

# How to grow a gut: ontogeny of the endoderm in the sea urchin embryo

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## Summary

Gastrulation is the process of early development that reorganizes cells into the three fundamental tissue types of ectoderm, mesoderm, and endoderm. It is a coordinated series of morphogenetic and molecular changes that exemplify many developmental phenomena. In this review, we explore one of the classic developmental systems, the sea urchin embryo, where investigators from different backgrounds have converged on a common interest to study the origin, morphogenesis, and developmental regulation of the endoderm. The sea urchin embryo is remarkably plastic in its developmental potential, and the endoderm is especially instructive for its morphological and molecular responsiveness to inductive cell interactions. We start by examining and integrating the several models for the morphogenetic mechanisms of invagination and tissue elongation, the basic processes of endoderm morphogenesis in this embryo. We next critique the proposed mechanisms of inductive gene regulation in the endoderm that exemplifies a concept of modular transcriptional regulation. Finally, we end with an examination of the current molecular models to explain cell fate determination of the endoderm. Recent progress at the molecular level should soon allow us to explain the seminal experimental observations made in this embryo over a hundred years ago. *BioEssays* 21:459–471, 1999. © 1999 John Wiley & Sons, Inc.

## Introduction

The basic body plan of most metazoans is one of protection on the outside (skin), digestion on the inside (gut), and support in between (bone and muscle). This basic body plan of ectoderm, endoderm, and mesoderm respectively, is acquired early in development in a dynamic process, both morphologically and molecularly, called gastrulation. Which

cells of the early embryo give rise to which tissue type, or primary germ layer, is an important decision in cell fate since, once committed to either an ectoderm, endoderm, or mesoderm fate, their progeny normally retain their germ-layer identity through adulthood. In order for embryos to gastrulate, several fundamental transitions must occur. These include: *Transcriptional activity*: Many embryos, like those of frogs, have either no or only low levels of transcription during cleavage divisions and embryogenesis relies strictly on the maternal contributions to the oocyte during oogenesis to provide the mRNA. Shortly before gastrulation in these embryos however, cell divisions slow and transcriptional activity begins. This new transcription is required for gastrulation, even though in some animals such as sea urchins and frogs, it is not needed prior to gastrulation. *Cell shape changes and motility*: In embryos of mammals and sea urchins, cells retain their positions throughout early cleavage divisions and only at gastrulation do they begin to change their relative positions by migration, rearrangement, and

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Abbreviations: ECM, extracellular matrix; PMC, primary mesenchyme cell; SMC, secondary mesenchyme cell

tissue folding. *Signaling systems*: In order to coordinate the above processes, communication between cells is crucial. Gastrulation is a particularly sensitive time to signaling processes and environmental perturbations have a major impact in the coordination of these events. Although the morphogenetic strategies used by embryos to gastrulate vary between different phyla, many of the cellular movements and molecular mechanisms are the same. Indeed, changes in the timing or direction of movement has a major impact on morphogenesis, and are common “targets” of evolutionary-directed changes in phenotype.<sup>(1)</sup>

This review will focus on the morphogenetic and molecular mechanisms used in the ontogeny of the endoderm lineage in the sea urchin embryo. This embryo has long been a model to study gastrulation because, in many species, it has excellent optical clarity to permit exacting observation, the embryo can be cultured easily and is resilient to molecular and surgical manipulations, and the cellular movements used during gastrulation in this embryo are shared throughout development in this and other embryos. Recent progress has identified new mechanisms and molecules of functional interest in this complex morphogenetic process.

#### Endoderm morphogenesis

Morphogenesis of the endoderm in the sea urchin is a three step process of invagination, elongation, and target recognition. The endoderm originates from the vegetal plate of the early embryo, a slightly thickened region that forms in the vegetal hemisphere at about 8 hours postfertilization.<sup>1</sup> The thickening of the epithelium at this site is a result of both increased cell adhesion between the cells<sup>(2)</sup> and cytoskeletal directed cell shape changes. It is morphologically similar to, for example, the ectodermal placodes that form the primordium of the ears and eyes in vertebrates.

Endoderm morphogenesis begins by an inward bending of the vegetal plate to form a pocket of a few hundred cells (Fig. 1). This process, called invagination, results in a short, squat stump of tissue, which contains most of the endoderm precursors as well as cells of the secondary mesenchyme<sup>2</sup> (SMC). Although invagination is seen in many tissues throughout phylogeny (for example, optic cup invagination in vertebrate eye development) the cellular mechanism of this movement is still debatable. Important experiments in the sea urchin<sup>(3,4)</sup> show that the motive force(s) for invagination are

resident in the vegetal plate e.g., the vegetal plate invaginates even when isolated from the embryo. Thus, the mechanical forces must be generated autonomously by cells of the vegetal plate, or by their near neighbors, and not by global forces generated throughout an embryo. This conclusion is generally true for invagination of tissues in other embryos<sup>(4)</sup> and focuses the research on mechanisms of invagination in the sea urchin to cellular changes within cells of the vegetal plate.

Several different cellular mechanisms have been proposed for generating the motive force(s) necessary for primary invagination (summarized in Fig. 2) and morphogenesis is likely to be the result of orchestration of these different mechanisms. These include changes in cell shape and cytoskeletal organization, filopodial and lamellapodial tractions, localized secretion and swelling, and changes in cell numbers. These and other proposed mechanisms of primary invagination were tested by computer simulations using three-dimensional finite element analysis.<sup>(5)</sup> The results of a modeled embryo show that each mechanism can generate an invagination of the tissue, that none of the models are biomechanically unrealistic. More importantly though, their results define ranges of physical parameters of the cells and the extracellular matrix (ECM) that are *required* in order for each mechanism to reproduce the observed behavior of the invaginating vegetal plate. Which mechanism or combination of mechanisms is actually utilized in the embryo might thus be answered by measuring the biomechanical properties of the cells and ECM in the vegetal plate during invagination to determine if their characteristics are compatible with the parameters derived from the computer-simulated models. Since none of the physical properties are known, we can not yet rule out any of the proposed mechanisms, but this modeling study shifts the focus to defining the properties of the proposed players and their roles. Because the sea urchin embryo can be induced to evaginate, or invagination blocked, the effects on the physical parameters can be modeled into an experimental framework. With the optical clarity and manipulations possible in this experimental model system, such definition appears possible for primary invagination.<sup>(5)</sup>

A new bend to the problem of invagination is the recent identification of a ring of bottle-like cells in the vegetal plate.<sup>(6)</sup> Bottle cells have been a major focus in *Xenopus* during gastrulation<sup>(7)</sup> and in ventral furrow formation in *Drosophila*<sup>(8)</sup> and are thought to have an active role in the invagination process in these organisms. Bottle cells, named for their appearance of a constricted apical neck with a bulbous basal region, form because of an apparent constriction of microfilaments in the apical cell cortex. While retaining junctions at the apex, the bulbous basal protrusion has the effect of buckling the tissue at the base. Of special interest for the bottle cells in the sea urchin is that they form transiently following thickening of the vegetal plate, and in a ring around the center of the vegetal plate.<sup>(6)</sup> Thus, they may have been overlooked by

<sup>1</sup>Various species of sea urchin have different developmental time tables. The times referred to here are for *Lytechinus variegatus* at 23°C.

