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TROPHECTODERM: THE FIRST EPITHELIUM TO DEVELOP IN THE MAMMALIAN EMBRYO

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Abstract

The first epithelium to appear during mammalian embryogenesis is the trophectoderm, a polarized transporting single cell layer that comprises the wall of the blastocyst. The trophectoderm develops concurrently with blastocoele fluid production as the morula develops into a blastocyst. The process whereby the morula becomes a fluid-filled cyst is called 'cavitation', which can be regarded as the first functional expression of the trophectoderm phenotype. The outer layer of cells of the morula comprise the nascent trophectoderm and are already morphologically polarized prior to the onset of cavitation. A major working hypothesis in the field of mammalian embryogenesis is that such polarization is a prerequisite for the initiation of cavitation. To test this hypothesis we examined morulae for their ability to cavitate during treatments that modify morphological polarity in nascent trophectoderm cells. These treatments included ouabain, different concentrations of extracellular K and Na, cytochalasins and colcemid. Ouabain and extracellular K and Na affect the activity of Na/K-ATPase, which has been implicated in the maintenance of morphological polarity of nascent trophectoderm cells. Cytochalasins and colcemid also modify apical-basal polarity of nascent trophectoderm cells and impair cavitation. The endpoints that were monitored included incidence of cavitation, rate of cavitation and the status of morphological polarity of nascent trophectoderm cells. Collectively, all of these treatments indicate that there is a functional association between an asymmetric distribution of organelles along the apical-basal axis of nascent trophectoderm cells and the ability of the embryo to produce nascent blastocoele fluid efficiently. In addition, the preimplantation embryo appears to possess two mechanisms for accumulating blastocoele fluid.

KEY WORDS: mouse preimplantation embryo, trophectoderm, polarity, cytoskeletal drugs, ion transport, Na/K-ATPase, ouabain, asymmetric cell contact.

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Introduction

Intraembryonic cavities and epithelial sheets are the first structures to form during mammalian embryogenesis and their subsequent modification establishes the embryo's three-dimensional architecture. The first cavity formed is the blastocoele and its wall, the trophectoderm, is the first epithelial sheet. Trophectoderm is a functionally polarized, transporting epithelium and is the only tissue that can implement implantation and develop into the immunological barrier of the placenta that prevents maternal immunological rejection of the embryo. The role(s) of the blastocoele and blastocoele fluid, on the other hand, are speculative. However, normal development of an embryo has never been observed in their absence, leading to the conclusion that they, too, play a critical role in embryogenesis, perhaps in the continued development of the inner cell mass (see Wiley, 1984a, 1987a).

A significant amount of attention has been given to the development of all three of these entities--trophectoderm, the blastocoele and blastocoele fluid--and the current view is that the development of all three is mutually interdependent. This review will present the working hypotheses on the development of these three embryonic entities. It is hoped that this discussion of the earliest example of epithelial development in the preimplantation embryo will facilitate an appreciation of the mechanisms that underlie epithelial morphology and behavior in the embryo during organogenesis and in the adult.

Overview of Preimplantation Development in the Mouse

A survey of metazoan phyla reveals that what typically follows fertilization is a characteristic number of reduction cleavages followed by cavitation into a cystic structure. In the case of the mouse preimplantation embryo, fertilization is followed by three reduction cleavages to form eight developmentally equivalent apparently non-polar blastomeres. 'Compaction' then occurs so that the blastomeres flatten against one another to obliterate all intercellular spaces (Lewis and Wright, 1935; Calarco and Brown, 1969; Calarco and Epstein, 1973; Ducibella et al., 1977). As a result of compaction the outer

blastomeres acquire a topographical polarity in the form of unapposed, microvillous apical surfaces and apposed, relatively smooth basolateral surfaces. The development of cell surface polarity is thought to result from asymmetric cell-cell contacts and will occur spontaneously in isolated blastomeres that are cultured on solid substrates (i.e., plastic or glass culture dishes; Johnson and Ziomek, 1981a, 1981b). As development continues with intact embryos, this topographical polarity is subsequently reinforced by the establishment of apical junctional complexes and molecular distinctions between apical and basolateral plasma membrane domains (Wiley and Eglitis, 1981; Vorbrodt et al., 1977; Izquierdo et al., 1980; Izquierdo and Ebensperger, 1982; DiZio and Tasca, 1977). As the apical and basolateral surface domains mature, several types of cytoplasmic organelles become localized predominantly in the apical or basal hemispheres of the outer blastomeres. Similarly, many cytoskeletal and contractile proteins become preferentially localized to the apical or basolateral cortical cytoplasm (Figure 1).

