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Dual Roles of Progesterone in Embryo Implantation in Mouse

Bojie Dai, Yujing Cao, Weimin Liu, Sumin Li, Yongjun Yang, Dayuan Chen, and Enkui Duan

State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Progesterone (P_4) is essential for the development of endometrial receptivity for blastocyst implantation and pregnancy maintenance. Many studies have demonstrated that P_4 restrains endometrial tissue breakdown by inhibiting the stimulation of matrix metalloproteinases (MMPs), which implies that P_4 impedes the invasion of trophoblast cells into endometrial tissue. To investigate the role of P_4 on the attachment and invasion of the trophoblast cells in the entire process of embryo implantation, we used two in vitro coculture systems of blastocysts on a monolayer of uterine epithelial cells and ectoplacental cone (EPC) on uterine decidual cells. The results indicated that all doses of P_4 significantly promoted blastocyst attachment, and that except for a concentration of 3.18×10^{-8} mol/L, P_4 markedly increased the percentage of blastocysts with outgrowth. However, all concentrations of RU486 clearly prevented blastocyst attachment, and except for a concentration of 10^{-8} mol/L, all doses of RU486 significantly inhibited the outgrowth of blastocysts. The effect of 3.18×10^{-6} mol/L of P_4 on the attachment and outgrowth of blastocysts was significantly blocked by all concentrations of RU486. All concentrations of P_4 retarded the attachment of EPC on uterine decidual cells and decreased the outgrowth area of EPC. Except for a concentration of 3.18×10^{-8} mol/L, all concentrations of P_4 had a significant inhibitory effect on the percentage of EPC outgrowth. Conversely, except for a concentration of 10^{-8} mol/L, all doses of RU486 had a significant stimulatory effect on the attachment of EPC. All concentrations of RU486 clearly promoted the outgrowth and outgrowth area of EPC. The inhibitory effect of P_4 on EPC was clearly blocked by all doses of RU486. In addition, P_4 promoted the activity of MMP-2 on blastocysts and EPC, but P_4 inhibited the activity of MMP-9 on EPC. In summary, P_4 played dual roles at the early and late stages of embryo implantation in mouse. When blastocysts interacted with the uterine epithelial cells, P_4 promoted the attachment and invasion of the primary trophoblast giant cells. When EPC was in contact with uterine decidual cells, P_4 inhibited the attachment and invasion of the secondary trophoblast giant cells. Furthermore, the role of P_4 was transduced through the classic nuclear receptor.

Key Words: Progesterone; RU486; coculture; embryo implantation; mouse.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Enkui Duan, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China. E-mail: duane@panda.ioz.ac.cn

Introduction

Mouse embryo implantation involves the invasion of the embryo into uterine epithelial cells at the early stage and the invasion of the secondary trophoblast giant cells of the ectoplacental cone (EPC) into the uterine deciduas at the late stage *(1,2)*. Progesterone (P_4) is essential for development of the endometrial receptivity for blastocyst implantation and embryonic survival throughout gestation *(3)*, such as transforming the prepared uterine endometrium into a secretory tissue and creating an environment within the uterine milieu that is conducive to embryo attachment *(4,5)*. The role of P_4 in controlling uterine secretory function is evident from the increase in protein accumulation within the uterine lumen as well as the qualitative changes in protein synthesis during the luteal phase of the estrous cycle of pregnancy *(6)*. Furthermore, P_4 plays an important role in protecting the fetus from the maternal immune system. P_4 has remarkable local antiinflammatory activity, as evidenced by the striking absence of inflammatory cells in the pregnant uterus *(7)*, and maintains pregnancy by stimulating the secretion of Th2 and reducing the secretion of Th1 cytokines *(8)*. RU486, a potent P_4 antagonist, binds with high affinity to P_4 and glucocorticoid receptors. RU486 inhibits P_4 action by competing for access to the P_4 receptor (PR).

P_4 is essential for initiating embryo implantation and maintaining pregnancy in eutherian mammals *(9)*. P_4 may preserve tissue integrity during the establishment and maintenance of pregnancy by limiting stimulation of matrix metalloproteinases (MMPs) from the endometrial tissue *(10–13)*. This implies that P_4 inhibits the invasion of embryo into the uterine endometrium. However, to date, the detailed effect and mechanism of P_4 on embryo adhesion and invasion in the whole process of embryo implantation is not clear. The adhesion and outgrowth of mouse blastocysts and EPCs in culture represent two in vitro models of trophoblastic

adhesion and invasion. In the present study, we established two coculture systems: i.e., blastocysts on a monolayer of uterine epithelial cells and EPC on uterine decidual cells. By using in vitro models, we investigated the effect of P_4 on the attachment and invasion of trophoblast cells in the entire process of embryo implantation.

Results

Effect of P_4 or RU486 on Blastocyst Attachment and Outgrowth on Monolayer of Uterine Epithelial Cells

An in vitro coculture system of blastocysts on a monolayer of uterine epithelial cells was used to mimic the in vivo process of embryo attachment and invasion to uterine epithelial cells. Data were collected at different times (36 and 72 h) after blastocysts were transferred onto the monolayer of uterine epithelial cells. Part of the blastocysts began to adhere to the monolayer of uterine epithelial cells after 36 h of coculture in both the control and treated cultures. All doses of P_4 significantly increased the percentage of blastocyst adhesion, whereas all doses of RU486 clearly decreased the percentage of blastocysts attaching. Outgrowth occurred at 72 h of coculture, and except for a concentration of 3.18×10^{-8} mol/L, all concentrations of P_4 significantly enhanced the outgrowth of blastocysts (Fig. 1A). Conversely, except for a concentration of 10^{-8} mol/L, all concentrations of RU486 significantly inhibited outgrowth (Fig. 1B). At 36 and 72 h of coculture, 3.18×10^{-6} mol/L of P_4 significantly promoted both attachment and outgrowth correspondingly. Interestingly, the effect of P_4 on the attachment and outgrowth of blastocysts was blocked by all concentrations of RU486 (Fig. 1C).

