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# A Model for Implantation: Coculture of Blastocysts and Uterine Endometrium in Mice[1]

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## ABSTRACT

**One of the limitations in embryo implantation research is the lack of an available in vitro model that faithfully replicates embryo-uterine interactions. In previous studies, embryos were cultured on a monolayer of either uterine epithelial cells or extracellular matrix substratum on which embryos could adhere and outgrow. However, these models failed to display embryonic invasion, primarily because of the shortage of critical structural and molecular supports that are available in vivo. In the present study, we used intact mouse uterine endometrium collected on Day 4 of pregnancy and placed in contact with blastocysts to initiate coculture experiments in a defined medium at the air-liquid interface. The culture medium was composed of Ham F-12/Dulbecco modified Eagle medium (1:1), 30% fetal calf serum, 63.5 nmol/L of progesterone, 7.14 nmol/L of estradiol-17β, 100 μg/ml of insulin, and 20 ng/ml of epidermal growth factor, whereas the incubation condition was mixed air of 50% oxygen, 5% carbon dioxide, and 45% nitrogen with a humidity of greater than 90% at 37°C. Our observations from 24 h of culture clearly demonstrated that embryos were capable of attachment to the uterine endometrium and displayed partial invasion into the endometrial stroma. Interestingly, no outgrowth of trophoblasts on the surface of uterine endometrium was seen, but embryos exhibited a pole-specific attachment. Overall, this model is capable of demonstrating a true invasion of embryo within the endometrial stroma and may be suitable in studies related to early embryo implantation.**

*embryo, implantation*

## INTRODUCTION

Embryo implantation is one of the earliest events in reproduction of humans and mammals that determines whether pregnancy will develop successfully. This process primarily begins with an intimate cross-talk between the embryo and the uterus. Numerous studies imply that the process of embryo implantation requires participation of numerous growth factors and cytokines to induce multifaceted cellular signaling pathways that, presumably, are regulated in a spatiotemporal manner both in the embryo and in the uterus. Uterine sensitivity to implantation has been defined into prereceptive, receptive, and nonreceptive (refractory) phases. The window of implantation in mice only lasts for a period of a few hours on Day 4, but it is considered to be prereceptive on Days 1–3 of pregnancy. The major factors specifying uterine receptivity in rodents are the ovarian progesterone and estrogens. On the first day of pregnancy in mice, uterine epithelial cells undergo proliferation under the influence of preovulatory estrogen secretion, whereas progesterone secreted from freshly formed corpora lutea induces stromal cell proliferation from Day 3. Further proliferation and subsequent differentiation of the stromal cells requires not only progesterone but also a small amount of ovarian estrogen on the morning of Day 4.

Because initiation of embryo implantation occurs within a short window, the so-called ''implantation window,'' conducting in vivo studies has been a major challenge. This has limited progress toward an understanding of detailed molecular mechanisms by which embryos invade into the uterus [1]. The successful development of in vitro models that faithfully mimic embryo-uterine interactions is expected to have potential to advance this field of research. However, years of effort to culture either the whole or some part of the mouse uterine horn have been in vain [2]. One of the main reasons for the failure is that the uterine horn is composed of heterogeneous cell types that are arranged spatially to mediate structural and functional integrity, whereas in vitro systems have not been supportive enough to maintain this arrangement. Another obstacle is the difficulty to localize microscopically embryos and subsequent implantation sites inside the cultured uterine lumen. Over the past decade, various types of embryonic implantation models have been established, among which mouse endometrial epithelial cells [3–7], stromal and decidual cells [8–10], and extracellular matrix (e.g., laminin and fibronectin) [11–14] were used as maternal components, respectively. Indeed, these models contributed greatly in relation to in vitro biological behavior of blastocyst, cellular interactions with epithelial and/or stromal/decidual cells and exploring roles of growth regulatory factors on embryo attachment. However, these models supported only the embryonic attachment and outgrowth on the maternal components, but invasion of embryos or trophoblasts could not be studied [15, 16].