During the next two rounds of cell division to the 16-32 cell stage fluid begins to accumulate between the blastomeres. Within the next 12 h this 'nascent' blastocoele fluid coalesces to occupy a single cavity, the blastocoele. When the blastocoele has grown to occupy a third of the diameter of the morula (about 75-80 microns), continued fluid accumulation causes the embryo diameter to expand until the diameter of the mature blastocyst is around 100 microns.

Meanwhile, the outer blastomeres develop into a squamous epithelium, the trophectoderm, that encloses the blastocoele while the inner blastomeres form the inner cell mass (ICM). As the outer blastomeres develop into trophectoderm, their mitochondria and numerous refractile lipid droplets co-localize to the basolateral cell borders. With the appearance of fluid between blastomeres the lipid droplets decrease in number and in diameter while the ICM cells retain their lipid droplets.

What is the relationship between cell polarity, trophectoderm differentiation and nascent blastocoele fluid formation? The assumption underlying a substantial body of experimental work is that polarization of the outer blastomeres represents the first morphological expression of the trophectoderm phenotype while the production of nascent blastocoele fluid represents the first physiological expression of the trophectoderm phenotype. Because morphological polarity precedes nascent blastocoele fluid formation, the major working hypothesis is that morphological polarity is a prerequisite for expression of the trophectoderm physiological phenotype. The major experimental question, then, is whether cell polarity is required for nascent blastocoele fluid production and cavitation. At this juncture it becomes helpful to review the three models of cavitation in the mouse preimplantation embryo.

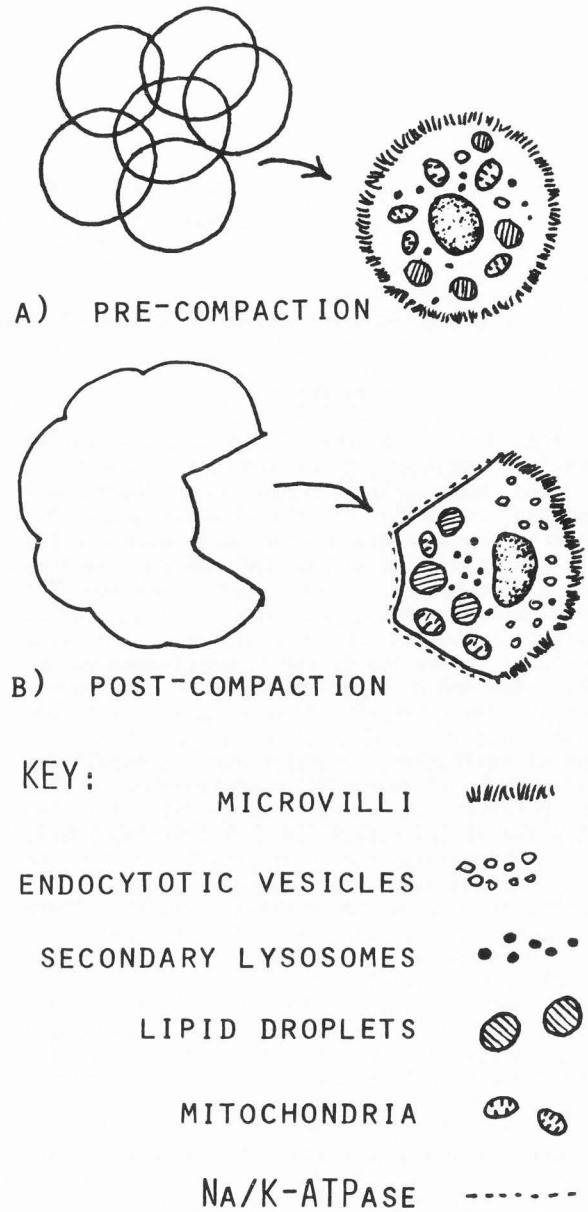


Figure 1. Cell surface and cytoplasmic asymmetries in the outer blastomeres of the mouse morula. (A) Prior to compaction, microvilli and numerous enzymes are globally distributed over the surfaces of outer blastomeres while cytoplasmic organelles show no overt apical-basal stratification. (B) However, following compaction, microvilli are limited to the apical membrane while several enzymes (i.e., Na/K-ATPase) become limited to the basolateral membrane. Similarly, several organelles become preferentially located in either the apical or basolateral hemisphere of outer blastomeres (See Wiley, 1987b, Table 5).