Effect of P_4 or RU486 on Adhesion and Outgrowth of EPC on Uterine Decidual Cells

An in vitro coculture of EPC on uterine decidual cells was used to observe the process of trophoblast attachment and invasion. At 24 h of coculture, all doses of P_4 significantly inhibited the adhesion of EPC on uterine decidual cells. At 48 h of coculture, except for a concentration of 3.18×10^{-8} mol/L, all concentrations of P_4 had a significant inhibitory effect on the percentage of outgrowth of EPC (Fig. 2A). Similarly, at 96 h of coculture, all concentrations of P_4 had an inhibitory effect on the outgrowth area of EPC (Fig. 3A). Contrary to the role of P_4, at 24 h of coculture, except for a concentration of 10^{-8} mol/L, all doses of RU486 clearly promoted the attachment of EPC on the uterine decidual cells. At 48 and 96 h of coculture, all concentrations of RU486 increased the outgrowth and outgrowth area of EPC (Figs. 2B and 3B). It is clear that 3.18×10^{-6} mol/L of P_4 decreased both the percentage of attachment and outgrowth and the outgrowth area of EPC. All doses of RU486 could significantly block the role of P_4 on the attachment, outgrowth, and outgrowth area of EPC on the uterine decidual cells (Figs. 2C and 3C).

Effect of P_4 or RU486 on Activity of MMP-2 and MMP9 of Blastocysts or EPC

To identify further that the role of P_4 is on blastocysts or uterine epithelial cells or EPC or decidual cells, only blastocysts or EPCs were cultured with different doses of P_4 or RU486 on fibronectin-coated dishes. At 24 h of culture, the activity of MMP-9 was not measured and all concentrations of P_4 promoted the activity of MMP-2 on blastocysts, but all doses of RU486 inhibited the activity of MMP-2 on blastocysts (Fig. 4). Different from that on blastocysts, all concentrations of P_4 inhibited the activity of MMP-9 but promoted the activity of MMP-2 on EPC (Fig. 5).

Discussion

Uterine receptivity is defined as a restricted period when the uterus supports blastocyst attachment. Although the molecular basis of this window of receptivity remains unclear, P_4 plays major roles in a species-specific manner. Mice lacking both PR isoforms are infertile, whereas selective deletion of PR-A allows ovulation but fails to rescue implantation *(14)*. P_4-regulated endometrial protein secretion would support rapid conceptus growth and attachment *(5)*. Administration of P_4 early in the estrous cycle of the recipient can effectively advance uterine receptivity for the transfer of older asynchronous embryos *(5)*. In general, P_4 is essential for the development of the endometrial receptivity required for blastocyst implantation. Only when the uterine endometrium is receptive do the trophoblast cells exhibit the ability to attach to the uterine epithelial cells and then interpose themselves among other cells by separating them and degrading extracellular matrix (ECM) components *(15)*.

In the present study, all doses of P_4 significantly promoted blastocyst attachment and except for a concentration of 3.18×10^{-8} mol/L, P_4 markedly increased the percentage of blastocysts with outgrowth. However, all concentrations of RU486 clearly prevented blastocyst attachment and except for a concentration of 10^{-8} mol/L, all doses of RU486 significantly inhibited the outgrowth of blastocysts. To our knowledge, this is the first report wherein P_4 significantly promoted the attachment and outgrowth of blastocysts on a monolayer of uterine epithelial cells.

P_4 can upregulate some important cytokines or growth factors for embryo implantation, such as leukemia inhibitory factor (LIF) and epidermal growth factor (EGF) *(16,17)*. LIF signaling permits uterine HB-EGF to interact with blastocyst ErbBs to prepare and direct blastocyst attachment *(14)*. LIF and EGF promoted the attachment and outgrowth of blastocysts *(18–20)*. This indicates that progesterone promotes the attachment and outgrowth of blastocysts indirectly by regulating some cytokines such as LIF and EGF. P_4-regulated endometrial protein secretion would also be established to support rapid conceptus growth and attachment *(5)*. Fazleabas and Strakova *(21)* observed that the first

