfaces of cells in the wall of the archenteron occur as its cells rearrange. Prior to the onset of secondary invagination, the basal surfaces of endoderm cells are rounded. Just prior to cell rearrangement, the basal surfaces of the cells in the wall of the gut rudiment undergo a dramatic change not visible in the conventional light microscope: long, highly oriented lamellipodial protrusions are extended by each cell toward the animal pole (Ettensohn, 1984a; Hardin, 1989; Fig. 19). The lamellipodia overlap one another, giving the archenteron a "shingled" appearance. They end in shorter,

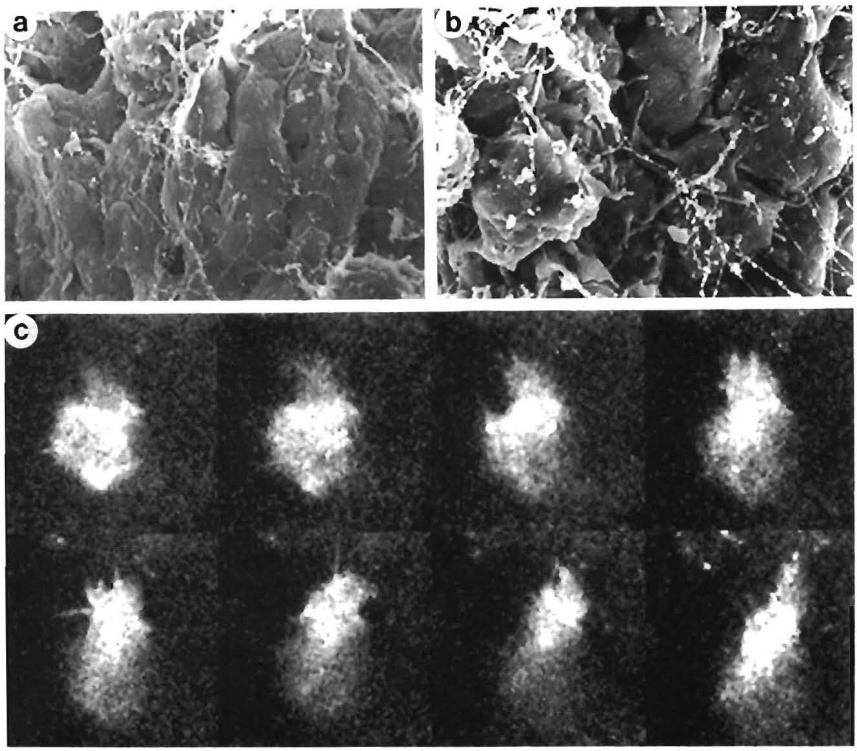


Fig. 19 Protrusive activity at the outset of archenteron elongation. (a) Scanning electron micrograph of the basal surfaces of cells in the wall of the archenteron of *L. pictus* just prior to elongation. (b) Basal protrusions in a slightly older embryo (a and b adapted from Hardin, 1989, with permission). (c) Basal view of a DiI-labeled endoderm cell just prior to archenteron elongation, viewed with a laser scanning confocal microscope. Note the broad lamellipodial protrusion and multiple fine filopodial protrusions.

filopodial protrusions that extend onto the basal surfaces of cells in overlying tiers, generally at an angle to the long axis of the archenteron (Hardin, 1989).

More recently, computer-assisted and laser scanning confocal microscopy of cells labeled with the lipophilic dye $DiIC_{16}$ have allowed additional documentation of the basolateral motility displayed by the rearranging cells (Laxson and Hardin, 1994; Fig. 19). As active rearrangement begins, the basal surfaces of cells in the archenteron extend numerous lamellipodial protrusions. In this sense, these protrusions resemble the basal "feet" transiently present on the basal surfaces of rearranging epithelial cells in insects during pupation (Locke and Huie, 1981; Nardi and Magee-Adams, 1986). How such motility is integrated to produce the directional rearrangement of the archenteron is unknown.

d. Mechanical Deformation of the Archenteron late in Secondary Invagination. As mentioned above, several lines of evidence indicate that SMCs can exert considerable tension during secondary invagination. Indeed, the thinning of the walls of the archenteron that occurs late in gastrulation was accounted for by Okazaki and colleagues (Okazaki, 1956; Dan and Okazaki, 1956) and by Gustafson and co-workers (Gustafson and Kinnander, 1956; Gustafson and Wolpert, 1963) largely by filopodial traction. More recently, morphometric measurements (Hardin, 1989; Hardin and Cheng, 1986) and mechanical simulations (Hardin and Cheng, 1986) have examined the possible mechanical effects of filopodial traction on the archenteron in greater detail.

Mechanical simulations of filopodial traction by Hardin and Cheng (1986) suggest that if filopodial traction is significant during archenteron elongation, then several observable changes in the shape of the embryo should take place, assuming that the embryo behaves visoelastically. First, the embryo should flatten along the animal–vegetal axis in response to the combined activity of SMCs. Second, in extreme cases, the animal pole ectoderm should be deflected due to filopodial traction. Third, the applied stress should widen the blastopore as secondary invagination proceeds. A broad survey of the shapes of gastrulae of different species reveals that in some cases precisely this sort of deformation is observed. For example, gastrulation in *Pseudocentrotus depressus* is accompanied by flattening of the embryo along the animal–vegetal axis and denting of large regions of the animal pole ectoderm, apparently in response to filopodial traction (Okazaki, 1956; see Fig. 16). Similar, though less dramatic, changes in the shape of *S. purpuratus* embryos have also been observed (Hardin and Cheng, 1986). However, such deformation is not observed in other species, such as *L. pictus* (Ettensohn, 1985; Hardin and Cheng, 1986). This difference could be due to a greater contribution in some species by active extension of the archenteron. An additional possibility is that the archenteron has significantly different mechanical properties than the ectoderm (Hardin and Cheng, 1986).

Despite the lack of overall deformation of *L. pictus* embryos during secondary invagination, there is evidence that the archenteron experiences considerable tension due to filopodial traction. Prior to the two-thirds gastrula stage, filopodial attachments of SMCs are predominantly lateral in embryos of the genus *Lytechinus* (Trinkaus, 1965; Hardin and McClay, 1990; Fig. 20). By the two-thirds gastrula stage, at the time that SMCs begin making attachments to the animal hemisphere, the cells of the archenteron wall of *L. pictus* begin to elongate along the animal–vegetal axis, and elongation continues through the three-fourths gastrula stage (Hardin, 1989). In the narrowest region of the archenteron, where tension appears to be the highest, cells are noticeably stretched along the axis of the archenteron (Hardin, 1989; Fig. 20). If SMCs are ablated with a laser microbeam, the cells in the wall of the archenteron rapidly shorten as the tension is relieved (J. Hardin, unpublished observations). The elongation is transient, however, as additional cell rearrangement appears to relieve the stresses within the archenteron (Hardin, 1989). That tension within the archenteron is high at the

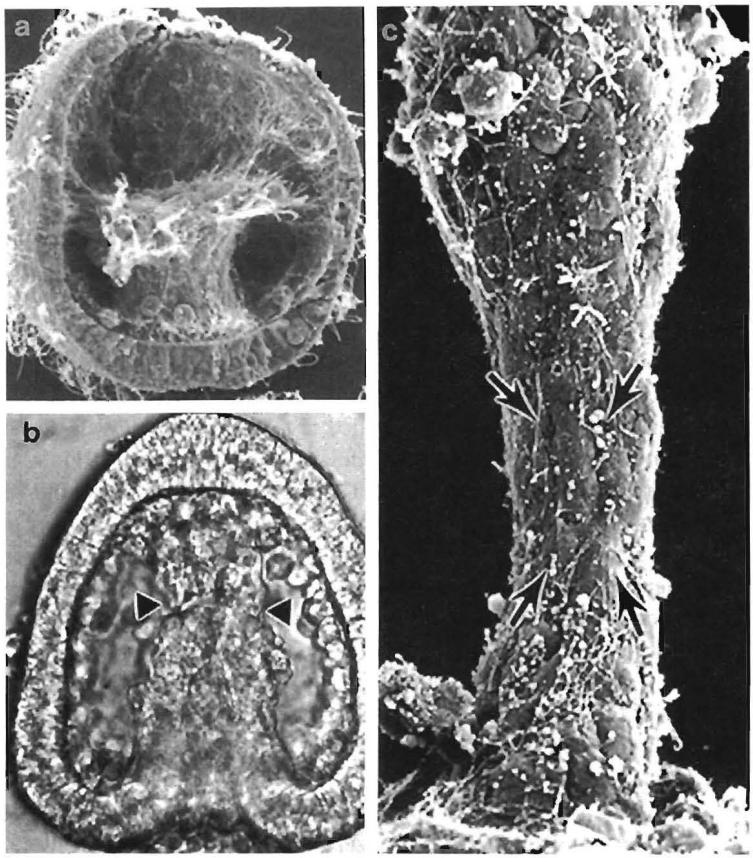


Fig. 20 Tension generation in the archenteron late in gastrulation. (a) Scanning electron micrograph of a *L. variegatus* midgastrula. Note that successfully attached filopodia have an exclusively lateral orientation (micrograph courtesy of J. Morrill). (b) *Melitta quiesquesperforata* gastrula. The distal portion of the archenteron has ripped loose from the remainder of the archenteron (arrowheads; J. Hardin, unpublished). (c) SEM of a *L. pictus* archenteron at the end of gastrulation. Note the narrowing of the archenteron in middle regions; in addition, the narrowest portion of the archenteron contains highly elongated cells (arrows). From Hardin (1989), with permission.

time is underscored by the tendency of the distal (animal) portion of the archenteron to rip loose from the base of the gut rudiment in sand dollar gastrulae (Dan and Okazaki, 1956; Fig. 20). Taken together, these observations suggest that tension within the archenteron may be largely responsible for the cell rearrangement seen late in gastrulation.

A cellular understanding of how cells in the archenteron rearrange when under axial stress is currently lacking. However, if such "passive rearrangement" does occur at this time, it is probably not a situation unique to sea urchins. In several

other situations in which extrinsic forces are thought to result in epithelial cell rearrangement, the rearranging cells are also elongated (reviewed in Keller and Hardin, 1987; Fristrom, 1988). In these cases, the tissue sheet is presumably deforming much like the plastic deformation of a polymer in response to stress in excess of the yield stress of the material. Unlike a polymer, however, in the case of biological tissue, the smallest mechanical element that can change position is the cell.

Recently, computer simulations have been performed by Weliky and Oster (1990) to simulate the behavior of an array of cells in which cell junctions are allowed to shift position under appropriate circumstances. In their models, cells are connected to neighbors along nodal boundaries (i.e., cell junctions), but these junctions are capable of remodeling, particularly when the stress on a given cell–cell connection is too high. When the stress exceeds a defined level, cells are free to rearrange. Their model has been applied to teleost epiboly (Weliky and Oster, 1990) and notochord formation in amphibians (Weliky *et al.*, 1991). When their model is applied to the archenteron of the sea urchin embryo, the resulting changes in position of cells, and their changes in overall shape, closely approximate what is actually observed in *L. pictus* (Fig. 21). Although a role for passive rearrangement late in sea urchin gastrulation is plausible, additional experiments need to be performed to investigate this possibility further. For example, stretching and necking of the archenteron should be abolished following laser ablation of SMCs. It may also be possible to ablate SMCs and then artificially distend the archenteron via micromanipulation or magnetic particles using techniques similar to those employed by Hiramoto in sea urchin eggs (discussed in Hiramoto, 1987).

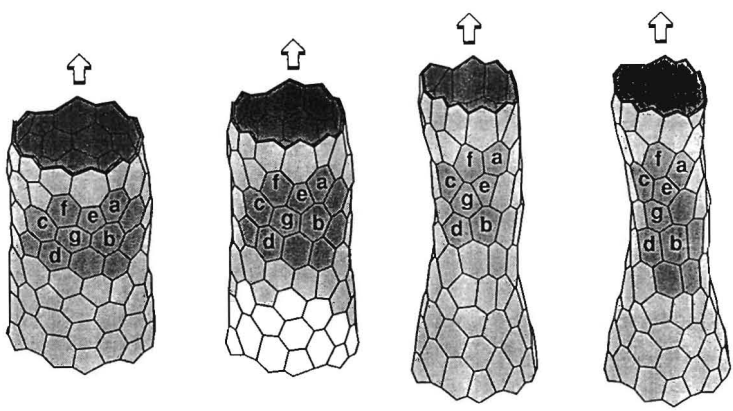


Fig. 21 Mechanical simulations by Weliky. When a model epithelial tube capable of undergoing cell rearrangement is pulled on from one end (arrows) and the other end if anchored in place, cell stretching and rearrangement occurs. Note that by the end of the simulation an array of 7 cells (labeled a–g) has rearranged and each cell has elongated. Adapted from Oster and Weliky (1990), with permission.

e. Blastopore Closure during Secondary Invagination. In addition to the cell rearrangement that occurs in the archenteron during secondary invagination, there is epithelial cell rearrangement at the blastopore. A plot of blastopore diameter vs archenteron length for *L. pictus* gastrulae at various stages of invagination clearly shows that blastopore closure occurs in two phases. During primary invagination, blastopore closure occurs at a greater rate than during secondary invagination. The blastopore decreases in diameter by ~35% during secondary invagination; this decrease is closely correlated with archenteron length. Scanning electron microscopy reveals that blastopore closure occurs by rearrangement of cells at the blastopore lip (Hardin, 1989; Fig. 22). Using the DiI technique, rearranging cells at the blastopore has been shown to extend numerous lateral lamellipodial protrusions; in many cases, these appear to be oriented toward the blastopore (Laxson and Hardin, 1994). The protrusions originate on the basolateral surfaces of the cells, and extend outward (apically) until they contribute additional material to the apical surface of the cell (Laxson and Hardin, 1994; Fig. 22). Thus, like the cells of the archenteron, these cells are "hybrid" sorts of cells, maintaining epithelial integrity while they change position during gastrulation.

f. Unanswered Questions about Cell Rearrangement in the Archenteron. Our current understanding of archenteron elongation in the sea urchin embryo is summarized in Fig. 23. While this model is consistent with the available data, many unanswered questions remain. First, the cellular basis of epithelial cell rearrangement is still unknown. The observed motility of cells within the archenteron and at the blastopore appears similar to that envisioned by the cortical tractor model originally proposed by Jacobson *et al.* (1986) to account for cell rearrangement within the neural plate during urodele neurulation. These authors propose that such basal blebs could intercalate between cells at their basolateral margins and ultimately cause the rearrangement of cells via the upward (i.e., basal-to-apical) rotation of the protrusions (see also a modified version of this model proposed by Fristrom, 1988). The cells in the archenteron display an impressive capacity for motility of this sort, and if the success of intercalating protrusions is somehow biased in the circumferential direction, then such motility may be a sufficient "motor" by which to drive cell rearrangement. However, the location of the protrusions observed thus far does not appear to be fully consistent with such models, since protrusive behavior, rather than occurring normal to the axis of extension of the tissue, occurs *parallel* to it. This is precisely opposite from the situation in *Xenopus*, where deep cells directly in contact with the superficial epithelium are strongly polarized normal to the overall axis of extensions of the marginal zone (Shih and Keller, 1992a,b). Similar morphology is seen during development of the notochord in chordates (Miyamoto and Crowther, 1985; Keller *et al.*, 1985, 1989; Thorogood and Wood, 1987) and in the dorsal hypodermis of *C. elegans* (Sulston *et al.*, 1983; Williams-Masson and Hardin, 1994). In the case of *Xenopus*, cells are thought to

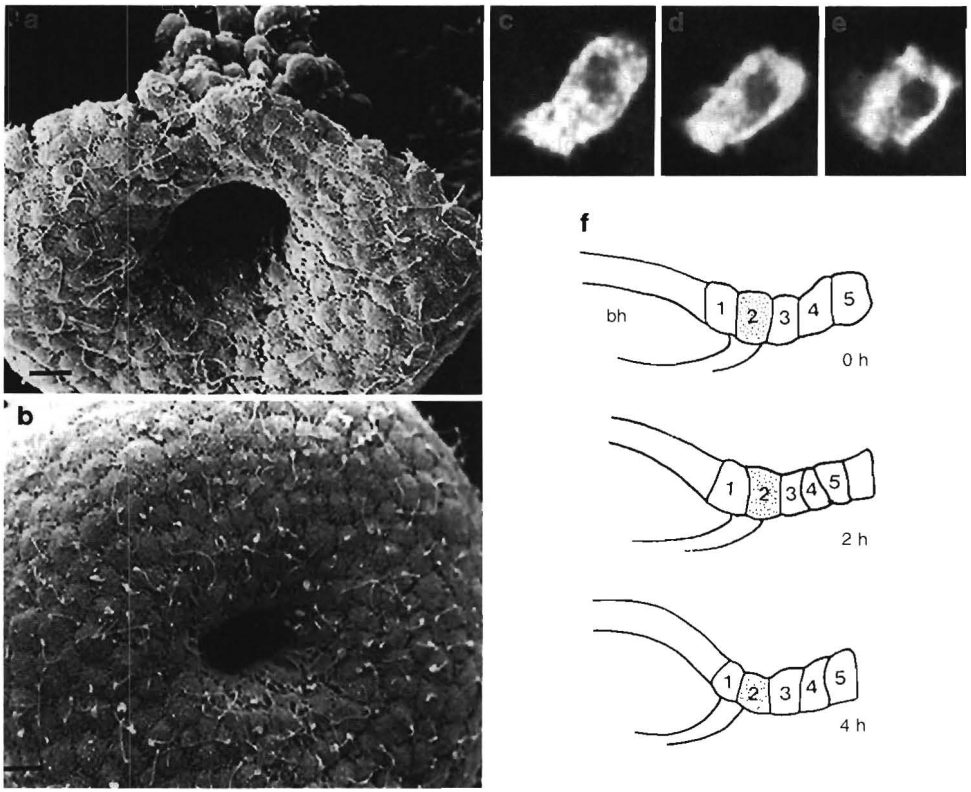


Fig. 22 Cell rearrangement at the blastopore in *L. pictus*. SEMs of the blastopores of embryos at the outset (a) and end (b) of secondary invagination (from Hardin, 1989, with permission). (c) DiI-labeled blastopore cell followed at 15-min intervals using confocal microscopy (E. Laxson and J. Hardin, unpublished). (d) Involution during secondary invagination, followed using videomicroscopy (from Hardin, 1989, with permission).

intercalate normal to the overall axis of extension via fine protrusions at their mediolateral margins (Keller *et al.*, 1992).