Three Models of Cavitation

Secretion Cavitation Model

Early in the twentieth century, Melissinos (1907) observed that the appearance of nascent blastocoele fluid is preceded by the basolateral localization of refractile cytoplasmic droplets. These droplets decrease in size and number as the blastocoele enlarges. Melissinos hypothesized that the droplets consist of blastocoele fluid and are secreted. Droplet-mediated secretion became the accepted explanation for the origin of nascent blastocoele fluid (Mintz, 1965; Calarco and Brown, 1969). In addition, cavitation is reversibly inhibited by anti-microtubule drugs (colcemid; Wiley and Eglitis, 1980), which inhibit the translocation of secretory vesicles and their subsequent exocytosis in a wide variety of cell types.

However, efforts to obtain morphological evidence of droplet exocytosis have failed (e.g., Calarco and Brown, 1969) and the droplets were found to consist of lipid (Calarco and Brown, 1969), primarily neutral lipid (Nadijcka and Hillman, 1975; Flynn and Hillman, 1978). Because blastocoele fluid is aqueous (see Borland et al., 1977), the question arose as to how lipid droplets could contribute towards the formation of an extracellular fluid that was aqueous?

Transport Cavitation Model

One way of approaching the above question was to invoke another mechanism for the production of nascent blastocoele fluid that did not involve lipid droplets. The Transport Cavitation Model is based on two features of the 16-32 cell stage morula. First, Na/K-ATPase activity of outer blastomeres is restricted to their basolateral plasma membrane domains. Second, apical junctional complexes, which become evident during compaction, gradually become zonular during blastocyst expansion.

Initial studies with permeability tracers suggested that these junctional complexes became zonular prior to the appearance of nascent blastocoele fluid (Ducibella et al., 1977) to form a permeability seal (Ducibella et al., 1977). The nascent blastocoele fluid was hypothesized to originate outside of the embryo in the form of ions (primarily sodium) and associated water that were drawn across the outer blastomeres into the intercellular spaces by the activity of Na/K-ATPase (and additional carriers?) and retained within the embryo by the permeability seal (Mintz, 1965; Ducibella et al., 1977).

However, later studies revealed that apical junctions do not become zonular tight junctions until 12-14 hours after the blastocoele has formed (McLaren and Smith, 1977). In addition, embryo diameter does not begin to increase until the diameter of the developing blastocoele consumes one third of the initial diameter of the embryo (Wiley and Eglitis, 1981). One is then left with the dilemma of accounting for an increase in intraembryonic fluid volume when there is no increase in embryo diameter. In addition, this model does not account for the observed decrease in cytoplasmic lipid within the outer blastomeres.

Metabolic Cavitation Model

This model invokes beta-oxidation of lipid as a source of nascent blastocoele fluid (water) and energy (ATP) to move fluid into the intercellular spaces (Wiley, 1984b). The Metabolic Cavitation Model is based on six observations, namely, that during nascent blastocoele fluid production, (1) cytoplasmic lipid in outer blastomeres decreases as nascent blastocoele fluid appears between blastomeres, (2) the lipid droplets consist of neutral lipids, including palmitic acid (Nadijcka and Hillman, 1975), (3) lipid catabolism increases (Flynn and Hillman, 1980), (4) basolateral Na/K-ATPase activity increases (Vorbrodt et al., 1977; DiZio and Tasca, 1977, (5) ATP utilization increases (Ginsberg and Hillman, 1973) and (6) prior to cavitation, lipid droplets and mitochondria co-localize to basolateral cell borders of outer blastomeres.

This model proposes that it is the juxtaposition of mitochondria, lipid droplets and Na/K-ATPase activity on the basolateral plasma membranes that is responsible for efficient nascent blastocoele fluid production. The purpose of this juxtapositioning would be to generate water and ATP in close proximity to the Na/K-ATPase activity. The water and ATP would be produced by the intra-mitochondrial beta-oxidation of the lipid (one mole of palmitic acid yields 131 moles of ATP and 146 moles of water). The ATP would be utilized by the Na/K-ATPase to pump sodium across the cells into the intercellular spaces. Water would follow passively to become nascent blastocoele fluid. Consequently, cytoplasmic lipid could be transformed into aqueous intercellular fluid in the absence of droplet exocytosis. In addition, there would be a decrease in cytoplasmic volume--via a decrease in cytoplasmic lipid and removal of water originating from the lipid into intercellular spaces--to allow for an accumulation of intercellular fluid without increasing embryo diameter. Then, when the apical junctions became zonular, continued Na/K-ATPase activity would result in blastocyst expansion, which has already been shown to be inhibited by ouabain (DiZio and Tasca, 1977).

