

2. Archenteron Morphogenesis in the Sea Urchin

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INTRODUCTION

The progression of development involves an impressive array of morphogenetic rearrangements, each of which involves the coordination of multiple cellular functions and molecular events. We have been studying gastrulation in the sea urchin embryo as a relatively simple model system in an attempt to understand the sorts of rules observed by an embryo as it performs a single morphogenetic event. To the observer, gastrulation in this embryo involves two major cell movements. First, primary mesenchyme cells ingress and display a series of migratory behaviors leading to the assembly of the larval skeleton. Second, invagination of the archenteron leads to the formation of the primitive gut. The ingression and subsequent behavior of primary mesenchyme cells is examined elsewhere in this volume (Ettensohn, Chapter 11). Here we review events associated with formation of the archenteron.

In echinoderm embryos, archenteron formation begins with an indentation of the vegetal plate, followed by elongation of the indented area until a tubular archenteron forms that extends into the blastocoel. Elongation continues until the tube reaches a defined, anatomically specific region on the wall of the blastocoel (Fig. 1). All through invagination, secondary mesenchyme cells at the tip of the archenteron extend filopodia that make contact with the wall of the blastocoel. This behavior of secondary mesenchyme cells continues until final contact is made with the anatomical target for archenteron extension. Later, the stomodeum forms, usually just ventral to the site of attachment of the archenteron.

More than twenty years ago, Gustafson and Wolpert (1963, 1967) published extensive reviews based on their time-lapse observations. These provide a descriptive background against which experiments can be performed. They also proposed a number of models suggesting how invagination might work. They proposed, for example, that the initial inward bending of the vegetal plate could be brought about by a cell shape change. The cells at the vegetal plate can be observed to change shape from cuboidal to columnar, and then

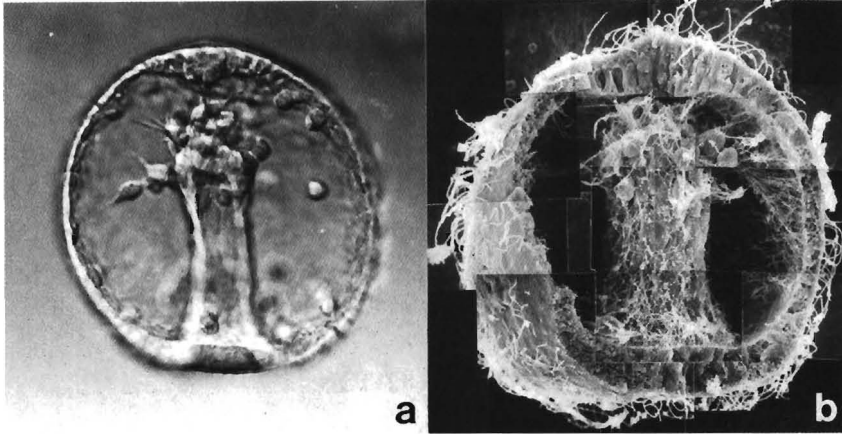


Fig. 1. Archenteron invagination in the sea urchin embryo. Cells of the endoderm rearrange as the archenteron extends from the vegetal plate toward the animal pole region. (a) In a micrograph using Nomarski optics, numerous filopodia can be seen to be extending from secondary mesenchyme cells at the tip of the archenteron before it reaches its target near the animal pole. (b) A scanning electron micrograph of an embryo at a similar stage to that shown in a. Numerous extracellular matrix fibers can be seen around the archenteron and lining the blastocoel wall.

some cells become keystone-shaped in profile (Fig. 2). Coincident with these shape changes, the vegetal plate begins to bend inward. Mechanistically, the shape change could be driven intrinsically by the cells of the vegetal plate, or there could be external forces imposing the cell shape changes. One intrinsic mechanism that has been proposed is apical constriction of cells (Rhumbler, 1902). Gustafson and Wolpert (1963, 1967) pointed out that changes in adhesion of cells in the vegetal plate could be the mechanism leading to the apical constriction; if cells lost adhesive affinity for the hyaline layer and gained affinity for the lateral surfaces of neighboring cells, the keystone shape might result. Alternatively, apical constriction was hypothesized to result from contraction of circumapical microfilament bundles during amphibian neurulation by Burnside (1971) and Baker and Schroeder (1967; see reviews by Ettensohn, 1985b; Fristrom, 1988 for further discussion). Later, stretch-activated constriction of apical microfilaments was presented as a model for the initial invagination of the archenteron in the sea urchin by Odell et al. (1981). The major requirement for these models is that the cell shape changes should be driven locally. Moore and Burt (1939) and later Ettensohn (1984) showed that isolated vegetal plates would invaginate on their own. This meant that global forces from the remaining embryo, or from sort of negative pressure within the blastocoel, were not crucial for invagination. In addition, the vital staining experiments of Hörstadius (1935) showed that there is no large global epibolous

