Ultrastructural studies of the mouse blastocyst substages

By MARY NADIJCKA AND NINA HILLMAN

From Department of Biology, Temple University, Philadelphia

SUMMARY

The mouse blastocyst stage covers approximately 2 days. During this period, embryonic development advances through four ultrastructurally distinct substages. Specific ultrastructural characteristics, such as changes in cell shape and/or the distribution of intracellular organelles, may be used to characterize the various cell types from each other at each substage (e.g. trophoblast from inner cell mass cells), as well as to distinguish the same cells from each other at different substages (e.g. substage 1 embryonic trophoblast cells from substage 2 embryonic trophoblast cells). A distinguishing characteristic of these blastocyst substages are the membrane contacts (tight junctions, desmosomes, focal tight junctions) between cells. The apical surfaces of adjacent trophoblast cells, from the time of cavity formation until the initial stage of implantation, are connected by a tight junction and a desmosome. The basal region of the apposed trophoblast membranes, however, vary both in their spatial interrelationship with each other and in their defined cellular contacts. Likewise, the cell contacts between the inner cell mass cells, and between these cells and the embryonic trophoblast cells, are distinctive for each blastocyst substage.

INTRODUCTION

Several light and electron microscopic studies of the mouse blastocyst have been reported (Enders & Schlafke, 1965; Reinius, 1965, 1967; Potts & Wilson, 1967; Potts, 1968; Enders, 1971; McReynolds & Hadek, 1972). However, descriptions of the continuous changes which take place between the time of completion of cavitation and the time when the embryo hatches from the zona pellucida and begins to implant are incomplete. The importance of the changes which occur during this period of development and the necessity for correct blastocyst staging and description are apparent in the light of several recent investigations. For example, Stern (1972) and Stern & Wilson (1972) found that mouse blastocyst cells are developmentally labile, and Gardner (1972) reported that the continued presence of the inner cell mass is necessary for trophoblast proliferation at the time of implantation. The studies which led to these conclusions utilized early blastocyst embryos which had not hatched from the zona pellucida. The continuous development of blastocyst embryos, however, may be expected to result in ultrastructural and physiological changes both within

1 Authors' address: Department of Biology, Temple University, Philadelphia, Pennsylvania 19122, U.S.A.
and between different embryonic cells from the time of blastocyst cavity formation until implantation. This, in turn, suggests that one may not be able to extrapolate the findings from studies utilizing early blastocyst embryos to include older blastocyst embryos. On the basis of ultrastructural evidence we have found that the two day developmental period between cavity formation and initial implantation may be divided into four substages of development. A description of these substages is presented so that mouse blastocyst staging may be more closely determined for experimental studies. Particular attention has been directed toward describing the types as well as the time of appearance of cell junctions during this embryonic period.

MATERIALS AND METHODS

Eight-week-old randomly breeding Swiss albino female mice were synchronously ovulated (Edwards & Gates, 1959) by intraperitoneal injections of 2.5 i.u. pregnant mare serum (Equinex, Ayerst), and 48 h later 2.5 i.u. human chorionic gonadotrophin (Pregnyl, Organon). Following the second injection, Swiss albino male mice were placed overnight with the females which were checked the following morning for the presence of copulation plugs. The pregnant females were designated as being in day 0 of pregnancy.

For the routine ultrastructural studies, embryos were allowed to develop in vivo to the desired developmental stage and were then removed from the uteri and fixed immediately. The blastocysts ranged from 3.5 to 5.25 days old, each litter containing embryos of variable blastocyst substages. Prior to settling into the crypts, blastocysts can be obtained by flushing excised uterine horns with Brinster's medium (Brinster, 1963). After the embryos have settled into the uterine crypts, it becomes increasingly difficult to obtain the embryos from the uterine horns by flushing with medium; however, they can readily be obtained by flushing with 3% glutaraldehyde (Bergström, 1972).

The recovered embryos were fixed, dehydrated and embedded as previously described (Hillman & Tasca, 1969; Hillman, Hillman & Wileman, 1970). The embryos were serially sectioned, taking alternate thick and thin sections. Thick sections were stained with methylene blue-azure II (Richardson, Jarett & Finke, 1960) and the thin sections, with lead citrate alone (Venable & Coggeshall, 1965) or with lead citrate preceded by uranyl acetate (Watson, 1958). Three hundred and forty-four blastocysts were processed and examined for this study.