Second, how the general motility of epithelial cells is biased to produce intercalation is not clear. One suggested mechanism for producing directionality is *adhesive disparities* between the cells. For example, Jacobson *et al.* (1986) have suggested that cells in the amphibian neural plate rearrange to maximize their contact with the neural plate/epidermis boundary, with the result that elongation of the neural plate occurs along the anterior/posterior axis. Mittenthal and Mazo (1983) have suggested that adhesive disparities could account for the pattern of cell rearrangement seen in cylindrical epithelial structures by minimizing the boundary between originally concentric zones of cells with adhesive dispari-

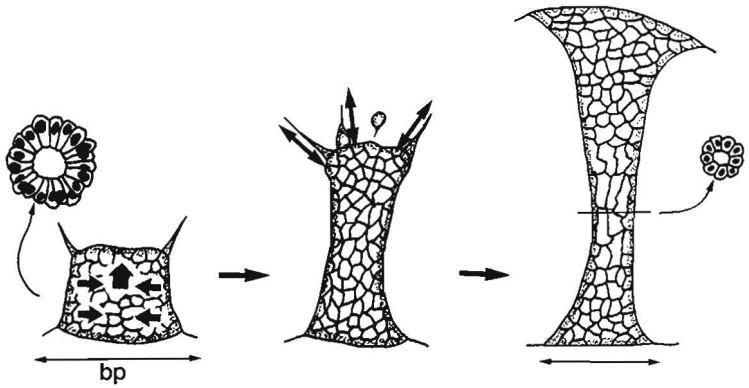


Fig. 23 Model of gastrulation in *L. pictus*. During the first phase of archenteron elongation, active rearrangement of epithelial cells results in an approximate doubling of the length of the archenteron. In the later stages of gastrulation, tension within the archenteron, generated by the combined effects of numerous SMCs, results in the complete elongation of the archenteron, accompanied by additional cell rearrangement and cell stretching.

ties; they postulate that a flat disc will be converted into a cylinder with the originally concentric regions lying in a proximal–distal sequence along the averted disc. A similar suggestion has recently been made regarding germ band extension in *Drosophila* (Wieschaus *et al.*, 1991; Irvine and Wieschaus, 1994). Late in sea urchin gastrulation the regions of the archenteron that will differentiate into the three compartments of the larval gut express spatial localized cell surface proteins (McClay *et al.*, 1983; Wessell and McClay, 1985), and if full archenteron elongation is prevented these markers are expressed in a compressed, concentric pattern. Although these results imply that there are concentric zones of differentiation within the archenteron, it is not known what role, if any, these regional differences might play in gastrulation.

g. Pharmacological Approaches to Studying Motility in the Archenteron. As a result of their extensive time-lapse studies, Gustafson and colleagues sought a biochemical basis for the changes in motility that occur during sea urchin gastrulation. Since muscle cells had been well studied, they chose to study substances that elicit muscular contractions in adult organisms. Augustinsson and Gustafson (1949) first demonstrated cholinesterase activity in sea urchin embryos, and Buznikov *et al.* (1964) found that levels of serotonin were elevated just prior to the onset of morphogenetic movements. Inhibitors of serotonin and acetylcholine biosynthesis inhibit mesenchyme migration and invagination of the archenteron in *P. miliaris* (Gustafson, 1969; Gustafson and Toneby, 1970). However, given the general nature of these treatments, it is difficult to draw specific conclusions about what processes are being affected in treated embryos.

3. Exogastrulation and the Normal Process of Invagination

The artificial induction of exogastrulation in sea urchin embryos was first reported by Herbst (e.g., 1892) and Driesch (1893). Using the vegetalizing agent LiCl, Herbst found that the critical period of sensitivity extended from fertilization through the blastula stage; at later stages it seems to exert toxic effects (Bäckström and Gustafson, 1954; refinements to the sensitive period are reviewed in Livingston and Wilt, 1990). Herbst (1892) found that a range of morphological abnormalities could result from this treatment, and he classified them into two types: those involving a simple redirection of the archenteron to produce a normally proportioned exogastrula, and those which produced an abnormally large amount of endodermal tissue. In the first type, the location and formation of the skeleton is essentially normal, and the gut rudiment differentiaties into three segments of normal size (Herbst, 1893; Herbst termed this *blosse Exogastrulation*, "simple exogastrulation"). In the second type, an extremely large endodermal structure was formed, with one or more large constrictions, which later came to be known as *vegetalized*. Vegetalized larvae have been shown to contain many more cells expressing endodermal marker proteins, including Endo 16 (Nocente-McGrath *et al.*, 1991; Ransick *et al.*, 1993) and Endo 1 (Hardin, unpublished observations), than normal embryos. However, the formation of septate junctional components occurs on a normal time course in such embryos (Spiegel and Howard, 1985).

Driesch (1893) was the first to show that simple exogastrulation did not depend on a specific effect of lithium ion on the embryo. He induced exogastrulation in *Sphaerechinus granularis* by transient heating from hatching through the early gastrula stage. Since then, many agents have been shown to induce exogastrulation. These include low-calcium seawater (Dan and Okazaki, 1956; Okazaki, 1956), sodium azide (Child, 1948), various dyes (Lallier, 1964), chloramphenicol (Hörstadius, 1973; Fujiwara and Yasumasu, 1974), inhibitors of cyclic adenosine monophosphate (cAMP) diesterase (Yoshimi and Yasumasu, 1978, 1979), and blastocoelic fluid components (Berg, 1972) and substances isolated from embryo homogenates (Suyemitsu *et al.*, 1989). Most of these agents affect very general processes, suggesting that the directionality of invagination is easily altered, but that the process of extension is not. This notion is supported by reports of the isolation of exogastrulation mutants, in which there is a tendency for the larvae to exogastrulate spontaneously at high frequency (Hinegardner, 1975). Such embryos appeared to be abnormally elongated along the animal–vegetal axis, and the vegetal plate tended to be curved outward prior to gastrulation, suggesting that an initial bias in the shape of the vegetal plate may influence the direction of the invagination (Hinegardner, 1975).

As mentioned above, exogastrulated archenterons are capable of limited extension, and their cells undergo many of the shape changes that occur in normal archenterons (see Fig. 24). Although this is the case, it is far from clear how

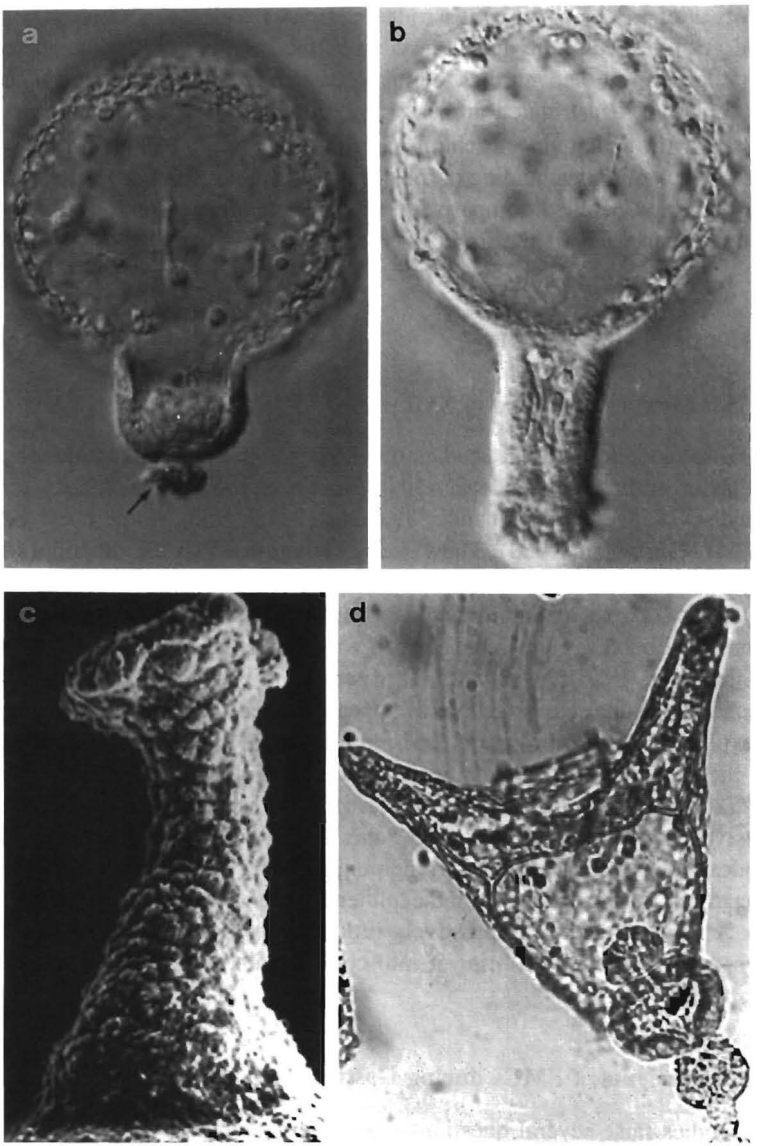


Fig. 24 Exogastrulation in *L. pictus*. (a) Midgastrula equivalent. (b) Late gastrula equivalent. (c) SEM of completely elongated, exogastrulated archenteron (J. Hardin, unpublished). (d) Exogastrulated pluteus. (a, b, d) Adapted from Hardin and Cheng (1986), with permission.

the process of extension in exogastrulae compares with the normal process. In everted gut rudiments, the apical surfaces of the cells are on the outside of the tube, rather than in their normal luminal location. However, cell rearrangement does occur in exogastrulated archenterons, although to a reduced extent commensurate with their reduced length (Hardin and Cheng, 1986; Hardin, 1988). Given our lack of understanding of the normal process of cell rearrangement, it is difficult to draw detailed comparisons between invaginated and evaginated gut rudiments; such analysis awaits further study.

D. Secondary Mesenchyme

1. The Motile Repertoire of SMCs

Time-lapse cinémicrographic studies by Gustafson and co-workers documented the behavior of these cells extensively in *Psammechinus miliaris* (Gustafson and Kinnander, 1956, 1960; Kinnander and Gustafson, 1960; Wolpert and Gustafson, 1961; Gustafson, 1963). These studies suggested that the filopodia of secondary mesenchyme cells "randomly" explore the blastocoel, undergoing continual cycles of extension, attempted attachment, and retraction (Fig. 25). The lifetimes of filopodia were found to vary widely, from 5 min to 2 hr (Gustafson and Kinnander, 1956). Eventually, as gastrulation ends, the cells at the tip of the archenteron undergo a change in appearance and behavior, largely ceasing their exploratory behavior, and often becoming more loosely associated with the tip of the archenteron (Dan and Inaba, 1968; Gustafson and Kinnander, 1960; Fig. 25). Finally, several hours after gastrulation is completed in *P. miliaris*, protrusively active cells at the tip of the archenteron make contact with the stomodeal invagination, apparently helping to bring it in contact with the oral ectoderm to form the mouth of the pluteus larva (Gustafson and Kinnander, 1960). In other species, comparatively little movement of the tip of the archenteron appears necessary to bring it into contact with the stomodeum (Dan and Inaba, 1968).

2. Attachments of SMCs during Gastrulation

These studies raise several questions regarding archenteron morphogenesis and the successful completion of gastrulation. First, how does the tip of the archenteron *localize* to the animal pole region at the end of gastrulation? Gustafson favored physical factors, including (1) differences in surface topography at contact sites between epithelial cells (Gustafson, 1963), (2) the proximity of available attachment sites (Gustafson and Wolpert, 1967; Gustafson, 1969), and (3) tissue curvature, which might concentrate nonspecific attachment sites for filopodia in some regions but not others (Gustafson, 1963; reviewed by Gustafson

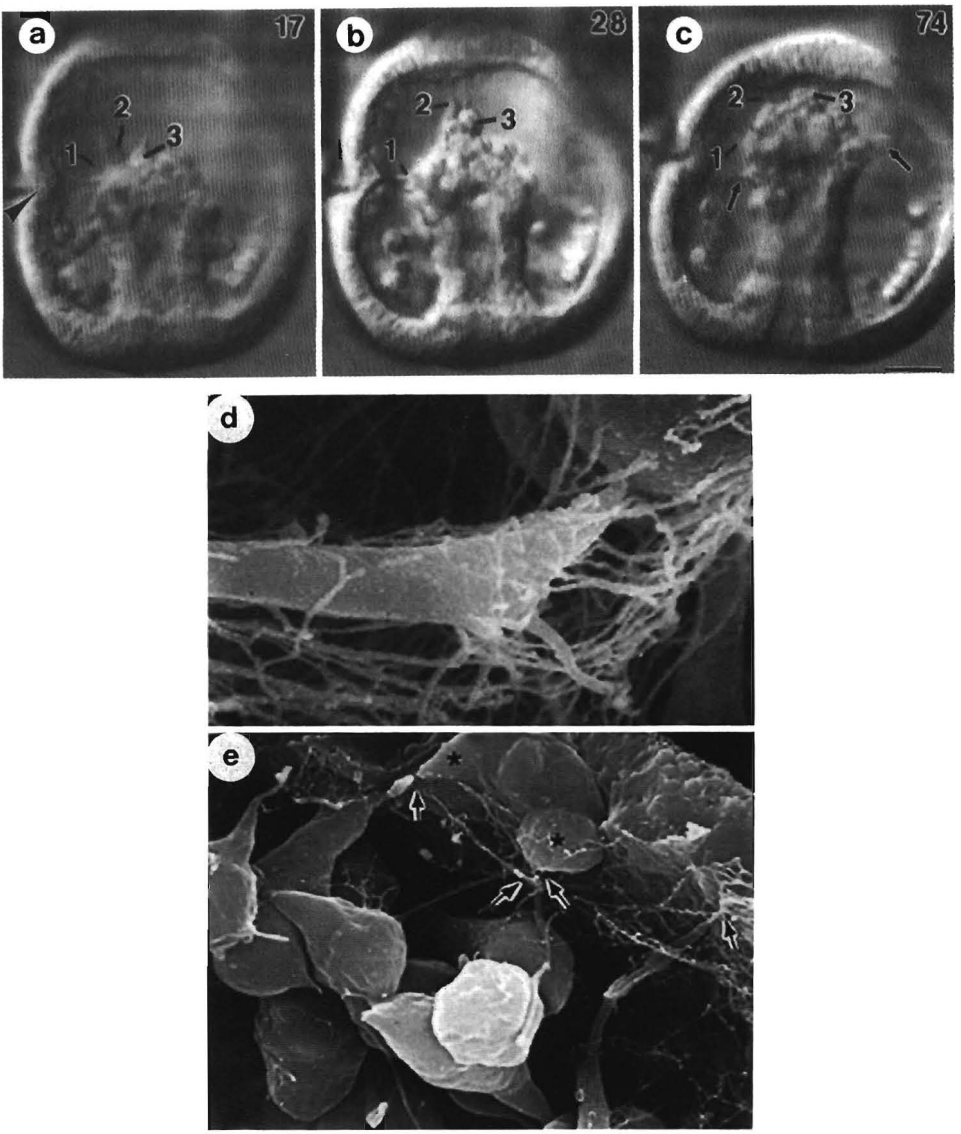


Fig. 25 Behavior of secondary mesenchyme cells as gastrulation proceeds in *L. variegatus*. (a–c) Time-lapse footage of SMC activity (times shown in minutes). Individual protrusions can be followed over extensive periods of time (e.g., cells labeled 1, 2, 3) until they make permanent contact with the animal pole region (c). (d) SEM of a filopodial protrusion in *L. variegatus*. Note the numerous extracellular matrix fibers (courtesy of J. Morrill). (e) High-resolution SEM of "cones of attachment" in *L. variegatus* (asterisks). Individual filopodial attachment denoted by arrows.

and Wolpert, 1967). In addition, Gustafson suggested that the ventral ectoderm might be more adhesive than the dorsal ectoderm; such adhesiveness might then account for localization of both primary and secondary mesenchyme cells (Gustafson, 1969). In several Japanese species (Dan and Inaba, 1968) and in *L. variegatus* (Trinkaus, 1965; reviewed in Trinkaus, 1984), filopodia initially extend laterally, and only late in gastrulation do they extend upward. This suggests that although the essential motile program of SMCs involves continual cycles of random filopodial extension, attachment, and eventual withdrawal, the pattern of attachments is modulated by the embryonic environment in significant ways. Specifically, is there a "target" near the animal pole for SMCs, i.e., a specific region which elicits a suite of stereotypical responses from these cells that results in attachment to the future oral region?

a. An Animal Pole "Target" Exists for SMCs. The attachment of SMCs to the animal pole region has been reexamined recently in detail (Hardin and McClay, 1990; Fig. 26). At the end of gastrulation, the tip of the archenteron makes contact with the ectoderm near a thickened region of epithelium, the apical plate. At this time, the exploratory behavior of the filopodia subsides. Real-time recording of residence times of attached filopodia indicates that protrusions in contact with the ectoderm near the apical plate region remain attached 20–50 times longer than filopodia attached at any other site along the blastocoel wall (Hardin and McClay, 1990; Fig. 26). The SMCs that attach to the animal pole region eventually change their behavior, flattening and spreading onto it. In some species, such as *L. variegatus*, this region lies near the animal pole; in other species, such as *S. purpuratus*, it is located on the ventral side of the animal hemisphere (Hardin and McClay, 1990).