movement of cells toward the vegetal plate as it bends inward. When the veg_2 layer of cells was stained, it was found that only the veg_2 layer contributed to the vegetal plate, and cells lateral to the inbending region do not move substantially toward it. These marking experiments were later refined by Etensohn (1984) to show that while there is some movement of marked regions of the vegetal plate during the early phase of invagination, only the vegetal plate contributes to the archenteron. Furthermore, dye marking and time-lapse videomicroscopy indicate that there is no involution during the subsequent elongation of the archenteron (Hardin, 1989). Thus, the forces of invagination are located near the vegetal plate and only cells of the vegetal plate contribute to the archenteron. Does this indicate that the forces for invagination are generated within each cell? Perhaps, although there has been no formal test of that hypothesis. Furthermore, it is not known what those forces might be. Are they cytoskeletal changes? Or, as suggested by Gustafson and Wolpert, are there changes in cell adhesion? Beyond these unanswered questions about the mechanism of invagination itself, there is no understanding of the controlling influences that help to initiate the invagination. Invagination begins after the primary mesenchyme cells (PMCs) have ingressed in most species, though removal of the PMCs from the blastocoel has no effect on the beginning of the invagination movements (Etensohn and McClay, 1988). Thus, while the descriptive information on the beginning phases of invagination is fairly complete, there are many local forces that are incompletely understood. Below, we discuss experiments that indicate a role for the synthesis of new embryonic proteins during gastrulation. This *de novo* synthesis shows that there is developmentally regulated, differential gene expression concurrent with gastrulation; however, the molecular regulatory apparatus that actually controls initiation of the inbending at the vegetal pole is completely unknown.

THE SIGNAL TO INVAGINATE

At the molecular level, recent studies have suggested that there are spatial and temporal regulatory molecules that activate genes at gastrulation (reviewed by Davidson, 1988, 1989, 1990). Presumably, DNA binding proteins and inductive signals are coordinated to activate the genes required for initiation of gastrulation. What are the signals and what genes are activated to initiate the process of invagination? One idea is that there is some kind of internal clock that directs presumptive endoderm cells at a certain time to begin their cell shape change (Spiegel and Spiegel, 1986). Such a clock might rely entirely upon *cis* regulatory elements that control entry into gastrulation as a result of certain rate constants intrinsic to the cells. In indirect support of such a model are the above experiments, in which only the presumptive vegetal plate region need be present for invagination to begin, implying localized signals. Other experiments, however, indicate that the timing model is probably incorrect

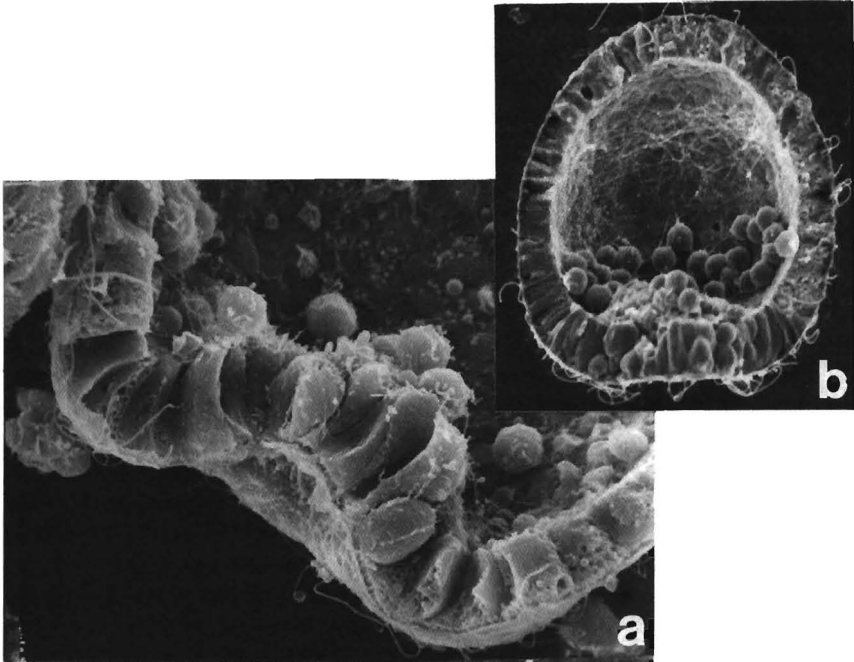


Fig. 2. (a) A scanning electron micrograph showing an embryo that has been split along a midsagittal plane. At the vegetal plate the early stages of invagination show a number of presumptive endoderm cells that have changed shape either causing or in response to the forces that initiate the invagination. (b) An embryo that has been cut lateral to the midsagittal plane to show the bulge in the vegetal plate and the primary mesenchyme cells surrounding the indentation and lining the blastocoel wall.