In order to analyze junctional complexes, other timed, pregnant females were killed, and the excised uteri were flushed with 3% glutaraldehyde in 0.2 M s-collidine buffer, pH 7.5. The collected embryos were washed with collidine buffer, and postfixed with a 2:1 mixture of 2% aqueous OsO₄ and collidine buffer for 1 h. These embryos were stored overnight in the buffer at 2 °C, and stained en bloc with 2% uranyl acetate for 1 h (Goodenough & Revel, 1970). They were then dehydrated, embedded in Epon, and sectioned for ultrastructural
In the present paper we use the terms tight junctions, adhering junctions, desmosomes and close junctions to denote cellular contacts. Tight junctions, also called zonulae ocludentes (Farquhar & Palade, 1963, 1965), are those junctions in which the outer laminae of adjacent cell membranes are fused, obliterating the intercellular space. Adhering junctions, also referred to as zonulae adhaerentes (Farquhar & Palade, 1963, 1965), are characterized as having dense cytoplasmic plaques subjacent to the plasmalemmata and an intercellular gap of greater than 25 nm. Desmosomes, referred to as maculae adhaerentes (Farquhar & Palade, 1963, 1965), are characterized by dense cytoplasmic plaques and microfilaments (tonofilaments) subjacent to the plasmalemmata and an intercellular space of approximately 20 nm. Close junctions are those in which focal areas of apposing cell membranes appear more electron dense than the remainder of the cell membrane and the intercellular space is less than 10 nm (Trelstad, Hay & Revel, 1967). Tight junctions have been described as focal tight junctions (Trelstad et al. 1967) and as zonulæ, maculae or fasciae ocludentes (Farquhar & Palade, 1963, 1965), the term used being dependent upon the extent of fusion of the apposing cell membranes.

The following terms are used in referring to specific embryonic areas: trophoblast, the peripheral single cell layer; embryonic pole, the region of trophoblast adjacent to the inner cell mass; abembryonic pole, the trophoblast cells opposite the inner cell mass; lateral trophoblast, those peripheral cells between the two poles; juxtaluminal, the surface of the trophoblast cell which is exposed to the external (uterine) environment; juxtacoelic, that surface of the cells exposed to the blastocyst cavity; inner cell mass (ICM), those clustered cells which are enclosed by the single layer of trophoblast cells.

**RESULTS**

Our studies show that blastocyst embryos may be placed into one of four developmental substages: substage 1, the blastocoelic cavity is formed but not fully expanded; substage 2, the cavity is fully expanded, however, the ICM is composed of only one cell type; substage 3, the ICM is divided into two morphologically distinct cell types, the epiblast and proximal (visceral) entoderm; substage 4, the distal (parietal) entoderm develops and forms a continuous layer around the blastocyst cavity. The first two substages of the blastocyst occur prior to hatching from the zona pellucida, the third substage is found immediately before and after hatching, and the fourth, always occurs after the embryo is free from the zona pellucida.

The nuclei in cells at every substage have two, rarely three, nucleoli which are elongated and totally reticulated. The nucleoli contain both fibrillar and granulo-
fibrillar areas. The fibrils range from 5–7 nm in diameter, and the granules are from 10–18 nm in diameter. Condensed chromatin is usually associated with the inner lamina of the nuclear envelope. At all stages, granules (approximately 15 nm in diameter) are attached to the outer lamina of the nuclear membrane, and the inner and outer laminae of the nuclear membrane are separated by a space measuring 33–44 nm. In all cells, the contour of the nucleus follows the shape of the cell. Overall, the nuclei and their component parts are the same as those described for late morula embryos (Hillman & Tasca, 1969).

In substage 1 (Fig. 1) the abembryonic and lateral trophoblast cells are elongated (Fig. 2). The embryonic pole trophoblast cells and the ICM cells are squamous-like and similar in shape (Fig. 3). Short microvilli are present on the juxtaluminal surfaces of all the trophoblast cells but are most numerous on the embryonic pole cells (Figs. 2, 3). The juxtacoelic surfaces of all cells are smooth and have few cytoplasmic projections.