The normal behavior of SMCs suggests that the animal pole region is a target for filopodial attachment, and several experiments indicate that SMCs respond uniquely to this region. First, when the animal pole region is pushed toward the tip of the archenteron with a micropipette or when embryos are trapped in Nitex mesh of the appropriate dimensions, SMCs interact with the animal pole earlier than they would normally do so, and their exploratory behavior ceases ahead of schedule. In some experiments, SMCs make precocious, stable attachments to the animal pole (Hardin and McClay, 1990; Fig. 26). SMCs make transient contacts with other areas of the blastocoel wall when they are indented, but the archenteron continues past such indentations, eventually attaching to the usual site. Second, when contact of SMCs with the animal pole is prevented by extruding embryos into capillary tubing so that SMCs cannot reach the animal pole, SMCs continue filopodial extension for abnormally long periods of time (Hardin and McClay, 1990; Fig. 26). If the archenteron is prevented from reaching the animal pole for several hours, some SMCs detach from the archenteron; those that migrate in the vicinity of the animal pole undergo the change in behavior seen in normal embryos, and they collect at the animal pole (Hardin and

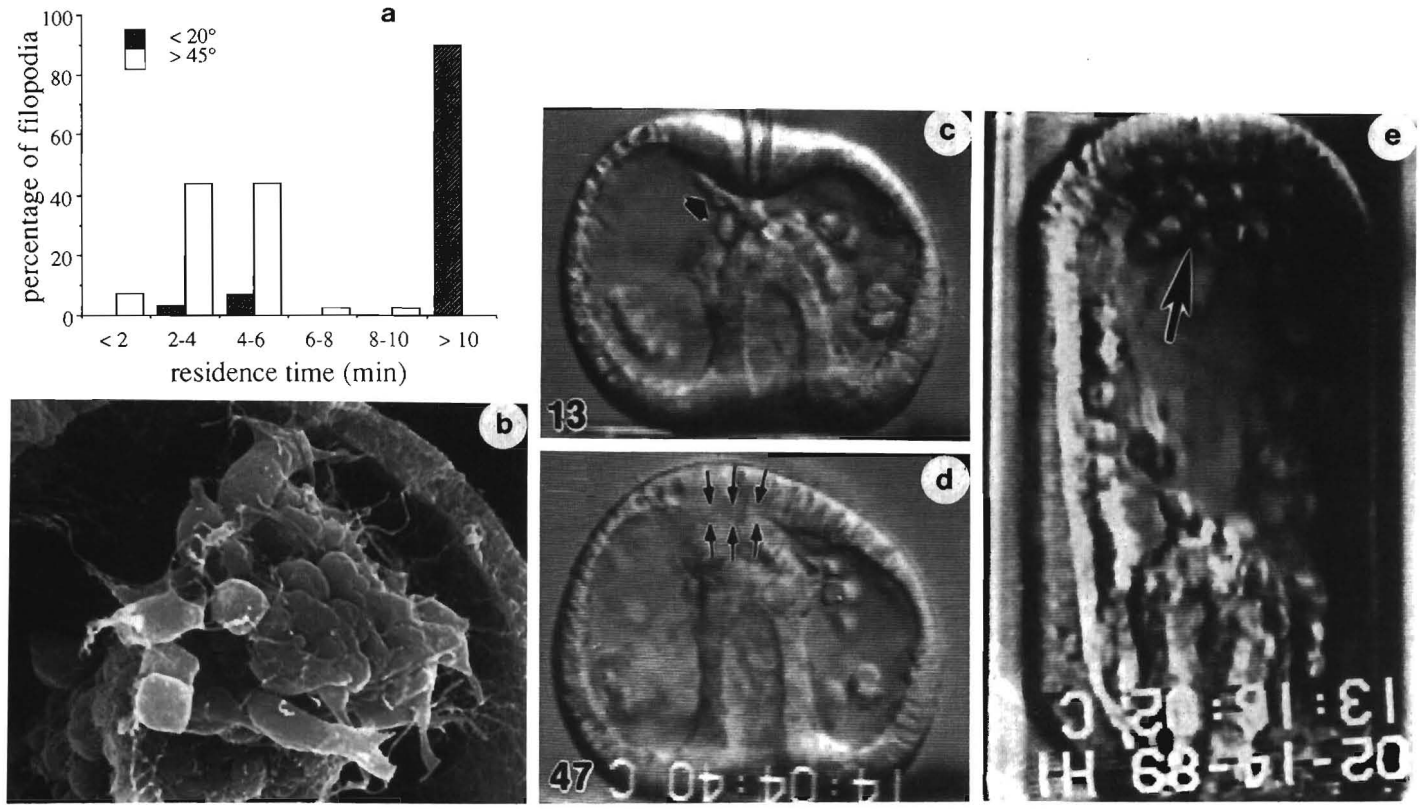


Fig. 26 Alterations in the behavior of SMCs following interaction with the animal pole "target" region in *L. variegatus*. (a) Distribution of filopodial attachment times in lateral (gray) and animal pole (hatched) regions. (b) Morphology of attached SMCs (the animal pole has been removed; J. Hardin, unpublished). (c, d) Precocious attachment of the archenteron following indentation of the blastocoel roof with a micropipet. (e) Prevention of attachment by elongation of an embryo in capillary tubing. Eventually, some SMCs detach from the tip of the archenteron and congregate near the animal pole (arrow). (a–d) From Hardin and McClay (1990), with permission.

McClay, 1990). Thus, SMCs appear to be programmed to continue extending filopodia until the appropriate target is reached on the wall of the blastocoel. Finally, when ectopic combinations of animal pole ectoderm and archenterons are created by producing fused multiple embryos, archenterons attach to the nearest available apical plate region (Hardin and McClay, 1990).

b. Spatial Extent of the Target. Based on time-lapse videomicrography, the target region is quite restricted in size and appears to be about the same size as the region that later forms the mouth (Hardin and McClay, 1990). More precise maps of the target region can be constructed by transplanting rhodamine-labeled SMCs into unlabeled hosts. When cells are removed from the tip of the archenteron of a labeled embryo and transplanted into an unlabeled midgastrula, the transplanted cells migrate and take part in pattern formation normally. Labeled SMCs localize to a discrete disc of ectoderm near the animal pole (Hardin, 1995). This region is 15–20 μm in diameter, agreeing well with the previous estimates based on differences in filopodial behavior.

c. Target Recognition and the Completion of Gastrulation. The experiments just described indicate that once filopodia can interact with the animal pole region, they rapidly attach to it, thereby anchoring the archenteron and helping to end gastrulation. As we have seen, active extension of the archenteron also occurs during gastrulation, particularly in *L. pictus*. How is target recognition related to the morphogenetic movements that occur simultaneously in the archenteron? Measurements indicate that the maximum length that filopodia can achieve in *L. variegatus* is ~35 μm (Hardin and McClay, 1990). However, in normal embryos, the tip of the archenteron is ~50 μm away from the animal pole when secondary invagination begins. As a result, even though filopodia extend in all directions, they only make successful attachments to lateral ectoderm (Hardin and McClay, 1990). In normal *L. variegatus* embryos, filopodia can reach the animal pole only at the two-thirds to three-fourths gastrula stage, when the distance to the animal pole is $\leq$ 35 μm. Since autonomous extension of the archenteron is occurring prior to this time, it seems likely that filopodia-independent extension is required to place filopodia close enough to the animal pole to allow them to attach to it. As the archenteron extends, the probability of a filopodium/target encounter is greatly enhanced by the proximity of the animal pole to the tip of the archenteron at the two-thirds to three-fourths gastrula stage. As stable filopodia remain attached, they pull the archenteron even closer toward the animal pole, thereby giving more filopodia the chance to make stable contacts with the animal pole region (Hardin and McClay, 1990). Thus, the animal pole region of the embryo focuses filopodial attachments as gastrulation ends. Since filopodia can attach to the animal pole several hours before they typically do and for several hours after they normally do, target recognition also allows for temporal flexibility during gastrulation. Target recognition by SMCs has other consequences as well: the animal pole region and attached archenteron bend

toward the ventral (oral) side of the embryo after gastrulation (Gustafson and Kinnander, 1960; Dan and Inaba, 1968), thereby moving the archenteron to the site where it fuses with the stomodeum.

Several general conclusions can be made regarding the nature of the interaction of SMCs with this target. First, physical cues, including the geometry of the embryo, are important nonspecific modulators of filopodial attachments. Second, target information in this case appears to be mainly contact-mediated, rather than produced by a long-range signal. This conclusion is based on the precise boundary between stable filopodial attachments and transient ones. However, it is exceedingly difficult to distinguish experimentally between very short-range diffusible signals and substrate-bound or cell-contact mediated cues (Trinkaus, 1984). Based on the experiments performed to date, it cannot be ruled out that secondary mesenchyme cells can use long-range sensing mechanisms. For example, primary mesenchyme cells appear to display long-range, directed migration in experimentally perturbed embryos, even though such behavior is difficult to detect in normal embryos (Gustafson and Wolpert, 1961; Okazaki *et al.*, 1962; Ettensohn and McClay, 1986). Third, attachment to the animal pole region appears to involve *increased adhesion*. When filopodia contact this region, they make very stable attachments to it; they spread onto it and maintain consistent contact with it throughout subsequent postgastrula morphogenesis. It is much more difficult to dislodge the tip of the archenteron from the animal pole *after* contact has been established for a short time than at the time the first contacts with the animal pole are being made, either by micromanipulation (J. Hardin, unpublished observations; D. McClay, personal communication) or in osmotically swollen embryos (Dan and Okazaki, 1956). Finally, target recognition by SMCs is a cell-type specific recognition event. This conclusion is based on the lack of attachment of several other mesenchymal cell types to this region, despite adequate opportunities for contact. These include pigment cell progenitors (Gibson and Burke, 1985; Ettensohn and McClay, 1988) and ectopic primary mesenchyme cells (Okazaki *et al.*, 1962; Ettensohn and McClay, 1986).

Despite the rather extensive cellular analyses of target recognition that have been performed to date, little is known about the molecular nature of this interaction. Several possibilities exist regarding the nature of the molecular determinants specifying target information. First, as has been suggested regarding neuronal pathfinding in vertebrates, negative, or *repulsive*, guidance cues could be operating. In this case, pathfinding cells would avoid repulsive areas; if the nonrepulsive area were sufficiently localized, this might be a means of guiding cells to the correct site(s). In the vertebrate trunk, peanut agglutinin binding moities appear to be a repulsive barrier to growth cone migration (Oakley and Tosney, 1991). Similarly, the collapsin family of proteins may serve as repulsive cues for growing neurites (Luo *et al.*, 1993). Second, positive, *attractive*, cues could lead to the attachment of SMCs. A host of such guidance molecules, including the recently characterized netrin family of proteins (Ishii *et al.*, 1992; Kennedy *et al.*, 1994; Serafini *et al.*, 1994) has been put forward as candidate

guidance molecules in neuronal pathfinding (reviewed by Dodd and Jessell, 1988; Tessier-Lavigne, 1992; Goodman and Shatz, 1993). At present, the dearth of molecular information in the sea urchin system does not allow us to adjudicate between repulsive or attractive mechanisms, or to determine what combination of such cues is operating. There are currently no genuine candidates for repulsive molecules in the sea urchin gastrula. However, one possibility is the protein product of the ECM3 gene, which appears to be expressed throughout the ectoderm, with the exception of a region near the animal pole (G. Wessel, personal communication). Likewise, there are no current candidate attractive molecules. Concanavalin A (con A) appears to bind preferentially to the basal lamina in the animal hemisphere at the gastrula stage in some species (Spiegel and Burger, 1982; Katow and Solursh, 1982; DeSimone and Spiegel, 1986). Furthermore, injection of con A into the blastocoel can interfere with filopodial attachment of SMCs, but not primary mesenchyme cells (Spiegel and Burger, 1982). However, the domain of expression of con A is much larger than that defined by the target region (see above). Identification of bona fide guidance molecules awaits further work.

d. Pattern Formation by Other Mesenchyme Cells. Little is known about how other populations of mesenchyme cells migrate or adopt patterned configurations in the embryo. Ruffins and Ettensohn (1993) have shown that at least some SMC progenitors are pluripotent at the late blastula stage; however, single labeled cells in the vegetal plate typically give rise to specific types of SMC derivatives. These include *pigment cells*, which produce the pigment *echinochrome* and are often found in association with the growing arm buds in the prism and early pluteus larva. These cells, which depart from the vegetal plate (Gibson and Burke, 1985) or the tip of the archenteron early in gastrulation (Ettensohn and McClay, 1986), appear to disperse widely within the embryo and invade the ectoderm (Gibson and Burke, 1987; Fig. 27). Gustafson and Wolpert mention that some secondary mesenchyme cells in *Echinocardium cordatum* cluster in certain regions of the embryo, and that this distribution is disrupted in radialized embryos produced by treatment with sodium dodecyl sulfate (Gustafson and Wolpert, 1967). Another population of cells, termed *blastocoelar cells*, has been described based on morphology (Dan and Okazaki, 1956; Okazaki, 1975) and immunostaining (Tamboline and Burke, 1992). These cells leave the tip of the archenteron after its initial invagination and distribute themselves within the blastocoel to form a network of cytoplasmic processes around the gut, along the skeletal rods, and within the arms of the pluteus larva. The suggestion has been made they have a function analogous to fibroblasts (Tamboline and Burke, 1992; Fig. 27), but little else is known about them.