and that epigenetic signals are necessary to launch the invagination program. If one disrupts the interaction of cells with the hyaline layer (Adelson and Humphreys, 1988), or with the basal lamina (Wessel and McClay, 1987; Butler et al., 1987), gastrulation is totally blocked. In both cases, the blockage is not permanent since the effects are reversible. Once the block is removed, gastrulation begins and normal larvae result. These data indicate that timing cannot be an explanation since the embryos can be held at the mesenchyme blastula stage for extended periods of time and then allowed to continue with normal development. Instead, the data suggest that the embryos are responsive to epigenetic signals for continuation of development.

What is the block to gastrulation and what does it tell us about the putative epigenetic signals that appear to be necessary for gastrulation? Presumptive endoderm cells adhere to at least two hyaline layer proteins, hyalin (McClay and Fink, 1982; Fink and McClay, 1985) and echinonectin (Alliegro et al.,

1988; Burdsal et al., 1991). A monoclonal antibody to hyalin was produced that blocked the interaction of cells to hyalin substrates (Adelson and Humphreys, 1988). When the antibody was added to embryos at low concentrations (5–10 $\mu\text{g/ml}$), invagination movements failed to occur. Blastomeres pulled away from the hyalin layer and the embryos appeared to shrink. By a number of criteria, the cells continued to metabolize and many genes continued to be expressed at the pregastrula levels (Adelson and Humphreys, 1988). The inhibitory effect could be reversed if the antibody were washed out of the culture medium.

While inhibition of gastrulation by blocking cell adhesion to hyalin was dramatic, a second kind of inhibition suggests the epigenetic signal is not specific to the cell–hyalin interaction. Treatment of embryos with β -aminopropionitrile (β APN) also inhibits gastrulation (Wessel and McClay, 1987; Butler et al., 1987). This lathrytic agent inhibits lysyl oxidase, the enzyme required for cross-linking of collagen. It was found that embryos would grow to the mesenchyme blastula stage in the presence of the β APN, but no further. If left in the drug, the embryos remained at the mesenchyme blastula stage (Fig. 3), and continued to express all genes measured, including collagen, at control mesenchyme blastula levels. If the embryos were removed from the drug, they resumed development to normal pluteus larvae. New transcription of endodermal genes failed to occur until the drug was removed. Of importance, embryos could be held in the arrested state for long periods of time (Fig. 3), yet when released they progressed through development normally from that point onward. The β APN treatment was shown to block the cross-linking of collagen, and other basal lamina proteins also failed to be assembled into the basal lamina during the treatment. These data indicate that an intact basal lamina is somehow important for initiation of gastrulation. From other studies it was known that cells of the mesenchyme blastula adhere to the basal lamina (Fink and McClay, 1985; Katow and Solursh, 1981; Solursh, 1986), but it is not known whether the lack of adhesion directly, or some other signal indirectly dependent upon adhesion, is the critical element missing from the blocked embryos. An interaction with the basal lamina might be instructive, or, more likely, simply permissive for further development. Whatever the reason for the inhibition of gastrulation by β APN, or for that matter with other inhibitors such as xylosides (Solursh et al., 1986; Lane and Solursh, 1988), the reversibility of the treatment indicates that a critical epigenetic event occurs at the beginning of gastrulation. Proper assembly of the basal lamina or the hyalin layer can be blocked at any stage of development up to gastrulation without any noticeable effect. Data have indicated that the sensitivity to these reagents exists for only about 2 hr. Addition of the reagents before the mesenchyme blastula stage has no effect until mesenchyme blastula stage, or, if added after the brief period of high sensitivity, the reagents have a reduced effect on further development (Fig. 3).