The types of organelles present and the ultrastructure of these organelles are the same in the trophoblast and ICM cells (Fig. 4). The cells contain a mixed population of mitochondria: the filamentous type, with parallel, transverse cristae together with the larger, round, multivacuolated mitochondria. These types are the same as those found in late morula mouse embryos (Calarco & Brown, 1969; Hillman & Tasca, 1969). Both trophoblast and ICM cells contain short lengths of rough endoplasmic reticulum (there is no enlargement of the endoplasmic cisternae in any cell) and crystalline inclusions. Free ribosomes are present in both trophoblast and ICM cells and polyribosomal clusters appear to be equally distributed between these two cell groups. Both groups contain

**Figures 1–4**

Fig. 1. Light micrograph of substage 1 blastocyst. The blastocoelic cavity (B) is formed but not fully expanded. The inner cell mass cells (ICM) are closely apposed to the overlying embryonic trophoblast cells (ET). Both the lateral (LT) and abembryonic trophoblast (AT) cells are elongated. × 400.

Fig. 2. Substage 1: An electron micrograph of an adjacent lateral trophoblast cell (LT) and abembryonic trophoblast cell (AT). Both cells are elongated. Note the presence of the few microvilli (MV) on the abembryonic cell surface. × 3800.

Fig. 3. Substage 1 embryonic trophoblast cells (ET) and an inner cell mass cell (ICM). Both cells are squamoid in shape. The juxtaluminal surface of these embryonic trophoblast cells contain numerous microvilli (MV). Note the close apposition of cells and the almost total lack of intercellular spaces. B, Blastocoelic cavity; ZP, zona pellucida. × 3800.

Fig. 4. A higher magnification of portions of a substage 1 embryonic trophoblast cell (ET) and of an adjacent inner cell mass cell (ICM). The types of organelles present and the ultrastructure of the organelles are the same in all trophoblast and ICM cells. These cells contain multivesicular bodies (mvb), two types of mitochondria (the multivacuolated (M1) and the elongated (M2)), degradation bodies (DB), perinuclearly located Golgi elements (g), crystalline inclusions (CI) and short lengths of rough endoplasmic reticulum (RER). These two cells are focally separated by an intercellular space (arrow). × 11500.
membrane limited degenerative bodies of varying sizes, lipid droplet inclusions and multivesicular bodies. There is a paucity of Golgi elements in the blastomeres; however, that which is present is perinuclearly located. Aggregates of short strands of fibrous material (Enders & Schlafke, 1965) are present in the cytoplasm of all cells prefixed in glutaraldehyde. Intranuclear annulate lamellae (IAL) are only occasionally found in the nuclei of this blastocyst stage. When present, the IAL are not limited to any specific cell type.

The lateral surfaces of adjacent trophoblast cells are closely applied (Figs. 2, 3)
and are connected at their apices by a tight junction and a desmosome (Fig. 5). These junctions are not contiguous. No junctions connect the basal edges of the closely apposed trophectodermal cells.

The ICM cells are connected to each other and to the overlying trophectodermal cells by focal tight junctions (Fig. 6), by desmosomes (Fig. 7), and by focal close junctions (Fig. 7A). Often a cluster or a chain of desmosomes, whose microfilaments overlap, connect the ICM to the trophectodermal cells, whereas only single desmosomes have been seen to connect adjacent ICM cells to each other (Fig. 7). Intercellular spaces are seldom seen between adjacent ICM cells or between these cells and apposing embryonic trophectodermal cells.

All the trophectodermal cells of the substage 2 blastocyst are elongate (Figs. 8–10). In contrast to the earlier stage, few microvilli are present on the juxtaluminal surface of any trophectodermal cell. Some cytoplasmic projections are found, however, on the juxtacoelic surfaces of trophectodermal cells, particularly on those cells which are abembryonically situated (Fig. 10).