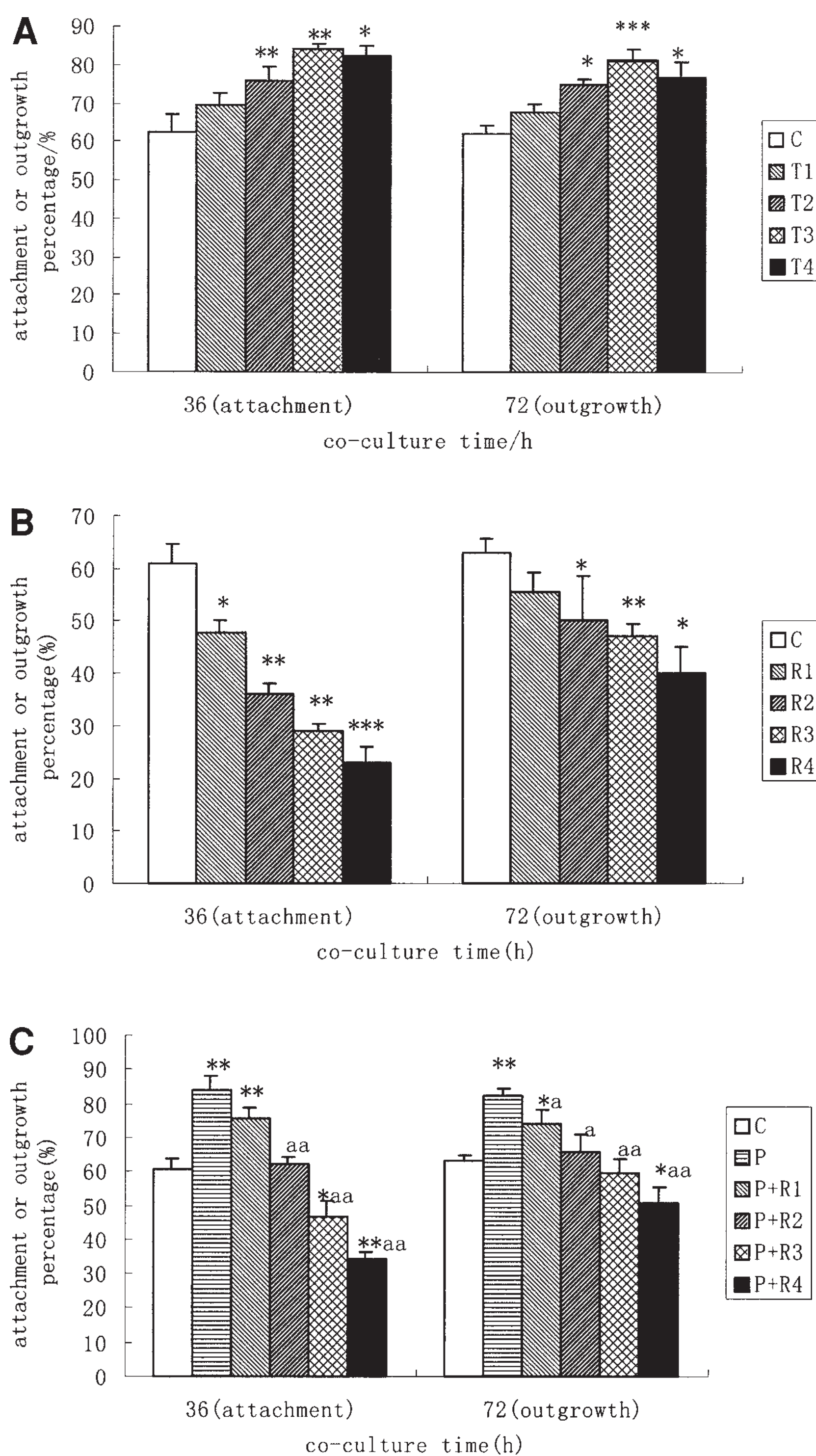

Fig. 1. Effect of P_4 or RU486 on attachment and outgrowth of blastocysts on a monolayer of uterine epithelial cells. (**A**) Effect of P_4. Blastocysts of d 4 mice were cocultured with a monolayer of uterine epithelial cells in a medium containing a range of P_4 concentrations: C, 0.0 mol/L; T1, 3.18×10^{-8} mol/L; T2, 3.18×10^{-7} mol/L; T3, 3.18×10^{-6} mol/L; T4, 3.18×10^{-5} mol/L. (**B**) Effect of RU486. Blastocysts of d 4 mice were cocultured with a monolayer of uterine epithelial cells in a medium containing a range of RU486 concentrations: C, 0.0 mol/L; R1, 10^{-8} mol/L; R2, 10^{-7} mol/L; R3, 10^{-6} mol/L; R4, 10^{-5} mol/L. (**C**) Blocking of role of progesterone on attachment and outgrowth of blastocysts by RU486. The experiment was carried out in a medium containing a range of RU486 concentrations (C, 0.0 mol/L; R1, 10^{-8} mol/L; R2, 10^{-7} mol/L; R3, 10^{-6} mol/L, R4, 10^{-5} mol/L, respectively) in the presence of 3.18×10^{-6} mol/L of progesterone. The effect of P_4 or RU486 on blastocyst attachment and outgrowth was determined by comparing the total number of blastocysts with the total numbers of attached or outgrowth blastocysts. Results are expressed as mean ± SE of three replicates ($n = 40$ blastocysts/well). *, **, and *** indicate significant differences from the control ($p < 0.05, p < 0.01, p < 0.001$, respectively). a and aa indicate significant differences for the P_4 (10^{-6} mol/L progesterone) ($p < 0.05$, $p < 0.01$, respectively).