The first demonstration of a successful coculture model for mouse uterine endometrium and blastocyst was reported by Shiotani et al. [17]. When placed in contact with blastocysts in the culture, the open uterine horns on Day 4 of pregnancy were able to demonstrate embryonic attachment and outgrowth of trophoblastic cells on the surface of endometrium. In another study, human endometrial biopsies were cultured together with blastocysts derived from in vi-

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tro fertilization. In these studies, blastocysts attached to endometrial epithelium of the biopsy and trophoblasts infiltrated into the stroma after 24 h of culture, although if kept in culture for longer periods, signs of tissue degeneration were observed. Moreover, plasmic serum purified from the same patient was added into the medium to maintain the biopsy tissue [18]. However, because of ethical issues, both human blastocysts and uterine tissue are difficult to obtain for research. In the present study, we have investigated the growth of both blastocysts and endometrium in a coculture model for mice using a specifically designed medium and air condition. A coculture model has been established that is more feasible for research on embryo implantation.

## MATERIALS AND METHODS

### *Animals and Endometrium Collection*

Adult NIH mice were obtained from the Laboratory Animal Center of Chongqing University of Medical Sciences, and investigations were conducted in accordance with the International Guiding Principles for Biomedical Researches Involving Animals as promulgated by the Society for the Study of Reproduction. Mice were housed under temperature- and light-controlled conditions (lights-on, 0700–1900 h) with free access to food and water. Females were mated with fertile males of the same strain to induce pregnancy. The first morning in which a vaginal plug was evident was considered to be Day 1 of pregnancy. Mice were killed at 1400 h on Day 4 of pregnancy by cervical dislocation after previous injection of sodium pentobarbital. Uterine horns were immediately dissected and flushed with saline. Morphologically excellent-looking blastocysts were collected for use, and endometrial tissues were squeezed out from uterine horns by use of a fine forceps [19]. The isolated tissues were cut into segments (length, ~2.0 mm) for their use in culture. For each trial, between five and eight mice were used, and the experiments were repeated three times.

### *Cradle Preparation*

To build an air-liquid interface and, therefore, ensure a supply of oxygen and nourishment for tissue and blastocyst culture, a cradle was made of a stainless-steel filter that was shaped like a platform. The size of the cradle was tailored to fit one well of a 12-cell culture plate. One piece of lens paper, which was soaked in advance with acetone and anhydrous ethanol for at least 12 h, was placed on the top of the cradle (Fig. 1). Both the cradle and lens paper were sterilized by autoclave before use.

### *Culture Conditions*

One culture cradle was set in the middle of one well of a 12-well culture plate, with each well filled by specifically designed Ham F-12/Dulbecco modified Eagle medium (1:1) containing 30% fetal calf serum, 63.5 nmol/L of progesterone, 7.14 nmol/L of estradiol-17β, 100 μg/ml of insulin, and 20 ng/ml of epidermal growth factor. Each well was filled with medium so that levels in the wells were exactly the same as the top of the cradle. The lens paper on the cradle was completely soaked in medium. Under a dissection microscope, endometrial segments were placed carefully, one by one, on the lens paper and gently stretched out with the epithelial surface facing upward. Subsequently, blastocysts were transferred gently with a mouth pipette onto each endometrial segment. The location of each blastocyst was recorded based on the grid number of the cradle. The whole system was then incubated for 24 h at 37°C and humidity greater than 90% in various concentrations of oxygen as follows: The first group comprised 95% oxygen and 5% carbon dioxide; the second group 75% oxygen, 20% nitrogen, and 5% carbon dioxide; the third group 50% oxygen, 45% nitrogen, and 5% carbon dioxide; and the fourth group 95% air and 5% carbon dioxide.

On the next morning, the cultures were taken out of the incubator to observe microscopically whether blastocysts already had been hatched out of the zona pellucida and attached firmly to the endometrium or floated off from the original location. If some blastocysts were missing from the surface of endometrium, they often could be recovered at the bottom of the culture well. After incubation for 24 h, culture plates were shaken or air was gently blown directly toward the blastocyst with a mouth pipette to confirm whether blastocysts were attached. Then, endometrial segments with attached blastocyst were fixed in fresh Bouin solution or 4% paraformaldehyde overnight and subjected to paraffin embedding. The deparaffinized sections (thickness, 5 μm) were examined after staining with hematoxylin-eosin under light microscopy. Another batch of tissue samples were fixed in 2.5% glutaraldehyde and subsequently treated with 1% osmium tetroxide, followed by embedding in epoxy resin and sectioning (thickness, ~50 nm) for ultrastructural observation.