<sup>2</sup>The secondary mesenchyme cells form a diverse array of cell types including muscle, coelom, basal cells, and pigment. Under certain experimental conditions, these cells can be induced to become skeletogenic cells (reviewed in Ettensohn et al., 1995).

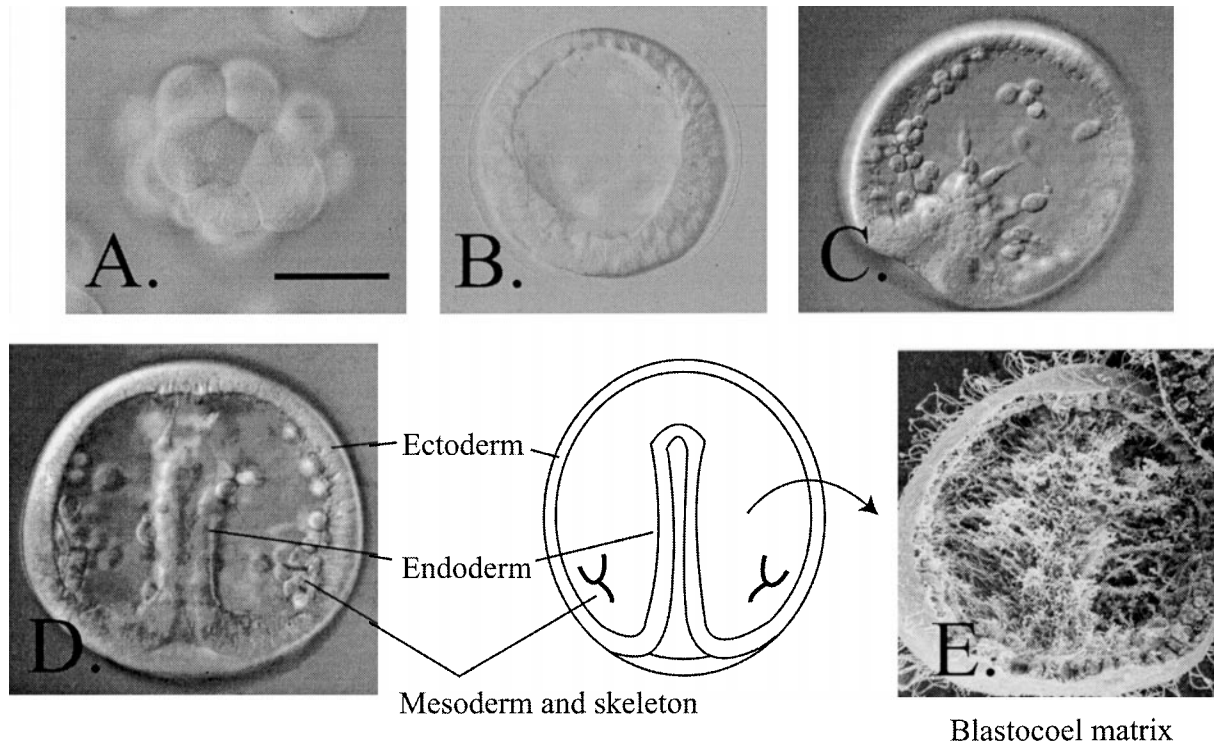
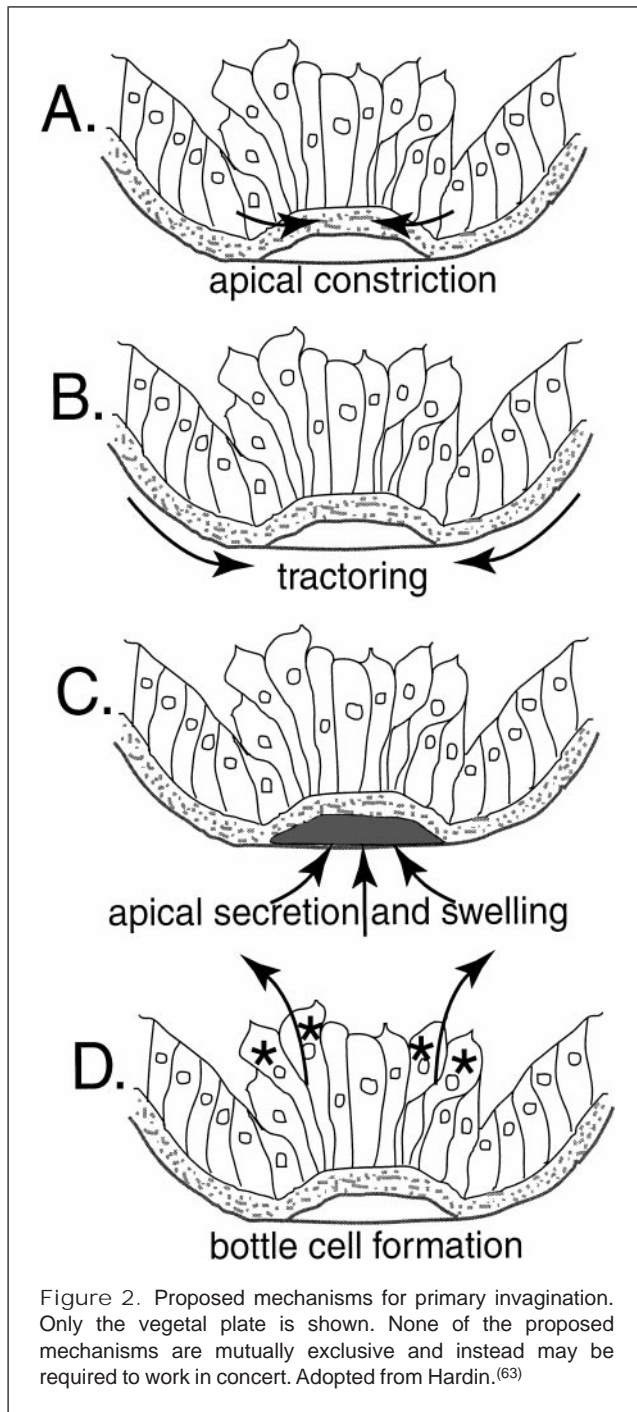