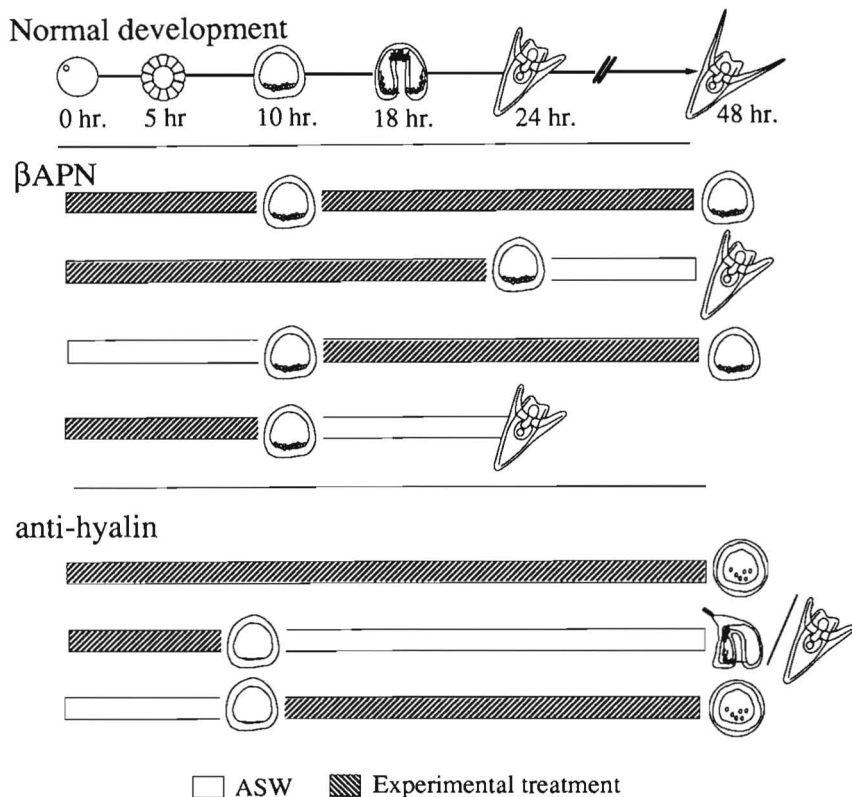


Fig. 3. Diagram summarizing treatments with β -aminopropionitrile (β APN) and antibody to hyalin. At the top, a time line shows several stages in development. The bars indicate embryos that were incubated in the presence (hash marks) or absence (open bar) of β APN or antihyalin. When left in β APN, the embryos reached the mesenchyme blastula stage on schedule but were arrested. The arrested behavior could be reversed (second bar) if the embryos were later washed in seawater without β APN. The critical period of sensitivity was at the mesenchyme blastula stage since embryos were arrested if β APN was added at the mesenchyme blastula stage (third bar), or they developed normally and on schedule if released from β APN at the mesenchyme blastula stage. Similar results were seen with antihyalin.

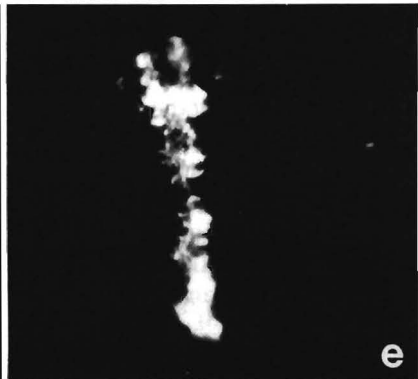
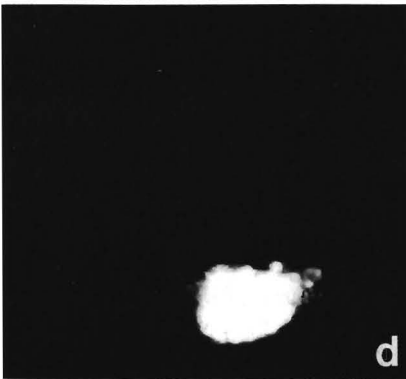
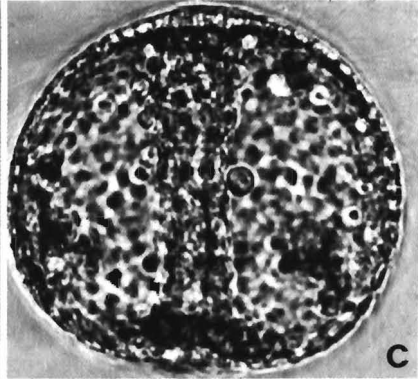
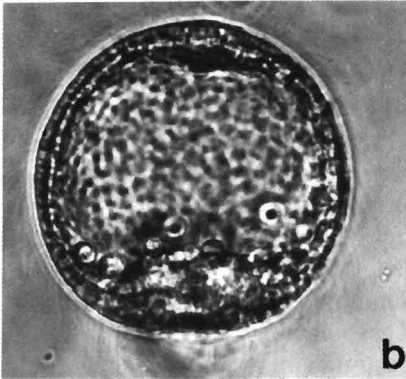
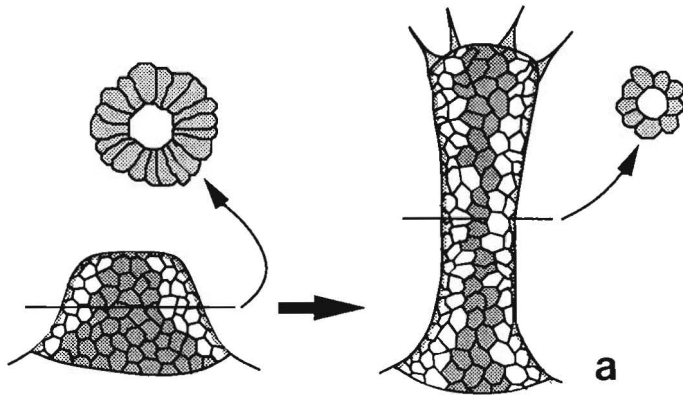
A number of marker proteins have been identified that are expressed in the endoderm at the beginning of gastrulation (McClay et al., 1983; Wessel and McClay, 1985; Wessel et al., 1989a; Nocente-McGrath et al., 1989). All appear to be dependent for their expression on the β APN-sensitive event. Among the genes expressed, presumably, are the genes required for the initiation of the invagination of the vegetal plate. Although the critical genes are not known, several properties of the invaginating tissue must be accounted for in the sequence of events that follow. First, the cells have apical-basal polarity (Schroeder,

