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In Vitro Systems for the Study of Human Trophoblast Implantation

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INTRODUCTION

The process of implantation can be viewed as a series of distinct events involving trophoblast-endometrial interactions. Soon after hatching of the blastocyst, there is apposition of the trophectoderm to the endometrial epithelial cell followed by attachment of these two cell types. In the human, the trophoblast cells subsequently intrude in between endometrial epithelial cells and come in contact with the basement membrane. Degradation of this barrier subsequently occurs, thus allowing the frank invasion of the trophoblast into the endometrial stroma. This process of migration through the stroma continues until invasion of the maternal vessels is achieved, establishing hemochorial placentation. The cellular and molecular mechanisms involved in these series of events are virtually unknown. It is postulated that specific signals involving cell adhesion molecules, substrate adhesion molecules, as well as paracrine and autocrine interactions are delicately orchestrated so that successful establishment of pregnancy is achieved. It is hypothesized that well-synchronized activation of cellular processes are involved in attachment and invasion, while reciprocal events are needed to arrest this process once placentation has been completed.

The progress in our understanding of the process of human embryo implantation has been extremely slow. This is principally due to the fact that the process of nidation is morphologically different in humans than in experimental and domestic animals.[1,2] Presumably, these morphologic variations have their basis on fundamental differences at the cellular and molecular levels. *In vivo* human experimentation for the study of implantation is not feasible, and *in vitro* systems would require the use of human embryos which are not available. Given the impressive advances in assisted reproductive technologies that have occurred over the last decade, as well as the increasing needs for establishment of methods for both contraception and contragestion, elucidation of the mechanisms of human implantation is imperative. The recent development of

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techniques for isolation of purified placental cytotrophoblasts have allowed characterization of their structural, morphologic and functional differentiation at the cellular and molecular levels (ref. 3; for review see 4). The accumulating evidence suggests that these cells possess many of the characteristics of the implanting trophoblasts of the blastocyst and thus can be used as surrogates in *in vitro* models of human implantation.[5-7] In the present report, our current understanding of the morphologic and functional differentiation of isolated human trophoblasts will be reviewed, and some preliminary observations of the behavior of these cells in several *in vitro* model systems will be described. We believe that understanding the behavior of these cells at the molecular level will shed light on the fundamental processes involved in nidation of the human blastocyst.

HUMAN TROPHOBLASTS IN CULTURE

Mononuclear cytotrophoblasts can be isolated from human placentae by a process of mincing, trypsin-DNase digestion of placental villi, and subsequent centrifugation of the digest through a discontinuous percoll gradient. A population of 96 to 98% pure mononuclear cells can be obtained at a density of 1.048–1.065. The isolated cells have been well-characterized and possess the morphologic and functional characteristics of cytotrophoblasts.[3] These cells can be viewed as the trophoblastic precursor cell, which upon differentiation through a process of aggregation and fusion can produce the terminally differentiated syncytial trophoblast. The cytotrophoblast is the mitotically active cell whose behavior with respect to both morphology and function depends upon its environment. The process of morphologic differentiation of the trophoblast from mononuclear cytotrophoblast to multinucleated syncytial trophoblast can be observed *in vitro* using purified cytotrophoblast preparations from human placentae. Using standard culture conditions and serum-containing media, the cells can be seen to migrate towards each other, form aggregates, and finally fuse to form syncytia over a period of 72 to 96 hours.[3] This process of morphologic differentiation can also be achieved in serum-free conditions if the culture surface has been precoated with extracellular matrix components such as fibronectin, laminin, and different types of collagen.[8] It appears that extracellular matrix components provide the lattice for movement of these cells. The presence, though, of extracellular matrix proteins is not obligatory for the process of morphologic differentiation since both aggregation and fusion of the cells can be achieved if the cells are cultured in serum-free media in *suspension*.[9] This culture system allows for random collision of these cells, which allows their aggregation and subsequent fusion. Morphologically, the cellular aggregates form cystic structures which upon subsequent plating on matrix components form outgrowths resembling villi. Under these conditions, scanning electron microscopic studies have indicated these aggregates to be covered with microvilli (FIG. 1). It has been clearly shown that this process of aggregation is protein synthesis dependent, and requires the presence of calcium ions.[9] Preliminary observations have indicated that the calcium-dependent cell adhesion molecule (CAM) E-cadherin is intimately involved in this process.[10,11] In addition, transmission electron microscopic studies have shown the establishment of desmosomes between the aggregating cells[9] and desmoplakins I and II (which are integral components of the desmosomal complex) have been demonstrated to be present at points of cell contact through immunohistochemistry.[11] This organization of the cell adhesion system is transient since the