Figure 1. Early endoderm morphogenesis. A: The fourth cleavage division (3 hours) is unequal and results in eight mesomeres (top tier, future ectoderm), four macromeres (middle tier, which when it divides again will form veg1 and veg2 tiers. Veg2 and some of the veg1 cells will form endoderm and mesoderm), and four micromeres, which at this time are irreversibly fated to become the primary mesenchyme cells and develop spicules. B: Blastula (6 hours), a single layer of cells surrounding the blastocoel. C: Early gastrula (10 hours) characterized by invaginating endoderm from the vegetal pole of the embryo. Note the mesenchyme cells within the blastocoel. D: Late gastrula (14 hours) with fully extended endoderm tube and aggregating mesenchyme cells early in construction of the skeleton. E: Late gastrula processed for scanning electron microscopy. Note the dense fibrillar arrays through which the mesenchyme cells migrate, and the endoderm invaginates. (E Courtesy of John Morrill) Bar is 50 microns.

other investigators measuring the overall apical surface area of an invagination to test the apical constriction model. Surprisingly, the interactions of cells in the vegetal plate with the apical lamina was necessary to induce formation of the bottle cells and that tracting of adjacent cells may be secondary to bottle cell formation.<sup>(6)</sup> High resolution fate mapping of the vegetal plate has allowed the identification of the bottle cell lineage. Remarkably, bottle cells appear not to be part of the developing endoderm, but of the secondary mesenchyme lineage.<sup>(9)</sup> Thus, the bottle cells have an origin and fate that is distinct from the endoderm, and behave differently during the initial phase of gastrulation. Similar bottle cells have now been seen in several different species of sea urchins so that this may be a general feature of invagination in this family of animals. One lesson that is clear from the analyses of gastrulation in other animals is that even seemingly simple morphogenetic movements can have very complex mechanisms acting in concert<sup>(7)</sup> and that none of the proposed mechanisms (Fig. 2) are mutually exclusive.

The forces of invagination move the future endoderm approximately 1/3 of the distance across the blastocoel. This short, stumpy tissue then elongates an additional 1/3 across the blastocoel by a mechanistically different process, referred to as secondary invagination. The motive force of secondary invagination results from a rearrangement of epithelial cells; a repacking of cells by interdigitation that converts girth into length. Cell lineage studies of the invaginating endoderm show that cell rearrangements occur only locally, with their immediate neighbors, and not by widespread mixing of the cells.<sup>(10,11)</sup> In addition, the integrity of the adherens and septate junctions are maintained,<sup>(4)</sup> as occurs in all systems where epithelial rearrangements occur.<sup>(12)</sup> Surprisingly, the entire process of secondary invagination is independent of microtubule function.<sup>(13)</sup>

Morphogenesis of the endoderm through secondary invagination appears to be independent, at least mechanically, from the ectoderm and mesoderm. In fact, the archenteron can bend and extend equally inward (invaginate as normal) or



outward (evaginate). The final 1/3 of archenteron extension, however, appears to come from the interaction of secondary mesenchyme cells (SMCs) with the ectoderm.<sup>(14)</sup> SMCs are at the tip of the archenteron and they extend filopodia to bridge it with the overlying ectodermal epithelium. Contraction of the SMC filopodia then bring the archenteron into close

apposition with the basal surface of the overlying ectoderm. Once apposed, the ectoderm and endoderm tissues fuse by cell rearrangements to form the mouth as the embryo begins feeding. The site on the ectoderm targeted by the SMCs is predefined and differs between species. The molecular basis for determining this site is unknown but it appears that the filopodia of the secondary mesenchyme cells have an adhesive preference for the “target site”.<sup>(14)</sup> The target site is morphologically apparent as a thickened ectodermal epithelium. The mesenchyme filopodia may contact or test either the basal lamina that underlies the ectodermal cells, or the cells themselves, since it is known that small, ectodermal processes extend through the basal lamina into the blastocoel. Although the basal lamina in this region appears morphologically similar to other regions of the ectoderm, it contains a different population of ECM molecules.<sup>(15,16)</sup> Surprisingly, the interaction of the SMCs and the ectoderm does not result in any detectable, wholesale inductive events as originally proposed.<sup>(2)</sup> Instead, the mouth region appears to be determined very early in gastrulation, prior to any contact with the secondary mesenchyme, and undergoes partial morphogenesis even in the absence of contact with mesenchyme or endoderm.<sup>(17,18)</sup>

Following fusion of the endoderm and ectoderm tissue, the endoderm differentiates into a functional digestive system with a hindgut (future intestine), midgut (future stomach), and foregut (future esophagus) marked morphologically by constrictions. This final morphogenesis begins at about the same time as mouth formation but is not dependent upon interactions with the ectoderm since a very similar constriction pattern is seen during evagination. In fact, gene expression within the endoderm is nearly identical between *invaginating* and *evaginating* endoderm.

Cell division does not appear to be required for the motive force of secondary invagination in most but not all sea urchin species examined.<sup>(19,20)</sup> However, a significant increase in the number of cells does occur during gastrulation, and persists until the prism stage.<sup>(21)</sup> The source of these new cells is likely due both to cell division<sup>(19)</sup> and to continued involution of *veg1* cells into the archenteron<sup>(22)</sup> and may explain the remarkable regulative capacity of this tissue so late in embryonic development.<sup>(23)</sup>

A critical concept in the regulation of morphogenesis at gastrulation is differential cell adhesion<sup>(2,24)</sup> and recently several molecules involved in cell adhesion and cell-cell signaling have been identified in the sea urchin embryo. Of special interest here are members of the cadherin/catenin family<sup>(25–27)</sup> and the notch family.<sup>(28)</sup> Both cadherin and catenin members are present throughout development (see below for additional commentary on catenin function), including the endoderm cells during invagination and elongation.<sup>(26,27)</sup> Thus, even though the cells change partners, their adherens junc-

tions are retained. Further observations<sup>(27)</sup> document that the sea urchin Lv-G cadherin identified (which contains 13 cadherin repeats instead of the 4–5 normally found in *Drosophila* and mammals) appears to be internalized in primary mesenchyme cells (PMCs) in the process of ingression. Thus, presumably, cadherin expression accompanies the loss of cell adhesion that is hypothesized to be required for ingression of PMCs.<sup>(24)</sup>

A single notch homologue has also been identified sea urchins.<sup>(28)</sup> This sea urchin notch protein is present throughout development, but is absent from the vegetal plate region early in development. During early gastrulation its abundance increases in vegetal plate cells, especially on the apical aspect of the endoderm cells and in a dorsal/ventral (D/V) axis of polarity within the embryo. This is the first evidence of D/V axis polarity in the endoderm and may help explain the direction of endoderm bending that occurs during secondary elongation which is characteristic within a species. Important to note is that those cells expressing the intense apical notch include those postulated to undergo apical tractoring to provide a motive force for gastrulation. Other cells of the embryo retain uniform notch labeling, and the pronounced endoderm labeling is coincident with formation of the fate boundary between secondary mesenchyme and endoderm. It is not clear how this apical preference is achieved, but it may indicate utilization of a different ligand. Surprisingly, experimental perturbations show that SMCs lose notch expression before they delaminate from the endoderm, resulting in a pattern similar to neuroectoderm/mesoderm fate determination in *Drosophila*.<sup>(29)</sup>