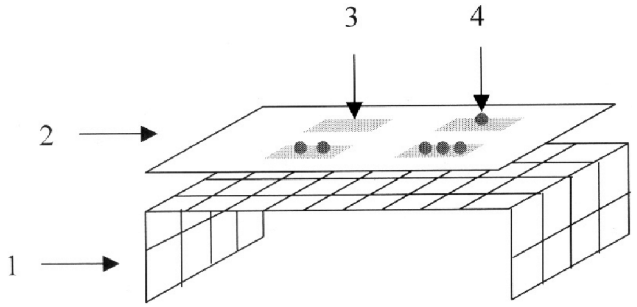


FIG. 1. Illustration of culture cradle. One piece of lens paper was placed on the top of a cradle made of a stainless-steel filter. Endometrial segments were placed carefully on the lens paper and gently stretched out with the epithelial surface facing upward. Blastocysts were then transferred gently with a mouth pipette onto each endometrial segment. 1, Platform; 2, lens paper; 3, endometrial segment; 4, transferred blastocysts.

### *Immunohistochemistry*

Paraformaldehyde-fixed endometrial sections were deparaffinized, hydrated in PBS, subjected to antigen retrieval by boiling the sections in 0.01 mol citrate buffer (pH 6.0) for 10 min, and then cooled to room temperature. Sections were incubated in blocking solution (PBS containing 5% normal donkey serum) for 30 min to prevent nonspecific binding and then incubated with mouse anti-human proliferating cell nuclear antigen (PCNA) monoclonal primary antibody, mouse anti-human progesterone receptor (PR; Boster Biological Technology Ltd., China), or goat anti-mouse laminin or goat anti-human leukemia inhibitory factor (LIF) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The sections were then incubated in biotinylated anti-donkey immunoglobulin (Ig) G for 30 min at room temperature, followed by incubation with avidin-biotin complex reagent (Zymed Laboratories) for 30 min at room temperature. After four washes in PBS (5 min each), the sections were incubated in substrate (3-amino-9-ethyl-carbazole) solution for 5–10 min to develop color. Positive staining was indicated by brown/red deposit. Negative controls were performed in parallel with addition of normal IgG by replacing the primary antibody. The sections were lightly counterstained with hematoxylin before clearing and mounting.

## RESULTS

### *Collection of Endometrium*

Endometrial tissues, as collected in our procedure, were composed primarily of intact epithelium and most of the stroma and glands (Fig. 2B). Interestingly, the orientation of the epithelium and the stroma are similar to that observed in vivo, in that epithelium is exposed outward for interaction with developing blastocysts. The luminal surface is an external body surface. Moreover, a crack was always observed from the center of stroma to the epithelial surface, which surprisingly was verified to localize along the mesometrial pole (Fig. 2A).

### *Culture of Endometrium*

After 24 h of culture, the structural integrity of endometrium in the first 95% oxygen group was maintained, including its epithelium, glands, capillaries, and stroma (Fig. 2C). Ultrastructural observations revealed no obvious signs of alterations in both cytoplasm and nucleus, except that mitochondria exhibited slight swelling (Fig. 2D). The quality of endometrial structure maintained in higher-oxy-