Recently, another population of patterned mesenchyme has been identified by its expression of the sea urchin member of the *snail* family of zinc finger transcription factors (C. Illingworth, and J. Hardin, manuscript in preparation). Other members of this family have been characterized in *Drosophila* (Boulay *et al.*, 1987), *Xenopus* (Sargent and Bennett, 1990), zebrafish (Hammerschmidt

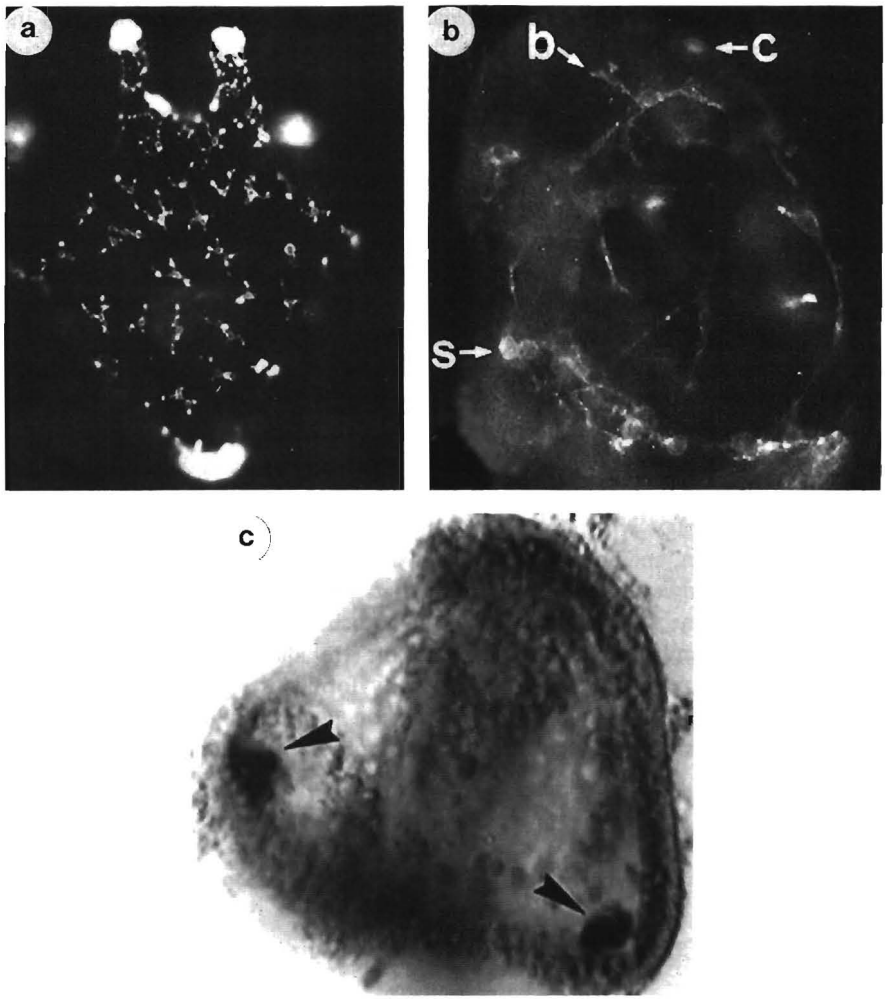


Fig. 27 Other nonspioculogenic mesenchyme. (a) Pigment cells immunostained with a monoclonal antibody (from Gibson and Burke, 1985, with permission). (b) "Blastocoelar" cells immunostained with monoclonal antibody Sp12; b, Blastocoelar cell; c, chromogenic mesenchyme (pigment cell); s, spiculogenic mesenchyme. From Tamboline and Burke (1989), with permission. (c) Whole mount *in situ* hybridization detection of cells expressing *snail* transcripts (arrows). From C. Illingworth and J. Hardin, unpublished.

and Nüsslein-Volhard, 1993; Thisse *et al.*, 1993), and mouse (Nieto *et al.*, 1992; Smith *et al.*, 1992). Based on sequence analysis, the only highly conserved region of the molecule in all of these cases is the putative DNA binding domain, which contains four or five zinc finger loops in all of the species examined (The mouse genes isolated thus far lack conservation of the first loop; there is

evidence from zebrafish that there are at least two different *snail* homologs in this species, one with four zinc finger domains and the other with five; Thisse *et al.*, 1993). Based on whole mount *in situ* hybridization, *u-snail* transcripts are first detectable midway through gastrulation in the archenteron. By the end of gastrulation, a cluster of cells, disposed asymmetrically with respect to the right–left axis of the embryo, expresses *u-snail* transcripts. After gastrulation, additional sites of *u-snail* expression are detectable as two clusters of 6–10 mesenchyme cells near the sites where the arm buds will grow later in development (Fig. 27). By the prism stage, staining disappears at the tip of the archenteron, and intense staining persists in the two clusters but eventually disappears by the pluteus stage. Interestingly, staining of what appears to be the same clusters is obtained with probes for the myogenic factor SUM-1 (J. Venuti, personal communication). Whether there is a causal connection between the two genes is unknown, although *snail* transcription is known to precede transcription of myogenic factors in mesodermal tissue in *Drosophila* and *Xenopus* as well (Leptin, 1991; Sargent and Bennett, 1990). What embryological function these clusters of cells play, whether they arise by migration of cells away from the tip of the archenteron, and how they localize in a bilateral pattern are unknown at the present time. However, it is of interest that a sea urchin homologue of the brachyury (T) gene, a gene expressed in the notochord of chordates, has recently been isolated from *Hemicentrotus pulcherrimus* (Harada *et al.*, 1995). Sea urchin *brachyury* localizes to presumptive and fully formed secondary mesenchyme cells. This result, combined with the data on *u-snail*, suggests that general features of mesoderm differentiation have been conserved among the deuterostomes.

E. Cell Adhesion and Gastrulation

1. Adhesive Changes Accompanying PMC Ingression

Given that PMCs must detach from the hyaline layer/apical lamina and neighboring cells in the vegetal plate, it might be expected that PMCs would lose adhesive affinity for these two surfaces. Experiments by Fink and McClay (1985) and Burdsal and colleagues (Burdsal *et al.*, 1991) indicate that such adhesive changes do occur. By isolating micromeres at the 16-cell stage and culturing them until the time PMCs from sibling embryos had begun or completed ingression, Fink and McClay were able to perform centrifugal adhesion assays to examine the adhesive properties of cultured PMCs on defined substrata. Micromere descendants undergo three simultaneous changes in adhesive affinity: (1) they lose affinity for proteins of the hyaline layer/apical lamina, including the protein hyalin, the major protein of the hyaline layer; (2) they lose affinity for monolayers of dissociated embryonic epithelial cells; and (3) they gain an affinity for

sonicated basal lamina "bags," as well as vertebrate fibronectin (Fink and McClay, 1985). Similarly, Burdsal *et al.* (1991) found that cultured micromere descendants lose affinity for the hyaline layer protein echinonectin at the time they ingress.

2. Adhesion to the Hyaline Layer

a. Hyalin. We have already seen that PMCs alter their adhesion to the hyaline layer at the time they ingress. The hyaline layer has been proposed to be important as a structural support and mechanical integrator of epithelial sheets in the sea urchin embryo, in a manner analogous to that at one time attributed to the "surface coat" in amphibians (Holtfreter, 1943; see Keller, 1986, for a critical evaluation of this notion in amphibians). Adding support to the notion that the hyaline layer might be a mechanical "integrator," Citkowicz (1971) used hyperosmotic solutions to show that the hyaline layer has structural integrity independent of its associated epithelial cells, forming a shell of relatively nondeformable material even when its epithelial cells are removed. As the archenteron elongates, at least some components of the hyaline layer remain associated with the archenteron (Fig. 28).

A major constituent of the hyaline layer is the protein *hyalin* (Kane, 1970; Adelson *et al.*, 1992). This large glycoprotein can be conveniently isolated by successive rounds of solubilization and precipitation via removal and readdition of calcium ions to seawater or other osmotically balanced media (Kane, 1970). Some of its structural properties have been characterized by affinity purification of hyalin using monoclonal antibodies followed by rotary shadowing (Adelson *et al.*, 1992) and more recently by isolating partial cDNAs from an expression library using the same antibody (McClay, 1992). Not surprisingly, hyalin appears to contain consensus calcium-binding motifs and nonidentical, but similar, modular repeats, similar to that seen in other large extracellular matrix molecules (McClay, 1991); it has also been shown to interact with calcium biochemically (Robinson *et al.*, 1992).

If the hyaline layer does serve a mechanical function during the blastula and gastrula stages, then perturbing the association of cells with it would be expected to block epithelial morphogenesis at the gastrula stage, particularly the invagination of the archenteron. When the hyaline layer is experimentally disrupted by incubating fertilized eggs in F_{ab} fragments of a monoclonal antibody that binds to hyalin, cell binding to the hyaline layer is disrupted. The hyaline layer visibly delaminates from the epithelium, the epithelium becomes abnormally thickened, and invagination of the archenteron is blocked (Adelson and Humphreys, 1988; Fig. 28). If blocked embryos are removed from the antibody, development resumes, and a normal pluteus larva results (Adelson and Humphreys, 1988). If the antibody is applied at the mesenchyme blastula stage, invagination is still blocked, but primary mesenchyme cells ingress into the blastocoel to produce spicules,

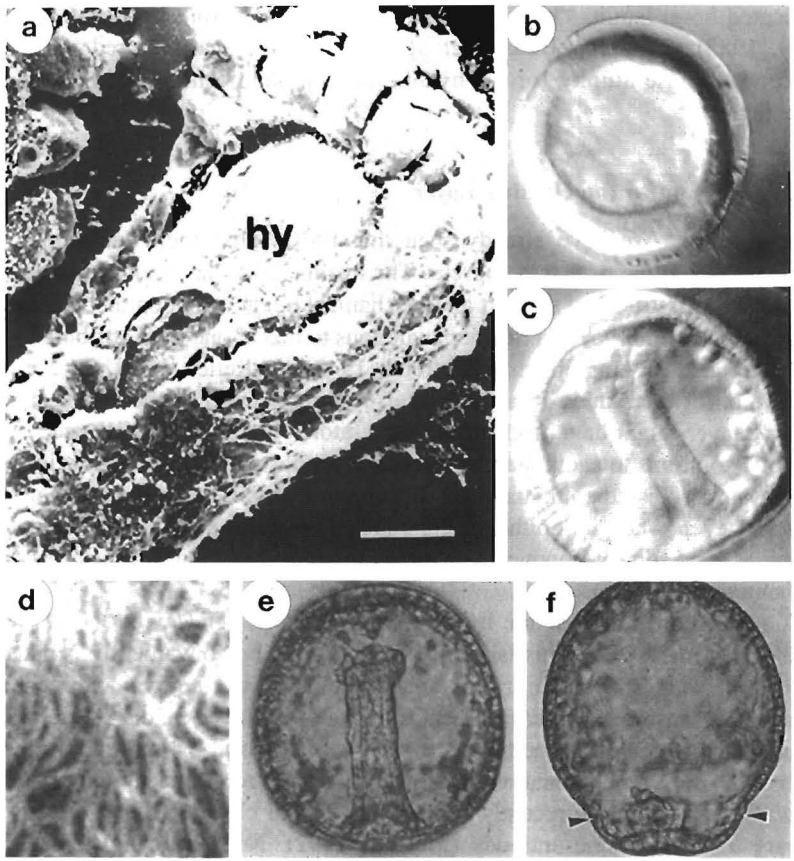


Fig. 28 The role of hyaline layer proteins in gastrulation. (a) SEM of a *L. pictus* archenteron in which a part of the epithelium has been removed to reveal the underlying extracellular matrix (hy, hyaline layer) (J. Hardin, unpublished). (b) *L. variegatus* embryo treated with a monoclonal antibody against the protein hyalin prior to gastrulation. Note the detachment of the epithelium from the hyaline layer, which is now visible at the periphery of the embryo. (c) Sibling untreated embryo (J. Hardin, unpublished). (d) High-magnification view of the apical extracellular matrix immunostained with a monoclonal antibody that recognizes fibropellins. (e, f) *S. purpuratus* embryos treated with control antisera (e) or antibodies against fibropellins (f). (d–f) From Burke *et al.* (1990), with permission.

and pigment cells eventually appear (J. Hardin, unpublished observations). These results suggest that the antibody interferes with the mechanical and structural integrity of the epithelium, but also with more general epigenetic cues necessary for the initiation of gastrulation.

b. Other Hyaline Layer Proteins. *Echinonectin* is a second abundant hyaline layer protein. Like hyalin, it is present in the unfertilized egg and at all

embryonic stages. Echinonectin is a 230-kDa dimer composed of two 116-kDa monomeric subunits joined by disulfide bonds, with a characteristic "bow-tie" structure in the electron microscope (Alliegro *et al.*, 1988; reviewed in Alliegro *et al.*, 1992). Echinonectin has lectin activity, allowing its chromatographic purification. As we have seen, based on *in vitro* adhesion assays, echnonectin is an adhesive substratum for cells; based on the adhesive properties of proteolytic fragments, each monomer may contain a single cell-binding domain (reviewed in Alliegro *et al.*, 1992). The spatial relationship of echinonectin to other hyaline layer molecules is unclear, based on currently available information (reviewed by Ettensohn and Ingersoll, 1992). Whether attachment to echinonectin is required for invagination of the archenteron is not known. Another constituent of the hyaline layer is *Ecto V*, a 350-kDa protein which localizes to the tips of microvilli of the oral ectoderm and to the foregut by the late gastrula stage, based on immunostaining (Coffman *et al.*, 1990). The function of this protein in the gastrula is unknown. However, it does become localized prior to the time that the oral ectoderm flattens late in gastrulation. Perturbational studies have not been performed to clarify the role of this molecule.

3. The Apical Lamina

In addition to the proteins of the outer laminae of the hyaline layer, a set of three proteins appears to localize to the inner region of the apical extracellular matrix, a region termed the *apical lamina* (Hall and Vacquier, 1982). Although the precise location of these components is not clear, there is evidence that they represent a distinct sublayer within the apical ECM, based on ultrastructural studies (Spiegel and Howard, 1983; Cameron and Holland, 1985) and on the relative resistance of these proteins to extraction when embryos are placed in calcium-free seawater (Hall and Vacquier, 1982). The three major components of the apical lamina are now termed *fibropellins*, and their sequence has been determined in *S. purpuratus* (Delgadillo-Reynoso *et al.*, 1989; Grimwade *et al.*, 1991; Bisgrove *et al.*, 1991; Bisgrove and Raff, 1993). They are produced from two genes, one of whose transcripts undergoes alternative splicing to yield two distinct products (fibropellins Ia and Ib); the other yields a single, smaller species (fibropellin III). The fibropellins possess varying numbers of epidermal growth factor-like repeats (Delgadillo-Reynoso *et al.*, 1989; Grimwade *et al.*, 1991; Bisgrove *et al.*, 1991; Bisgrove and Raff, 1993); fibropellin III contains the fewest such repeats (Bisgrove and Raff, 1993). Sequence analysis reveals several glycolsylation sites for both O- and N-linked carbohydrates (Bisgrove *et al.*, 1991; Bisgrove and Raff, 1993). Immunostaining indicates that the fibropellins form an extracellular, filamentous meshwork on the exterior of the embryo (Burke *et al.*, 1991; Bisgrove *et al.*, 1991; Bisgrove and Raff, 1993; Fig. 28).

The functional role of fibropellins has been explored using monoclonal antibodies. When *S. purpuratus* embryos are treated with purified monoclonal antibodies raised against these same proteins, perturbation of primary invagination

results (Burke *et al.*, 1991; see above). Whether these effects result from perturbation of cell binding to one or more of the fibropellins, or reflect structural alterations in the extracellular matrix near the embryo surface, is not currently known. Interestingly, the effects of external application of the antibodies seem largely confined to primary invagination in *S. purpuratus* (Fig. 28). Many other aspects of gastrula and postgastrula development appear to occur reasonably normally. It is not known whether these proteins play a role in subsequent steps of archenteron morphogenesis, since the lumen of the archenteron is not easily accessible to exogenous molecules.

4. The Basal Extracellular Matrix

a. Collagen. Collagen is a major structural component of the basal lamina in the sea urchin embryo. Collagen synthesis, measured by hydroxyproline incorporation, can be detected at the midblastula stage and increases through the prism stage, when typical striated fibrils are visible within the blastocoel via transmission electron microscopy (Golob *et al.*, 1974; Gould and Benson, 1978; Crise-Benson and Benson, 1979; Katow and Solursh, 1979). Partial biochemical purification of collagen-like molecules has been carried out as well (Pucci-Minafra *et al.*, 1972; Shimizu *et al.*, 1990). Molecules sharing immunological cross-reactivity with vertebrate type IV collagen have also been reported (Wessell *et al.*, 1984). In addition to collagen itself, the enzymatic activity of lysyl oxidase, which oxidizes peptidyl lysine residues to reactive semialdehydes as part of a multistep covalent cross-linking of collagen fibrils, has been examined. Lysyl oxidase activity shows a six- to sevenfold increase in activity at the gastrula stage (Butler *et al.*, 1987). More recently, collagen genes have been characterized in several species of sea urchin. Genomic clones isolated from *S. purpuratus* using mouse type IV cDNAs have been characterized; *in situ* hybridization indicates that transcripts produced from this gene are expressed in mesenchyme cells (Angerer *et al.*, 1988). In addition, there is evidence that multiple transcripts are recognized by exon probes derived from the genomic sequences (Nemer and Harlow, 1988). A putative collagen gene has also been cloned from a *Paracentrotus lividus* genomic library using *C. elegans* collagen gene as a probe (D'Alessio *et al.*, 1989, 1990; Esposito *et al.*, 1992).

Collagen and other components associated with the basal lamina appear to be at least a permissive requirement for several events associated with sea urchin gastrulation. One method for demonstrating this requirement has been to employ the lathyritic agent β-aminoproprionitrile (BAPN), an inhibitor of lysyl oxidase, to prevent appropriate crosslinking and assembly of collagen. BAPN treatment has been shown to result in an increase in the pool of soluble collagen present in embryos (Butler *et al.*, 1987), as well as structural alterations in the basal lamina, as assessed by scanning electron microscopy (Hardin, 1987). BAPN treatment affects several morphogenetic processes in sea urchin gastrulae: treatment of

gastrulae with the drug results in defects in mesenchymal migration and a flaccid archenteron (Butler *et al.*, 1987; see below). Cultured micromeres treated with the lathyritic agent cannot synthesize spicules *in vitro*, yet when they are supplied with exogenous type I collagen produced in a bacterial expression vector, they are able to do so (Wessel *et al.*, 1991). Similarly, a subpopulation of dissociated cells from mesenchyme blastula stage embryos expresses endoderm-specific genes when cultured on artificial extracellular matrices in the presence of serum (Wessel, 1991).