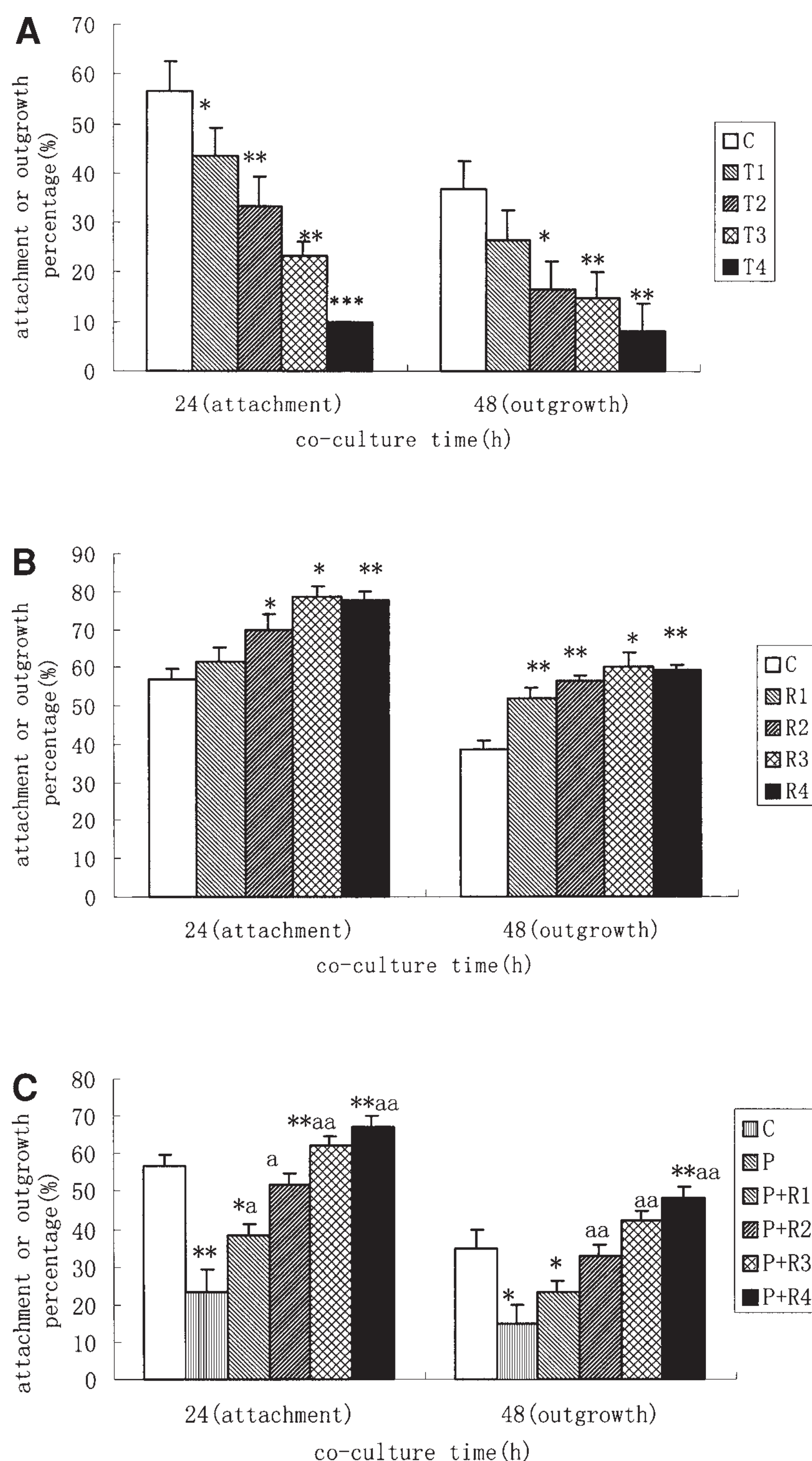

Fig. 2. Effect of P_4 or RU486 on attachment and outgrowth of EPC on a monolayer of uterine dedicual cells. (**A**) Effect of P_4. EPCs of d 8.5 mice were cocultured with a monolayer of uterine decidual cells in a medium containing a range of P_4 concentrations: C, 0.0 mol/L; T1, 3.18×10^{-8} mol/L; T2, 3.18×10^{-7} mol/L; T3, 3.18×10^{-6} mol/L; T4, 3.18×10^{-5} mol/L. (**B**) Effect of RU486. EPCs of d 8.5 mice were cocultured with uterine decidual cells in a medium containing a range of RU486 concentrations: C, 0.0 mol/L; R1, 10^{-8} mol/L; R2, 10^{-7} mol/L; R3, 10^{-6} mol/L; R4, 10^{-5} mol/L. (**C**) Blocking of role of P_4 in attachment and outgrowth of EPC by RU486 on uterine decidual cells. The experiment was carried out in a medium containing a range of concentrations of RU486; C, 0.0 mol/L RU486; R1, 10^{-8} mol/L; R2, 10^{-7} mol/L RU486; R3, 10^{-6} mol/L RU486; R4, 10^{-5} mol/L RU486, respectively, in the presence of 3.18×10^{-6} mol/L P_4. The effect of P_4 or RU486 on EPC attachment and outgrowth was determined by comparing the total number of EPC with the total numbers of attached or outgrowth EPC. Results are expressed as mean ± SE of three replicates ($n = 20$ EPC/well). *, **, and *** indicate significant differences from the control ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively). a and aaa indicate significant differences for the P_4 (10^{-6} mol/L progesterone) ($p < 0.05$, $p < 0.001$, respectively).

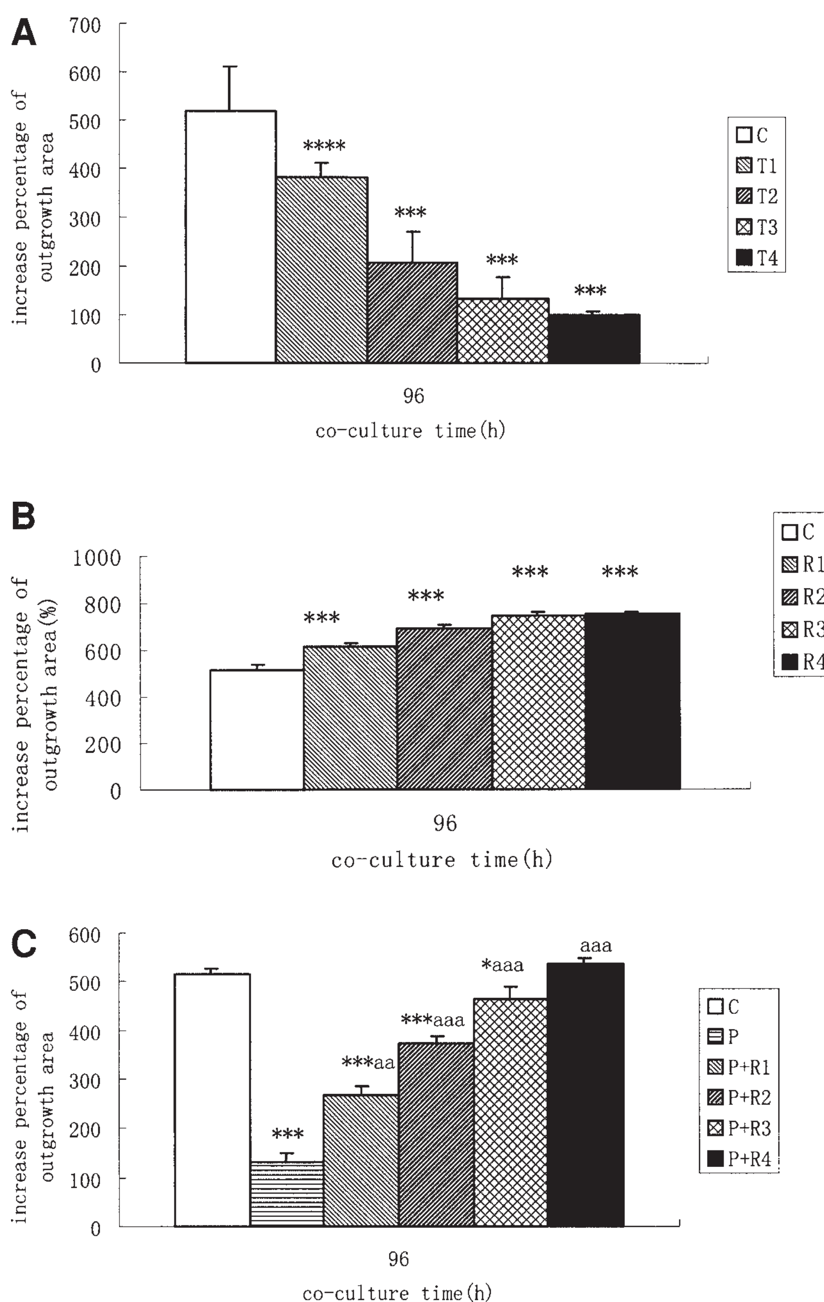

Fig. 3. Effect of P_4 or RU486 on outgrowth area of EPC on uterine decidual cells. (**A**) Effect of P_4. EPCs of d 8.5 mice were cocultured with uterine decidual cells in a medium containing a range of P_4 concentrations: C, 0.0 mol/L; T1, 3.18×10^{-8} mol/L; T2, 3.18×10^{-7} mol/L; T3, 3.18×10^{-6} mol/L; T4, 3.18×10^{-5} mol/L P_4. (**B**) Effect of RU486. EPC of d 8.5 mice were co-cultured with uterine decidual cells in medium containing a range of concentrations of RU486: C, 0.0 mol/L RU486; R1, 10^{-8} mol/L RU486; R2, 10^{-7} mol/L RU486; R3, 10^{-6} mol/L RU486; R4, 10^{-5} mol/L RU486. (**C**) Blocking of role of P in the outgrowth area of EPC by RU486 on uterine decidual cells. The experiment was carried out in a medium containing a range of concentrations of RU486: C, 0.0 mol/L RU486; R1, 10^{-8} mol/L RU486; R2, 10^{-7} mol/L RU486; R3, 10^{-6} mol/L RU486; R4, 10^{-5} mol/L RU486, respectively, in the presence of 3.18×10^{-6} mol/L P_4. The effect of P_4 or RU486 on the outgrowth area was determined by dividing the surface area at 96 h by the surface area at 0 h × 100. Results are expressed as mean ± SE of three replicates (*n* = 20 EPC/well). ** and *** indicate significant differences from the control ($p < 0.01$, $p < 0.001$, respectively). aaa indicates significant differences from the P_4 (10^{-6} mol/L P_4) ($p < 0.001$).