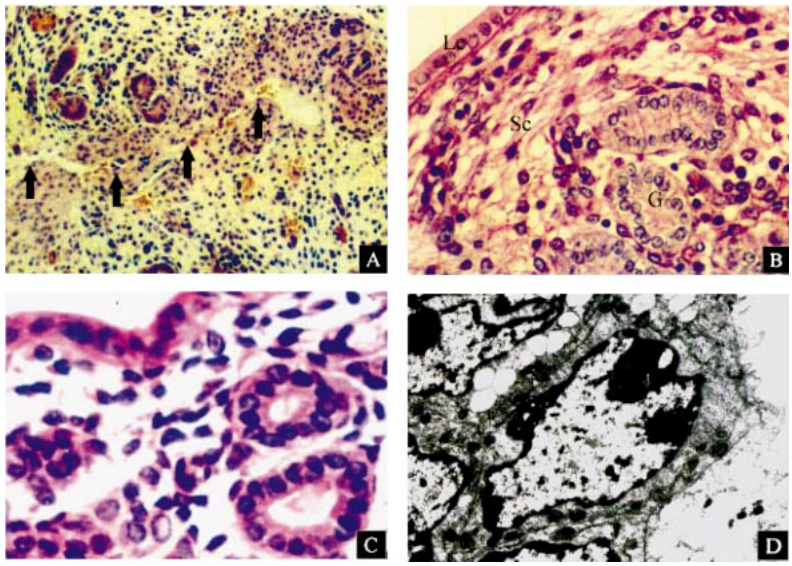


FIG. 2. Structure of mouse uterine endometrium on Day 4 of pregnancy. The endometrial segments were fixed in fresh Bouin solution (**A**) or 4% paraformaldehyde (**B** and **C**) overnight and subjected to paraffin embedding. The deparaffinized sections (thickness, 5 μm) were stained with hematoxylin-eosin. Tissue samples (**D**) were fixed in 2.5% glutaraldehyde and subsequently treated with 1% osmium tetroxide followed by embedding in epoxy resin. A crack (arrows) is observed inside the tissue, corresponding to the position of the mesometrial pole (**A**). Collected tissue contains epithelium, stroma, and glands (**B**), and the structure was maintained intact after 24 h of culture (**C** and **D**). G, Gland; Le, lumen epithelium; Sc, stromal cells. Original magnification ×40 (**A**), ×200 (**B** and **C**), or ×5000 (**D**).

gen-concentration groups was better than that in the lower groups.

### *Expression of Receptivity Markers in the Cultured Endometrium*

The PCNA was shown immunohistochemically in most stromal cells and glandular epithelial cells in both controlled and cultured endometrial explants (Fig. 3, A and B). As one of the most important implantation markers among mammalian species, nuclear immunostaining of PR was detected in stromal cells and glandular epithelial cells. In cultured endometrial explants, the staining degree of PR in stromal cells was more intense than in controls (Fig. 3, C and D).

The LIF is expressed transiently in uterine glands on Day 4 of pregnancy in mice, suggesting its role in implantation [20]. The presence of LIF in cultured endometrial tissue was revealed by immunohistochemical analysis. No significant difference was observed in staining as compared to tissues before the initiation of culture (Fig. 3, E and F).

Among the adhesion molecules, several extracellular matrix components are up-regulated in the peri-implantation endometrium including fibronectin, laminin, and collagen type IV [21]. The staining for laminin was expressed in both the fresh and the cultured tissue (Fig. 3, G and H), indicating its unchangeable capability of binding to integrins, which primarily mediate interactions between endometrium and trophoblasts.

### *Attachment of Blastocyst to the Uterine Epithelium*

Synchronously developing blastocysts were transferred onto the epithelial surface of endometrial tissue, and coculture experiments were initiated under four different oxygen concentrations as described in *Materials and Methods.* To judge whether blastocysts had adhered to the epithelium, the culture system was gently shaken horizontally for a few times. If blastocysts remained adhered to the surface, they were considered to be successfully attached. As shown in Table 1, the ratios of embryonic attachments were 30.9%, 42.4%, 47.9%, and 54.1% by a gradual decrease in oxygen concentration. These results indicate that high concentration of oxygen was harmful to the development of blastocysts for attachment. Furthermore, the occurrence of most embryonic attachment was revealed after the coculture had been going for around 22 h. At this time, any unattached blastocysts still possessed the characteristic of attachment to the epithelium if they were retransferred.

In mice, blastocysts attach to the antimesometrial luminal epithelium, with their inner cell mass (ICM) directed toward the mesometrial pole. Our in vitro coculture experiments confirmed that mouse blastocysts attach to the endometrium with their mural trophoblasts, although the mesometrial versus antimesometrial orientation of endometrial tissue was unable to be defined (Fig. 4, A and B).

### *Invasion of Blastocyst into the Uterine Endometrium*

For invasion studies, the coculture experiment was carried out in a mixed air condition of 50% oxygen, 45% nitrogen, and 5% carbon dioxide, because 50% oxygen is a selective concentration that is relatively beneficial to the maintenance of uterine endometrium and growth of blastocysts based on the results described in the present study. Following the termination of culture with the attachment by 24 h, no outgrowth of trophoblastic cells was displayed along the surface of tissue, which normally takes place on a hard surface, like a plastic well, once the blastocyst escaped from the zona pellucida. In contrast, the invasion of blastocysts into endometrial tissue indeed occurred (Fig. 4, C–F). The localization of ICM was noted at the right side of the invading blastocyst's chamber (Fig. 4, E and F).