The ICM cells, which are squamous-like in shape, are usually divided into two layers. These cells are indistinguishable from each other (Fig. 9). The juxtacoelic surfaces of these ICM cells are smooth. Both trophectodermal and ICM cells contain increased numbers of polyribosome clusters when compared with substage 1 embryos. There is, however, a decrease in the fibrous strands which were so predominant in the younger staged embryo. Rough endoplasmic reticulum is increased in both the trophectodermal cells and the ICM cells of substage 2, with more being present in the former cells. Both trophectodermal and ICM cells contain fewer multivesicular bodies than do those of the earlier substage, and IAL are only infrequently found in either group of cells. Both cell types contain lipid droplets, membrane limited degenerative bodies, perinuclearly located Golgi elements, and a mixed population of mitochondria. Crystalline inclusions are present in both cell types but are found more frequently in the trophectodermal than in ICM cells.

Adjacent embryonic and lateral trophectodermal cells are connected apically by tight junctions and by desmosomes. These connexions show the same spatial relationship to each other as in the previous stage. No other junctional complexes or interdigitations are found on the apposed surfaces of these cells. The abembryonic trophectodermal cells are also connected to each other by apical junctional complexes. In addition, the more basal portion of these apposed cell surfaces interdigitate.

The ICM cells are closely applied to each other and to the overlying embryonic trophectodermal cells. Only infrequently are small intercellular spaces found between adjacent ICM cells. Desmosomes, close junctions and tight junctions connect the trophectodermal cells to the ICM cells as well as the adjacent ICM cells to each other (Fig. 11). Frequently a tight junction and a desmosome form a complex which connects an embryonic trophectodermal cell with an adjacent ICM cell (Fig. 12). Also, as in substage 1, clusters of desmosomes connect these diverse
within the ICM, however, neither complexed junctions nor clusters of desmosomes connect apposing cells.

Laterally and at the embryonic pole the trophoblast cells of substage 3 are elongate and flattened (Figs. 14, 15). At the abembryonic pole they are of varying shapes, but are usually spindle-shaped (Fig. 16). There are no microvilli present on the juxtaluminal surfaces, but there are both juxtaluminal and
Fig. 11. In addition to desmosomes, tight junctions (tj) frequently connect adjacent substage 2 ICM cells with each other. ×66000.

Fig. 12. This micrograph shows a complexed tight junction and desmosome connecting a portion of a substage 2 inner cell mass (ICM) cell with a portion of an overlying embryonic trophoblast (ET) cell. ×66000.

Fig. 13. A cluster of desmosomes connecting a substage 2 ICM cell with an embryonic trophoblast (ET) cell. Neither desmosome clusters nor tight junction-desmosome complexes are found between adjacent ICM cells. ×66000.

Fig. 8. A light micrograph of a substage 2 blastocyst. The blastocoele (B) is expanded and all the trophoblast cells (embryonic, ET; lateral, LT; abembryonic, AT) are elongated. Intercellular spaces are infrequently present in the inner cell mass (ICM). ×400.

Fig. 9. An electron micrograph of a portion of a substage 2 inner cell mass (ICM) and embryonic trophoblast cells (ET). Note the elongation and flattening of the embryonic trophoblast cells (compare with Fig. 3). In distinction to substage 1, few microvilli are present on the juxtaluminal surfaces of these trophoblast cells. Note the extensive lengths of rough endoplasmic reticulum (arrows) in the trophoblast cells and the junctional complex (JC) between the trophoblast cells. Degradation bodies (DB), lipid droplets (L) and diverse ultrastructural types of mitochondria (M) are present in all cell types. B, Blastocoelic cavity. ×6600.

Fig. 10. An ultramicrograph of substage 2 abembryonic (AT) and lateral trophoblast (LT) cells. Cytoplasmic projections (CP) are present on the juxtapacoelic surfaces of trophoblast cells, particularly on those located abembryonically. B, Blastocoelic cavity. ×2600.
Studies of mouse blastocysts

juxtacoelic cytoplasmic projections (particularly in the abembryonic region) which appear in cross-section as membrane limited vacuoles (Fig. 16). Rough endoplasmic reticulum is present in all trophoblast cells but is more extensive in the embryonic region. Crystalline inclusions and multivesicular bodies are found only infrequently at this stage. Degenerative bodies are almost totally restricted to the abembryonic and lateral trophoblast cells; and lipid droplets, present throughout the trophoblast, are found most frequently in the abembryonic cells. The two types of mitochondria described in younger blastocyst cells are retained by both the ICM and trophoblast cells in substage 3 embryos. IAL have not been observed in the nuclei of substage 3 embryos.