Testing the Metabolic Cavitation Model

The Association Between Na/K-ATPase Activity (Functional Polarity) and Cavitation

There are three predictions from the Metabolic Cavitation Model that are based on the proposed role of basolateral Na/K-ATPase activity. The first prediction is that if vectored Na transport is involved in intercellular fluid (water?) accumulation, then treatments that modify Na/K-ATPase activity should modify nascent blastocoele fluid formation. Second, if Na is leaving the outer blastomeres by crossing their basolateral surfaces, the Na should be leaking into the blastomeres by crossing their apical surfaces. This transcellular apical-basal Na flow should be detectable as transcellular ionic current loops around the outer blastomeres.

Third, if such Na-dependent ionic currents are important in nascent blastocoele fluid production, then treatments that reduced the magnitude of Na movement across outer blastomeres should reduce the rate of intercellular fluid accumulation.

Effects of Ouabain and Extracellular Potassium on Cavitation. To test the first prediction, 16-32 cell stage mouse morulae were incubated in different concentrations of ouabain ($10 M^{-7}$ to 10^{-4} M) and extracellular potassium (K_o, 25 mM, 6.0 mM; Wiley, 1984b). Potassium competes with ouabain for binding sites on Na/K-ATPase and can be used to test the specificity of ouabain's effect on Na/K-ATPase activity. The concentration of K_o also affects plasma membrane potentials--i.e., voltage gradients across the plasma membrane--to provide us with another way of altering the magnitude of putative transcellular ionic currents. Decreasing K_o would hyperpolarize the plasma membrane and thus increase voltage gradients across it and therefore increase inward apical movement of Na ions and Na/K-ATPase activity; increasing K_o would have the opposite effect.

At 10^{-5}M, ouabain increased the rate of cavitation while 10^{-4}M ouabain decreased the rate of cavitation and the percentage of morulae that cavitated. This biphasic response is indicative of ouabain's specificity as an inhibitor of Na/K-ATPase (Glynn, 1964). The magnitude of this response decreased at higher concentrations of K_o and was enhanced at lower concentrations of K_o, verifying the specificity of ouabain's effect on Na/K-ATPase activity. Finally, in the absence of ouabain, cavitation rate was inversely related to the concentration of K_o, implying that hyperpolarization enhanced fluid accumulation while depolarization impaired fluid accumulation. All of these observations suggest that the production of nascent blastocoele fluid is linked to Na/K-ATPase activity and to the magnitude of the apical-basal transcellular movement of Na ions (and other ions?) across outer blastomeres.

Transcellular Ionic Current Loops Around Outer Blastomeres. To test the second prediction, a microvibrating probe apparatus was used in an attempt to detect and define current loops through isolated outer blastomeres from mouse morulae (Nuccitelli and Wiley, 1985). Such loops were indeed found. They entered the apical aspects, left the basal aspects and returned to the apical aspect by passing over the lateral surface of the blastomeres. Current amplitudes were reduced to almost zero when 75% of extracellular Na (Na_o) was replaced by equimolar amounts of choline chloride, indicating that the majority of the current was carried by Na ions.

Reducing the Concentration of Na_o Impairs Cavitation. The above experiment where K_o was varied was an indirect test of the third prediction. To directly test this prediction, 4-cell stage mouse embryos were cultured for 66 h in different concentrations of Na, achieved by replacing NaCl with equimolar amounts of choline chloride (Table 1). The incidence of blastocyst formation was found to be directly related to the concentration of Na_o, compatible with the idea that the magnitude of the apical-basal Na flow was directly related to the rate of blastocoele fluid accumulation.

Table 1. Effect of extracellular Na concentration on the incidence of cavitation after 66 h of culture from the 4-cell stage.[a]

mM Na	incidence of cavitation[b] % blastocysts/total No. embryos
49.8	0%
74.2	6%
98.5	11%
147.2	97%

[a]4-cell stage embryos collected 48 h after hCG injection.
[b]Each sodium concentration was tested 6 times using 10-12 embryos per test.

The Association Between the Asymmetric Distribution of Organelles (Morphological Cell Polarity) and Cavitation

One of the observations or which the metabolic cavitation model is based is that prior to cavitation lipid droplets and mitochondria co-localize to the basolateral cell borders. A prediction can then be made that treatments which accelerate cavitation should intensify basolateral localization of mitochondria and lipid. Conversely, those treatments that impair cavitation should also impair this basolateral localization of organelles.