phase, regulated by estrogen and P_4, is characterized primarily by changes in both the luminal and glandular epithelial cells in preparation for blastocyst apposition and attachment, and if the action of P_4 is antagonized, these changes are inhibited and the uterus is maintained in a prereceptive state. This further suggested at the early stage that P_4 was indeed helpful for the attachment and invasion of embryo. The key to regulating embryo invasion is MMPs; MMP-9 in glandular epithelial cells was greatest during the late proliferative phase, when the concentration of P_4 was also highest *(22)*.

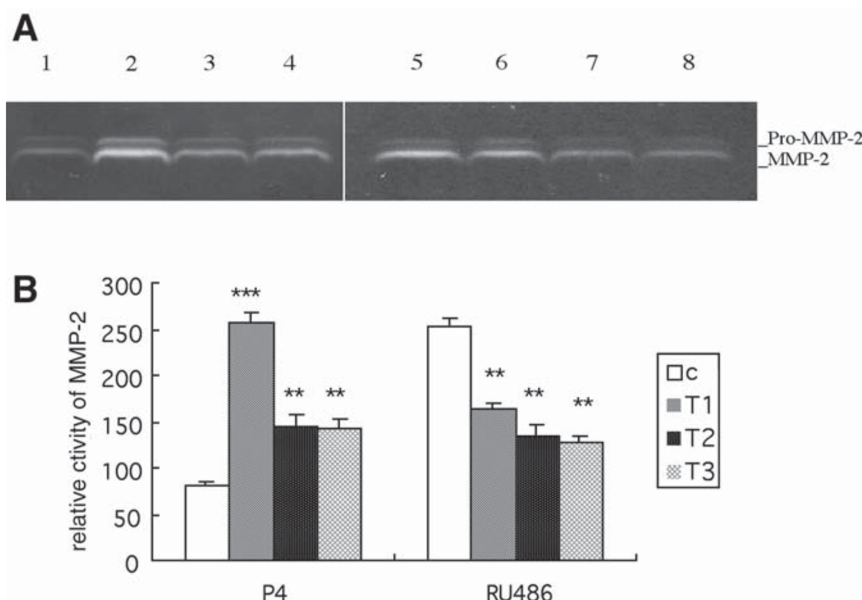

Fig. 4. Effect of P_4 or RU486 on MMP-2 and MMP-9 activities 24 h of culture on blastocysts on fibronectin-coated dishes. (**A**) Samples from the medium were analyzed for gelatinase activity. Bands correspond relative molecule of MMP-2. Lanes 1–4, samples from the medium containing a range of P_4 concentrations: C, 0.0 mol/L; T1, 3.18×10^{-5} mol/L; T2, 3.18×10^{-6} mol/L; T3, 3.18×10^{-7} mol/L at 35 h, respectively. Lanes 5–8, samples from the medium containing a range of P_4 concentrations: C, 0.0 mol/L; R1, 10^{-7} mol/L; R2, 10^{-6} mol/L; R3, 10^{-5} mol/L. (**B**) Total activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by computer-aided densitometry. Results are expressed as mean ± SE of four replicates (*n* = 40 blastocysts/well). ** and *** indicate significant differences from the control ($p < 0.01$, $p < 0.001$, respectively).

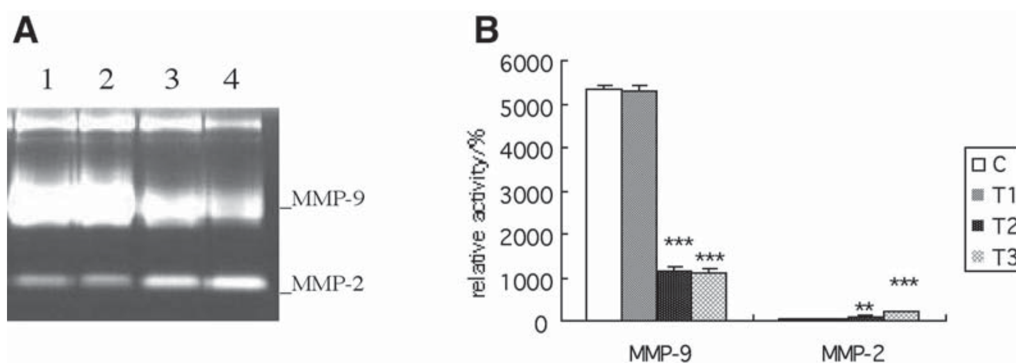

Fig. 5. Effect of P_4 on MMP-2 and MMP-9 activities at 24 h of culture of EPC on fibronectin-coated dishes. (**A**) Samples (6 µg) from the culture medium were analyzed for gelatinase activity. Bands correspond to the relative molecular masses of MMP-9 and MMP-2. Lanes 1–4, samples from the medium containing a range of P_4 concentrations: C, 0.0 mol/L; T1, 3.18×10^{-7} mol/L; T2, 3.18×10^{-6} mol/L P_4; T3, 3.18×10^{-5} mol/L P_4 at 36 h, respectively. (**B**) Total activities of MMP-2 and -9 detected by gelatin zymography were quantified by computer-aided densitometry. Results are expressed as mean ± SE of four replicates (*n* = 10 EPC/well). ** and *** indicate significant differences from the control ($p < 0.01$, $p < 0.001$, respectively).

This indicates that at the early stage of embryo implantation, P_4 may promote embryo invasion by enhancing the activity of MMPs. Our results showed that P_4 upregulated the activity of MMP-2 when only blastocysts were cultured, and it was reported that the uterine epithelial cells did not secrete MMPs *(23)*. This suggested that the stimulatory effect of P_4 at the early stage was on the blastocysts.