#### Gene activity in the endoderm during gastrulation

Several gene products have been identified that are expressed specifically by endoderm cells at gastrulation (Table 1) and Endo 16 has been best examined to understand the mechanisms of gene expression in these cells. Endo 16 is initially expressed in the entire vegetal plate but is then down-regulated during gastrulation in the secondary mesenchyme cells that delaminate from the tip of the invaginating archenteron. In *plutei*, Endo 16 is also down-regulated in the fore- and hindgut so that its expression is restricted to the midgut of the larvae.<sup>(30)</sup> The Endo 16 gene regulatory region reveals the presence of several interacting *cis*-modules (Fig. 3).<sup>(11,31)</sup> A complete map of the high specificity target sites for DNA-binding proteins was made by gel shift assays and oligonucleotide gel shift competitions that resulted in the identification of 38 high specificity DNA-protein interactions in the Endo 16 promoter region.<sup>(32)</sup> Nine different protein factors bind at unique sites of the promoter region, and five other proteins bind at multiple sites within the Endo 16 regulatory domain. Both positive-acting modules and repressor modules

TABLE 1. Genes Expressed Selectively by the Endoderm Lineage

Gene	Protein	Characteristics and function
Endo 16	extracellular matrix glycoprotein	gene active in all regions of the gut during gastrulation, then restricted to midgut in larvae <sup>40</sup> ; protein in excess of 300 kDa accumulates specifically in the basal lamina of the endoderm <sup>30</sup> ; binds calcium <sup>66</sup> ; multiple splice forms of mRNA <sup>67</sup>
ECM 18	extracellular matrix glycoprotein	ECM 18 protein is expressed selectively by endoderm cells during gastrulation and is required for both primary and secondary invagination, temporal regulation of expression controlled by translation <sup>68</sup>
LvN1.2	apical cytoplasmic protein	gene activity restricted to mid and hindgut during and after gastrulation, associated with vesicles in apical cytoplasm <sup>69</sup>
Endo 1	cell surface glycoprotein	gene activity restricted to mid and hindgut during and after gastrulation, associated with cell surface, approximately 320 kDa <sup>70</sup>
Msx	transcription factor, member of the homeobox family	gene active in vegetal plate prior to gastrulation, in endoderm during gastrulation, and in the "target site" late in gastrulation, <sup>18</sup> over-expression of Msx inhibited endoderm differentiation <sup>71</sup>
HphnF3 and Spfkh1	transcription factors, members of the forkhead, winged helix, family	members of the class I and class IV groups of forkhead/HNF-3 family respectively; expressed in vegetal plate, then in gut during gastrulation, mostly in cells surrounding blastopore <sup>72,73</sup>
SpHMX	transcription factor, member of the homeobox family	endoderm expression during gastrulation, then pigment cells in <i>plutei</i> <sup>74</sup>
SpKrox	transcription factor, member of the zinc-finger family	zinc-finger transcription factor restricted in expression to cells that give rise to the vegetal plate, starting with the macromeres. Accumulation greatest during gastrulation to cells surrounding the blastopore then disappears in <i>plutei</i> <sup>75</sup>

were defined<sup>(31)</sup> and these studies showed that synergy of module interaction is crucial for normal Endo 16 expression. Thus, for example, in the seven modules defined in the Endo 16 regulatory region and referred to as modules A-G, module A is sufficient for normal expression *prior* to gastrulation, and is enhanced by modules B and G, whereas *following* gastrulation, module B is the major positively acting module and is enhanced by module G, but not A. Modules E, F, and DC are used instead to *repress* ectopic expression of the gene. Furthermore, treatment of embryos with lithium chloride, which results in a shift in cell fates to endoderm and increased Endo 16 expression requires interaction of module A with either module E or F for expression: module A or B by itself is insufficient for lithium responsiveness. The complexity of this promoter and its modularity is thought to reflect the plasticity of endoderm differentiation in response to inductive interactions. The implication from these studies is that different regulatory domains may be utilized in different combinations (once bound by protein regulatory factors) by cells at different times, embryonic positions, or in response to different cell interactions. One may expect that these different modules are sensitive to signals such as cell-cell or cell-ECM interactions that are critical for endoderm ontogeny. Furthermore, since the vegetal plate contains precursors for not only endoderm, but also many secondary mesenchyme cell populations that cease Endo 16 expression during gastrulation, some of the modules may repress Endo 16 transcription in non-endoderm lineages following invagination.

Recent work has shown that a single *cis* element in the A module of the Endo 16 transcriptional control region is essential for expression of Endo 16 in the vegetal plate.<sup>(33)</sup> By using DNA affinity chromatography the factor that binds this *cis* element was purified and shown to be the orthodenticle-related homeodomain transcription factor SpOtx. SpOtx was initially identified as the factor that bound four redundant *cis* elements essential for the transcriptional activation of the aboral ectoderm-specific *Spec2a* gene.<sup>(34)</sup> Now it is known that SpOtx is present in the nuclei of all cells of the early embryo and its essential and specific function in expression of the Endo 16 and *Spec2a* genes is not regulated simply by its own expression. Instead, it is likely that the DNA elements interacting with the Otx sites within the Endo16 and *Spec2a* control regions discriminate in gene expression. For example, module A of Endo 16 contains binding sites for several other DNA-binding proteins besides Otx, including four redundant elements termed CG sites, which are bound by the same protein.<sup>(31,33)</sup> These CG sites are required for maximal transcription from the Endo 16 control region.<sup>(32,33)</sup> The CG4 site matches the consensus binding sequence for the transcription factor LEF1/TCF [(A/T)A/T)CAAAG]. LEF/TCF, which functions as a heterodimer with  $\beta$ -catenin, may only be functional in transcriptional activation in vegetal plate cells where  $\beta$ -catenin is nuclear (see below). Thus, vegetal plate-

specific expression of Endo 16 could result from a synergistic interaction of SpOtx and LEF-1/TCF- $\beta$ -catenin on the Endo 16 control region.