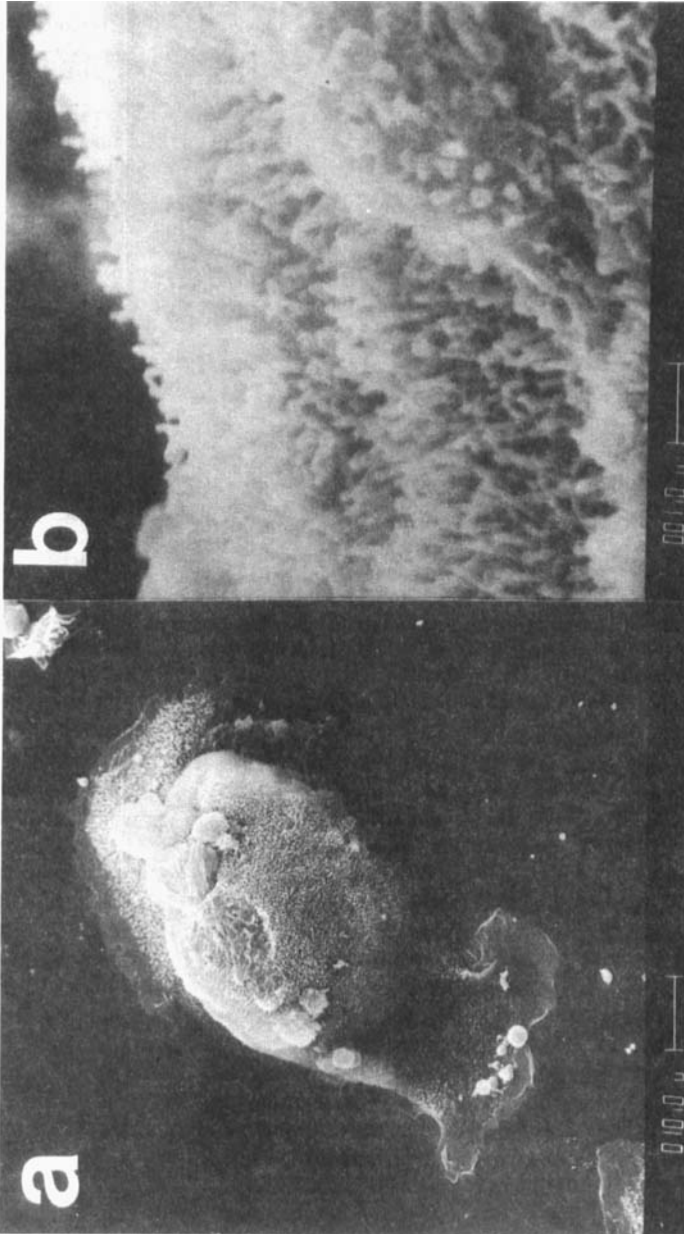

FIGURE 1. Scanning electron micrographs of a human trophoblast aggregate in outgrowth culture. Aggregates were prepared by overnight incubation of purified human cytotrophoblasts in a shaking suspension culture (see ref. 9). The aggregates were subsequently allowed to attach to glass coverslips in serum containing media and fixed and prepared for scanning EM after 24 h of incubation. The aggregates project from the culture surface (**a**) and are covered by microvilli (**b**).

trophoblast cells subsequently fuse and the cell surface expression of E-cadherin and desmoplakins disappears.[10,11]