The ICM in substage 3 is composed of two morphologically distinct cell types – the epiblast and the proximal entoderm (Fig. 15). The epiblast cells are found in two cell layers and are arranged either in regular stacks or in irregular clusters. The proximal entodermal cells form a single continuous layer beneath the epiblast (Figs. 14, 15). These entodermal cells, whose lateral edges often overlap (Fig. 15), assume a variety of shapes but usually are either cuboidal or spindle-shaped. The rough endoplasmic reticulum of proximal entodermal cells is more extensive than that found in either the epiblast or the embryonic trophoblast cells. Enlarged cisternae of the rough endoplasmic reticulum in the entodermal cells distinguish these cells at this substage (Fig. 17A). The epiblast cells contain the least extensive rough endoplasmic reticulum in substage 3.

Adjacent trophoblast cells both at, and immediately lateral to, the embryonic pole are connected apically by a junctional complex (tight junction and desmosome) and basally by an additional desmosome (Fig. 18). Continuing laterally to the abembryonic pole, the trophoblast cells are connected apically by a

Figures 14–17

Fig. 14. A light micrograph of a substage 3 blastocyst. The inner cell mass is divided into two cell types, the epiblast (EP) and proximal entoderm (P.Ent.). The embryonic (ET) and lateral (LT) trophoblast cells are still elongate, whereas the abembryonic (AT) cells are irregularly shaped. B, Blastocoelic cavity. × 400.

Fig. 15. A section through a substage 3 embryonic area. The embryonic trophoblast (ET), which has few juxtaluminal cytoplasmic projections, overlies the ICM which is composed of the epiblast (EP) and proximal entoderm (P.Ent.). Note the intermingling of cell processes and the numerous intercellular spaces. × 7500.

Fig. 16. This ultramicrograph shows substage 3 abembryonic trophoblast (AT) cells. Note the lipid inclusions (L), degradation bodies (DB), and vacuoles (V). B, Blastocoelic cavity. × 3400.

Fig. 17. (A). A higher magnification of portions of adjacent epiblast (EP) and proximal entodermal (P.Ent.) cells. The organelles of these cells are the same but the increased size of the cisternae of entodermal rough endoplasmic reticulum (RER) distinguishes the two cell types. M, Mitochondria; cj, close junction. × 15000. (B). This insert shows the amorphous material (AM) present on the juxtacoelic epiblast (EP) cell surface. This material is also present, in patches, on the juxtacoelic surfaces of the trophoblast cell surfaces adjacent to the inner cell mass. × 35000.

EMB 32
Figures 18-20

Fig. 18. Portions of adjacent substage 3 embryonic (ET) and lateral (LT) trophoblast cells. These cells are connected apically by a junctional complex (JC) and basally by a desmosome (d). This embryo is still enclosed by a zona pellucida (ZP). x 15700.

Fig. 19. Portions of adjacent substage 3 abembryonic trophoblast cells. In this region the adjacent cells are connected apically by a junctional complex (JC). The remainder of the apposing cell surfaces interdigitate. Note the presence of amorphous material (AM) on the juxtacoelic surface. B, Blastocoelic cavity. x 32800.

Fig. 20. Tight junctions (arrow) connect substage 3 embryonic trophoblast (ET) cells with underlying epiblast cells (EP). Desmosomes are only rarely seen between epiblast cells, proximal entodermal cells or between these two cell types. x 66000.

The embryonic cells are not as closely applied to the embryonic trophoblast cells as they were in the younger substages. However, the epiblast and trophoblast cells are still connected to each other by focal tight junctions and,
Studies of mouse blastocysts

infrequently, by desmosomes. Patches of amorphous material, most likely the beginning of the basement membrane, may be found in the intercellular spaces between the ICM and embryonic trophoblast as well as on the juxtaacoelic surfaces of lateral and abembryonic trophoblast cells (Fig. 19). This amorphous material is also found between adjacent epiblast and entodermal cells (Fig. 17 B).