Treatments affecting collagen synthesis or processing also block archenteron morphogenesis. Incubation of fertilized eggs in BAPN does not prevent embryos from developing normally to the mesenchyme blastula stage, but the archenteron fails to invaginate. If the BAPN is removed, even after the embryos have been arrested at the mesenchyme blastula stage for more than 24 hr, the embryos begin to gastrulate and complete development normally (Butler *et al.*, 1987; Wessel and McClay, 1987). Similar effects are observed when embryos are treated with other agents known to perturb earlier steps of collagen synthesis or assembly, including *cis*-hydroxyproline and α–α' dipyridyl (Mizoguchi *et al.*, 1983a,b; Wessel and McClay, 1987). In the case of BAPN, synthesis of collagenous molecules appears unaffected, and the drug does not interfere with the *de novo* expression of a number of genes in the ectoderm (Wessel *et al.*, 1989). In contrast, molecules that are normally expressed in the archenteron fail to appear as long as the embryos are incubated in BAPN (Wessel and McClay, 1987; Wessel *et al.*, 1989). If the drug is applied after gastrulation has commenced, then invagination proceeds, but secondary mesenchyme cells show impaired motility, and the archenteron is often flaccid (Butler *et al.*, 1987; Hardin, 1987; Fig. 29).

In light of the similar, reversible effects that BAPN and anti-hyalin antibodies have on gastrulation, it appears that in addition to the direct mechanical or structural effects that disruption of the basal lamina may have, a critical period precedes gastrulation during which the vegetal plate must be in normal contact with both the basal lamina and the hyaline layer in order for gastrulation to commence. In the case of the basal lamina, some of the observed ability of extracellular matrices to support differentiation may be due to the presence of bound peptide growth factors. Support for this notion comes from experiments employing vertebrate platelet-derived growth factor (PDGF): when BAPN-treated embryos are treated with vertebrate PDGF, they can overcome the block to gastrulation to produce a gut (though it is often everted) and a skeleton (Ramachandran *et al.*, 1993). Conversely, embryos treated with antibodies against vertebrate PDGF receptor are prevented from proceeding through gastrulation (Ramachandran *et al.*, 1994).

b. Laminin. The first convincing evidence for the presence of laminin in the basal lamina of sea urchin embryos came from studies in *Sphaerechinus granularis* using a monoclonal antibody (MAb BL1) by McCarthy *et al.* (1987).

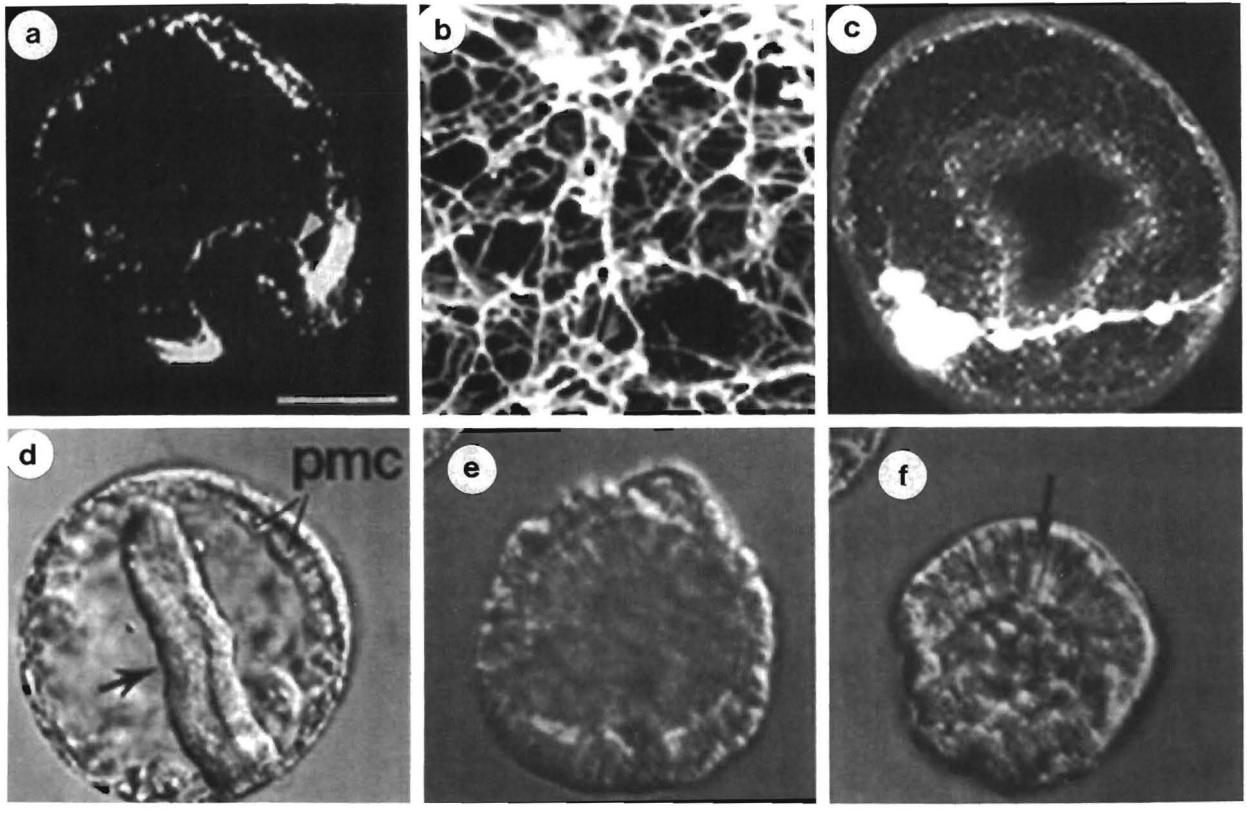


Fig. 29 The role of the basal lamina and blastocoel matrix in gastrulation. (a) Immunostaining of a *L. variegatus* embryo with antibodies against the ECM 1 epitope (from Ingersoll and Ettensohn, 1994, with permission). (b) SEM of fibrillar matrix material in *S. purpuratus* (from Hardin, 1987). (c) Blastocoel matrix material revealed by immunostaining with a PMC-specific antibody in *L. pictus*, visualized with confocal microscopy (J. Hardin, unpublished). (d) Flaccid archenteron (arrow) resulting from treatment of *L. pictus* gastrulae with β-aminoproprionitrile (from Butler *et al.*, 1987, with permission). (e, f) *Sphaerechinus granularis* mesenchyme blastulae injected with BL1 antibodies. The arrow in (f) denotes the thickening of the epithelium (from McCarthy *et al.*, 1987, with permission).

The antibody localizes to the basal lamina in blastulae and gastrulae (McCarthy and Burger, 1987) and immunoprecipitates two proteins of 260 and 480 kDa from basal lamina preparations. Rotary shadowing of affinity-purified fractions of sea urchin basal laminae reveals cruciform molecules with an appearance strikingly similar to that of vertebrate laminin. However, MAb BL1 does not cross-react with vertebrate laminin (McCarthy *et al.*, 1987). McCarthy and Burger (1987) injected MAb BL1 into the blastocoel of *S. granularis* blastulae and gastrulae. The antibody had profound effects on epithelial morphology and morphogenesis. Epithelial cells transiently thickened after antibody treatment and eventually displayed impaired lateral cell–cell association (McCarthy and Burger, 1987; Fig. 29). Treated embryos eventually recovered from the treatment. If BL1 recognizes a genuine laminin, these studies suggest that LN is required for epithelial remodeling during the blastula and gastrula stages. Attempts have been made to perturb morphogenetic movements in sea urchin gastrulae using synthetic peptides thought to mediate cell binding to vertebrate laminin, particularly variants of the sequence YIGSR and IKVAV (Yamada and Kleinman, 1992; but see Ruoslahti, 1991). Hawkins *et al.* (1995) found that peptides containing the sequences YIGSR and IKVAV added to cultures of embryos shortly after fertilization inhibited archenteron formation and other aspects of morphogenesis. Exogenously added peptides had less pronounced effects if added progressively laterin development. Crawford and Burke (1994) found that addition of YIGSR to isolated basal lamina "bags," which contain isolated basal lamina and trapped mesenchymal and endoderm cells, stimulated migration of mesenchyme out of the bags. Unfortunately, it is not clear how much significance should be attached to these studies. First, in *L. variegatus* (Laxson and Hardin, 1993) and *S. purpuratus* (Hawkins *et al.*, 1995) the amino acid sequence at this putative binding site in the B1 chain is not conserved at the second and fourth positions. Second, there is considerable debate about whether these sequences represent genuine or cryptic cell attachment sites; recent work favors the G domain of the A chain as a primary cell attachment domain for laminin (Ruoslahti, 1991; Tryggvason, 1993). More recently, cDNAs have been isolated from both *S. purpuratus* and *L. variegatus* coding regions of the A and B1 chains of sea urchin laminins (S. Benson, pers. commun.; Laxson and Hardin, 1993). At the amino acid level, the sea urchin LN B1 chain appears to bear roughly the same similarity to vertebrate laminin as does *Drosophila* LN (Laxson and Hardin, 1993).

c. Fibronectin. The presence of fibronectin (FN) has been suggested by immunostaining using polyclonal antibodies against vertebrate plasma fibronectin. However, depending on the immunostaining method used, the described distribution of cross-reactive material appears markedly different (Katow and Solursh, 1982; Spiegel *et al.*, 1983; Wessel *et al.*, 1984). DeSimone *et al.* (1985) identified a 220-kDa protein that comigrates with human plasma FN on sodium dodecyl sulfate (SDS) gels run under reducing conditions and is weakly detected

on Western blots by antibodies against vertebrate FN. Iwata and Nakano (1981) used gelatin–agarose chromatography to isolate a putative FN from sea urchin ovaries; antibodies raised against this protein recognize molecules in the basal lamina of the ovary, as well as in the basal lamina of gastrulae. However, the protein is not recognized by antibodies raised against vertebrate FN (Iwata and Nakano, 1981). At present, the existence of a bona fide FN in the sea urchin is inconclusive.

In addition to attempts to characterize FN directly, other studies have been performed to examine a possible role for FN in supporting PMC migration. Cultured PMCs plated on human plasma FN appear to attach more avidly and migrate more extensively than when cultured on uncoated plastic or collagen (Venkatasubramanian and Solursh, 1984). Katow and colleagues (Katow *et al.*, 1982; Katow and Hasyashi, 1985; Katow, 1990) have suggested that PMCs require FN for migration, based on immunostaining of PMC cell surfaces and on stimulation of migration of PMCs *in vitro*. In addition, migration of PMCs on FN *in vitro* can be blocked by addition of RGDS-containing peptides (Katow, 1990). However, the assessment of these studies is problematic for several reasons: (1) the actual measured migration distances are quite small in these studies (translocation of less than a single cell diameter was scored as a migration event); (2) the *in vitro* competition experiments do not examine endogenous FN; and (3) the location of putative FN is unclear in these studies (i.e., is it bound to the PMC cell surface or is it attached to the migratory substratum?). More recently, Katow (1990) has introduced antibodies against human plasma FN and RGDS-containing peptides into the blastocoel of *Clypeaster japonicus* embryos by creating a mechanical rupture in the vegetal plate at the mesenchyme blastula stage using cytochalasin D, and he reports perturbation of PMC migration. The interpretation of these experiments would be greatly aided by the identification of a bona fide FN in a sea urchin embryos.

Little is known about putative receptors for extracellular matrix molecules in the sea urchin embryo. Recently, several laboratories have reported the cloning of putative α- and β-integrin subunits using degenerate primers and the polymerase chain reaction (Marsden *et al.*, 1993; Susan and Lennarz, 1993; P. Hertzler and D. McClay, personal communication). Hopefully the further characterization of these receptors and the identification of the ligands will lead to a better understanding of the ways in which motile cells in the sea urchin gastrula interact with the extracellular matrix.

d. The Blastocoel matrix

Structural properties of the blastocoel matrix. The blastocoel of echinoderm embryos is not simply filled with a low-viscosity liquid similar in composition to seawater, as one might suppose based on cursory observations. A number of studies indicate that the molecular constituents of the blastocoel are considerably more complex. For example, Katow and Solursh (1979) described a mesh-

work of fibrous and granular material which is structurally continuous with the basal lamina in direct contact with cells. Similar material is observed in well-preserved specimens prepared for scanning electron microscopy (e.g., Morrill and Santos, 1985; see Fig. 29). Burke *et al.* (1990) identified a fibrous component of the blastocoel matrix in *S. purpuratus* using a monoclonal antibody (MAb SP14). Whole-mount immunostaining with certain PMC-specific antibodies often reveals a similar dense network of fibers and granules in the blastocoel (J. Hardin, unpublished observations; Fig. 29). More recently, Cherr *et al.* (1992) have used high pressure and quick-freezing techniques to reveal an oriented, fibrillar meshwork within the blastocoel. Strathmann (1989) demonstrated that this material has structural integrity apart from the epithelial tissue of the larva; gel-like material in the blastocoel of a number of echinoderm larvae is sufficiently dense to exclude ink and other small particles, even when the overlying epithelium is dissected away.

The implications of a structured blastocoel matrix have not been thoroughly explored. Given the apparent fibrillar nature of some of this material, however, it is reasonable to suppose that one function of this material is to provide structural support to mesenchyme cells as they extend filopodia. Given the prodigious length attained by the filopodia of SMCs, for example, a fibrillar scaffolding could provide lateral stability to these protrusions as they extend. In support of this idea, Burke *et al.* (1990) visibly disrupted the organization of the blastocoel matrix of *S. purpuratus* gastrulae by injecting the SP14 antibody into the blastocoel. Some minor effects were observed on migration of SMCs away from the tip of the archenteron, apparently due to the inability of these cells to migrate until they made direct contact with the ectoderm and associated basal lamina of the animal pole. Additional evidence for the structural support of the blastocoel matrix comes from archenterons explained *in vitro*. Such explants possess SMCs that can extend protrusions up to 35–40 μm in length, but these protrusions are confined to the solid substratum upon which the archentera are explanted; filopodia extended above the substratum are limited in length to considerably less than this (J. Hardin, unpublished).

Proteoglycans. Histochemical techniques demonstrated the presence of sulfated glycans in the blastocoel 40 years ago (Immers, 1956, 1961). Stained material shows a particularly strong association with PMCs (Immers, 1961; Sugiyama, 1972). Incorporation of $^{35}SO_4^{2}$ peaks at the time when PMCs appear (Karp and Solursh, 1974). Sulfated macromolecules in the blastocoel and basal lamina are heterogeneous, including heparin-like (Kinoshita, 1971) and dermatan sulfate proteoglycans (Solursh and Katow, 1982) and sulfated glycoproteins (Heifetz and Lennarz, 1979). The majority of the sulfated components in the blastocoel are very large proteoglycans; based on Sepharose chromatography, most are $>2 \times 10^4$ kDa (Solursh and Katow, 1982). Ultrastructurally, strands consisting of 30-nm granular subunits are found in the blastocoel matrix, often associated with PMCs (Katow and Solursh, 1982).

Since sulfated components are a conspicuous constituent of the blastocoel matrix/basal lamina, it is perhaps not surprising that sulfate deprivation perturbs postblastula development and morphogenesis in the sea urchin embryo. Such studies date back to Herbst (1904). More recently, the selective effects of sulfate deprivation on proteoglycans have been analyzed; sulfated glycans are in fact reduced in sulfate-deprived embryos (Immers, 1956; Sugiyama, 1972; Karp and Solursh, 1974; see Solursh, 1986, for a review), as is 30-nm granular material in the blastocoel (Katow and Solursh, 1979). As we have seen, migration of PMCs is reversibly blocked in sulfate-deprived embryos (Karp and Solursh, 1974; Katow and Solursh, 1981; Solursh, 1986). As mentioned above, this may result from deficiencies in the synthesis of molecules associated with the PMC surface. Sulfate deprivation also results in failure of invagination of the archenteron and defects in secondary mesenchyme attachment and motility (Akasaka *et al.*, 1980). lacking specific molecular probes for individual components of the sulfated material, it is difficult to draw any more specific conclusions about the role of sulfated molecules in sea urchin gastrulation.

More recently, Ingersoll and Ettensohn (1994) have characterized an N-linked carbohydrate-containing epitope on several high-molecular-weight basal lamina glycoproteins recognized by a monoclonal antibody (ECM-1). The epitope is expressed predominantly in the extracellular matrix in the vegetal region of the embryo prior to and during gastrulation (Fig. 29) and eventually localizes to the ventral region of the embryo. Injection of ECM-1 antibodies or Fab fragments blocks archenteron elongation in gastrulae of the genus *Lytechinus* and in *S. purpuratus*, and there is a concomitant lack of additional cell rearrangement in the archenteron (Ingersoll and Ettensohn, 1994). The specific role of molecules expressing the ECM-1 epitope is not clear, although Ingersoll and Ettensohn suggest that these molecules may be involved in signaling the cells of the archenteron to undergo additional rearrangement.