In parallel to this process of morphological differentiation functional differentiation of the cells with respect to endocrine activity also occurs. The freshly isolated mononuclear cytotrophoblasts do not synthesize or secrete the hormones characteristic of the placenta (*e.g.*, chorionic gonadotropin, placental lactogen, progesterone, and estrogen) while the resultant multinucleated syncytial structures do.[3] This process mimics the *in vivo* situation where the mononuclear cytotrophoblasts do not exhibit this characteristic endocrine function while the syncytial trophoblasts do. Interestingly, the processes of morphologic and endocrine differentiation of the cells can be uncoupled by the addition of cyclic AMP analogues to the culture media or of agents which increase intracellular cyclic AMP.[12] The involvement of this classic "second messenger system" in the functional differentiation of these cells may suggest a role for paracrine or autocrine factors in the function of these cells *in vivo.*

TROPHOBLAST-EXTRACELLULAR MATRIX INTERACTIONS

As described above, morphologic differentiation of the trophoblast cells under standard culture conditions requires extracellular matrix components which presumably provide the lattice for movement of the cells. The intruding/invading trophoblast *in vivo* comes in contact with various extracellular matrix components through its journey from the endometrial epithelium to the maternal vessels. Initially, binding and subsequent degradation of basement membrane needs to be accomplished. This involves interactions of the trophoblast with type IV collagen as well as laminin and proteoglycans. In the *in vitro* system described, precoating of the culture surface with type IV collagen or laminin, allows for binding and movement of these cells.[8] In addition, preliminary immunohistochemical observations using monoclonal antibodies against various integrins have indicated the presence of the α^6-β_1 heterodimer, which corresponds to a laminin receptor (Coutifaris, unpublished observations). Recent studies have indicated that plating of these cells on a critical thickness of matrigel not only allows for the morphologic differentiation of these cells through aggregation and fusion, but also promotes the elaboration of enzyme(s) which degrade the laminin component of this basement membrane construct.[13] This process may involve the elaboration of urokinase-type plasminogen activator and its inhibitors, which have been shown to be secreted by these cells under the described culture conditions.[14,15] It is of interest to note that *in vivo*, urokinase has been implicated to be of importance in the process of implantation.[16] Further, recent evidence suggests the elaboration by these trophoblastic cells of metalloproteinases, which can degrade gelatin and specifically ones with collagenolytic activity (ref. 17; Abisogun & Coutifaris, unpublished observations). Therefore, these trophoblastic cells isolated from placentae not only have the capacity and molecular mechanism to attach to the basement membrane through specific cell-substratum adhesion molecules, but also possess the cellular mechanism for degradation of this structural component which separates the epithelium from the stroma.

Another extracellular matrix protein which the trophoblast encounters is fibronectin. The α^5-β_1 integrin has been demonstrated to be present on the surface of the purified cytotrophoblasts and shows specific distribution along adhesion plaques between the substrate and the cell (Coutifaris, unpublished observations). Normal motility of these cells with subsequent aggregation and fusion has been demonstrated if the culture

surface has been precoated with fibronectin,[8] and placement of aggregates onto millicell filters precoated with fibronectin shows a prompt attachment with subsequent degradation of the fibronectin surrounding the trophoblast aggregates (FIG. 2). This is a time-dependent phenomenon, which terminates in the detachment of the trophoblast aggregate from the culture surface once the extacellular matrix has been completely degraded, since the millicell filter cannot support attachment. It appears, that although addition of cyclic AMP inibits the degradation of laminin by these cells,[13] the degradation of fibronectin is further enhanced, thus suggesting a possible differential regulation of proteolytic processes by the stage of differentiation of these cells.

HETEROLOGOUS CO-CULTURE SYSTEMS

The first interaction between trophoblast and endometrium involves the adhesion of trophoblast to the endometrial epithelial cell, followed by intrusion of the trophoblast in between these cells. Characterization of both a two- and a three-dimensional co-culture system have provided the tools for study of these processes at the cellular and molecular level.[5-7]