1988; Nelson and McClay, 1988), and secrete matrix components toward both surfaces. The signal that launches the inward folding, if present inside each of the cells that change shape, must be responsive to that polarity. Second, as described below, the cells begin to rearrange within the plane of the cell sheet. This requires the onset of cell motility to drive the cell neighbor changes, and could require synthesis of new cell adhesion molecules. Third, directionality must somehow be present. The cells move in a net inward direction and so there must be some kind of signal imparted to the cell sheet that gives it that directionality. Finally, the movements must somehow be coordinated. Clearly, all of these processes are launched early in gastrulation, but the molecular identity of the components involved still are not known in any system.

DEEPENING THE INVAGINATION

The archenteron begins to elongate following the initial inward bending of the vegetal plate. The first half to two-thirds of the invagination are brought about largely by cell rearrangements described as "convergence and extension" in other morphogenetic systems (Fristrom, 1988; Keller, 1987) (Fig. 4a). Several kinds of experiments demonstrate that convergence-extension is a major mechanism of archenteron elongation. First, by counting cells in cross sections of the tube, it was observed that the number of cells in any given circumference steadily declined (Ettensohn, 1985a; Hardin and Cheng, 1986). Second, dye marking experiments showed that the elongation is local, i.e., there is no recruitment of cells from areas lateral to the site of invagination (Hardin, 1989). The only way to explain these observations is a rearrangement of the cells. Third, a patch of presumptive endoderm was marked with a fluorescent tag and observed throughout gastrulation. Cells in the patch shifted position relative to one another during gastrulation, resulting in a highly elongated patch of labeled cells (Fig. 4) (Hardin, 1989). Fourth, time-lapse films of the archenteron have detected shifts in cell position along the axis of elongation (Hardin, 1989). Finally, the one other major mechanism proposed for primary elongation of the archenteron, as suggested by Gustafson and Wolpert, was a pulling force due to traction of the secondary mesenchyme cells. The necessity for such traction is ruled out since exogastrulae extend archenterons without benefit of secondary mesenchyme pulling, and laser ablation experiments destroyed filopodia but the archenteron continued to extend until it reached up to two-thirds its final length (Hardin, 1988).

Cell adhesion changes also occur during the time of invagination as demonstrated by aggregation and sorting experiments (McClay et al., 1977; Bernacki and McClay, 1989). These changes readily show that endoderm cells develop the capacity to recognize one another relative to ectoderm and mesoderm. However, it is not known whether these experimentally observed adhesive changes might actually be important for the invagination process. The germ layers really



do not sort out from one another during normal invagination since the cells already are physically isolated from one another. The adhesion changes may reflect newly acquired properties that contribute to endodermal cell-cell rearrangements. In cell aggregates, endodermal cells quickly rearrange to form a tubular structure, demonstrating their tendency to interact with one another and to form a cell sheet one cell deep (Spiegel and Spiegel, 1975; Bernacki and McClay, 1989).