Embryo implantation is an immunologic act, although it is still a mystery how the blastocyst escapes maternal immune surveillance at the time of implantation *(14)*; obviously, P_4 plays an important role in this aspect. P_4 acts as an important immunosuppressant, which not only inhibits specific functions of T-lymphocytes and macrophages *(7)* but also blocks natural killer cell activity *(8)*. This further suggests that, in immunology, P_4 provides the possibility for the attachment and outgrowth of blastocysts by preventing rejection of the fetal allograft.

At the initial stage of embryo implantation in mice, trophoblasts begin to attach to and then invade the receptive uterine epithelium, after which the trophectoderm prolifer-

ates to form the EPC, and later the spongiotrophoblast *(24)*. The outermost trophoblasts of the EPC differentiate into secondary trophoblast giant cells, which invade the maternal decidua. The coculture system of EPC on the uterine decidual cells provides a good method with which to investigate the effects of P_4 on the invasion of trophoblast cells into decidual cells. In the present study, our results demonstrated that P_4 inhibited the attachment and outgrowth of EPC on uterine decidual cells. Conversely, RU486 promoted the attachment and outgrowth of EPC on it and the role of P_4 was blocked by RU486, which suggested that the role of P_4 was transduced through the classic nuclear PR. It was reported that P_4 inhibited activation of MMPs from decidual cells *(10)*, and our results showed that P_4 inhibited the activity of MMP-9 but promoted the activity of MMP-2 on EPC. These results suggested that the effect of P_4 at the late stage is on both EPC and decidual cells. Since MMP-2 is the prevailing metalloprotease at the early stage of embryo implantation and MMP-9 is the principal protease at the late stage *(25)*, the inhibitory effect of P_4 on MMP-9 resulted in the blocking of EPC outgrowth on decidual cells.

The invasion of trophoblast cells into the maternal endometrium is highly controlled and coordinated. On one hand, the trophoblast cells do not stop invading the maternal endometrium during embryo implantation. On the other hand, the trophoblast cells cannot invade without restriction. There might be two sets of molecules to regulate the process. One set of molecules is used to promote the invasion of the trophoblast cells, and the other to inhibit it. MMPs produced by the trophoblast cells degrade ECM and penetrate basement membrane so that the trophoblast cells have the ability to invade. Tissue inhibitors of metalloproteinase (TIMPs) are natural inhibitors of MMPs. The equilibrium between MMPs and TIMPs is an important guarantee for the strict regulation of trophoblast invasion *(26)*. P_4 blocks the secretion and activation of MMPs from the endometrial tissue *(10–13)*, Conversely, P_4 can enhance the expression of TIMPs, especially TIMP-3, in the endometrial stroma *(27)*. Protease inhibitors can inhibit the attachment and outgrowth of trophoblasts on a monolayer of decidual cells *(28)*. In addition, P_4 restrains endometrial tissue breakdown by regulating other molecules involved in the invasion of trophoblast cells. For example, interleukin-1 (IL-1) and IL-6, which play an important role in the invasion and proliferation of trophoblasts, are inhibited by P_4 *(29)*. P_4 regulates the expression of transforming growth factor-β, insulin-like growth factor binding protein-1, and fibronectin (FN) in the endometrial stroma, which restrain the invasion of trophoblast cells *(27)*.

These data further support the views that P_4 restrains the invasion of trophoblast cells into deciduas by regulating the expression of related molecules in trophoblast and decidual cells. The different effect of P_4 on the epithelial cells and decidual cells will make a different effect on the trophoblast at the early and late stage. On one hand, P_4 action in target tissues is mediated through two PR isoforms, PR-A and PR-B, which display different regulatory functions in target cells. Relative expression ratio of these isoforms varies depending on cell and tissue types *(30)*. On the other hand, the expression of some genes in different tissue is differently mediated by P_4. For example, *Hoxa10* expression is induced in the stroma and decreased in the myometrium by P_4. The differential tissue-specific response of this gene in response to P_4 is likely mediated by sex steroid receptor coactivators or corepressors *(31)*. The detailed molecular mechanism of the dual role of P_4 requires further investigation.

Taken together, these data show that in the entire process of embryo implantation, P_4 plays dual roles in the attachment and invasion of the trophoblast cells. When trophoblast cells came into contact with uterine epithelial cells, P_4 promoted the attachment and outgrowth of blastocysts. When the trophoblast cells interacted with the uterine deciduas, P_4 restrained the attachment and outgrowth of EPC. The role of P_4 was transduced through the classic nuclear PR.

Materials and Methods

Animals

Kunming white strain mice (Experimental Animal Center, the Genetic Institute of Chinese Academy of Sciences) were housed in the animal facility of the State Key Laboratory of Reproductive Biology. Adult female mice (25–30 g, 5–8 wk old) were mated with males of the same strain at room temperature and with a constant photoperiod (14-h light/10-h dark cycle). Food and water were freely available.

Preparation and Culture of Uterine Epithelial Cells In Vitro

On d 4 of pregnancy, a monolayer of uterine epithelial cells was prepared using the method described by Zeng and Cao *(32)*. Briefly, female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin and 48 h later with 5 IU of human chorionic gonadotropin (hCG). Following hCG injection, each female was caged with a male mouse overnight. The first morning that a vaginal plug was found was designated d 1 of pregnancy. Uteri from d-4 pregnant mice were split longitudinally to expose the epithelial cell surface and digested with 0.6% trypsin (Sigma, St. Louis, MO) solution at 4ºC for 2 h, followed by another 0.5 h at 25–30ºC. Tissues were gently shaken to dislodge the epithelium from the endometrial bed. The epithelial cells and fragments were collected by centrifuging at 500*g* for 10 min. Cells were washed three times with Ham's F-12 (Gibco, Rockville, MD) containing 2.20 m*M* calcium lactate, 2.05 m*M* glutamine, 12.5 m*M* $NaHCO_3$, and 400 IU/mL of gentamycin sulfate, and then the cells were resuspended in Ham's F-12 with 10% fetal calf serum (FCS) (Sigma). The cell suspension was adjusted to 1×10^6 cells/mL and placed on 24-well Falcon plates and incubated at 37ºC, 5% CO_2 in a humidified chamber. The culture medium was changed to remove unattached cells and cell debris after 24 h.