Intercellular spaces are present between all adjacent embryonic cell mass cells (Fig. 15). Cytoplasmic processes from adjacent cells intermingle regardless of cell type. In this substage, desmosomes are rarely found between ICM cells. However, focal close junctions and tight junctions between like and unlike cells are more numerous than in younger blastocyst substages (Fig. 20).

In substage 4 (distinguished by the formation of distal entoderm), the only trophoblast cells which are elongated are those of the embryonic trophoblast (Fig. 24). The remaining are either spindle-shaped or randomly shaped (Figs. 21–23). Cytoplasmic projections are present on the juxtaluminal membranes of the trophoblast, the projections being most numerous at the abembryonic pole. Juxtaluminal microvilli are also present, particularly in the abembryonic region; and the abembryonic cells contain numerous lipid droplets and large degenerative bodies (Fig. 22). Few crystalline inclusions have been observed in any cells at this developmental stage. The proximal and distal entodermal cells contain the most extensive rough endoplasmic reticulum found in this substage and in both of these cell groups the cisternal spaces are enlarged (Fig. 25). The rough endoplasmic reticulum in the epiblast cells is more extensive than in the previous blastocyst substage, while the rough endoplasmic reticulum in the trophoblast cells is similar in appearance and amount to that found in the trophoblast cells of substage 3. Lipid droplets are present in both ectodermal and entodermal cells, whereas degradation bodies are usually absent. The shape and cristal orientation of the mitochondria are variable, and the mitochondrial matrix is condensed. The mitochondria in all the cell types have the same diverse ultrastructural appearances.

Adjacent trophoblast cells are connected apically by a tight junction and a desmosome. In addition, interdigitations are found between all lateral and abembryonic trophoblast cells (Fig. 25). A basement membrane is present on the juxtacoelic surfaces of all trophoblast cells and appears to be continuous (Fig. 25) except at some focal points where tight junctions still attach the embryonic cell mass to the overlying trophoblast.

Within the embryonic cell mass, both the epiblast cells and entodermal cells are separated from each other by large intercellular spaces, but cytoplasmic extensions of adjacent cells do intermingle (Fig. 24). Only tight junctions have been found to connect the epiblast cells, proximal entoderm cells and these diverse cells to each other (Fig. 26). Desmosomes have not been found either among or between these two distinct cell types. Also, only tight junctions have been noted connecting adjacent distal entodermal cells.
DISCUSSION

Mouse embryonic development from the two-cell stage to beginning blastocoelic cavity formation covers approximately 2 days and may be divided into six ultrastructurally distinct developmental stages (2-, 4-, 8-cell, early morula, late morula, early blastocyst). It is reasonable, therefore, that the blastocyst stage which encompasses the subsequent two days of development should also show ultrastructural differentiation and that a description of the changes which take place during this period could result in subdividing the blastocyst into distinct and separate substages. The findings from the present study do in fact show that the blastocyst stage may be divided into four substages which differ from each other morphologically.

Detailed studies on the ultrastructure of rat blastocysts, both before (Schlafke & Enders, 1967) and after (Enders & Schlafke, 1967) the shedding of the zona pellucida are available. Although the mouse blastocyst and rat blastocyst are similar in their ultrastructural morphologies, the temporal and sequential development of these embryos is distinctive. For example, in the rat blastocyst the proximal entoderm does not form until after the zona pellucida is shed whereas in the mouse, this layer is usually present prior to hatching. In addition, desmosomes are not present in the ICM of the rat blastocyst until after the formation of proximal entoderm, at which time they are found only between entodermal cells. Later they may be seen between ectodermal cells. In the mouse, desmosomes are present in the ICM prior to proximal entoderm formation after which they begin to diminish in number. Prior to the formation of desmosomes in the rat blastocysts, adjacent cells are connected by 'primitive' desmosomes, which appear to be the same as the close junctions described here in mouse.

FIGURES 21–24

Fig. 21. A light micrograph of a substage 4 blastocyst. These blastocysts are never enclosed in a zona pellucida. The blastocoelic cavity (B) is fully expanded and distal entoderm (D.Ent.) is formed. The embryonic (ET) and lateral (LT) trophoblast cells are elongated and spindle-shaped whereas the abembryonic cells (AT) are of variable shapes. The ICM is separated into epiblast (EP) and proximal entodermal cells (P.Ent.). × 400.