## DISCUSSION

Through repeated comparative studies, we have screened the special culture medium, which was beneficial to the maintenance of mouse uterine endometrium in vitro. High concentration of oxygen (e.g., 95%) was necessary for endometrial culture; otherwise, it might interrupt the embryonic metabolism by producing excessive free radicals, which results in cellular damage and death of the embryos [22, 23]. However, high concentration of oxygen was harmful to embryonic growth. In our study, when we cultured blastocysts in the air-tight incubator filled with the mixed gas composed of 95% oxygen and 5% carbon dioxide, most blastocysts started degenerating after 12–24 h of culture. The blastocyst cavity appeared to disappear and remain encased in the center of the zona pellucida. Interestingly,

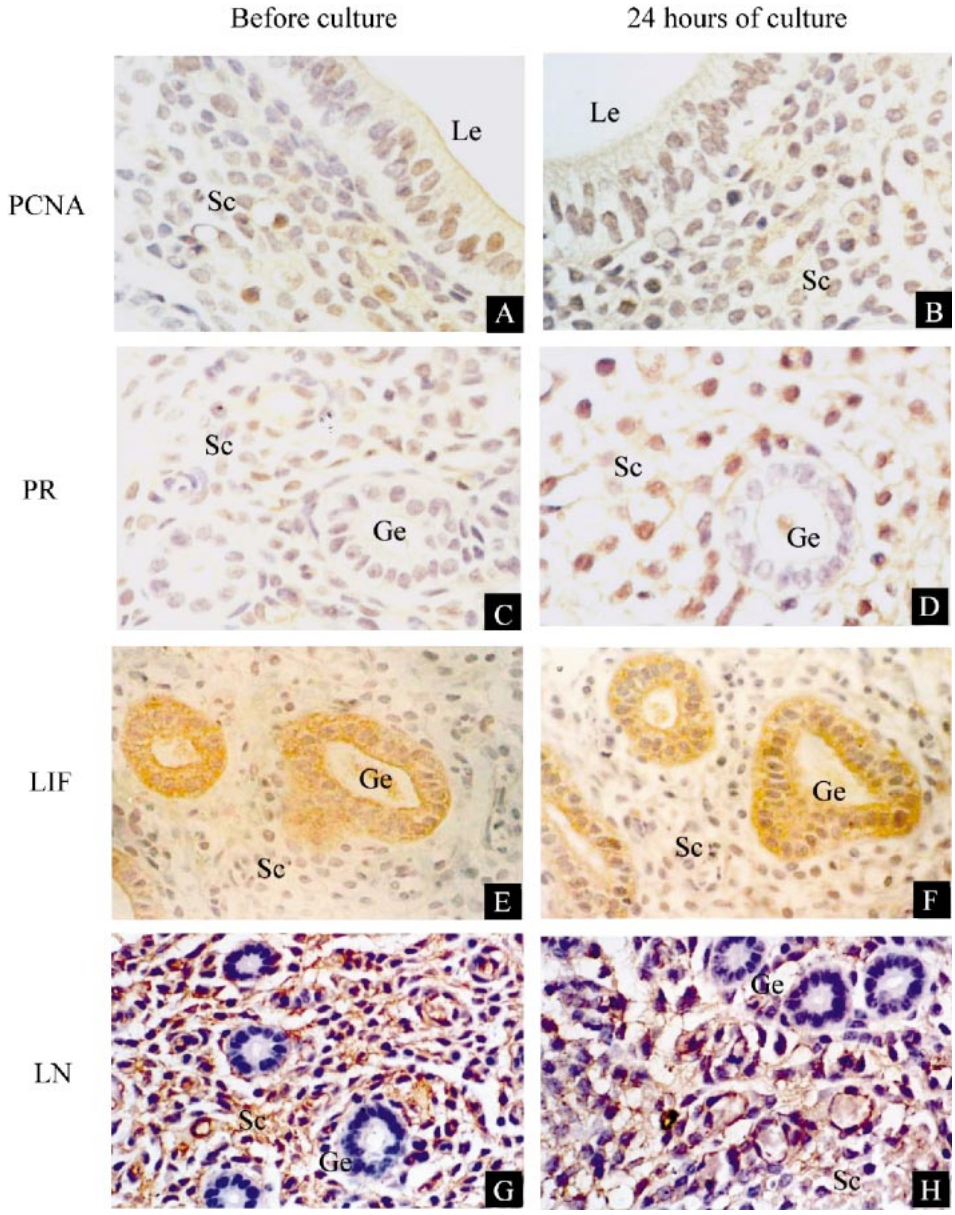


FIG. 3. Immunohistochemistry of PCNA, PR, LIF, and laminin (LN) in mouse uterine endometrium. The photomicrographs of representative uterine sections are shown for PCNA (**A** and **B**), PR (**C** and **D**), LIF (**E** and **F**), and laminin (**G** and **H**). Brown/red deposits indicate positive nuclear immunostaining for PCNA and PR and uterine gland and cytoplasmic staining for LIF and LN, respectively. These experiments were repeated three times with similar results. Ge, Glandular epithelium; Le, luminal epithelium; Sc, stromal cells. Original magnification ×200.

when the blastocysts were cultured on the endometrium in the same air condition as described above for 24 h, no alteration was observed in the structure of blastocysts, indicating that the presence of endometrial tissue was beneficial for the development or survival of blastocysts [24, 