The most elusive property of gastrulation, and one of the most critical, is the directionality of invagination. If one examines the basal end of the endodermal cells during gastrulation, they can be seen to overlap adjacent cells in the direction of net movement of the archenteron (Hardin, 1989). How do the cells know which way is which? Apparently information along the animal-vegetal axis is all that is needed since the elongation continues when the dorsal-ventral axis has been destroyed (Hardin et al., in preparation). The signal probably is local (as opposed to global) since the archenteron continues to elongate in embryos that have had the animal hemisphere removed. This point of reference in space is an important element of all pattern-forming systems. Many experiments in the sea urchin embryo point to the existence of such directional signals; however, in this and in other embryonic systems, the identity of such signals remains obscure.

SECONDARY MESENCHYME CELL BEHAVIOR DURING ARCHENTERON FORMATION

Films of gastrulation show the dramatic sequence of filopodial extension and retraction exhibited by secondary mesenchyme cells (Fig. 5). Although it was shown experimentally that filopodial activity is not required for the first two-thirds of the extension of the archenteron (Hardin, 1988), completion of extension requires filopodia (Hardin, 1988, 1989). Indeed, toward the end of the extension process when filopodia finally reach the point at which the archenteron attaches to the wall of the blastocoel, one can observe a transient cell shape change in endodermal cells suggestive of a stretching in the direction of archenteron movement (Hardin, 1989). As indicated above, even though the filopodia are extended all through gastrulation, they apparently are not crucial until the last phases of the extension.

Fig. 4. Rearrangement of endoderm during archenteron elongation. (a) Diagrams of the cross section of the archenteron at an early and of a later stage of invagination. Many more cells line the lumen early in invagination compared with later. This observation enabled Etnensohn (1985a) to deduce that endoderm cells rearrange during invagination. (b,c) Phase micrographs of (d) and (e). A patch of presumptive endoderm was tagged with rhodamine isothiocyanate. Early in gastrulation (b,d) the patch was rounded and at the vegetal plate. Late in gastrulation (c,e) the patch had rearranged so that cells were found in a long thin linear array (Hardin, 1989), providing evidence for the cell rearrangement that occurs during invagination.