#### Regulative capacity of endoderm ontogeny

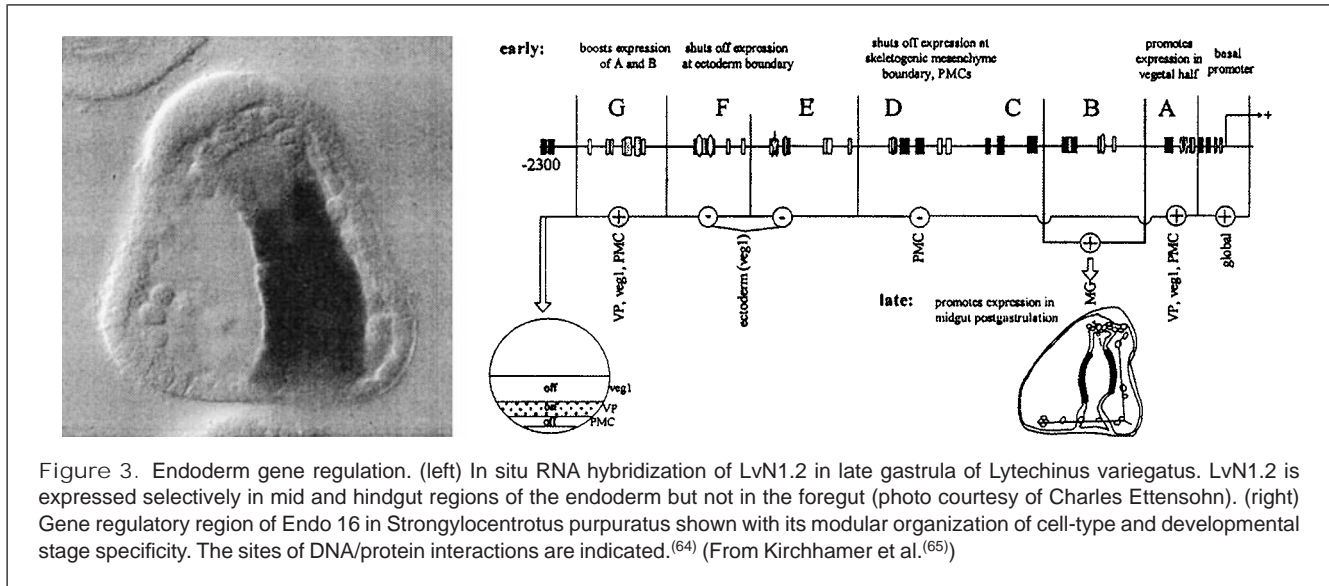
The sea urchin embryo is famous for its properties of regulative development. Before the turn of the last century, this animal demonstrated to us that embryonic cells were not fixed in their fates, and that cell interactions were important for the proper cell fate determination.<sup>(35)</sup> In the 1920s and '30s, Horstadius demonstrated several inductive interactions during development and their involvement in cell fate determination in early embryogenesis. A key observation from these classical experiments was the regulative nature of endoderm development in the sea urchin embryo. Recent investigations have operationally defined the developmental progression of endoderm ontogeny that includes: 1) formation of the vegetal pole in oocytes and eggs; 2) conditional specification during cleavage; 3) commitment prior to gastrulation; and 4) differentiation during and after gastrulation (Fig. 5). This continuum is defined currently only by experimental operation and no solid molecular basis for the phenomenon are yet known. Likely the mechanism(s) will not only include presence or absence of key regulatory elements, but also subtle alterations of combinations of molecules, the net result of which is to steer daughter cells along different, and divergent pathways.

#### *Animal-vegetal axis formation in the egg*

Boveri showed, as early as 1901, that in the sea urchin, the egg has a distinct animal-vegetal axis at fertilization and that the pole opposite the site of the meiotically derived polar bodies is the eventual site of endoderm cell formation and invagination.<sup>(35)</sup> Although mRNAs have been localized that define these axes in the egg,<sup>(36)</sup> and vegetal determinants have been functionally identified,<sup>(37)</sup> it is still not clear what the mechanism of A/V axis formation is in this egg.

#### *Conditional specification of endodermal precursor cells*

The initial observations and fate map experiments of Boveri and Horstadius<sup>(37)</sup> showed that the vegetal plate descendants, as all cell types of the embryo, are derived from specific tiers of blastomeres along the animal-vegetal axis of the early embryo (Fig. 4). During cleavage, the endodermal lineage precursors are derived solely from the macromeres of the 16 cell stage embryo, and following two more cell divisions, these cells form yet two more specific tiers of cells, the veg1 and veg2 tiers. Recent evidence shows that descendants of both the veg1 and veg2 tier contribute to the endoderm.<sup>(22,38)</sup> The recruitment of veg1 progeny to the endoderm lineage occurs relatively late during cleavage and is not thought to be part of the initial specification events.<sup>(38,39)</sup>



Horstadius is perhaps most famous for his classical demonstration that the 16 cell stage micromeres have a dominant, instructive effect on neighboring cells to organize the fundamental animal-vegetal axis of the embryo and to specify the endoderm lineage. Thus, when he isolated animal hemispheres from early embryos, he found they would develop only into an ectodermal-like ciliated ball, whereas if he opposed these hemispheres with more vegetally derived tiers of cells, he rescued more normal looking embryos, complete with guts and skeletons. By detailed mapping procedures he documented that transplanted cells induced new cell fates, and did not just contribute to new cell fates.<sup>(37)</sup> His experiments illustrate that cells of the early embryo have exceptional plasticity in their developmental fate. Ransick and Davidson<sup>(40)</sup> advanced the classical findings of Horstadius to show that micromeres are sufficient to also induce endoderm-specific gene activity. Since these investigators labeled the donor micromeres, they know that the micromeres themselves did not form endoderm. Instead, the micromeres altered the fate of adjacent cells for a complete molecular and morphological transformation. Furthermore, they showed that these inductive interactions occur normally in vivo.<sup>(41)</sup> Presumptive endoderm cells from early embryos also induce repatterning within the embryo that includes ectoderm and skeletal elements, acting as an “organizer” of ectopic axial structures.<sup>(64)</sup>

Lithium chloride treatment of embryos mimics the inductive capacity of micromeres, an observation originally made by Herbst in 1896<sup>(35)</sup> in sea urchins, and subsequently shown to have teratogenic effects in embryos of many species.<sup>(e.g.,42,43,55)</sup> Bathing sea urchin embryos in lithium results in an expansion of the vegetal plate territory in vivo, as would be expected were the micromere effect increased and lead to

vegetalized embryos that develop with an excess of endoderm and mesoderm.<sup>(44)</sup> Importantly, when the embryo is immersed either in lithium or phorbol esters, it does not change completely into vegetal plate derivatives like endoderm as might be expected. Instead, a shift of cell fates occurs along a continuum to more vegetal fates. Treating embryos with lithium results in a fate transition so that the entire veg1 tier of cells, which normally also contribute to ectoderm, follows a strict endodermal cell fate.<sup>(45)</sup> Lithium can also mimic another effect of micromeres and change the developmental fate of isolated mesomeres, from ectoderm to endomesoderm.<sup>(46)</sup> This ability to alter cell fates is transient within a host embryo, beginning at the eight cell stage and lasting for only a few cleavages. By midblastula, the effect of these treatments, as with micromeres, is lost.<sup>(37)</sup> Perhaps cells no longer have signal pathways to respond to treatment or, more likely, their fate is fixed to some other lineage.