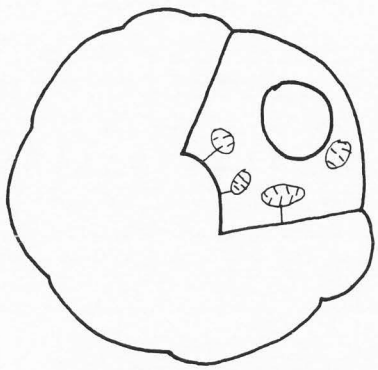
Effect of Ouabain and the Concentrations of K_o and Na_o on Mitochondrial Distribution in Outer Blastomeres. During the study on the effects of ouabain and K_o on cavitation, morphometric analyses were performed on thin sections of morulae from the different treatment groups (Wiley, 1984b). Historically, it has been the basolateral localization of the large (1 μm in diameter) refractile lipid droplets that has been studied in the cavitating embryo because they are easy to follow under a compound microscope (eg., Melissinos, 1907; Mintz, 1965; Wiley and Eglitis, 1980). However, the number of droplets per thin section is insufficient for statistical analyses of morphometric data. Since mitochondria co-localize with the lipid droplets and one thin section contains numerous mitochondria, they were chosen to quantify the effects of these treatments on the basolateral localization of organelles preceding cavitation (Figure 2).

Those treatments that impaired cavitation also decreased the population density of mitochondria along basolateral cell borders and increased their average distance from the nearest basolateral plasma membrane. Those treatments that accelerated the rate of cavitation had the opposite effect: They increased the population density of mitochondria along basolateral cell borders and decreased their average distance from the nearest basolateral plasma membrane (Wiley, 1984b).

Figure 2. Morphometric analyses at the electron microscopic level: determining the average distance of mitochondria from the basolateral cell borders of mouse morulae. Midsagittal sections of treated morulae were overlaid with tissue paper on which were traced cell outlines. Each mitochondrion was identified by a dot. Dots were connected to the nearest cell border by straight lines, which were then measured, averaged and subjected to statistical analyses.

In the study using choline chloride to vary Na_o concentration, a similar morphometric analysis was performed (unpublished data). Decreasing the concentration of Na resulted in a significant increase in the average distance of mitochondria from basolateral cell borders (Table 2).

Table 2. Effect of extracellular Na concentration on the localization of mitochondria to basolateral cell borders in morulae cultured from the 4-cell borders in morulae cultured from the 4-cell stage.



average distance in microns X ± S.D. (total no. embryos) (total no. cells) 98.5 mM Na	147 mM Na
8.38 ± 5.01* (10) (48) (1512)	6.52 ± 4.1* (12) (66) (1588)

* $P < 0.1$

Collectively, the morphometric data from both experiments reveals a positive correlation between population density of mitochondria (and presumably lipid droplets) along basolateral cell borders and the rate at which the embryo accumulates nascent blastocoele fluid. This correlation is consistent with the hypothesis that the morphological polarity of outer blastomeres is somehow associated with the expression of the physiological phenotype of the trophectoderm in terms of nascent blastocoele fluid accumulation.

Finally, both colcemid (Wiley and Eglitis, 1980) and cytochalasin B (Granholm and Brenner, 1976) impair cavitation and the basolateral localization of organelles (Wiley and Eglitis, 1981). At appropriate concentrations, both drugs can reversibly inhibit nascent blastocoele fluid production for as long as 12 h with no cell death, indicating that a reduction in cell viability *per se* cannot fully explain the lack of cavitation in the presence of these drugs. These observations strengthen the association between cell polarity and cavitation. In addition, they implicate cytoskeletal elements in the process of cavitation, perhaps in the implementation and/or maintenance of the asymmetric distribution of organelles along the basolateral axes or in the asymmetric distribution of plasma membrane components between the apical and basolateral membrane domains (Wiley and Eglitis, 1981; Wiley, 1984a,b, 1987a).

Cavitation May Occur By Two Mechanisms

We have reviewed the three experimental models of cavitation, the observations that inspired them and the evidence that either favors or disputes their major tenets. The model that best accounts for the phenomenological observations on mouse preimplantation embryos and which also has consistent experimental support is the metabolic cavitation model. This model embodies two mechanisms for the accumulation of blastocoele fluid. One mechanism involves the metabolic conversion of cytoplasmic lipid into water and ATP, which then interacts with NA/K-ATPase activity confined to the basolateral membrane domains to result in fluid accumulation between blastomeres. This mechanism is developmentally regulated, operates during a finite time period somewhere between 12 to 20 h in length, occurs only once during embryogenesis (so far as we know), and is normally initiated after the fifth cleavage, approximately 80 h after fertilization.

The other mechanism for fluid accumulation also involves Na/K-ATPase activity as well as the activity of additional plasma membrane ATPases and asymmetric ion fluxes across apical and basolateral membrane domains (see Nuccitelli and Wiley, 1985). Experimental evidence suggests that this mechanism is responsible for continued fluid accumulation that occurs during blastocyst expansion (DiZio and Tasca, 1977; Benos, 1981a, 1981b). However, this mechanism--or portions of it--are not developmentally regulated and are evidently constitutive functions that can be experimentally revealed as early as the two-cell stage when the blastomeres are adhered together with certain plant lectins, wheat germ agglutinin in particular (Johnson, 1986). Under these conditions, 2-cell stage embryos will accumulate fluid between their two blastomeres and the production of fluid is unaffected by cytoskeletal drugs and a wide variety of metabolic inhibitors, but is inibited by ouabain (Johnson, 1986).