25]. Because high concentration of oxygen was required for the maintenance of endometrium but harmful to embryonic growth, we attempted to resolve this conflict by performing the coculture of the embryo and endometrium under four conditions with different concentrations of oxygen. The results showed that the ratio of embryonic attachment was improved (approximately >50%) by maintaining the oxygen concentration at approximately 20%.

In the present study, we cultured blastocysts and endometrial luminal epithelium at the air-liquid interface, which guaranteed the supply of oxygen and nourishment of both the embryo and the endometrium simultaneously. Lens paper was placed on the stainless-steel grid not only to reduce the damage to tissue when endometrium was outspreaded onto the cradle but also to nourish the tissue through absorption of medium onto the paper, even when the level of medium was low because of evaporation.

Although the mechanics of implantation are yet to be established, it is known that during embryo implantation in mice, following the onset of luminal closure, blastocysts are attached to endometrial luminal epithelium at the antimesometrial side of the lumen along the uterine axis. Shortly after the luminal closure, zona-encased blastocysts, located in the implantation chamber, undergo random orientation of the ICM. However, with the beginning of the at-

TABLE 1. Attachment rate of blastocysts under different oxygen concentrations.

| Group | Concentration of oxygen (%) | Total no. of blastocysts | No. of attached blastocysts | Attachment rate (%) |
|---|---|---|---|---|
| 1 | 95 | 113 | 35 | 30.9 |
| 2 | 75 | 118 | 50 | 42.4 |
| 3 | 50 | 117 | 56 | 47.9 |
| 4 | 20 | 122 | 66 | 54.1 |

