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Dynamic distribution of epidermal growth factor during mouse embryo peri-implantation

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Abstract

Embryo implantation depends on the synchronized development of the blastocyst and the endometrium. This process is highly controlled by the coordinated action of the steroid hormones: estrogen and progesterone. By autocrine, paracrine or juxtacrine routes, some growth factors or cytokines are involved in this steroidal regulation pathway. Here we report the effects of epidermal growth factor (EGF) on embryo implantation in the mouse, the expression and distribution patterns of EGF protein in the mouse blastocyst, ectoplacental cone (EPC) and peri-implantation uterus on days 1–8 of gestation.

By RT-PCR and dot blot, we found that EGF and its receptor (EGFR) are co-expressed in the blastocyst and peri-implantational uteri of pregnant days 2–8 (D2–D8) mice. Injection of EGF antibody into a uterine horn on the third day of pregnancy (D3) significantly reduced the number of mouse embryos that implanted on D8, indicating EGF have a function in the mouse embryo implantation.

Further investigation by using indirect immunofluorescence and confocal microscope was made to trace EGF and EGFR protein localization during the mouse embryo implantation. EGF and EGFR are co-localized in the blastocyst, and in the secondary trophoblastic giant cells (SGC) of the EPC. At the pre-implantation stage, the distribution of EGF protein in the mouse uterus changes from epithelium to stroma. On D1 of pregnancy, EGF is mainly distributed in uterine stroma and myometrium. On D2, it is present in the uterine epithelium. On D3, it changes again from the uterine epithelium to the stroma. By D4, EGF is predominantly in the stroma. This dynamic distribution correlates with the proliferation activity of uterine cells at each period. On D6–D8 of embryo implantation, EGF 3 protein accumulates at the uterine mesometrial pole, a region that contributes to the trophoblastic invasiveness and placentation.

This temporal and spatial localization of EGF protein in the mouse uterus implicates the cytokine in the regulation of trophoblastic invasiveness and uterine receptiveness.

Keywords: Embryo implantation; Epidermal growth factor; Mouse

1. Introduction

The mechanism of embryo implantation has long been a scientific enigma. It is essential for development of the mammalian embryo and requires a series of cellular and molecular events. Two crucial factors determine the success of the implantation process: the invasiveness of the blastocyst and the receptivity of the endometrium. A pre-implantation stage embryo must develop to a specific stage before it can penetrate the endometrium. At the same time, uterine cells proliferate and differentiate to be ready to accept the embryo [1].

It is well established that estradiol (E_2) and progesterone (P_4) from the ovary control the implantation process [2]. At the peri-implantation stage, ovarian steroid hormones (E_2 or P_4) specifically up-regulate gene expression of growth factors or cytokines, such as leukemia inhibitory factor [3], members of the epidermal growth factor (EGF) family [4–6], colony stimulating factor [7] and interkeukin-1 [8]. These locally produced

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factors mediate the maternal-fetus interaction and control the implantation process.

EGF family growth factors are hypothesized to participate in multiple developmental, physiological and pathological processes [9]. Earlier work reported that EGF deficiency during pregnancy causes the intra-uterine growth retardation or abortion in mice [10,11]. By replacing estrogen, EGF can initiate embryo implantation in the rat [12,13]. Knockout of the 4 EGF receptor in the mouse leads to death before implantation [14]. A large number of studies have demonstrated that EGF affects mouse embryo development in vitro [15–20], and promotes trophoblast differentiation needed for implantation [21–23]. EGF receptors are expressed in the embryo at very early stages [24], and also in the peri-implantation uterus [25], indicating that EGF is involved in the development of the embryo and the uterus throughout the implantation process. However, the distribution of the EGF protein during the peri-implantation period has not been studied. To achieve this, we used intra-uterine injection, reverse transcription-polymerase chain reaction (RT-PCR), indirect immunofluorescence and confocal scanning microscopy, to study EGF distribution in the mouse embryo and the peri-implantational uterus.

2. Results

2.1. Expression of EGF and EGFR in the mouse blastocyst

We used RT-PCR and dot blot techniques to detect the presence of EGF and EGFR mRNA and protein during the mouse embryo implantation, also to verify the specificity of the antibody. Saliva gland tissue was used as a positive control, since it is the source tissue for EGF expression. As Fig. 1 shows EGF and its receptor are co-expressed in the blastocyst and peri-implantational uteri. RT-PCR products were cloned and sequenced, and they all turned out to be the correct sequences (data not shown).