If the cavitating morula has (at least) two mechanisms for accumulating aqueous fluid, do adult epithelial cysts also have multiple means of accumulating their aqueous lumenal contents? Do they also have constitutive mechanisms and additional ones whose activity is temporally regulated?

Stability of Apical and Basolateral Membrane Domains of Nascent Trophectodermal Cells

Cultured single isolated outer blastomeres from 16-cell stage morulae retain their original apical-basolateral surface distinctions (i.e., the presence of a well-defined microvillous

apical domain and a smooth basolateral domain until they divide (up to 12 h from when they are removed from the embryo; Ziomek and Johnson, 1981; our unpublished observations). In addition, the aforementioned apical-basal transcellular ion currents measured around isolated 16-cell stage blastomeres remain stable for periods of 55-156 minutes (Nuccitelli and Wiley, 1985), indicating that the apical-basal distribution of ion pumps/enzymes generating the currents also persist during this time. Finally, the native apical concentration of receptors for Concanavalin A remains stable on these blastomeres regardless of whether they are incubated at room temperature or at 35°C, in the absence of Concanavalin A. In the presence of Concanavalin A, however, the apical concentration of receptors decreases upon warming to 35°C and this decrease is prevented by cytochalasin D (Nuccitelli and Wiley, 1985).

In contrast to single isolated 16-cell stage morula outer blastomeres, single isolated mouse intestinal epithelial cells lose their original morphological apical-basal surface polarity within minutes of being isolated (Ziomek et al., 1980). The native apical concentration of microvilli becomes greatly reduced in extent after a 40 minute incubation at 0°C and higher temperatures. In addition, the native apical restriction of two marker ectoenzymes--alkaline phosphatase (AP) and leucine aminopeptidase (LAP)--is relieved to permit their diffusion into the basolateral domains within minutes of cell isolation regardless of whether the cells are held at 0°C or higher temperatures (although these decreases occur more slowly at the lower temperatures).

What, then, are the differences between the morula 16-cell stage outer blastomere and the mouse intestinal epithelial cell that account for this apparent difference in stability of surface domains? With single isolated outer morula blastomeres, cytochalasin D prevents the decrease in concentration of apical Concanavalin A receptors in the presence of Concanavalin A (Nuccitelli and Wiley, 1985). However, cytochalasins (as well as a variety of other inhibitors) have no effect on the diffusion of these two enzymes out of the apical domain into the basolateral one of single isolated mouse intestinal epithelial cells (Ziomek et al., 1980). This difference is consistent with the possibility that in the blastomere the maintenance of distinctions between the apical and basolateral domains might rely differently--qualitatively and/or quantitatively--on cell surface-cytoskeletal interactions than in the intestinal epithelial cell.

There is some evidence during cleavage for a trend towards increasing membrane fluidity (or molecular mobility within the plasma membrane), which becomes blatantly obvious between 8-cell and 16-cell stages. When isolated outer blastomeres of a compacted 8-cell stage embryo are labelled with Concanavalin A, their apical microvillous domains retain their distinction from the smoother basolateral domains with respect to the concentration of lectin within the apical domain and the population density of microvilli, regardless of whether the blastomeres are incubated at room temperature or at 35°C. However, as mentioned above, similar treatment of an outer blastomere that is isolated from a 16-cell stage embryo and incubated at 35°C results in a rapid decrease in the concentration of apical lectin receptors and in the population density of apical microvilli (our unpublished observations).

There is evidence suggesting that cleaving mouse embryos incorporate lipids into their cell membranes in a manner that is compatible with enhanced plasma membrane fluidity (see Pratt, 1978; Wiley, 1979). In other cell types, many transport proteins require a relatively fluid membrane environment for their optimal activity (Lee, 1975). Perhaps these observations are related to the increase in the membrane pump rate for potassium and for the change in Na:K flux stoichiometry from 2.7:1 to 1.7:1 that are both observed when the mature oocyte is compared with the 2-cell stage embryo (Powers and Tupper, 1977). As cleavage continues, the activities of Na/K-ATPase (Vorbrodt et al., 1977), Na/K-ATPase-dependent amino acid transport (DiZio and Tasca, 1977), ion pumps/channels for Na and K and activities of carriers involved in the transport of sugars (Wales and Biggers, 1968) and nucleosides (Daentl and Epstein, 1971) all increase as the embryo attains the morula stage of development.