Endometrial epithelial cells can be isolated from human endometrium using a procedure developed by Satyaswaroop *et al.*[18] The endometrial epithelial cells plated under usual culture conditions on glass or plastic, form islands of cells, which have been characterized to be epithelial in nature by their characteristic cytokeratin immunostaining. After establishment of these cells in culture, their interaction with trophoblastic cells can be studied by the addition of purified human cytotrophoblasts. When this is done, the trophoblasts are observed to aggregate around the islands of endometrial epithelial cells, intrude in between them and penetrate deep into these cell islands.[5] This process appears to be gentle rather than destructive and allows the gradual separation of the junctions between the epithelial cells by the intruding trophoblasts. The endometrial epithelial cells subsequently remodel themselves around the trophoblasts, eventually completely encasing them. This process, which occurs in this two-dimensional co-cuture system, is reminiscent of the early intrusive process of trophoblasts *in vivo*, during which the trophoblast intrudes between endometrial cells rather then destroying them. Once the *in vivo* process of intrusion through the epithelium is complete, the implanting embryo is completely imbedded in the endometrium and covered by epithelium at the original implantation site. As is the case *in vivo*, the described *in vitro* system requires the transient dissolution of the adhesion system between the endometrial epithelial cells. Preliminary immunohistochemical observations show the disappearance of desmoplakins I and II from the cell surface at the points of endometrial epithelial cell contact as the intruding trophoblast is allowed to penetrate the epithelial cell islands. The mechanism by which this cellular dissociation is achieved with no apparent destruction of the epithelial cells is unknown at the present time.

The process of implantation *in vivo* is a three-dimensional event. Thus, the establishment of a three-dimensional system is imperative in order to have the capability of studying the regulation of attachment of the trophoblast to the endometrium. If human endometrial epithelial cells are isolated and placed onto extracellular matrix coated platforms (type IV collagen, laminin, matrigel) a confluent cell layer can be established, which shows distinct polarization of the epithelial cells with a basal region adjacent to the matrix-coated platform and an apical region equivalent to the