Several recent reports now indicate that the developmental effects of lithium can be attributed to its activation of the wnt-signaling pathway by inhibition of the serine/threonine protein kinase glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a negative regulator of  $\beta$ -catenin.<sup>(42,47)</sup> GSK-3 $\beta$  normally phosphorylates  $\beta$ -catenin and targets it for degradation resulting in low cytoplasmic levels of  $\beta$ -catenin in cells with active GSK-3 $\beta$ . Inhibition of GSK-3 $\beta$  by lithium, or by wnt in its signal transduction pathway, leads to the stabilization of  $\beta$ -catenin and therefore its accumulation.  $\beta$ -catenin is thus able to enter the nucleus and dimerize with DNA binding proteins of the high mobility group (HMG) family such as LEF-1 and TCF.<sup>(48)</sup> This complex then binds target genes and activates new transcription. In sea urchins,  $\beta$ -catenin protein appears in the nuclei of vegetal blastomeres as early as the 16 cells stage, putting it in the right place at the right time to play a role in

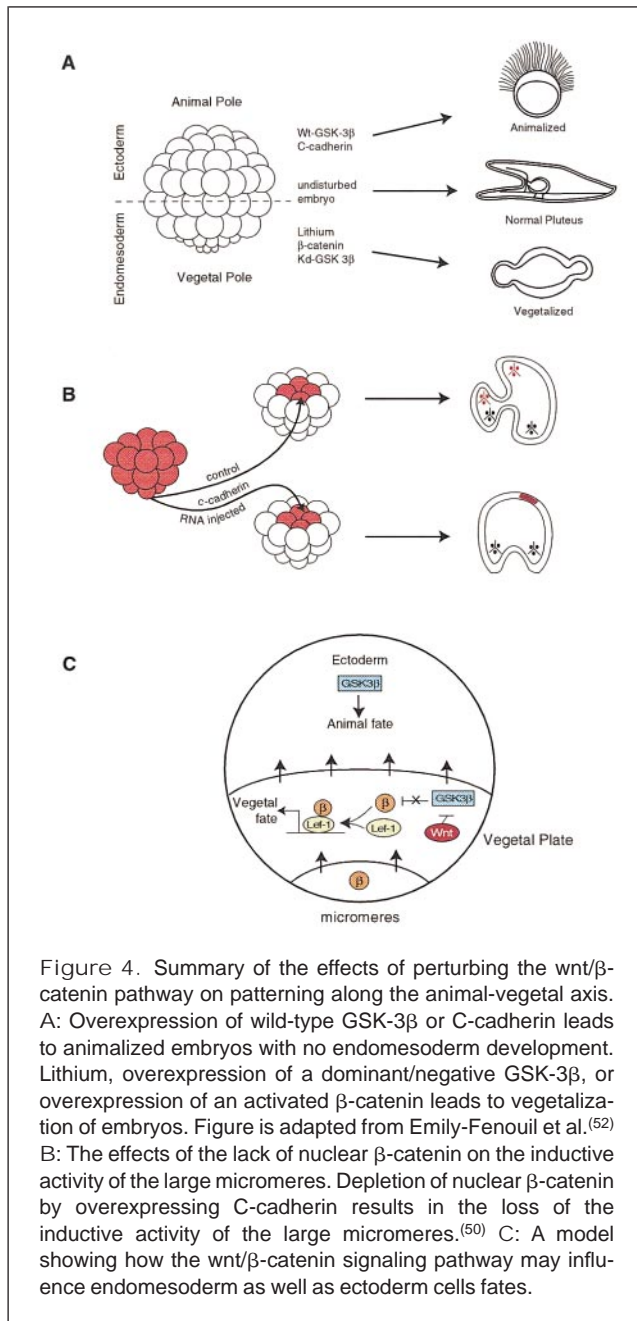


Figure 4. Summary of the effects of perturbing the wnt/ $\beta$ -catenin pathway on patterning along the animal-vegetal axis. A: Overexpression of wild-type GSK-3 $\beta$  or C-cadherin leads to animalized embryos with no endomesoderm development. Lithium, overexpression of a dominant/negative GSK-3 $\beta$ , or overexpression of an activated  $\beta$ -catenin leads to vegetalization of embryos. Figure is adapted from Emily-Fenouil et al.<sup>(52)</sup> B: The effects of the lack of nuclear  $\beta$ -catenin on the inductive activity of the large micromeres. Depletion of nuclear  $\beta$ -catenin by overexpressing C-cadherin results in the loss of the inductive activity of the large micromeres.<sup>(50)</sup> C: A model showing how the wnt/ $\beta$ -catenin signaling pathway may influence endomesoderm as well as ectoderm cells fates.

specification of endomesodermal cell fates.<sup>(49,50)</sup> Additionally, treating embryos with lithium drives high levels of  $\beta$ -catenin into veg1 nuclei<sup>(49)</sup> indicating a link between the nuclearization of  $\beta$ -catenin and the change in the fate of veg1 cells.<sup>(37,45)</sup>

The hypothesis that the wnt/ $\beta$ -catenin pathway participates in fate determination of the vegetal plate has recently been tested, and the results clearly identified a direct role for the wnt pathway in the specification of both endoderm and mesoderm cells, and in patterning of the animal-vegetal axis (Fig. 4). In one study, over expression of an activated form of

$\beta$ -catenin by RNA injection resulted in embryos that morphologically resembled those treated with lithium chloride.<sup>(51)</sup> That is, the embryos were vegetalized and developed an excess of endomesodermal cell types. In addition, expressing the activated form of  $\beta$ -catenin in animal-half explants was sufficient not only to induce endoderm in these cells, but to also induce cells that normally only contribute to ectodermal lineages, to invaginate and begin gastrulating. Furthermore, blocking the endogenous signaling pool of  $\beta$ -catenin by over expressing C-cadherin, which binds  $\beta$ -catenin via its cytoplasmic domain, resulted in animalized embryos with complete absence of endomesodermal cells, indicating that  $\beta$ -catenin was required for their specification.<sup>(50,51)</sup> Embryos with a depleted signaling pool of  $\beta$ -catenin also lacked any molecular markers for endoderm and mesoderm differentiation. In a second study, Gache and colleagues<sup>(52)</sup> over expressed a dominant-negative form of GSK-3 $\beta$  and showed that this mutated protein caused embryos to become vegetalized, much like those treated with  $\beta$ -catenin or with lithium chloride and over expression of the wild-type GSK-3 $\beta$  produced animalized embryos that lacked any endomesodermal cells. Results from these studies show that the activity of two components of the wnt/ $\beta$ -catenin pathway produce essentially identical phenotypes when over expressed, strongly suggesting that this evolutionarily conserved pathway plays an essential role in patterning the animal/vegetal axis and in specifying endomesodermal cell fates.