It is possible, then, that the plasma membrane of adult epithelial cells has progressed further along a general trend for increasing mobility of plasma membrane components compatible with mature transport rates and a concomitant domain destabilization that reflects a departure from the embryonic form of plasma membrane organization. A closer examination of this embryonic form, as it exists and evolves during the development of nascent trophectoderm from cleavage stage blastomeres, may provide us with a better appreciation of the functional control of adult cell surface and cytoplasmic asymmetries (see Pratt, 1985).

Summary

The nascent trophectoderm appears to possess two mechanisms for the accumulation of nascent blastocyst fluid. One of these may involve the metabolic conversion of cytoplasmic neutral lipid into water and ATP, which then interact with the basolateral Na/K-ATPase activity to result in aqueous fluid accumulating between the blastomeres. This mechanism is developmentally regulated, under temporal control and may be unique to the nascent trophectoderm. The other mechanism is also dependent on Na/K-ATPase activity, appears to be operative as early as the 2-cell stage and may be shared with adult epithelia that accumulate aqueous lumenal fluid.

The developing trophectoderm may also be distinguished from adult epithelia by a difference in relative stability of plasma membrane domains. The molecular basis for this difference is not yet appreciated, but may be related to the

stage-specific, spontaneous development of cell surface asymmetries that become morphologically and functionally obvious with compaction, but whose genesis resides in subtle molecular asymmetries that are already evident when the embryo consists of just two blastomeres (see Pratt, 1985).

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Discussion with Reviewers

Reviewer II: Richard Schultz's laboratory has recently reported that cAMP can stimulate the rate of increase of volume of the blastocoele cavity in mouse embryos (Manejwala et al., 1986). How does this finding integrate into your Metabolic Cavitation Model? Are the magnitudes of the transcellular ion current loops that were measured around outer blastomeres consistent with the current magnitudes you would predict if an apical-basolateral sodium flow were involved in the production of blastocoele fluid? Since these currents were measured around isolated cells, what effect would multiple cell-cell contacts and zonular tight junction formation have on current magnitudes?

Author: The study by Manejwala et al. is rather interesting and describes the finding that mouse morulae possess an exogenously excitable adenylate cyclase activity which increases between the morula and blastocyst stages. As suggested by the authors of this study, this cyclase activity could be involved with enhancing Na transport as occurs with other fluid transporting epithelia. If this suggestion proves correct, then we would have a mechanism for the increase in Na transport that would be predicted by the increase in Na/K-ATPase activity that occurs during cavitation and increase in Na-coupled transport activity mentioned in the review above. However, the presence of increasing levels of activatable cyclase activity does not necessarily mean that in the normal morula cyclase activity actually increases and that cAMP levels increase. In fact, earlier work revealed that the actual cAMP levels drop within the mouse embryo as it develops from the 2-cell stage into a blastocyst (Fisher and Gunaga, 1975). Perhaps the presence of activatable cyclase activity is a preparation for implantation and has no purpose during earlier events such as cavitation.

It is difficult to predict the magnitude of putative sodium-carried transcellular ion current loops in situ because there are no reliable data on the magnitude of the Na/K-ATPase activity and the Na ion fluxes across outer blastomeres of cavitating morulae during the time nascent blastocoele fluid production occurs. In addition, there are probably other apical-basal ion fluxes that are involved with the generation of these current loops and there is insufficient data on these as well. It must be remembered that the current mapping around isolated blastomeres was performed on artificially enlarged blastomeres and it is difficult--if not impossible--to extrapolate from our data to probable current magnitudes in situ. What this experiment on current mapping does show, however, is that the currents exist and that they are vectored and are carried largely by Na.

We have no data on the effect of cell-cell contact on current magnitude. However, the associated voltage for a given current magnitude would increase with cell-cell contact because of increasing resistance to current flow (Ohm's Law, V = IR) and the development of junctions would increase resistance even further to produce further increases in voltage for a given current magnitude (see Jaffe, 1981). It is the voltage generated by the currents as well as the currents themselves (perhaps by the associated electro-osmotic drag of water?) that is postulated to be an effector of cell surface and cytoplasmic asymmetries (see Jaffe, 1981).

Reviewer II: With respect to the Metabolic Cavitation Model, how much lipid is potentially available in an embryo for blastocoele fluid formation? Is it sufficient to account for the amount of fluid that is accumulated within the blastocoele? What effect do variations in sodium concentration of the culture medium have on aspects of embryonic development other than blastocoele formation, e.g., cell division?