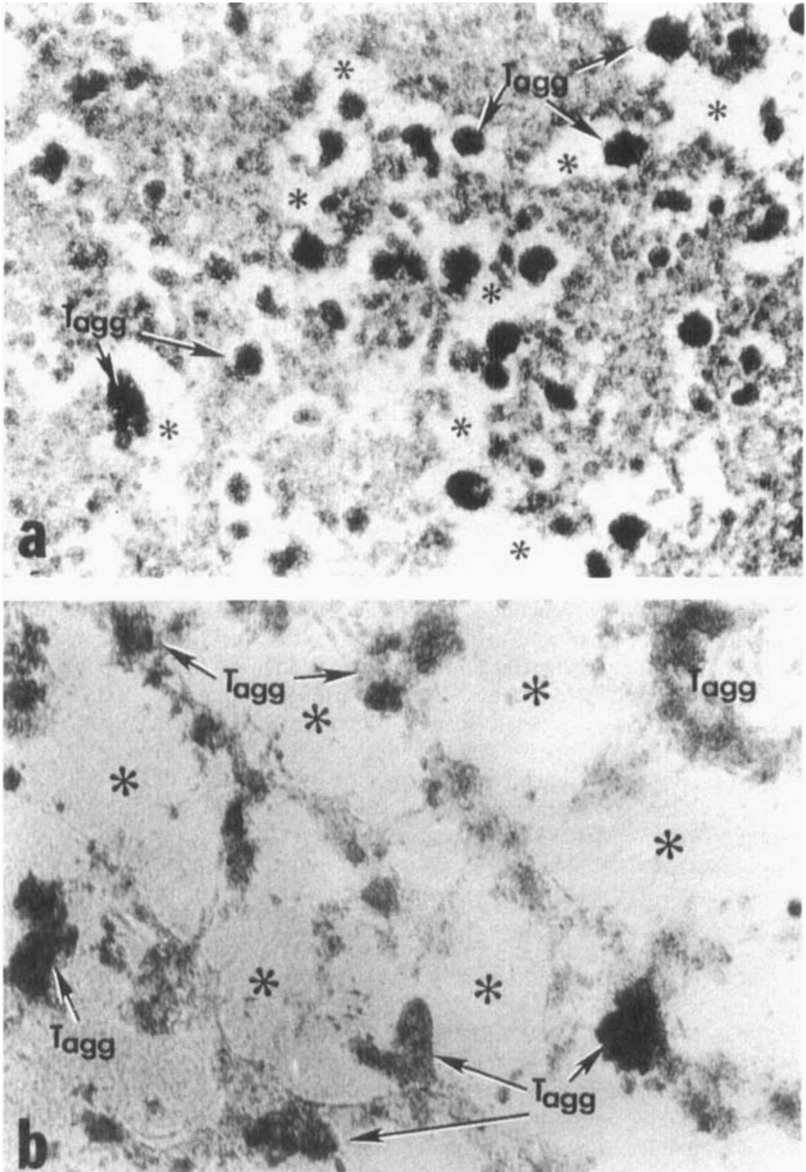

FIGURE 2. Culture of trophoblast aggregates (Tagg) on fibronectin-coated millicell-CM filters. Aggregates were prepared as described in ref. 9, placed onto fibronectin precoated millicell-CM filters, incubated in serum-free media in humidified 95% air–5% CO_2, and observed under an inverted microscope at time intervals up to 96 h (*magnification:* ×100). **a.** At 48 h of incubation, note areas of clearing of the granular fibronectin layer (*) around trophoblast aggregates. **b.** At 96 h, extensive areas of clearing of fibronectin (*) are evident and trophoblast aggregates have detached from the culture surface.

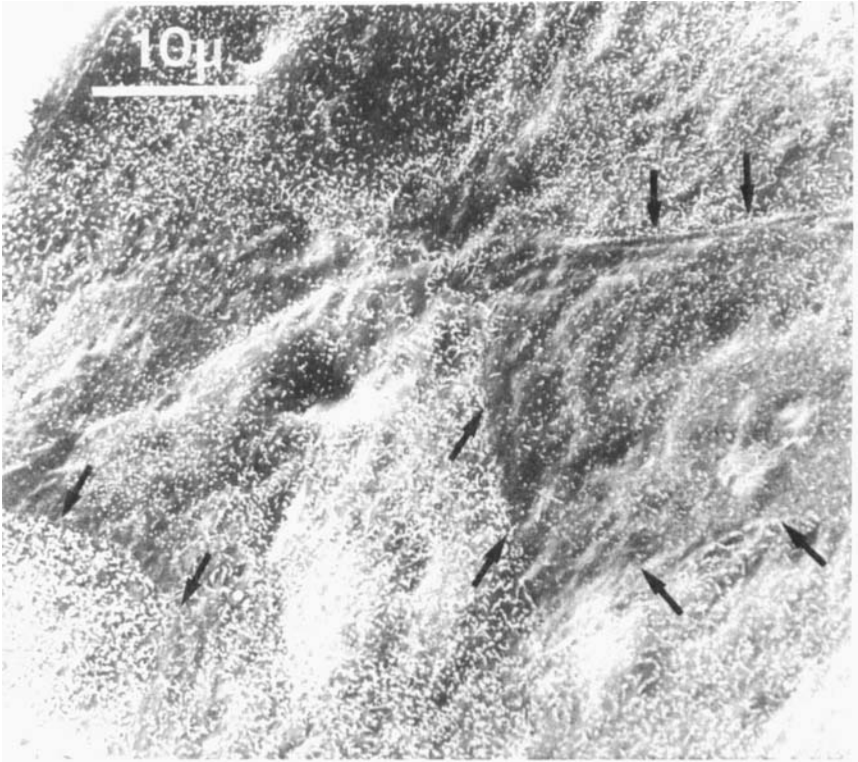

FIGURE 3. Scanning electron micrograph of human endometrial epithelial cells cultured on a collagen membrane (Cellagen,® ICN Biochemicals). Note the extensive surface microvilli. Individual cell borders in this confluent cell culture are also discernible (*arrows*).

endometrial luminal compartment. In contrast to the epithelial cells cultured on plastic or glass, the polarized cells show a luminal surface rich in microvilli (FIG. 3) and a functional polarity showing transport of labeled amino acids from the basal compartment with preferential secretion into the luminal compartment. Transmission electron microscopy indicates the presence of desmosomes near the apical region between adjacent epithelial cells as well as other elements characteristic of polarized epithelium. Of interest, is the observation that trophoblast aggregates prepared as described earlier and placed onto these polarized epithelia show prompt attachment (FIG. 4), which transmission electron microscopic studies have shown to be mediated by desmosomes (FIG. 5). It is reasonable to conclude that this attachment is specific and is mediated by a cell adhesion molecule. This hypothesis as well as the regulation of the described process awaits confirmation and further elucidation.