In the early embryo (60–120 cells),  $\beta$ -catenin has a graded nuclear distribution from the vegetal pole to the animal pole, with the highest concentration residing in the nuclei of the most vegetal cells, the micromeres.<sup>(49,50)</sup> This graded nuclear distribution of  $\beta$ -catenin suggests that the nuclear localization may be modulated by a morphogen released by the micromeres. Some recent studies by McClay and colleagues have shown, however, that removal of micromeres from 16 cell stage embryos has no effect on the nuclear localization of  $\beta$ -catenin in macromere descendants, indicating that nuclearization of  $\beta$ -catenin in these cells is not dependent simply on interactions with the micromeres.<sup>(50)</sup> These workers also found that transplantation of micromeres to the animal pole of host embryos induced secondary gut formation, but without translocation of  $\beta$ -catenin into the nuclei of mesomeres, in contrast to what one would postulate based on endogenous micromere activity. Several explanations may resolve this discrepancy. For example, perhaps the micromeres act downstream of the  $\beta$ -catenin pathway in vegetal plate specification, and do not themselves change  $\beta$ -catenin levels. Alternatively,  $\beta$ -catenin signaling may synergize with signals from the micromeres to specify the vegetal plate in the undisturbed embryo. Davidson et al.<sup>(39)</sup> have recently argued that both  $\beta$ -catenin signaling and the micromere signaling are required for endomesoderm specification in the undisturbed embryo, but that the threshold for endomesoderm formation may be

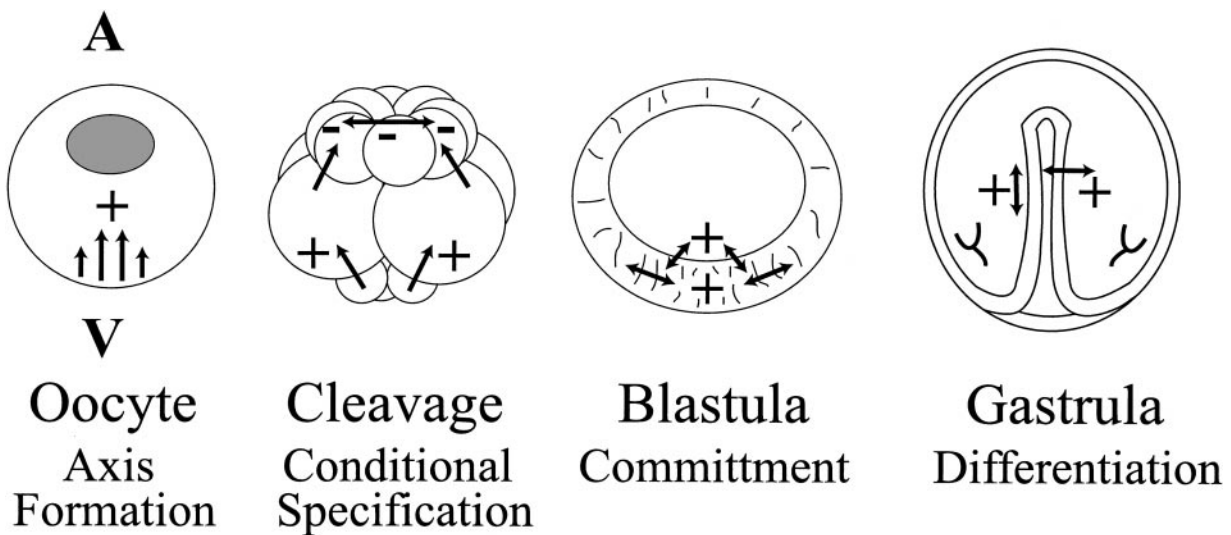


Figure 5. Summary of proposed interactions during endoderm ontogeny. The animal/vegetal axis is formed during oogenesis creating a vegetal pole with morphogenetic potential for endoderm. Micromere/macromere interactions during early cleavage conditionally specify descendants from macromeres to develop into vegetal plate, and then endoderm. Interactions between macromeres and mesomeres, and interactions within mesomeres repress an endodermal potential. Cells within the vegetal plate become committed to endoderm and interactions with the extracellular matrix appear necessary to manifest that fate, both in blastula and during gastrulation. Planar interactions along the endodermal epithelium are present in endoderm during and following gastrulation that probably contribute to the regionalization of the gut. Although each of these steps have no discreet molecular explanation, each is experimentally separable, and should be viewed as a continuum.

lower in the animal half. Thus, either  $\beta$ -catenin or transplanted micromeres can induce endomesoderm independent of the other pathway, in the animal half. This explanation would also fit with the developmental result of bathing embryos in lithium, that is, the animal pole cells retain an animal pole identity.

Another striking result from these studies by Logan et al.<sup>(50)</sup> is the requirement in the micromeres themselves for  $\beta$ -catenin. In undisturbed embryos, the micromeres show a high concentration of  $\beta$ -catenin in their nuclei suggesting a role for this protein in the function of these cells. Depleting the nuclear pool of  $\beta$ -catenin in micromeres by overexpressing C-cadherin, compromises the ability of these micromeres to induce an ectopic gut when transplanted to the animal pole of a host embryo.<sup>(50)</sup> Additionally, these nuclear  $\beta$ -catenin depleted micromeres assumed an ectoderm-like morphology and became incorporated into the animal-half ectoderm. This result reveals why primary mesenchyme cells do not form in embryos animalized by overexpressing C-cadherin or GSK3 $\beta$ , and also reveals a non-cell autonomous effect of  $\beta$ -catenin in the sea urchin embryo. Although these studies have highlighted the importance of  $\beta$ -catenin in early embryogenesis of sea urchins, what is not known at this time is the signal or the molecules that lead to the nuclear localization of  $\beta$ -catenin. It is possible that in micromeres, lateral signaling initiates and

maintains the nuclear pool of  $\beta$ -catenin in vegetal cells and that the molecules responsible are localized to these cells. Identifying components upstream of  $\beta$ -catenin and GSK3b is important since the nuclear localization of  $\beta$ -catenin in vegetal cells is one of the earliest molecular asymmetries seen in the sea urchin embryo, and molecules modulating GSK3b and  $\beta$ -catenin in the early embryo may provide important insights into the initial specification of the animal-vegetal axis. Obvious candidate molecules for mediating the nuclear localization of  $\beta$ -catenin are wnts, and although heterologous wnt molecules are powerful inducers of endoderm (A. Wikramanayake, unpublished observations) and maternal wnts have been isolated from sea urchins (S. Dayal, unpublished observations), the role of wnts in specification of the vegetal plate or the animal-vegetal axis remain to be elucidated. With the wealth of information available on the control regions of genes expressed in the vegetal plate such as Endo16, studying the mechanisms of transcriptional synergism between maternal factors such as  $\beta$ -catenin and ubiquitous transcriptional factors that may be modified by micromere signaling (ex. Otx) will continue to be a fruitful area of study.