Author: The mouse blastocoele contains approximately 0.40-0.45 nl of fluid (Dickson, 1966; Biggers, 1972). The following calculations are based on the assumption that the vast majority of this fluid is water (the remainder consisting primarily of salts). Using the values of 0.45 nl of water for volume, 18 for the molecular weight of water and 6.02×10^{-23} for Avogadro's number, this volume corresponds to 1.5×10^{-9} moles, or, 1.5×10^{14} molecules of water. It is estimated that the amount of lipid in the mouse oocyte is 0.003 µg of which about half consists of cholesterol and its esters (Loewenstein and Cohen, 1964; Pratt, 1978, text reference). Assuming that the remainder consists of neutral lipid, this leaves the oocyte with 0.0015×10^{-9} g neutral lipid. Taking triacylglycerol containing palmitic acid as a generic neutral lipid, whose molecular weight is 374, 0.0015×10^{-9} g corresponds to 1.5×10^{-9} moles or 24.4×10^{-11} molecules of lipid of which 21.7×10^{-11} molecules consist of palmitic acid, the remainder being triglycerol. For every 16-carbon molecule of palmitic acid that is catabolized via beta oxidation, 146 molecules of water are generated

(Lehninger, 1972). Consequently, from 0.0015 g neutral lipid (using palmitic acid as an example) that is present in the oocyte, the embryo can produce 10^{14} molecules of water. However, in addition to this potential source of lipid, the cleaving embryo can also take up neutral lipid (palmitic acid) from the culture medium--and presumably, therefore, also from oviductal fluid (see Nadijcka and Hillman, 1975 text reference) and can synthesize significant amounts of lipid (Wales and Whittingham, 1970), especially as it nears the morula stage (Flynn and Hillman, 1978 text reference). Together with the potential store of lipid that it could inherit from the oocyte, the uptake of exogenous lipid that is available in the environment and the lipid that is synthesized during cleavage could provide sufficient lipid for the production of the estimated 10^{14} molecules of water in the blastocoele.

We have some unpublished data on the effect of varying the concentration of Na of the culture medium on development. To come to our conclusion regarding the effect of reduced Na_o on the cortical localization of mitochondria to basolateral cell borders, we considered the data obtained with 147.2 mM, the control concentration of Na_o, and 98.5 mM, which impaired cavitation and the basolateral cortical localization of mitochondria. In 98.5 mM Na, 2-cell stage embryos developed into grossly morphologically normal morulae, which at the E.M. level, exhibited no overt signs of cell degeneration. However, 98.5 mM Na also impaired mitosis so that after 66 h of culture from the 4-cell stage embryo cell number was approximately 14 (in 147.2 mM Na embryo cell number was close to 80). The external concentration of Na can influence cell proliferation in vitro (see Rozengurt and Mendoza, 1980) and glucose transport in the generic mammalian cell (i.e., co-transport is dependent on Na in the generic mammalian cell (see Lehninger, 1972). Prior to the morula stage, carbohydrate (glucose, pyruvate, lactate) transport in mouse embryos is not related to Na transport (Powers and Tupper, 1977 text reference) and we are unaware of any data on whether this situation is also true for the mouse morula. Mouse morulae do take up and metabolize exogenous glucose (see Brinster, 1965a,b), and the possibility therefore exists that glucose uptake by the mouse morula could be sensitive to Na_o. So the effect we observed of 98.5 mM Na_o on cavitation, mitochondrial localization and cell division could have resulted from energy substrate (glucose) uptake insufficiency. Consequently, we cultured embryos in the absence of glucose, leaving pyruvate and lactate in the culture medium containing either 147.2 mM or 98.5 mM Na_o. With 147.2 mM Na_o, neither embryo diameter nor cell number nor blastocoele formation was affected. With 98.5 mM Na_o, embryo diameter remained unaffected, while cell division and cavitation were still impaired. Reduced embryo cell number *per se*, does not inhibit cavitation (Smith and McLaren, 1977) and under certain conditions, 2-cell stage embryos will accumulate intercellular fluid and "cavitate", in the absence of any further DNA replication (Johnson, 1986 text reference). Taken together, all of these observations suggest that the effect of reduced Na_o on cavitation was not a (direct?) result of altered exogenous carbohydrate energy substrate availablility to the embryo. At this time we are left with the observation that reduced Na_o impairs cavitation and basolateral cortical localization of mitochondria--and cell division--but we cannot not yet appreciate the nature of the connection between impaired cell division and the rest of these observations. Interestingly, cell division rate in 98.5 mM Na_o is not impaired until the fourth division, so the effect that reduced Na_o may have on cell division is not evident during earlier divisions.

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