General cell surface requirements during gastrulation. Although bona fide cell–cell adhesion molecules have not been conclusively isolated from the sea urchin embryo (but see below for promising preliminary evidence), several studies have examined general requirements for cell adhesion during gastrulation. Heifetz and Lennarz (1979) demonstrated that several new classes of sulfated, N-linked glycoproteins are synthesized at gastrulation. Different amounts and species of glycoproteins are synthesized by ectodermal and mesendodermal cell populations (Lennarz, 1986). Inhibition of protein glycosylation using tunicamycin causes developmental arrest of embryos at the early gastrula stage (Schneider *et al.*, 1978), suggesting that synthesis of specific glycoproteins is a necessary prerequisite to further morphogenesis.

Using the cell adhesion assay developed for use with primary mesenchyme cells, McClay and Matranga (1986) have characterized the adhesion of gastrula stage cells to one another *in vitro*. Adhesion involves a rapid initial adhesion step that occurs at 4°C, and a second, permanent step that occurs at physiological temperatures. The second step requires ATP, an intact actin cytoskeleton, and the

presence of calcium (McClay and Matranga, 1986). It is not known what relation such events *in vitro* have to the modulation of adhesive contacts between cells in the normal embryo.

Candidate cell adhesion molecules expressed in the archenteron. Several proteins that localize to specific regions within the archenteron have been characterized. The two best studied of these are the proteins Endo 1 and Endo 16. Endo 16 has consensus calcium binding motifs, as well as a RGD sequence (Nocente-McGrath *et al.*, 1989). Endo 16 appears on the surface of cells in the archenteron during gastrulation, and it is eventually restricted in expression to the mid- and hindgut regions of the gut (Nocente-McGrath *et al.*, 1989). Endo 16 appears to bind calcium and may be secreted and bound into the extracellular matrix near the archenteron (Soltysik-Espanola *et al.*, 1994). Endo 1, which has only been characterized immunologically, is a cell surface protein with a pattern of expression quite similar to that of Endo 16, including restriction to the mid- and hindgut after gastrulation (Wessell and McClay, 1985). Based on the expression patterns of other endoderm-specific genes, this restriction may be fairly general (Wessell *et al.*, 1989; Coffman and Davidson, 1992; Kingsley *et al.*, 1993). Despite the provocative location of these proteins, no function during invagination has thus far been demonstrated for them.

More recently, attention has focused on isolation of potential cadherin family members in the sea urchin embryo. Ghersi and colleagues have characterized antigenic determinants recognized by antibodies directed against specific cadherins or against domains shared by all cadherins (Ghersi and Vittorelli, 1990; Ghersi *et al.*, 1993). When embryos are incubated with antibodies against mammalian E-cadherin, invagination of the archenteron is blocked, and epithelial morphogenesis appears perturbed (Ghersi *et al.*, 1993). Whether the protein(s) displaying immunological cross-reactivity recognized by vertebrate anti-cadherin antibodies are actually cadherins or not remains to be determined. However, a genuine cadherin superfamily member has been cloned recently in *L. variegatus* using degenerate primers against conserved sequences shared by the cytoplasmic domain of vertebrate cadherins (Miller and McClay, 1994). How many cadherins are produced by sea urchin embryos and whether or not they are expressed in a tissue-specific manner as they are in vertebrates (for example, see Takeichi, 1991) awaits further study. However, it appears that it will now be possible to study the role of specific adhesion molecules with well-defined molecular identities in the sea urchin embryo.

V. Cell Interactions Regulating Gastrulation

A. Sequential Cell–Cell Interactions May Establish Targets for Mesenchyme Cells in the Sea Urchin

The two major populations of nonpigmented mesenchyme, PMCs and SMCs, engage in distinctly different pattern-forming processes during and immediately

after gastrulation. As we have seen, PMCs form two large clusters on the ventral side of the embryo, and intervening PMCs adopt a stereotypical pattern (reviewed in Decker and Lennarz, 1988; Ettensohn, 1990, 1992; Benson and Wilt, 1992). SMCs, on the other hand, are a heterogeneous (or pluripotent) population of cells that give rise to various cell types (Tamboline and Burke, 1992; Ettensohn and Ruffins, 1993; Ruffins and Ettensohn, 1993). As we have seen, the pattern adopted by these two populations of mesenchyme appears to be largely controlled by the ectoderm. However, what developmental interactions generate this pattern? Based on studies by Hörstadius and Cameron and colleagues (reviewed in Cameron and Davidson, 1991; see above), it is clear that lineage founder cells that give rise to the major tissue territories of the embryo can be distinguished by the fifth to sixth cleavage. It is also clear from classic and more recent studies that local cell–cell interactions between cells from different tissue territories can influence the expression of particular cell fates in dramatic ways (Hörstadius, 1939, 1973; Davidson, 1990, 1993).

One cell interaction that may influence patterning of PMCs is the ability of micromeres to alter the fate of neighboring cells. Two potential effects of micromeres on nearby cells have been investigated (reviewed by Livingston and Wilt, 1990; Davidson, 1990). First, Wilt and colleagues have shown that mesomeres can produce gut and skeletal structures when in contact with micromeres (Livingston and Wilt, 1990; Wilt *et al.*, 1995). Second, transplants by Hörstadius (1935) and Ransick and Davidson (1993) have shown that micromeres transplanted to ectopic locations in either 16- or 32-cell embryos can induce nearby tissue to form an archenteron, even though the fate of the tissue under normal circumstances is to form ectoderm (Fig. 30). The induced tissues express appropriate mRNAs and/or proteins consistent with their morphology (Khaner and Wilt, 1990; Ransick and Davidson, 1993; Wilt *et al.*, 1995). Implantation of ectopic micromeres has an additional consequence: one or more supernumerary skeletal elements are produced (Hörstadius; 1935; Ransick and Davidson, 1993). When Ransick and Davidson (1993) transplanted micromeres to the animal pole of an early 16-cell stage embryo, the resulting embryos possessed complete, bilateral skeletons formed by the implanted cells. The labeled micromere descendants formed the skeleton around the unlabeled, induced archenteron, aligned with the dorsoventral axis of the host (Ransick and Davidson, 1993; Fig. 30).

The experiments of Ransick and Davidson and Hörstadius raise several possibilities regarding how ventrolateral patterning sites for PMCs arise: (1) they could arise via inductive signals produced directly by the micromeres; (2) they could arise via a cascade of interactions, requiring induction of presumptive gut tissue followed by the induction of ventrolateral patterning sites via lateral signaling from presumptive gut tissue; or (3) a combination of such signals could be involved. Experiments suggest that sequential interactions can account for the induction of ventrolateral patterning sites within the ectoderm. Hörstadius transplanted labeled veg_2 cells (the cells that normally produce archenteron and anal

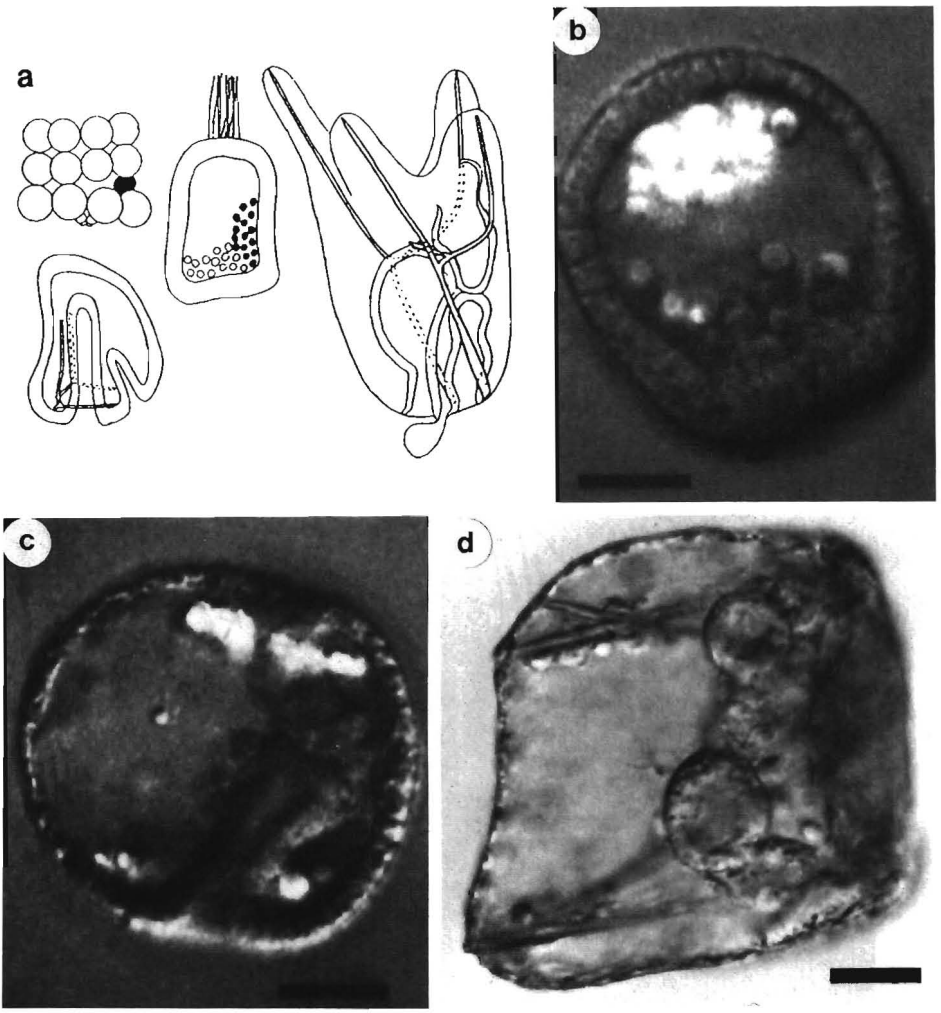


Fig. 30 (a) Implantation of micromeres into the lateral region of a 32-cell stage *P. miliaris* embryo by Hörstadius. A second archenteron is induced from unlabeled tissue by the implanted material. (b–d) Implantation of rhodamine-labeled micromeres at the animal pole of a late 8-cell embryo, by Ransick and Davidson. (b) The implanted cells ingress at the mesenchyme blastula stage (b) and engage in skeletogenesis (c). In addition, a second axis is produced (c, d) with an induced, unlabeled archenteron and associated supernumerary skeletal elements. (a) From Hörstadius (1935). (c–e) From Ransick and Davidson (1993), with permission.

ectoderm, but not ventrolateral ectoderm) to ectopic locations. The implanted cells formed a second archenteron autonomously; Hörstadius also noted that ectopic skeletal rods formed from unlabeled, host PMCs (Hörstadius, 1935; Fig. 31). Hardin *et al.* have repeated and extended these results by adding single, rhodamine-labeled veg_2 cells or their descendants to unlabeled host embryos (reviewed in Hardin, 1995). Such ectopic cells generate an additional archenteron, but they also induce two new bilateral sites of spicule formation. The ectoderm underneath the new skeletal elements is from the host; this implies that lateral induction of host ectoderm by the incorporated cell produces new patterning sites (Fig. 31).

Another major site of mesenchymal pattern formation is the preumptive oral region of the larva, a region that maps to the ventral ectoderm near the animal

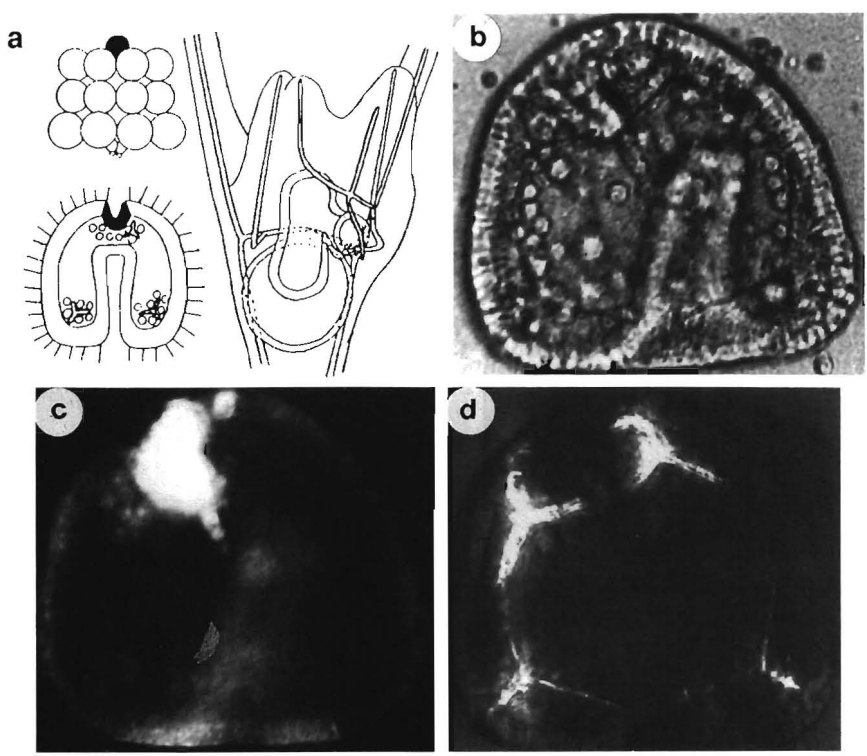


Fig. 31 Implantation of veg_2 cells into the animal pole of a 64-cell *P. miliaris* embryo by Hörstadius (1935). The labeled tissue invaginates, and a small spicule forms from host tissue near it. (b–d) Incorporation of a rhodamine-labeled macromere descendant in a chimeric *L. variegatus* embryo. The implant produces a second archenteron (b, c), and a bilateral skeletal array flanks the ectopic rudiment (Hardin *et al.*, 1994). (a) From Hörstadius (1935).

pole of the embryo (Hörstadius, 1973; Cameron and Davidson, 1991). Experiments indicate that additional territorial interactions occur to establish mesenchymal patterning information for both PMCs and SMCs in the oral field. In his classic isolation studies, Hörstadius found that the cells destined to give rise to the bulk of the ectoderm (the "an_1" and "an_2 tiers," in his terminology) form *Dauerblastulae* when isolated at fifth or sixth cleavage. However, when the next, more vegetal tier (the veg_1 cells) is included, a stomodeal invagination is produced (reviewed in Hörstadius, 1939; Fig. 32). This suggests that interactions between the veg_1 progeny and the adjacent animal cells result in the eventual induction of the oral field. The experiments of Hörstadius did not establish when the oral field is specified. Isolation of animal caps by Hardin and Armstrong (1991; J. Hardin and N. Armstrong, manuscript in preparation) indicates that the oral field is not determined until relatively late in development. Animal caps isolated prior to the early gastrula stage do not produce stomodeal invaginations. Instead, the remaining vegetal tissue regulates to produce a new oral field, including parallel skeletal rods that flank the mouth. When isolated at the early gastrula stage, animal caps can produce a stomodeum, and the vegetal fragments no longer have this capacity (Fig. 33). Transplantation of PMCs into animal caps isolated at various times indicates that the pattern information required for the production of the parallel skeletal rods flanking the mouth is coordinately regulated with the oral field; there is a sharp increase in the ability of PMCs to form parallel skeletal rods when transplanted into animal caps isolated at the early gastrula stage (Hardin and Armstrong, 1994).

Given that the boundary of the region to which the archenteron attaches at the end of gastrulation is limited in diameter, it is of interest to ask whether the oral field in general has precisely mapped subregions within it by the gastrula stage.