In addition to the positive inductive signals seen from micromeres described above, evidence is also available for repression of vegetal plate fates. Mesomeres and their descendants of the early embryo only develop ectodermal

fates in an undisturbed embryo. Separation of the mesomeres however reveals a latent capacity of the cells to develop vegetal fates.<sup>(53)</sup> Micromeres (or lithium or TPA) may reveal this latent capacity—either by a positive effect or by inhibition of a putative repressive signal from vegetal cells. GSK-3 $\beta$  could also fit into this model, since keeping it active, either by blocking the wnt or protein kinase C pathways, or by activating phosphatases that would dephosphorylate an inhibitory phosphoserine or phosphothreonine, would repress vegetal fate. This means that the same pathways could be used in both positively and negatively inductive events, and differential utilization of such a pathway would have a dramatic impact on cell fate determination.

### *Commitment*

Once endoderm progenitors are specified during early development, they become committed to develop into endoderm cells several hours before gastrulation. As with micromere induced specification, this transition in the endoderm lineage is defined only experimentally: embryos dissociated in development were tested in culture for their ability to activate endoderm genes (LvN1.2/Endo 1) independent of direct cell contacts.<sup>(54)</sup> Cells derived from embryos just prior to mesenchyme blastula stage showed no ability to express endoderm markers in vitro, whereas beginning at early mesenchyme blastula stage, shortly after formation of a morphologically distinct vegetal plate, the cultured, presumptive endoderm cells expressed both endoderm markers. Importantly, proper endoderm gene expression still depended on an extracellular matrix (ECM). Commitment in these experiments is only operationally defined—no marker has yet been identified to indicate commitment in these cells, though expression characteristics of the Msx transcription factor (see Table 1) is an intriguing candidate. The developmental transition to commitment is a temporally distinct phenomenon from *specification*, resulting from micromere induction and well before differentiation (as measured by morphogenesis or cell-type specific gene expression) of the endoderm at gastrulation.

A detailed fate map of the vegetal plate of the sea urchin embryo was recently constructed using the fluorescent lipophilic dye Dil(C<sub>18</sub>), which shows that organization of the regionalized gut is also complete well prior to invagination<sup>(55)</sup> and may reflect one aspect of commitment. At the mesenchyme blastula stage, the vegetal plate contains approximately 155 endodermal precursor cells that reside in concentric rings, between 20–45°, from the future blastopore center. The future endodermal cells of the foregut are internal to those of the midgut, which in turn are internal to those of the hindgut. During invagination and elongation, this regionalized pattern unfurls; each labeled endodermal cell gives rise to a small population of cells, but only to cells within one region of the gut. Progeny never overlapped boundaries between the

future esophagus and stomach, or the stomach and intestine. Thus, at the mesenchyme blastula stage already, the endodermal precursor cells have a defined fate that is restricted to a specific subregion of the endoderm that requires distinct fates of the cells.<sup>(55)</sup>

### *Differentiation*

Invagination of the endoderm at gastrulation is a transition to differentiation in the ontogeny of the endoderm, revealed by a morphologically and molecularly distinct cell lineage from other residents of the vegetal plate, and by a difference in cell function. Functionally, at gastrulation the endoderm begins to synthesize digestive enzymes, like amylase and alkaline phosphatase, and to endocytose contents from the endodermal lumen. Morphologically, endoderm cells have a different phenotype from other cells in the embryos (cuboidal cell shape with basal lamellapodia). Molecularly, endoderm cells express genes such as LvN1.2 and Endo 1, which have strict specificity for endoderm at gastrulation.

The endoderm invaginates through the extracellular matrix of the blastocoel, a hydrated, diffuse matrix, surrounded by a specialized, condensed extracellular structure, the basal lamina. This extracellular environment contains a diverse array of ECM molecules including homologues of ECM molecules identified in vertebrates e.g., fibrillar and non-fibrillar collagens, laminin, proteoglycans, as well as proteins with sequences that are unique so far to this embryo.<sup>(56)</sup> Several different types of studies show that endoderm cell interactions with the extracellular matrix are required for differentiation and morphogenesis at gastrulation.<sup>(56,68)</sup> For example, Endo 1 is not expressed in embryos with disrupted ECM, but re-expression occurs normally following removal of the ECM inhibitors.<sup>(57)</sup> At least for LvN1.2, the response to the ECM is at the level of transcription (Chen and Wessel, in preparation). Thus it, like another gene in this embryo, LvS1 of the ectoderm, may have an ECM response element within the genetic regulatory system.<sup>(58)</sup> Godin et al.<sup>(59)</sup> have shown that the block to endoderm development by disrupting the ECM is after the initial specification of the vegetal plate. Another intriguing functional aspect of the early ECM in this embryo may be in binding or localizing growth factors important for endoderm differentiation. For example, a combination of platelet-derived growth factor (PDGF) and transforming growth factor  $\alpha$  (TGF $\alpha$ ) was found to be sufficient to rescue gastrulation in embryos whose ECM had been disrupted and would otherwise not gastrulate<sup>(60)</sup> and a dominant/negative PDGF receptor would selectively disrupt gastrulation and oral-aboral ectoderm differentiation.<sup>(61)</sup> The implications from these findings are important. First, it shows a critical juncture in endoderm development in which the extracellular matrix becomes essential for continued development. Second, even though sequences for these growth factors and their recep-

tors have not been identified, this is the first functional demonstration of growth factor involvement for morphogenesis in this embryo, and it suggests that more than one growth factor type is important for endoderm morphogenesis. Third, the results imply a functional role of the ECM in growth factor interaction either directly in binding and presentation of the growth factor or indirectly in the activation or regulation of ECM expression. Fourth, a family of integrins has recently been identified and at least one subunit, beta G, is enriched in the invaginating endoderm<sup>(62)</sup> and serves as a candidate for endoderm-ECM interactions.

Surprisingly, the regulative capacity of the endoderm does not stop following gastrulation. When McClay and Logan<sup>(23)</sup> surgically removed the endoderm either during gastrulation or even after gastrulation was complete, they found that the embryo remaining was able to reform a functional gut similar to non-manipulated embryos. When the whole gut was removed, or when pieces were dissected, the remaining descendants of either veg1 or veg2 tiers expanded their normal fates to include the removed endodermal regions, complete with normal endoderm gene expression patterns. A major contributor to the regulated endoderm appears to be descendants of the veg1 tier, previously thought to yield only ectodermal derivatives, but now it is clear in several sea urchin species that veg1 derivatives contribute normally, and under experimental conditions to endodermal fates.<sup>(22,38)</sup>

So we have come full circle, both within this review and in the experimental sense, from the early embryo and its mechanisms of morphogenesis to the differentiated embryo, and back to the mechanisms of fate specification. It is clear from what we have learned recently that gene regulatory regions and embryonic developmental mechanisms are modular and overlapping. At different times, in different cells, or under different conditions, shared mechanisms are used and reused by an embryo, and between embryos. What gives the sea urchin embryo so much regulative capacity is its ability to integrate many different morphogenetic mechanisms and signaling systems that gradually mold the embryonic cells in to the wonderful instructive device we call development.

#### Note added in proof

These references relevant to endoderm ontogeny appeared during production of this article.

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