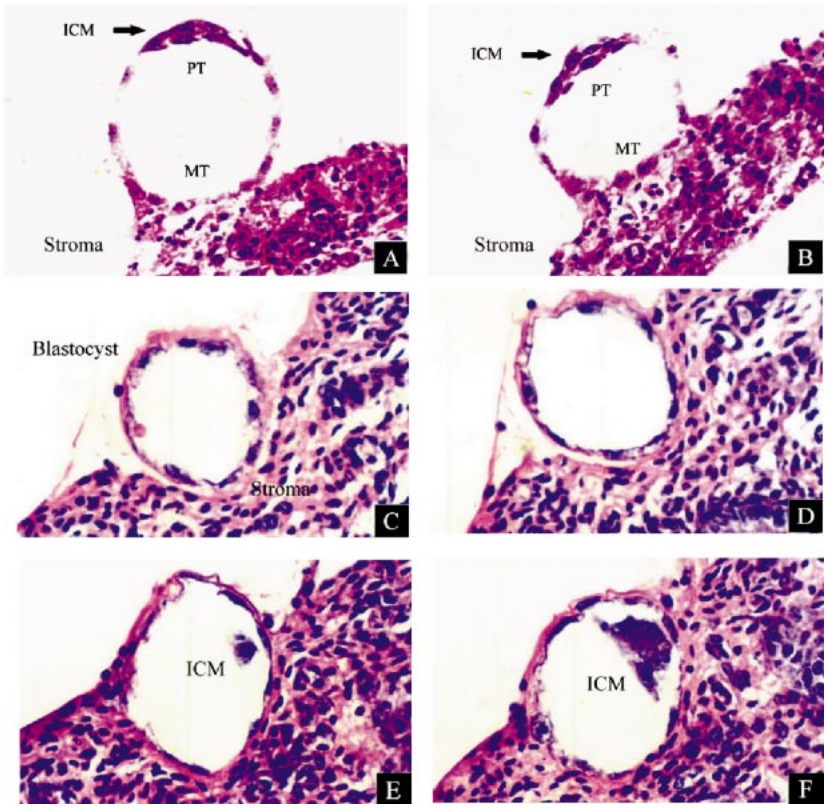


FIG. 4. Coculture of mouse blastocysts with endometrium. The stroma and attached blastocyst were fixed in 4% paraformaldehyde overnight and subjected to paraffin embedding. The deparaffinized sections (thickness, 5 μm) were stained with hematoxylin-eosin. **A** and **B**) Two individual experiments. Note that blastocysts attached to the endometrium by its mural trophectoderm cells. **C** and **D**) Serial sections of blastocyst invasion into the endometrium. **E** and **F**) Note that the ICM of the blastocyst was located on the right side of cavity. MT, Mural trophectoderm; PT, polar trophectoderm. Original magnification ×100.

tachment reaction, blastocysts are oriented correctly, with their ICM directed toward the mesometrial pole. This observation suggested that the trophectoderm of the entire blastocyst surface has the potential for attachment to the luminal epithelium and that attachment occurs randomly immediately after loss of the zona pellucida [26, 27]. Evidence presented here suggests that the correct orientation of the blastocyst is achieved by free movement of the ICM. However, further investigation is required to resolve this issue. In the present study, we observed, surprisingly, that not only mural trophectoderm cells but also the pole trophectoderm cells or trophoblastic cells near it attached to the endometrium, suggesting that many sites of the trophectoderm could initiate attachment in vitro. The reason for the discrepancy of the in vitro results with the in vivo results requires further exploration. Likely, the simplified culture conditions result in the loss of specificity of attachment orientation of embryonic trophoblastic cells in vitro.

With respect to the underlying mechanism for proper attachment of blastocyst to the uterine luminal epithelium, the role of endometrium itself likely is involved. Uterine endometrium at the mesometrial pole appears to undergo relatively poor proliferation, with less stroma as compared to the antimesometrial pole. A remarkable feature that this coculture model established was that no outgrowth of trophoblastic cells occurred onto the endometrium. Outgrowth of embryo most likely was a special phenomenon in vitro when embryos were growing on the solid surface of glass slides or plastic wells, on which embryos were unable to implant but could develop by stretching out along the support surface after attachment.

In our studies, the embryonic attachment reaction took place after approximately 22 h in culture. In mice, normal implantation occurs around 2300–2400 h on Day 4. The apparent delayed attachment in our coculture system may result from the less favorable culture conditions in vitro. First, it is quite difficult to mimic the complicated uterine environment to satisfy the maintenance and development of both blastocysts and endometrium because of the huge number of participating factors, such as steroid hormones, cytokines, and growth factors as well as the undefined relationships that span the interdependent phases of implantation [28–31]. Second, precise dialogue between embryo and uterus during implantation is critical to embryonic apposition, attachment, and invasion to endometrium [32–34]. Experimental manipulation of both blastocysts and endometrium might influence this cross-talk for implantation.

Collectively, we have developed a tridimensional culture model of embryo implantation using the mouse blastocysts and uterine endometrial cells in which the blastocyst ultimately attaches and invades the endometrial wall. Immunohistochemical results of PCNA, PR, LIF, and laminin confirmed the viability, biochemical integrity, and responsiveness to implantation events of the cultured endometrium. To our knowledge, the present study describes the most advanced and physiologically relevant in vitro model yet developed for embryo implantation, and it provides an opportunity for the study of embryo implantation.

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