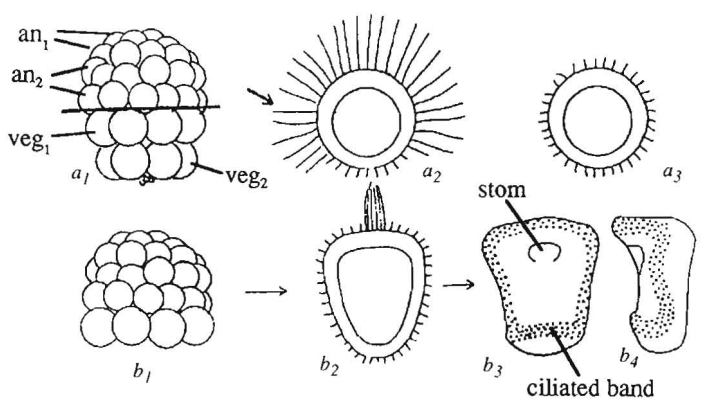
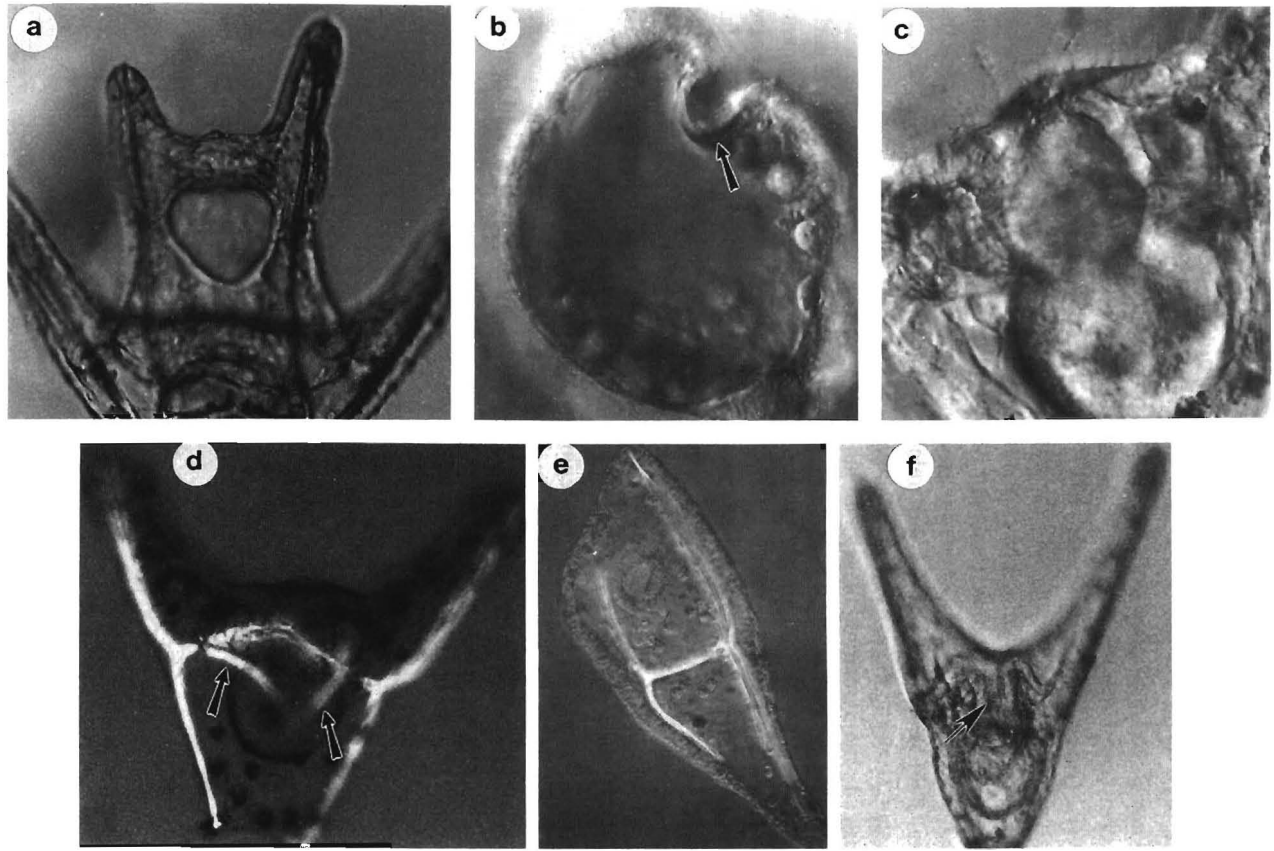


Fig. 32 Isolation of an_1 and an_2 derivatives or an_1 + an_2 + veg_1 derivatives. In the latter case, a stomodeum and ciliated band are produced. Adapted from Hörstadius (1935).


a
b
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e
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In order to address this question, embryos were partially ligated at the early gastrula stage, when the oral field is known to be determined, based on the above animal pole isolation experiments (Hardin and Armstrong, 1991). In such partial ligations, the presumptive oral field tissue is twisted and distorted. When such ligated embryos are allowed to develop to the pluteus stage, the mouth is stretched and distorted in a corresponding fashion, indicating that the boundaries of the tissue that will fuse with the archenteron are determined in a precisely mapped configuration by the midgastrula stage.

B. Signal Transduction Events and the Establishment of Mesenchymal Patterning Sites

It has been known for nearly a century that lithium chloride has dramatic effects on differentiation of sea urchin embryos, simultaneously shifting the boundaries between presumptive ectoderm and endoderm and patterning sites for PMCs (see above). It has recently been hypothesized that the effects of lithium are mediated via the inositol triphosphate (IP_3) and/or protein kinase C (PKC) intracellular signaling pathways (Livingston and Wilt, 1990, 1992; Wilt *et al.*, 1995). Two different experiments have demonstrated that both pathways may be involved. First, injection of myoinositol into lithium-treated mesomeres prevents them from differentiating into endoderm, apparently by relieving the block in recycling of IP_3 to inositol (Wilt *et al.*, 1995; B. Livingston, personal communication). Second, treatment of early embryos with phorbol esters, apparently via the overstimulation of the PKC pathway, results in overproduction of endoderm and skeleton (Livingston and Wilt, 1992; the reasons for the latter are accounted for via the "conversion" process; see below). Such experiments indicate how intracellular signaling may be involved in regulating very early interactions between blastomeres, but it cannot address later signaling events that may occur. In an attempt to address such questions, Cameron *et al.* (1993) have injected mammalian serotonin receptor mRNAs into early sea urchin embryos. By adding serotonin at various times during development, they have attempted to stimulate signal transduction pathways that may be usually employed by endogenous receptors and ligands involved in establishing patterning sites. When stimulated with exogenous serotonin, injected embryos display a range of defects. In one

Fig. 33 Production of oral structures in microsurgically isolated fragments. (a) Normal oral hood, including mouth and parallel skeletal elements. (b) Animal cap isolated at the early gastrula stage. Note the stomodeum (arrow). (c) Corresponding remaining tissue forms virtually all structures, except for a mouth (c) and correctly positioned oral rods (d). (e) When PMCs are transplanted into an animal cap isolated at this time, parallel rods flanking the stomodeum form. (f) When the animal cap is removed at the mesenchyme blastula stage, the vegetal fragment regulates to produce a mouth. (J. Hardin and N. Armstrong, manuscript in preparation).

class of defective embryos, the skeletal rods are radialized, despite apparently normal distributions of oral and aboral ectoderm (Cameron *et al.*, 1993). It is possible that signal transduction events required for differentiation of ventrolateral ectodermal patterning sites have been disrupted in these experiments.

C. Cell Interactions between Mesenchyme Cells

As a result of experiments performed by Fukushi (1962) and, systematically, by Ettensohn and colleagues (Ettensohn and McClay, 1988; Ettensohn, 1990; reviewed by Ettensohn, 1992), it is clear that primary mesenchyme cells exert a remarkable repressive influence on a subpopulation of secondary mesenchyme cells as gastrulation proceeds. When all PMCs are removed from an embryo, a number of SMCs migrate away from the tip of the archenteron to the ventrolateral ectoderm and other normal sites of PMC localization, and become spiculogenic mesenchyme (Ettensohn and McClay, 1988; Fig. 34). This "conversion" response involves the expression of PMC-specific genes (Ettensohn and McClay, 1988; Guss and Ettensohn, 1994), and the converted cells appear indistinguishable from normal PMCs, with the exception that skeleton production is delayed by several hours, compared to control embryos (Ettensohn and McClay, 1988). The converted cells appear to respond to pattern information in the external environment in a manner identical to normal PMCs in normal embryos (Ettensohn and McClay, 1988) and in experimentally perturbed embryos (Hardin *et al.*, 1992).

The specific means by which this repressive interaction is mediated is not known; however, a number of specific features of the interaction have been characterized. First, the replacement of missing PMCs by SMCs is quantitative; when a portion of the PMCs are removed, an essentially equivalent number of SMCs converts (Ettensohn and McClay, 1988). Second, the period during which SMCs can convert is limited in duration. By replacing the normal complement of PMCs by an equivalent number of rhodamine-labeled PMCs, Ettensohn was able to photoablate PMCs at various times during development and then assay for the ability of such embryos to display the conversion response. The period during which conversion can occur ends roughly at the time the archenteron makes contact with the animal pole at the end of gastrulation (Ettensohn, 1990). Third, there is some evidence that the interaction may require physical contact between the two populations of mesenchyme. High-resolution videomicroscopy indicates that at least some PMCs and SMCs make contact with one another via filopodia. Furthermore, SMCs appear to withdraw filopodia when they are contacted by PMCs (Ettensohn, 1992). When PMCs are prevented from migrating by injection of wheat germ agglutinin into the blastocoel, or when the blastocoel is inflated using sucrose, conversion occurs. In both cases, PMCs are physically prevented from making contact with SMCs (S. Ruffins and C. Ettensohn, personal commu-

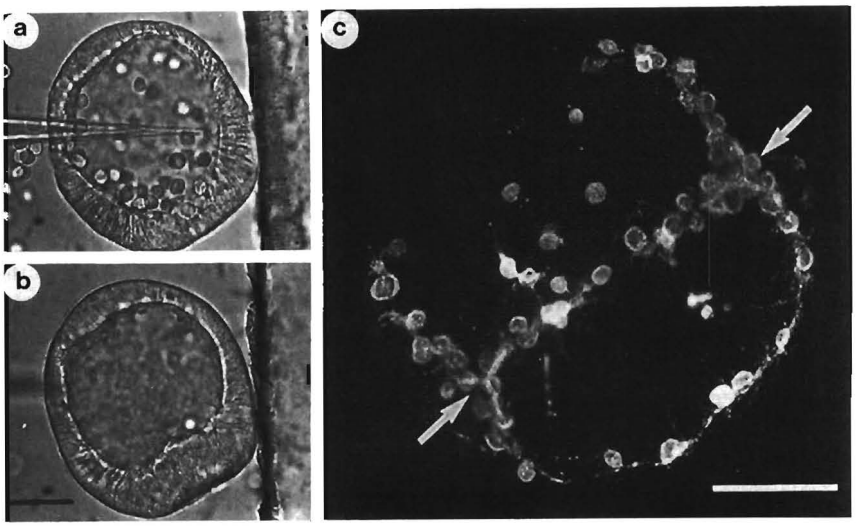

Fig. 34 "Conversion" of secondary mesenchyme cells. (a) PMCs are removed by flushing the blastocoel with a micropipet. (b) The same embryo after removal is complete. (c) SMCs convert and adopt the spiculogenic program of differentiation, including expression of PMC-specific cell surface proteins (in this case, the msp130 protein), as well as secrete skeletal elements (arrows). (a, b) From Ettensohn and McClay (1988); (c) from Ettensohn (1992), with permission.

nication). However, the experiments performed to date do not rule out short-range interactions involving substrate-bound or diffusible molecules (reviewed in Ettensohn, 1992). Recently, Ettensohn and Ruffins (1993) have shown that of the various derivatives of SMCs, only pigment cells show decreased numbers in PMC-depleted embryos, implying that the converted cells come from a population of presumptive pigment cells.

The conversion response also appears to account for several other phenomena observed in experiments aimed at elucidating cell–cell interactions along the animal–vegetal axis. Hörstadius performed micromere removal experiments and made anecdotal reference to SMCs migrating away from the tip of the archenteron to form the skeleton (Hörstadius, 1973). More recent micromere removal experiments in *Dendraster* and *S. purpuratus* suggested the same thing (Langelan and Whiteley, 1985; Ransick and Davidson, 1995). Isolated macromeres and half-macromeres produce small spicule rudiments yet have no PMCs (Hörstadius, 1936), so presumably skeletogenic cells are produced via conversion in this case as well. Finally, treatment of embryos (Livingston and Wilt, 1992) or cultured cells (M. Truschel, pers. comm.) with vegetalizing agents such as lithium chloride or phorbol esters results in overproduction of spiculogenic cells, apparently because some SMCs are no longer capable of responding to repressive signals from PMCs.

D. Cell Interactions Regulating Invagination

Little is known about regulatory interactions within the archenteron as it invaginates. Following the demonstration by Driesch (1891) that dwarf larvae could arise from single blastomeres at the two- and four-cell stages, Morgan (1895a) and later Driesch himself (1900a,b) investigated their morphogenesis. Although dwarves contained one-half or one-quarter the normal complement of cells, they nevertheless completed gastrulation successfully. Such behavior implies that regulation of target information for mesenchyme as well as overall aspects of gastrulation must be under regulative control, but aside from this, little else can be said. Isolation experiments indicate that the cells that give rise to the archenteron display considerable autonomy regarding the "program" leading up to invagination. Isolated macromeres, which normally generate anal ectoderm and one-quarter of the archenteron, form embryoids in which an archenteron successfully invaginates (Hörstadius, 1936). Although macromeres do not usually give rise to spiculogenic cells, isolated macromeres produce a small tiradiate spicule. This presumably results from "conversion" of presumptive secondary mesenchyme cells (see above). Similarly, isolated veg_2 quartets form gut-like structures (Hörstadius, 1939).

The autonomous ability of endoderm to differentiate and engage in morphogenesis has been studied by several investigators. Isolated vegetal plates do not continue morphogenesis beyond primary invagination (Ettensohn, 1984); however, this may represent inadequate culture conditions. Endoderm cells transplanted ectopically into the blastocoel of an intact host embryo appear to display some protrusive activity, although the purity of the transplanted cells was difficult to assess in the one reported study (Fukushi, 1962). Control of the timing of gastrulation was investigated by Morgan (1895b), who generated fused double embryos by shaking eggs together. Although such fused twins had confluent blastocoels, two invaginations usually formed. In addition, the invaginations were often initiated at different times, suggesting that despite the confluent blastocoel, the timing of gastrulation is an inherent property of the constituent embryos in the twin. This conjecture was confirmed more rigorously in the case of starfish embryos by Mita and Sato (1982). Similar results are obtained in heterchronic chimeras; implanted macromere descendants invaginate on an apparently autonomous schedule (J. Hardin, unpublished observations). Isolations performed at the gastrula stage suggest that further restrictions in cell fate occur as gastrulation proceeds. Pieces of gastrulae cut with fine scissors undergo morphogenesis in what appears to be a regionally specific manner; in those cases in which the vegetal plate is bisected and allowed to heal, a bifurcated archenteron results (Jenkinson, 1911). Logan and McClay (1994) removed the archenteron from an otherwise intact *L. variegatus* embryo, resulting in the formation of a new archenteron by the ectoderm adjacent to the removed tissue. The differences from Jenkinson's results may be accounted for by inclusion of substantial amounts of ectoderm by Logan and McClay.

VI. Phylogenetic Variation, Reliability, and Variability during Gastrulation

A. Apparent Heterotopies during Archenteron Elongation

Based on the experiments and observations described above, the shape of the gastrula can have a significant effect on cell behaviors during gastrulation. Gustafson suggested that this might be the case in *Psammechinus miliaris*, in which he proposed that the shape of the embryo might restrict attachment of secondary mesenchyme cells to the dorsal ectoderm (Gustafson, 1964). The analysis of gastrulation in *L. variegatus* presented above (Hardin and McClay, 1990) likewise suggests that in some cases a cell is incapable of completing its normal function during gastrulation without the simultaneous performance of other morphogenetic functions by other cells. An example of this sort of limitation is the interplay between filopodial exploration and autonomous extension of the archenteron which appears to operate in *L. variegatus* and *L. pictus* (Hardin, 1988; Hardin and McClay, 1990). The combination of a small set of relatively simple cell behaviors (e.g., autonomous cell rearrangement, random exploration by filopodia, and target recognition) allows gastrulation to be completed successfully despite variations in embryonic shape and the positions of interacting tissues in different species.