Fig. 22. Abembryonic trophoblast cells of a substage 4 blastocyst. Note the irregular shape of the cells, the cytoplasmic projections and the numerous lipid droplet (L) inclusions. B, Blastocoelic cavity. × 2200.

Fig. 23. Lateral trophoblast (LT) cells of a substage 4 blastocyst. Microvilli and some cytoplasmic projections are present on the juxtaluminal surfaces. Some lipid droplets are found in these cells. Portions of distal entodermal cells (arrows) are adjacent to the lateral trophoblast layer. × 2700.

Fig. 24. Embryonic trophoblast (ET), epiblast (EP) and proximal entoderm (P.Ent.) of a substage 4 embryo. The embryonic trophoblast cells are elongated and flattened. Large intercellular spaces are found between like and unlike cells. B, Blastocoelic cavity. × 3100.
Studies of mouse blastocysts

blastocysts. Additionally, rat embryos have not been processed for the study of tight junctions. The present study is the first, therefore, in which tight junctions have been described in mammalian embryos.

The importance of correct staging of the mouse blastocyst is apparent when one considers the experiments by Gardner (1972), which show that the presence of ICM cells is necessary for the proliferation of abembryonic trophoblast cells at the time of implantation. At the stage tested, substage 2, few ultrastructural differences distinguish abembryonic trophoblast cells from other trophoblast cells, and the ICM is still closely adherent to the embryonal trophoblast. In substage 3, however, the abembryonic trophoblast cells show several distinct morphological features which distinguish them from lateral and embryonic trophoblast cells and by substage 4, the ICM and trophoblast are almost totally separated from each other by amorphous material. Such ultrastructural distinctions and/or changes in cellular apposition may be prerequisites for trophoblast proliferation. Therefore, abembryonic trophoblast cells isolated from either substage 3 or 4 might not be dependent upon the presence of the ICM for proliferation in receptive uteri.

The necessity for correct staging of the mouse blastocyst is also evident when one examines the reaggregation experiments by Stern (1972) and Stern & Wilson (1972). The embryos used by these investigators correspond to substage 2, or perhaps substage 3, blastocysts. None of the embryos were developed to the stage in which distal entoderm is formed. The experimental blastocysts were, however, composed of at least three, and possibly four, morphologically distinct cell types (abembryonic trophoblast, embryonic trophoblast, epiblast and proximal entoderm). After dissociation and reaggregation these cells are rechannelled into a second pattern of development, a pattern determined by their position in a new spatial orientation.

Our studies show that substage 2 and substage 3 cells are attached to each other via differing cell contacts. Stern (1972) suggests that the continuation of blastocyst development after dissociation and reaggregation is attributable to the

Figures 25 and 26

Fig. 25. Adjacent lateral trophoblast (LT) cells are connected by junctional complexes (JC) apically, and basally interdigitate. An amorphous substance (AM) is present between the trophoblast cells and the distal entoderm (D.Ent.). This substance appears to be continuous around the juxtacoelic surface of the trophoblast. The distal entoderm, like proximal entoderm, has enlarged cisternae of the rough endoplasmic reticulum (RER). × 22 300.

Fig. 26. Substage 4 embryonic pole showing portions of embryonic trophoblast (ET), epiblast (EP) and proximal entodermal cells (P.Ent.). The embryonic cells are almost totally separated from the trophoblast cells. Although both tight junctions and desmosomes connect adjacent trophoblast cells, the plane of this section shows only the tight junction (arrow). Tight junctions also connect the like and unlike embryonic cells to each other and focally to the embryonic trophoblast. Desmosomes are rarely seen among these diverse cell types. × 9000.
complete disruption of cellular junctions followed by the refusion of cells by similar junctions following restoration of cell contact. If this hypothesis is correct, then the types of junctions and their spatial distribution between randomly reaggregated cells would not be dependent upon the type of cell occupying a specific position, but would be determined by the position itself.