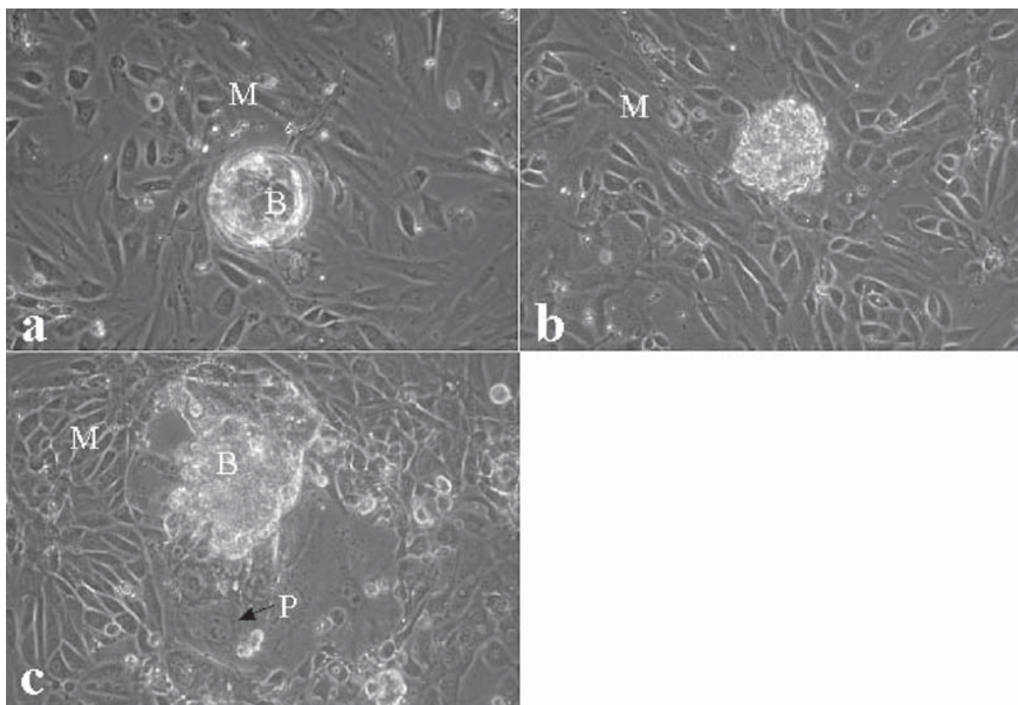

Fig. 6. Attachment and outgrowth of mouse blastocysts on a monolayer of uterine epithelial cells. (**A**) Nonattached blastocysts that floated over the monolayer (×200); (**B**) attached blastocysts (×200); (**C**) trophectoderm outgrowth (×200). M, monolayer of uterine epithelial cells; B, blastocysts; P, primary trophoblast giant cells.

Collection of Embryos

Preimplantation blastocysts were flushed from the uterus of d 4 pregnant mice with Hank's solution and rinsed three times with Ham's F-12 medium. Then they were transferred in droplets of preheated medium (the same as that used for cell culture, except that 10% FCS was replaced by 0.4% bovine serum albumin [BSA]; Sigma) and incubated under standard culture conditions.

Coculture of Embryos and Uterine Epithelial Cells

Epithelial cells harvested as described earlier were placed in 24-well sterile plastic plates and cultured under the same conditions. The next morning, a monolayer of the uterine epithelial cells had formed. This monolayer was further identified by immunostaining for cytokeratin and hematoxylin and eosin staining and comprised 94.8 (±1.0%) epithelial cells. After the monolayer was rinsed three times in Ham's F-12 medium, the coculture medium was added, which contained Ham's F-12 medium supplemented with 0.4% BSA and a specific concentration of P_4 or RU486. At the same time, blastocysts were placed in corresponding wells with the monolayer of uterine epithelial cells, respectively (about 40 blastocysts/well), and their attachment and outgrowth were observed.

Collection of Decidual Cells

Decidual cells were isolated and maintained as previously described *(33)*. Briefly, on d 8.5 of pregnancy, the mice were killed and the deciduas collected. After the deciduas were rinsed three times in phosphate-buffered saline, they were minced and digested with 1 mg/mL of collagenase I (Sigma) at 25ºC for 90 min. Digestion was stopped by the addition of 2 vol of Ham's F-12 medium. After washing, the dispersed cells were filtered through a nylon sieve to remove the gross core residues. The filtered cell suspension (1 to 2 mL) was then added to Ham's F-12 medium and collected by centrifuging at 500*g* for 10 min. Cells were washed three times with Ham's F-12 and then resuspended in Ham's F-12 with 10% FCS. The purified deciduas were planted on 24-well Falcon plates at 1×10^6 cells/mL and incubated at 37ºC, 5% CO_2 in a humidified chamber. The culture medium was changed to remove unattached cells and cell debris after 24 h. Immunocytochemistry studies revealed that >98% of the cells exhibited positive staining for vimentin.

Coculture of EPC on Decidual Cells

EPC from the mice on d 8.5 of pregnancy was dissected out under sterile conditions and placed into corresponding wells on uterine decidual cells (approx 20 EPC per well). Cultures were kept in the incubator at 37ºC, in an atmosphere of 5% CO_2 for 96 h, after which their attachment and outgrowth were observed. The coculture medium was added, which contained Ham's F-12 medium supplemented with 0.4% BSA and a specific concentration of P_4, RU486, or their combinations.