CONCLUSIONS

The process of human implantation involves a well-orchestrated series of complex events culminating in successful hemochorial placentation and establishment of preg-

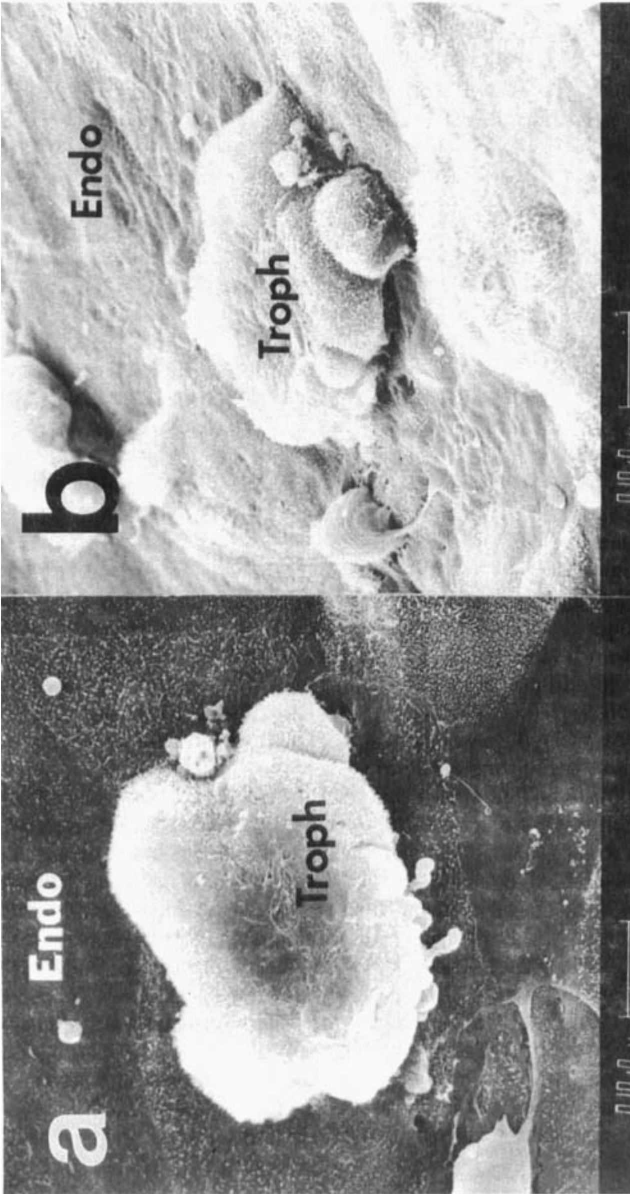

FIGURE 4. Scanning electron micrographs of a trophoblast aggregate (troph) adherent on a confluent layer of endometrial epithelial cells (endo) cultured on a collagen membrane. Both micrographs represent the same aggregate (a. top view; b. 45 degree angle view from the right). Note the microvilli on both the trophoblast and endometrial cell surface.