Variability during gastrulation in several sea urchin species can be accounted for by differences in embryonic shape and target placement (Hardin and McClay, 1990; Fig. 35). These differences, in turn, appear to modulate the simple cell behaviors outlined above. Modes of archenteron elongation include "central elongation," in which the archenteron is equidistant from all lateral ectodermal surfaces (e.g., *L. variegatus, L. pictus*), "dorsal crawling," in which the dorsal ectoderm is near the tip of the archenteron (*P. miliaris, Echinus microtuberculatus*), and "ventral crawling," in which the ventral side is closer (e.g., *S. purpuratus*). In other species, such as the cidaroid, *Eucidaris tribuloides*, and the Japanese sand dollar, *C. japonicus*, filopodia do not reach the animal pole, apparently because in these species the animal pole is simply out of reach (Okazaki, 1975; Hardin, 1989). In *Eucidaris*,when the animal pole is dented down, filopodia make transient contacts with the animal pole within 2–5 min, but they never make any stable contacts of the kind seen in *L. variegatus* (J. Hardin, unpublished observations). In these species, filopodia extend directly toward the stomodeal invagination (Schroeder, 1981); in this respect, they resemble starfish gastrulae (cf. Crawford and Abed, 1983), and so this mode of gastrulation has been termed "central elongation/pause/oral contact" (Hardin and McClay, 1990). In all of the species examined, the simple cell behaviors that are responsible for archenteron elongation and attachment appear to be flexible enough to permit phylogenetic variations in embryonic shape. Phylogenetic variations point out another salient feature of a cellular analysis of gastrulation: cells

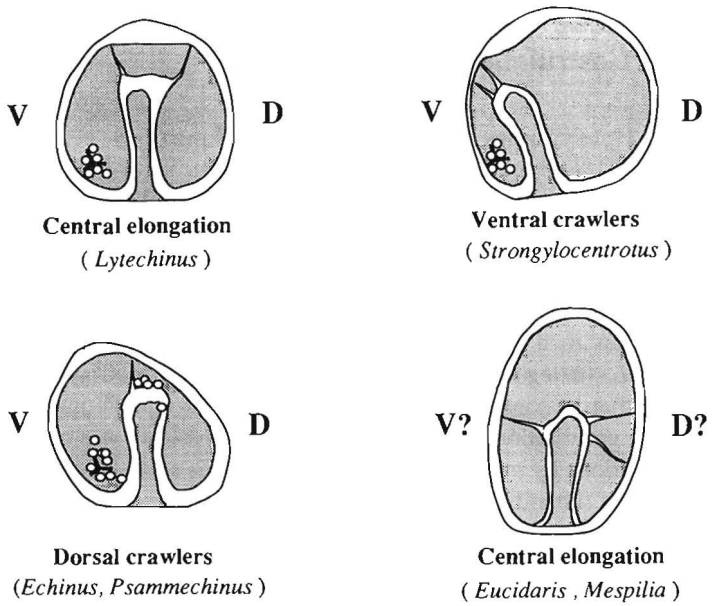

Fig. 35 Different modes of gastrulation revealed by a comparison of a number of species of sea urchin and sand dollar (from Hardin and McClay, 1990, with permission). For further description, see the text.

with the same fate may play different *morphogenetic* roles in different species. As an example, secondary mesenchyme cells seem to be important mechanically in many euechinoid species, yet in *Eucidaris* they have little or no mechanical role during gastrulation (Hardin, 1989). Despite this difference in morphogenetic function, molecular markers indicate that these cells give rise to similar, differentiated mesenchymal populations in euechinoids and *Eucidaris* (Wray and McClay, 1989).

B. Phylogenetic Variations Involving Spiculogenic Mesenchyme

In addition to variations in embryonic shape, there are also variations in the timing of developmental events with respect to one another (*heterochronies*; Wray and Raff, 1990). Events that are temporally correlated in one species but functionally independent might be expected to display heterochrony when the same processes are analyzed in other species. A good example is the ingression of spiculogenic cells. Embryos in which micromeres have been removed (Hörstadius, 1973; see above) do not produce PMCs; hence, PMC ingression is dissociable from invagination of the archenteron. Spicule-producing cells ingress at the mesen-

chyme blastula stage in many sea urchin embryos, but in *Eucidaris*, ingression of spiculogenic cells occurs many hours after invagination of the archenteron has begun (Wray and McClay, 1988). Interspecific transplantations have been performed to study the phylogenetic conservation of regulatory mechanisms involving these cells as well. Interspecific transplantation of PMCs from closely related species shows that they can recognize the same cues for localization to the vegetal plate and ventrolateral clusters when placed into a heterospecific environment. However, these abilities are lost when the species are too distantly related phylogenetically (Ettensohn, 1991) (Ettensohn, 1991). Armstrong and McClay (1994) have shown that PMCs in a heterospecific environment participate in skeletal formation in heterospecific hosts, but make short segments of skeleton, with the fenestrations typical of their species of origin (see above).

C. Radical Alterations in Gastrulation in Species with Direct Development

Sea urchin embryos are a good system in which to study ontogenetic conservation and flexibility during early development. The *Echionoidea* have an extensive adult and larval fossil record (reviewed in Raff, 1992a,b), and the many extant members of this class provide numerous opportunities for comparative studies at the morphological and molecular levels. Despite the high degree of conservation of the pluteus larva among typical indirect developing species, radical alterations in early development have occurred among those species with either direct development or a nonfeeding larval stage (reviewed in Raff, 1992a,b). In the case of the Australian genus *Heliocidaris*, indirect- and direct-developing species are congeneric, yet the early development of the two species is radically different. *Heliocidaris tuberculata* develops from a small egg (~100 μm) via a typical feeding pluteus. *H. erythrogramma*, on the other hand, has dispensed with a feeding larva altogether; its large eggs develop rapidly into a juvenile urchin (Fig. 36). This then raises the question as to how species with such radically different modes of early development can converge onto the same adult body plan. In one view, direct development is a novel mode of development in part generated by the initial suppression of the patterns of development found in indirect developers (Raff, 1992). One way for this initial suppression to take place may involve oogenesis; indirect developers typically have eggs less than 150 μm in diameter, whereas direct developers have eggs 300–1500 μm in diameter (Raff, 1987, 1992a,b). Species with eggs of ~300 μm seems to be on the threshold; in one instance, their larvae have been demonstrated to be facultative feeders (Emlet, 1986).

In addition to its large size, the embryos of *H. erythrogramma* are quite different from those of the typical *H. tuberculata*. Rather than the smooth, hollow blastula typical of indirect developers, *H. erythrogramma* produces a

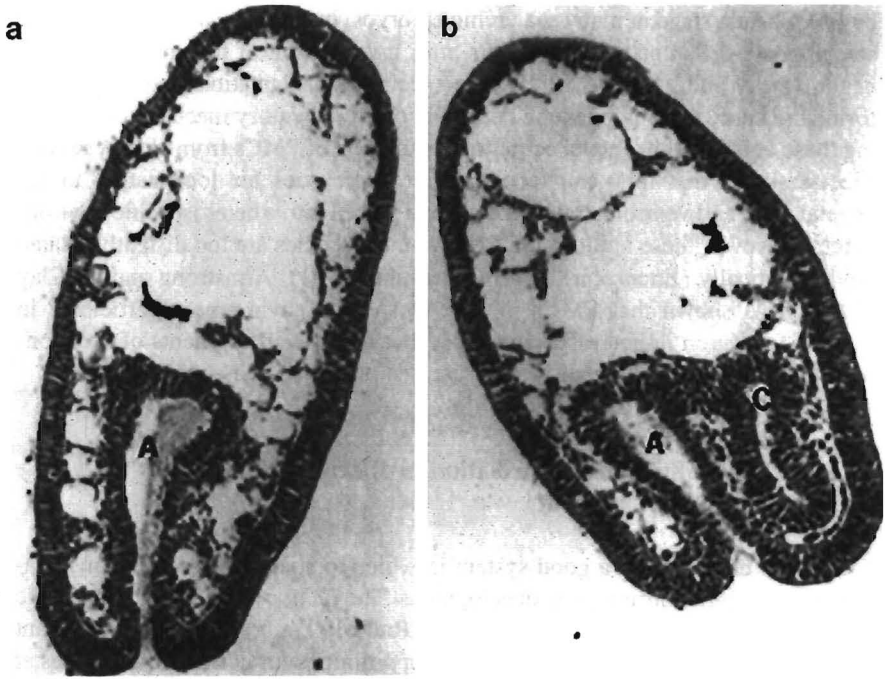

Fig. 36 Gastrulation in the direct-developing species, *Heliocidaris erythrogramma*. (a) Late gastrula. (b) Coelom formation. A, archenteron; C, coleomic rudiment. From Raff (1992), with permission.

wrinkled blastula (Raff, 1992a,b), which is characteristic of echinoderms with large eggs and of a few with smaller eggs (Dan and Inaba, 1968). Maternal localization of developmental potential, especially the dorsoventral axis, has been modified (Henry *et al.*, 1990; Jeffery, 1992); initially, cleavages are radial but equal, with no visible asymmetry in the vegetal hemisphere, as is true with indirect developers (Wray and Raff, 1990). Lineage founder cell relationships have also been pervasively altered (Wray and Raff, 1990; Fig. 37). Much of the postgastrula development devoted to production of the pluteus has been altered or deleted (reviewed in Raff, 1992a,b).

Not surprisingly, gastrulation has also been extensively modified in this species. The number of ingressing mesenchyme cells and the number and origin of cells that generate the archenteron have been drastically altered. In contrast to indirect developers, gastrulation in this species is immediately followed by coelom formation in *H. erythrogramma*; the coleomic tissue ultimately generates the mesodermal structures of the hydrocoel, which, together with the vestibular ectoderm on the surface of the larva, forms much of the juvenile urchin. The recruitment of large numbers of cells from the tip of the archenteron to coelom

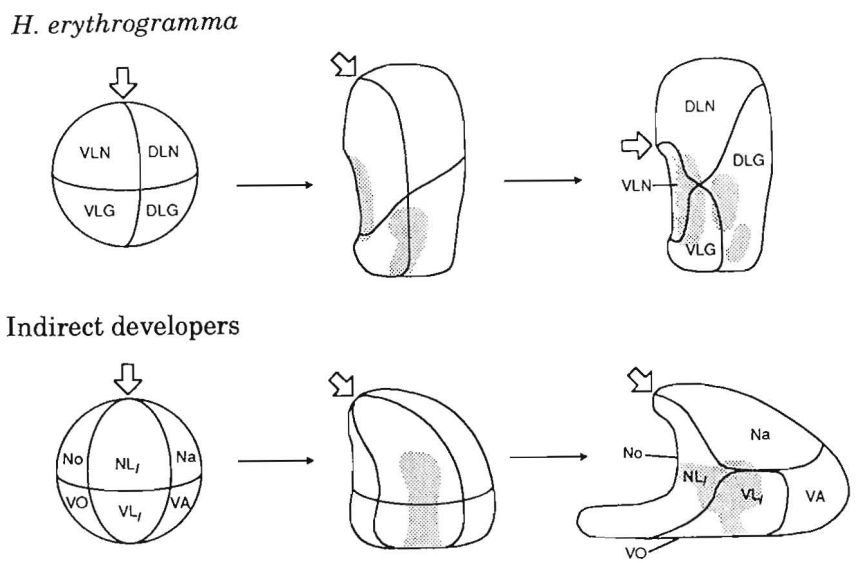

Fig. 37 Alterations in the ectodermal fate map in direct developers. A consensus ectodermal fate map is shown for indirect-developing species (largely derived from the work of Cameron and colleagues) and compared with that for *H. erythrogramma*. Notations for *H. erythrogramma*: VLN, ventral, left, animal; VLG, ventral, left, vegetal; DLN, dorsal, left, animal; DLG, dorsal, left, vegetal. Adapted from Wray and Raff (1990), with permission.

production is in distinct contrast to the case in indirect developers, in which only a small number of cells contribute to the left and right coelomic pouches (Pehrson and Cohen, 1986; Cameron *et al.*, 1991). Extensive distortions of the ectodermal fate map also occur (Wray and Raff, 1990; Fig. 37).

In contrast to the typical mode of gastrulation described above, involving an initial invagination accompanied by some involution and a second phase in which the archenteron elongates with little or no additional involution, gastrulation in *H. erythrogramma* involves two phases of involution, based on lineage tracer injections into single blastomeres at the 2-cell stage (Wray and Raff, 1991). In the first phase, involution is symmetric, as it is during primary invagination in direct developers (however, it should be pointed out that there are 20,000 cells in the *H. erythrogramma* gastrula, as opposed to roughly 1000 in indirect developers). In the second phase, extensive asymmetric involution occurs (Wray and Raff, 1991; Fig. 38). The second phase of involution in *H. erythrogramma* appears to be required to produce the tissue mass needed for production of the large coelomic rudiment. The mechanisms by which such bulk tissue movements are generated at the cellular level are unknown. This single well-studied example points out in stark fashion the surprising flexibility possible

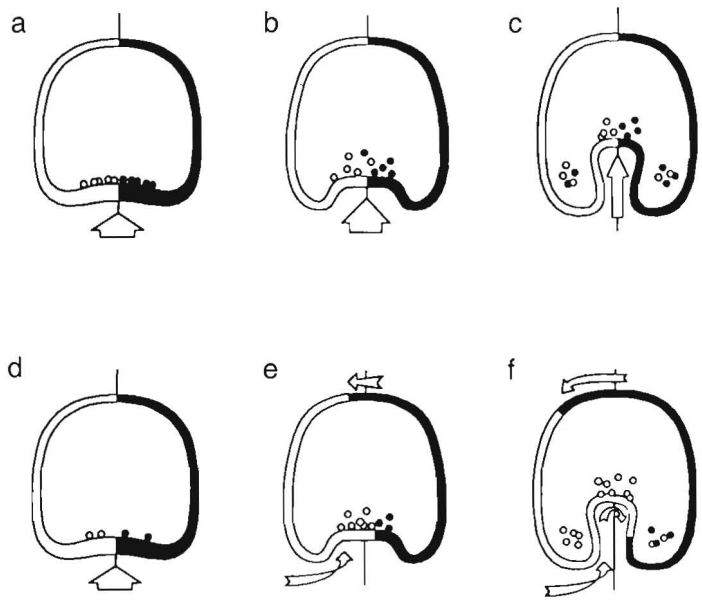

Fig. 38 A comparison of involution during gastrulation in indirect and direct developers. (a–c) Involution in indirect developers is symmetric and not particularly pronounced. (d–f) In *H. erythrogramma*, extensive, asymmetric involution occurs as the archenteron deepens. From Wray and Raff (1991), with permission.

in mechanisms of gastrulation. Among amphibians, similar radical changes seem to have occurred in the embryos of "marsupial" frogs, in which gastrulation occurs within a blastodisc, rather than within the spherical embryo characteristic of many amphibians (del Pino and Elinson, 1983; reviewed by Keller, 1986).

VII. Conclusion—Prospects for the Future

The foregoing discussion has attempted to review what is known and still unknown about the cellular mechanisms of morphogenesis in the sea urchin gastrula. Several salient features of such an analysis emerge. First, mechanical interactions are significant during gastrulation. Forces capable of shaping the embryo may be produced by single cells (e.g., secondary mesenchyme cells) or groups of cells (e.g., rearranging cells in the wall of the archenteron). Understanding the response of other cells to such forces must also be a part of the analysis of gastrulation (e.g., the deformation of the archenteron during secondary invagina-

tion). Our current understanding of the biomechanics of embryonic tissues is rudimentary at best; recently, however, there have been attempts to address this issue (Adams *et al.*, 1990; Koehl, 1990; Oster and Weliky, 1990; Moore and Keller, pers. commun.). How stress is transmitted through an embryonic tissue, whether cells in embryonic epithelia can indeed respond to applied stress by rearranging, and what mechanical role extracellular matrices play in influencing the composite mechanical behavior of epithelia are questions which may be answered in simple systems such as the sea urchin embryo.

Second, behaviors exhibited by a cell may serve multiple functions during gastrulation. A clear example of such multiplicity is the role played by secondary mesenchyme cells. Their mechanical influence seems to be important for elongating the archenteron, but they are also required for correct attachment and positioning of the tip of the archenteron. Both consequences of SMC activity are mediated by a single basic behavior: cyclical extension, attachment, and shortening of filopodia.

Third, an analysis of cells *as populations* is necessary for understanding gastrulation. For example, cell rearrangement events, by definition, necessitate studying more than one cell at a time; it seems equally clear that coordinated morphogenetic movements cannot be fully understood by reducing them to the study of single cells. As we have also seen, specific interactions between cells in time and space are important. This point has been recognized as crucial in the case of inductive interactions during early vertebrate development (e.g., reviewed in Kimelman *et al.*, 1992; Gurdon, 1992; Sive, 1993), but it is also an important factor during morphogenetic movements. During sea urchin gastrulation, the proximity of various regions of the embryo to the archenteron affects the function of secondary mesenchyme cells and the sort of mechanical contribution they can make to elongation of the archenteron. Such context-sensitive behavior is not restricted to sea urchin gastrulation. For example, Keller and colleagues have shown that the various region-specific behaviors at work during *Xenopus* gastrulation must operate in a temporally and spatially coordinated manner to complete gastrulation (Keller, 1986; Keller and Winklbauer, 1992; Keller *et al.*, 1992). These sorts of sequential and hierarchical interactions are the hallmarks of the process of gastrulation.

Clarifying the role(s) that specific molecules play in morphogenetic movements is one of the great achievements of modern biology. However, while the interactions of specific proteins certainly underlie morphogenesis, it is also clear that properties of tissues are not always easily predicted from molecular data. As our understanding of the cellular interactions during gastrulation improves, it will be possible to ask increasingly specific questions about the molecular mechanisms underlying morphogenesis. As molecular analyses gain sharper focus, they will in turn lead to an even deeper understanding of the cellular basis of gastrulation.

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NOTE ADDED IN PROOF. Miller *et al.* (1995) used high resolution Nomarski imaging to examine thin filopodia (0.2–0.4 μm in diameter) extending from primary mesenchyme cells. The thin filopodia can extend to more than 80 μm in length, and appear to be highly dynamic, rapidly changing from extension to resorption. Miller *et al.* suggest that these filopodia mediate cell–cell interactions between PMCs and other tissues. Nickel treatment alters the normal position-dependant differences in the thin filopodia, and reciprocal transplants like those done by Armstrong *et al.* (1993) indicate that the ectodermal environment is mainly responsible for influencing filopodial length. It is possible that the main function of such protrusions, rather than producing motive force, is to mediate signal transduction between PMCs and patterned ectoderm. Additonal studies will be required to test this hypothesis experimentally.

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