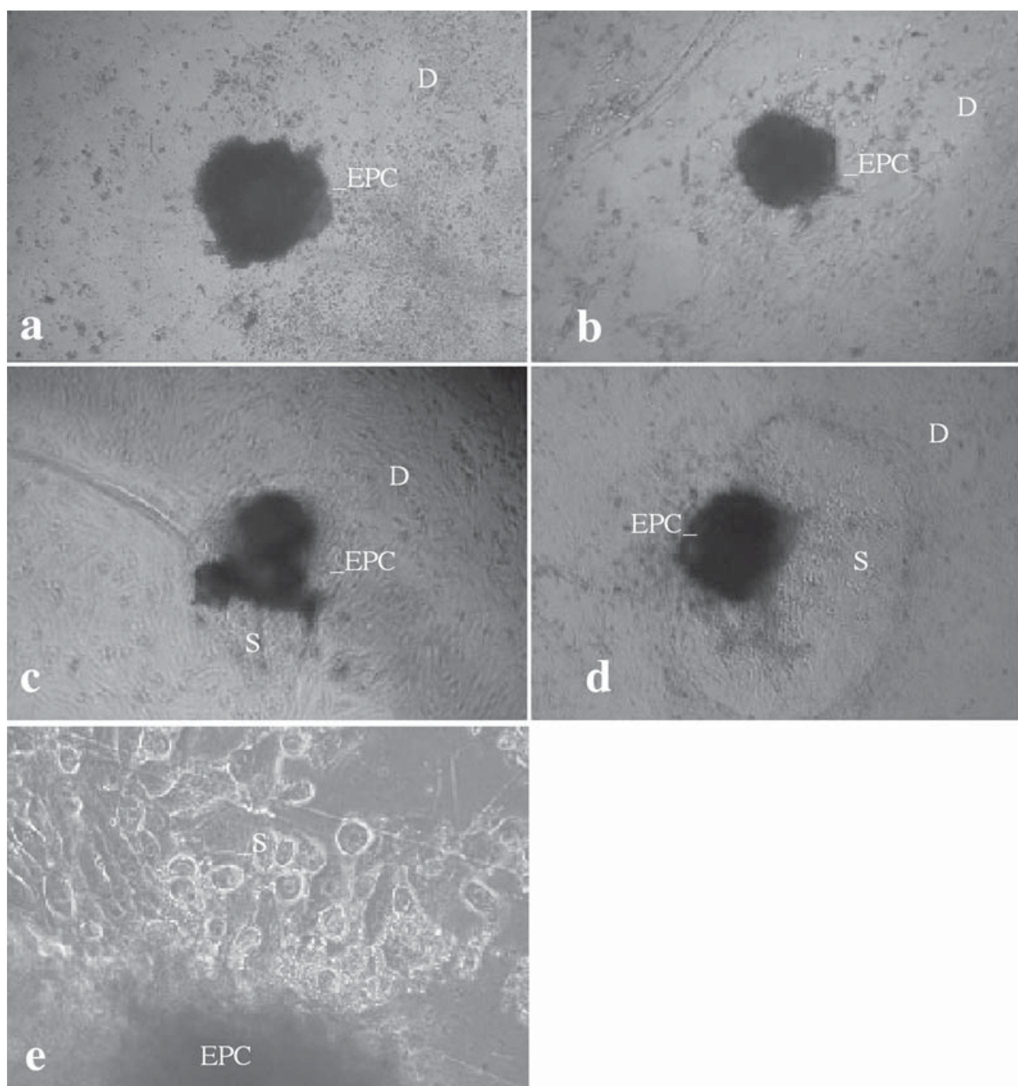

Fig. 7. Attachment (×40) and outgrowth of mouse EPC on uterine (×40); decidual cells. (**A**) Nonattached EPC (×40); (**B**) attached EPC at 24 h of coculture (×40); (**C**) EPC with outgrowth at 48 h of coculture (×40); (**D**) EPC with outgrowth at 96 h of coculture (×40); (**E**) magnification of periphery of EPC after 96 h of coculture (×200). D, uterine decidual cells; EPC, ectoplacental cone; S, secondary trophoblast giant cells.

Criteria for Attachment and Outgrowth of Blastocysts or EPC

Attachment or outgrowth was observed using phase-contrast microscopy (Olympus, Japan) and defined according to the following criteria: After the plate was shaken for 20 s with one rotation per second, if the blastocyst or EPC was found to stay at the same place, this blastocyst was considered as "attachment"; if not, it was designated as "nonattachment." After attachment, blastocysts began to outgrow outward. When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocysts as "outgrowth" (Figs. 6 and 7). The outgrowth area of blastocysts is the area occupied by primary giant trophoblast cells. The percentage increase in outgrowth area of EPC was defined as the surface area at 96 h of coculture divided by the surface area at 0 h of coculture. The outgrowth area was recorded and measured by a digital camera.

Gelatin Zymography

The culture medium (6 µg) at 36 h was mixed with 4× sample buffer (8% sodium dodecyl sulfate [SDS] [w/v], 0.04% bromophenol blue [w/v], 40% glycerol [v/v], 0.25 *M* Tris) and then subjected to electrophoresis in a 10% polyacrylamine gel containing 0.5 mg/mL of gelatin (Sigma). The gel was washed in 2.5% Triton X-100, 50 m*M* Tris-

HCl, at pH 7.5 for 1 h to remove the SDS and incubated for 18 h in calcium assay buffer (50 m*M* Tris, 200 m*M* NaCl, 10 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 1% Triton X-100, pH 7.5) at 37ºC. After staining with 0.2% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid, the gel was destained with 10% acetic acid. Estimation of molecular weight was possible owing to the concurrent electrophoresis of molecular weight markers. Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by computer-aided densitometry.

Experimental Design in Two Sets of Cocultures

The coculture study was divided into six experiments depending on the type of coculture and the inclusion of P_4 or RU486. P_4 (product no. 80K0885; Sigma) and RU486 (the Third Pharmaceutical Factory of Beijing) were dissolved in ethanol and diluted with Ham's F-12 culture medium, respectively. The final ethanol concentration was <0.1%, and the control contained the same concentration of ethanol. Each experiment consisted of several groups at different concentrations. The P_4 experiments in two kinds of cocultures were composed of five groups: C (0.0 mol/L of P_4), T1 (3.18×10^{-8} mol/L of P_4), T2 (3.18×10^{-7} mol/L of P_4), T3 (3.18×10^{-6} mol/L of P_4), and T4 (3.18×10^{-5} mol/L of P_4). The RU486 experiments in two kinds of cocultures were also composed of five groups: C (0.0 mol/L of RU486), R1 (10^{-8} mol/L of RU486), R2 (10^{-7} mol/L of RU486), R3 (10^{-6} mol/L of RU486), and R4 (10^{-5} mol/L of RU486). To identify whether the role of P_4 was transduced through a specific nuclear receptor protein, blastocysts or EPCs were cultured with R1 (10^{-8} mol/L of RU486), R2 (10^{-7} mol/L of RU486), R3 (10^{-6} mol/L of RU486), and R4 (10^{-5} mol/L of RU486), respectively, in the presence of 3.18×10^{-6} mol/L of P_4 in both kinds of cocultures. To identify further that the role of P_4 is on blastocysts or uterine epithelial cells or EPC or decidual cells, only blastocysts or EPCs were cultured with different doses of P_4 or RU486 on fibronectin-coated dishes, and at 24 h of culture, the activity of MMP-2 and MMP-9 in the culture medium was detected by gelatin zymography.

Statistical Analysis

All the results are presented as mean ± SE. All the data were analyzed using Dunnet test of one-way analysis of variance in SPSS software. A value of $p < 0.05$ was considered significant.

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