There is evidence which supports this hypothesis. Giudice & Mutolo (1970) have shown that sea-urchin blastulae can be disaggregated and randomly reaggregated. Following reaggregation, normal terminal bars are found between the reorganized epithelial cells. Likewise, Wilson (1907) and Galtsoff (1925) have shown that dissociated sponge cells will reaggregate into normal sponges. Loewenstein (1967) has found that electronic coupling is rapidly restored between cells in these reaggregated sponges. These observations clearly show that specialized cell contacts are reinstated after cells are separated and allowed to randomly re-establish contact.

The presence of tight junctions in embryos has been assumed as a result of numerous electrophysiological studies. However, these specialized cell contacts have been described only in the chick (Trelstad et al. 1967) and in Fundulus (Lentz & Trinkaus, 1971). In the chick, tight junctions are first present after head-process formation, while in the teleost they are found in the junctional complexes between surface cells of blastular and gastrular embryos. Focal tight junctions in chick embryos are found simultaneously between cells of the same as well as between cells of different tissues. The tight junctions between like cells become more extensive as development progresses while those between unlike cells become less frequent. This dissolution of tight junctions coincides with the formation of basement laminae between different tissues. Although desmosomes are not present in head-process staged chick embryos, they are present in later developmental stages and are particularly well formed in the epithelium.

The mouse blastocyst is similar to the chick in having distinct changes in the types of cellular attachments between like and unlike cells during development. In the younger blastocyst embryos, when the cells are still closely adhering, numerous desmosomes and some focal tight junctions concurrently join the ICM cells to each other and to the overlying embryonal trophoblast. In the older-staged blastocysts, desmosomes are infrequently seen (except between adjacent trophoblast cells), and tight junctions become more numerous. Both the chick and mouse embryos, therefore, show a sequential appearance of specialized cell junctions; however, the order of appearance of these junctions differs between the two species. In the chick embryo, focal tight junctions are found before the appearance of desmosomes, whereas in the mouse blastocyst, desmosomes and tight junctions are simultaneously present with the tight junctions remaining after most desmosomes disappear.

It may be postulated that the formation of tight junctions at the apical surface of adjacent trophoblast cells in the mouse embryo is prerequisite for the formation and expansion of the blastocoelic cavity. The tight junctions may act
as a seal or barrier between two zones which are constitutively different (i.e. the lumen of the uterus and the blastocyst cavity). The trophoblast would thus resemble an epithelial transport system with the component parts of the junctional complexes, an apical tight junction and a basally situated desmosome, comparable to those of many epithelia (Farquhar & Palade, 1963; Dewey & Barr, 1970). Unlike epithelia, however, the embryonic junctional complex does not contain an intermediate close junction.

Nevertheless, the work of Gamow & Daniel (1970) supports the hypothesis that the trophoblast functions as an epithelium. As the blastocoelic cavity forms, the trophoblast layer actively transports water into the blastocyst cavity. This transport and subsequent expansion of the cavity is dependent upon the concentration of Na\(^+\) in the external medium. More recently, Borland & Tasca (1974) have found a Na\(^+\)-dependent pump for the active transport of certain amino acids (methionine and leucine) in both late morula and blastocyst stages. A Na\(^+\)-dependent active transport mechanism is not present in younger cleavage-staged embryos. The transport system becomes active immediately prior to and during cavity formation and expansion, a time coincidental with the formation of junctional complexes between trophoblast cells.

In addition to the hypothesis that specialized cell contacts are necessary for transport, several other suggestions for the importance of cell contacts and junctional complexes in embryos have been advanced (Trelstad et al. 1967; Lentz & Trinkaus, 1971). It has been postulated that both tight junctions and desmosomes function in cellular adhesion, and that tight junctions play an additional role in contact inhibition. It has also been suggested that in *Fundulus* embryos the tight junctions, as well as apical junctions (composed of a tight junction, adhering junction and desmosome), electrically couple adjacent peripheral cells. This latter hypothesis is suspect, however, in the light of recent studies suggesting that gap junctions and not tight junctions are responsible for electrical coupling (Bennett, 1973). The specific function(s) of these diverse contacts found between embryonic cells remains, therefore, highly speculative.

The research was supported by U.S. Public Health Research Grant HD-00827. Miss G. Wileman and Miss M. Morris provided excellent technical assistance.

**REFERENCES**


Studies of mouse blastocysts


(Received 6 April 1974, revised 17 May 1974)