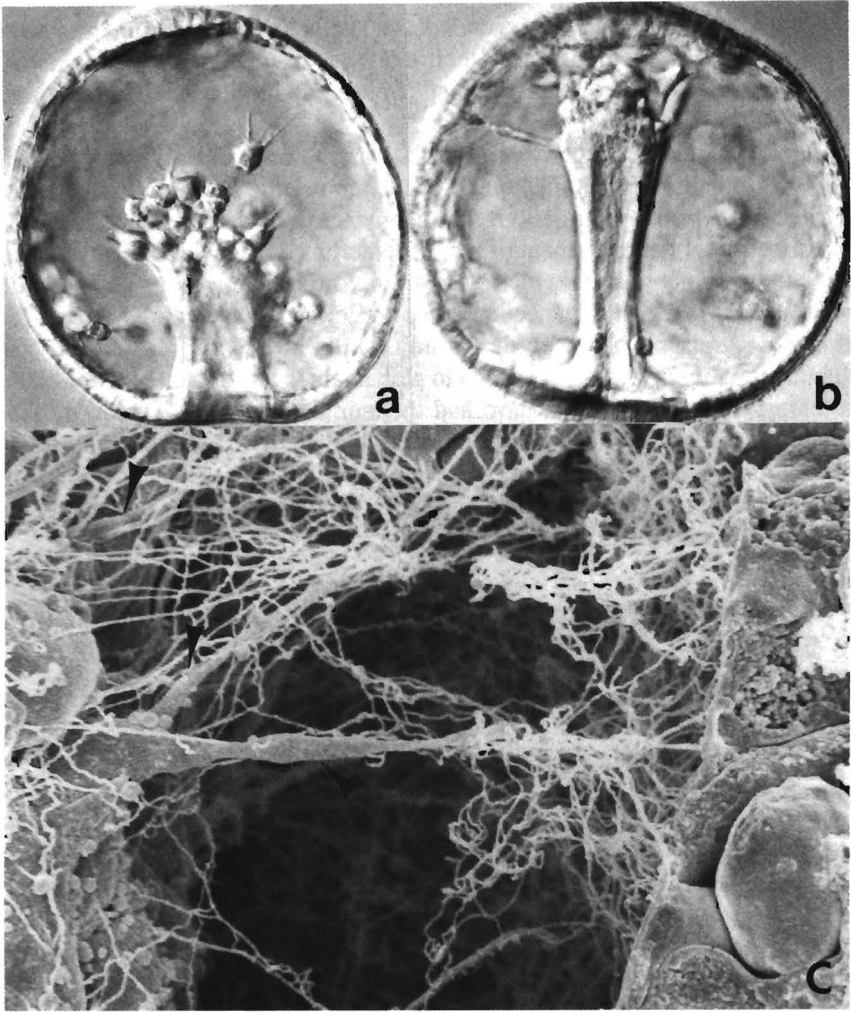


Fig. 5. Secondary mesenchyme cells extend filopodia for the duration of invagination. (a) A *Lytechinus pictus* midgastrula stage showing numerous filopodia. (b) A late gastrula embryo showing the secondary mesenchyme cells reaching the target, and one long filopodium at the left. (c) A scanning electron micrograph showing the extension of three filopodia (arrows) from the archenteron (at left) to the blastocoelic wall (at right). The tips of the filopodia are enmeshed in extracellular matrix fibers.

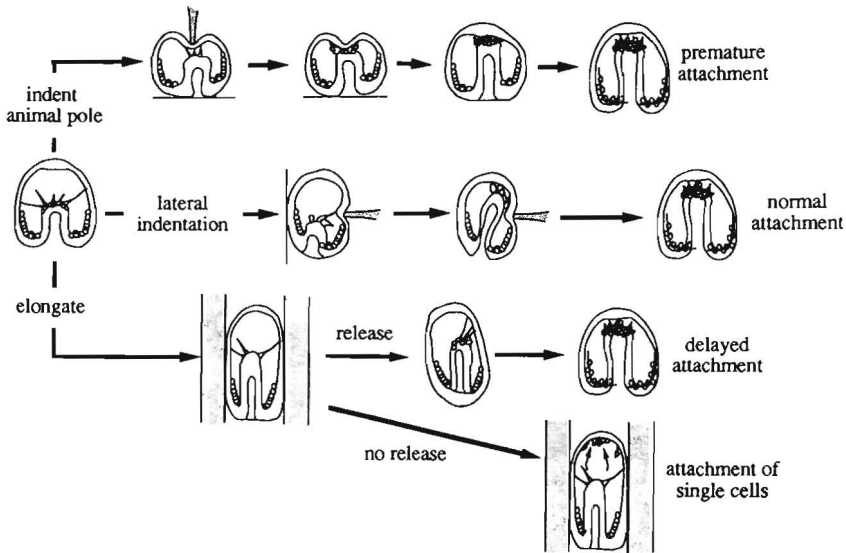


Fig. 6. Summary diagram of experiments demonstrating the existence of a target for archenteron extension (Hardin and McClay, 1990). If embryos are indented at the animal pole, there is a premature attachment to the target region. If the indentation is made laterally, the archenterons make contact but continue to move until the target region is reached. If the embryos are experimentally elongated, the archenteron fails to reach the target region, filopodial extension continues, and this behavior continues until embryos are released, allowing the archenteron to attach to its target region. Only when the target is reached do the secondary mesenchyme cells change their behavior by ending the repetitive extension of filopodia and assuming a flattened phenotype.

A curious characteristic of secondary mesenchyme cells was that many filopodia were extended without making contact with a substrate (Fig. 6) (Hardin and McClay, 1990). A careful study of filopodial behavior revealed that when contact was made with the wall of the blastocoel, the cells held that contact for intervals lasting from 2 to 10 min. If cells did not reach a substrate within about 35 μm , the filopodia were withdrawn. This behavior continued for the duration of gastrulation, until contact was made with a region of the blastocoel wall near the animal pole. When contact with this region was made, the behavior of secondary mesenchyme cells changed dramatically. The duration of contact was extended much beyond the 10 min maximum that was observed up until then, and in many cases the filopodia were never withdrawn from the animal pole region. In addition, the secondary mesenchyme cells changed phenotype and took on a more fibroblastic appearance.

Was there something special about contact with the animal pole region of the embryo that led to the behavior change? Or was the change in the secondary mesenchyme cells independent of a contact-mediated stimulus that might

exist in the animal pole region? The experiments that follow show that contact with a specific target region is necessary for the phenotypic change to occur. Comparative studies in other species, described below, also indicate that for each species there is an anatomical target for attachment of the archenteron. The experiments that reveal the existence of the target and some of the properties of that target are described below.