2.2. Intra-uterine injection

We further tested the requirement for EGF in mouse embryo implantation by injecting the EGF antibody

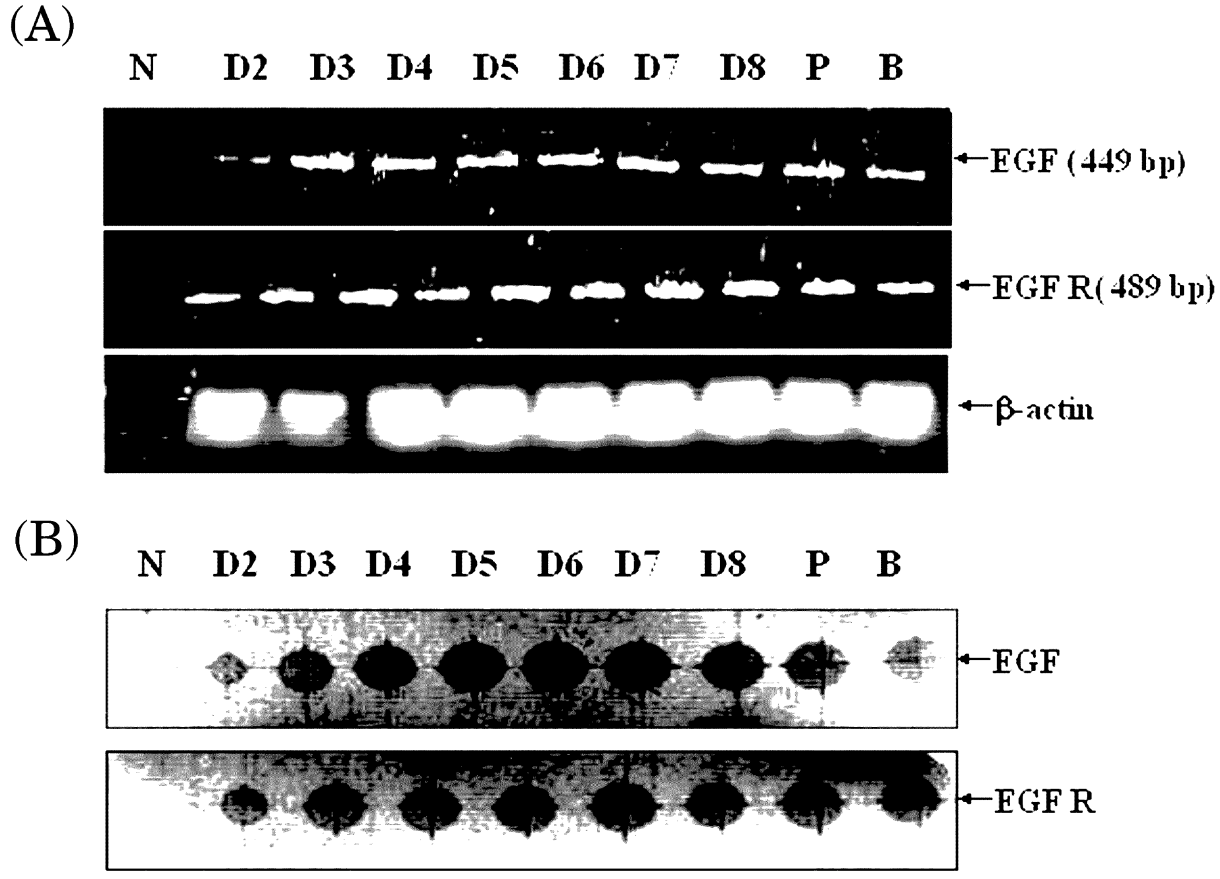


Fig. 1. Expression of EGF and EGFR in the blastocyst and peri-implantational uteri. Tissues from the mouse blastocysts and the uteri of pregnant days 2–8 were subjected to RT-PCR to detect the mRNA expression (A), and dot blot to detect the proteins of EGF and EGFR (B). P is a positive control by using the mouse saliva gland isolated as the EGF marker tissue, B stands for blastocysts and N is a negative H_2O control.

into the lumen of the uterus. As shown in Fig. 2, injection of the EGF antibody reduced the number of embryos that were implanted by day 8 ($P < 0.001$), thus implicating a role for EGF in embryo implantation.

2.3. Distribution of EGF and its receptor in the mouse blastocyst and ectoplacental cone (EPC)

EGF and its receptor, EGFR/Erb-B1, are co-localized in the mouse blastocyst trophoblast (Fig. 3A1–B1–C1 and A2–B2–C2). EGF protein is localized in the expanding SGCs of EPC culture, expanding outwards from EPC, while EGFR protein is localized in both the EPC and its SGC (Fig. 3A3–B3–C3 and A4–B4–C4). The co-localization of EGF and its receptor supports the assumption that EGF regulates blastocyst development and trophoblast differentiation/invasiveness by autocrine pathway, making the blastocyst capable of penetrating the uterine endometrium.