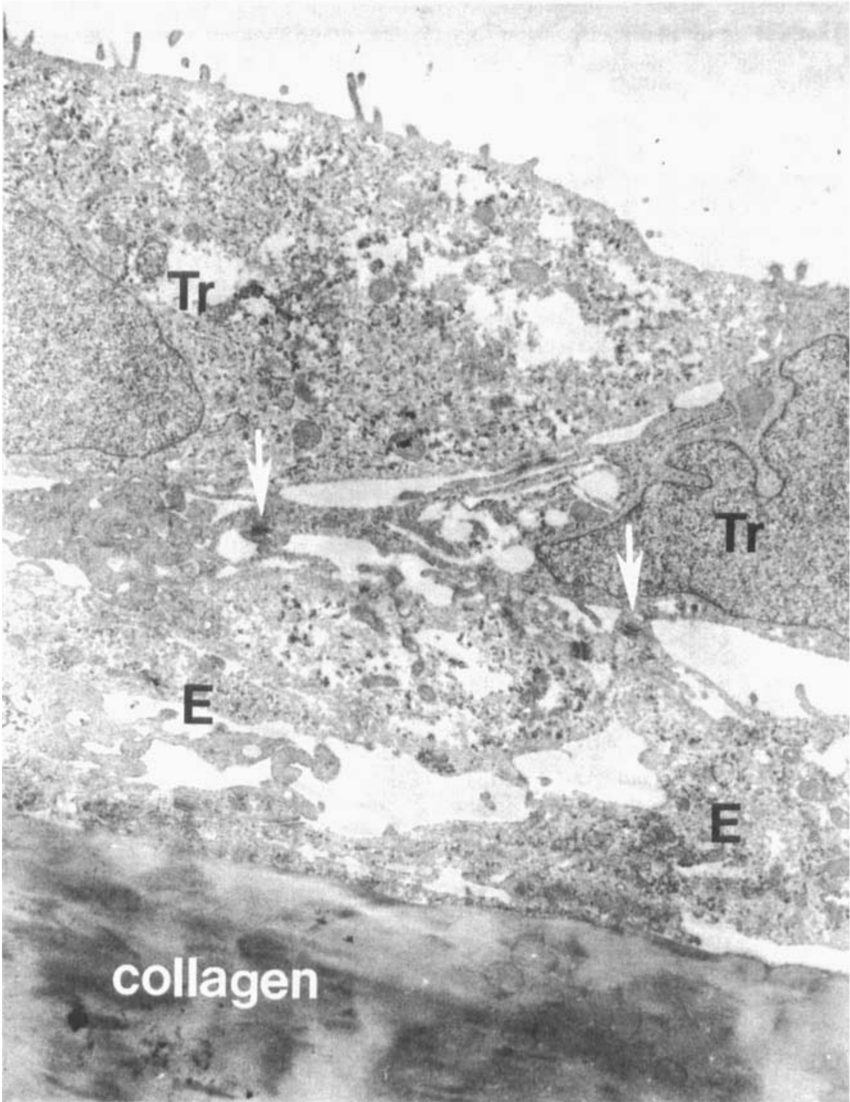

FIGURE 5. Transmission electron micrograph of trophoblasts (Tr) on a confluent epithelial endometrial cell layer (E) cultured on a collagen membrane (× 10,140). Note the presence of desmosomes at points of contact of trophoblasts with the endometrial cells (*arrows*).

nancy. Since its study cannot be achieved *in vivo*, *in vitro* systems designed to study each individual step at its cellular and molecular level is necessary. Is it justified to use the purified mononuclear cytotrophoblasts from human placentae as a surrogate for the implanting trophoblast? Our data to date suggests: (1) that these cells can differ-

entiate *in vitro* to form syncytial trophoblasts through a process of aggregation and fusion; (2) aggregates in suspension form cystic structures with evidence of functional and morphologic polarity; (3) these cystic structures form outgrowths in culture reminiscent of chorionic villi; (4) the differentiated syncytial structures secrete the characteristic hormones of the syncytial trophoblast, chorionic gonadotropin, placental lactogen, estrogens and progesterone. Thus, these cells undergo the morphologic differentiation seen in cytotrophoblasts *in vivo*. Most importantly though, the behavior of these cells in the described *in vitro* systems is also remarkably reminiscent of the behavior of the implanting human trophoblast *in vivo*: 1) they attach to polarized endometrial epithelial cells; 2) they are capable of intrusive behavior, insinuating themselves in between endometrial epithelial cells in culture by gently separating the cells without destroying them, and finally, 3) they have the capacity to attach, degrade and remodel extracellular matrix elements which are integral components of the endometrial basement membrane and stroma. We believe that the use of the described *in vitro* models in the study of human trophoblasts, may generate fundamental information at the cellular and molecular levels on the regulation of the processes which result in normal and abnormal human implantation.

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