A TARGET FOR ARCHENTERON EXTENSION

Since the filopodial behavior, described above, continues for the duration of gastrulation until contact with the blastocoelic wall is made, the question arises as to whether the change in secondary mesenchyme cell phenotype could be induced precociously by early contact with the animal pole region. By simply pushing the animal pole region into contact with the archenteron tip, the secondary mesenchyme cells changed phenotype much earlier in response (relative to control embryos of the same age). If contact with the animal pole region were prevented by preventing the secondary mesenchyme cells from reaching the animal pole region, one might predict (if contact were essential) that secondary mesenchyme cells would continue their filopodial extension behavior for an abnormally prolonged time. This prediction was tested by inserting embryos into narrow-bore glass tubes so that the embryos were elongated (Fig. 6). It was observed that filopodial extension continued for hours longer than the behavior occurred in the control embryos. If the embryos were released from the tubes, they rounded up, contact with the animal pole region was made, and the phenotypic change occurred. Thus, until contact with the animal pole was made, the secondary mesenchyme cells continued their filopodial extension behavior (Hardin and McClay, 1990).

The contact-induced change in phenotype occurred when the secondary mesenchyme cells reached the animal pole. Was that the only region of the blastocoelic wall capable of inducing the phenotypic change? To address this question, the tip of the archenteron was brought into contact with other regions of the blastocoelic wall by denting the wall from the side (Fig. 6). Although extensive filopodial contact was made with these indentations, the filopodial contact was brief (in the 2–10 min range), and the archenterons continued to extend until contact with the animal pole was made. Thus, only contact with the animal pole region brought about the phenotypic change.

Secondary mesoderm cells give rise to pigment cells, cells of the coelomic pouches, and muscle (Gibson and Burke, 1985). Normally these cells do not participate in the production of the larval skeleton, although experiments have shown that secondary mesenchyme cells retain the capacity to produce spicules until quite late in gastrulation (Ettensohn and McClay, 1988; Ettensohn, 1990). This lineage conversion is a property of the secondary mesenchyme cells that can be induced experimentally by depriving the embryo of

cells of the primary mesenchyme cell lineage from very early in development (Hörstadius, 1939), through gastrulation (Ettensohn and McClay, 1988), until about the time of target contact (Ettensohn, 1990). In lineage mapping, studies have yet to determine when secondary mesenchyme cells are specified. The experiments of Ettensohn (1990) suggest that the lineage is finally committed irreversibly sometime around the end of gastrulation, or at about the time the secondary mesenchyme cells undergo the contact-mediated phenotypic change. The striking correlation between Ettensohn's experimental results showing loss of lineage conversion capacity and the phenotypic change observed in the present experiments suggest that at least one consequence of striking the target at the animal pole might be commitment toward the restricted fate of the secondary mesenchyme cell lineage.

The process of archenteron extension appears to have two critical control steps, based upon the experiments described above. At the beginning of invagination the cells are very sensitive to interactions with the extracellular matrix. Expression of a number of genes that normally are transcribed coincident with the beginning of invagination fails to occur unless, or until, contact with the extracellular matrix is established. At the end of gastrulation, target contact may be involved in cell lineage restriction of secondary mesenchyme cells (although this notion is entirely correlative at present). As the archenteron extends, cells appear to follow a simple set of instructions that may be given once at the beginning of gastrulation. The endoderm cells rearrange, and the secondary mesenchyme cells send out filopodia until further notice. The "further notice" appears to be contact with the target region. The accuracy of this model remains to be tested rigorously. Nevertheless, the behavioral data gathered, the inhibitor studies, the lineage conversion studies, and the gene expression studies that have been performed during gastrulation all support this idea.

What is meant by the phrase "set of instructions," as used above? Taking the behavior of the endodermal cells as an example, it is clear that the cells perform many behavioral subroutines, each of which must be coordinately regulated at the molecular level with the others. Indeed, at the beginning of gastrulation it is known that a group of genes is transcribed coordinately in the endoderm (McClay et al., 1983; Wessel et al., 1989a; Nocente-McGrath et al., 1989) (although expression of those genes has not been linked directly to the behaviors observed by the cells). Cells become motile and undergo circus movements that appear to jostle the cells as viewed by time lapse. They rearrange in an ordered sequence that involves initiating and breaking cell adhesions. They converge and extend the archenteron directionally. All through elongation the cells retain contact with the luminal matrix and the basal lamina. Each of these component cell behaviors must have a molecular basis, and that group of molecules must be under coordinate regulation. Thus, while it is easy to state that the behavior is a simple programmed sequence, this incomplete list of functions indicates that a complex network of molecular events underlies the process.

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