2.4. Distribution of EGF in the mouse peri-implantation uterus

On D1 of pre-implantation, EGF protein is distributed in the uterine stroma and myometrium at the basal level. By D2, the uterine epithelium is producing high levels of EGF. By D3, the site of EGF protein expression has changed from the epithelium to the stroma, while on D4, the EGF protein is localized in the stroma (Fig. 4). This distribution pattern correlates with variation of the uterine cell proliferation activity at the pre-implantation stage, suggesting the involvement of EGF in this process.

On D6–D8 at the implantation stage, EGF is localized at the mesometrial pole (Fig. 5). The fluorescent signals increased gradually as implantation occurred. At these stages EGF may play a role in the establishment of the placenta.

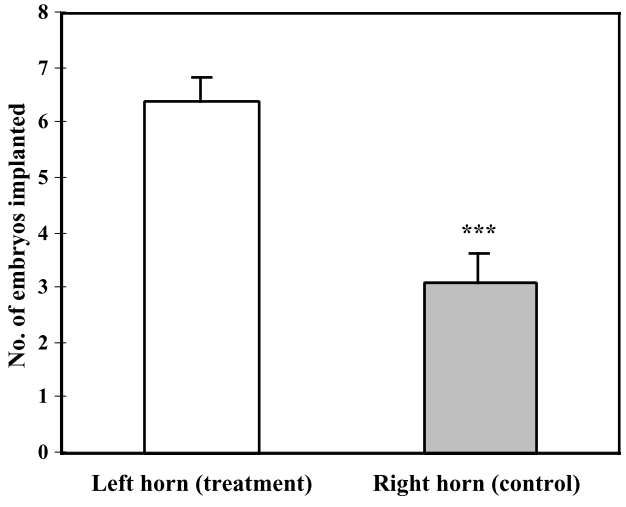


Fig. 2. Effect of intra-uterine injection of EGF antibody on embryo implantation in the mouse. The uterine horn of D3 mice was injected with 5 ml EGF antibody solution (left horn, treatment) or goat IgG (right horn, control). D8 animals ($n = 12$) were killed to count the number of embryos implanted. Results are expressed as the mean ±SE. *** indicates a significant decrease in the number of embryos that were implanted compared to the control ($P < 0.001$).

3. Discussion

Cytokines or growth factors have been implicated in the regulation of embryo implantation [2]. The synthesis of these factors is induced by ovarian steroid hormones, and is restricted to the uterine epithelia or the stroma that surrounds the penetrating blastocyst adjacent to the blastocyst attachment site [13,19,20] where they regulate fetal–maternal development and interaction. The evidence in this idea comes from the gene knockout experiment [3,7] or the interference with embryo implantation by application of a specific antibody [8,26,27].

In this study, we tested EGF's function by injecting EGF antibody into the uterine horn on day 3, just before the blastocyst implantation. This assay was used before to block the action of other cytokines, such as interleukin-1 [27] and leukemia inhibitory factor [26]. The inhibitory effect of this intra-uterine injection is possibly due to the hindrance of both trophoblast invasiveness and the uterine receptivity. Since the presence of EGF in the blastocyst and peri-implantational uterus is arguable [28,29], we clarified this question in various ways: confocal immunostaining, RT-PCR and dot blot from both protein and mRNA level. Also, positive control tissue, such as saliva gland, was used to confirm the specificity of EGF antibody used in this study. Co-expression of EGF and its receptor implicates that by the autocrine pathway, a blastocyst can regulate its own invasiveness, in a way similar to tumors. This conclusion is supported by Paria et al., who reported that a blastocyst lacking EGFR is dormant and can be activated by restoring the EGFR activity [30]. Possible mechanism is that EGF regulates the expression of some proteinases essential for trophoblast penetration [22]. Thus, at the implantation stage, EGF and other factors promote the development and maturation of the blastocyst, enabling it to penetrate the uterine wall. The epithelium may not be considered as the direct target tissue for the actions of EGF-related growth factors since EGFR mRNA is expressed in the stroma, deciduum and myometrium, but not in the epithelium [31].

During peri-implantation stage, the uterine cells undergo proliferation and differentiation, resulting in the establishment of a receptive state in the uterus for embryo implantation. Ligands of EGF family members were recently reported to be associated with this process

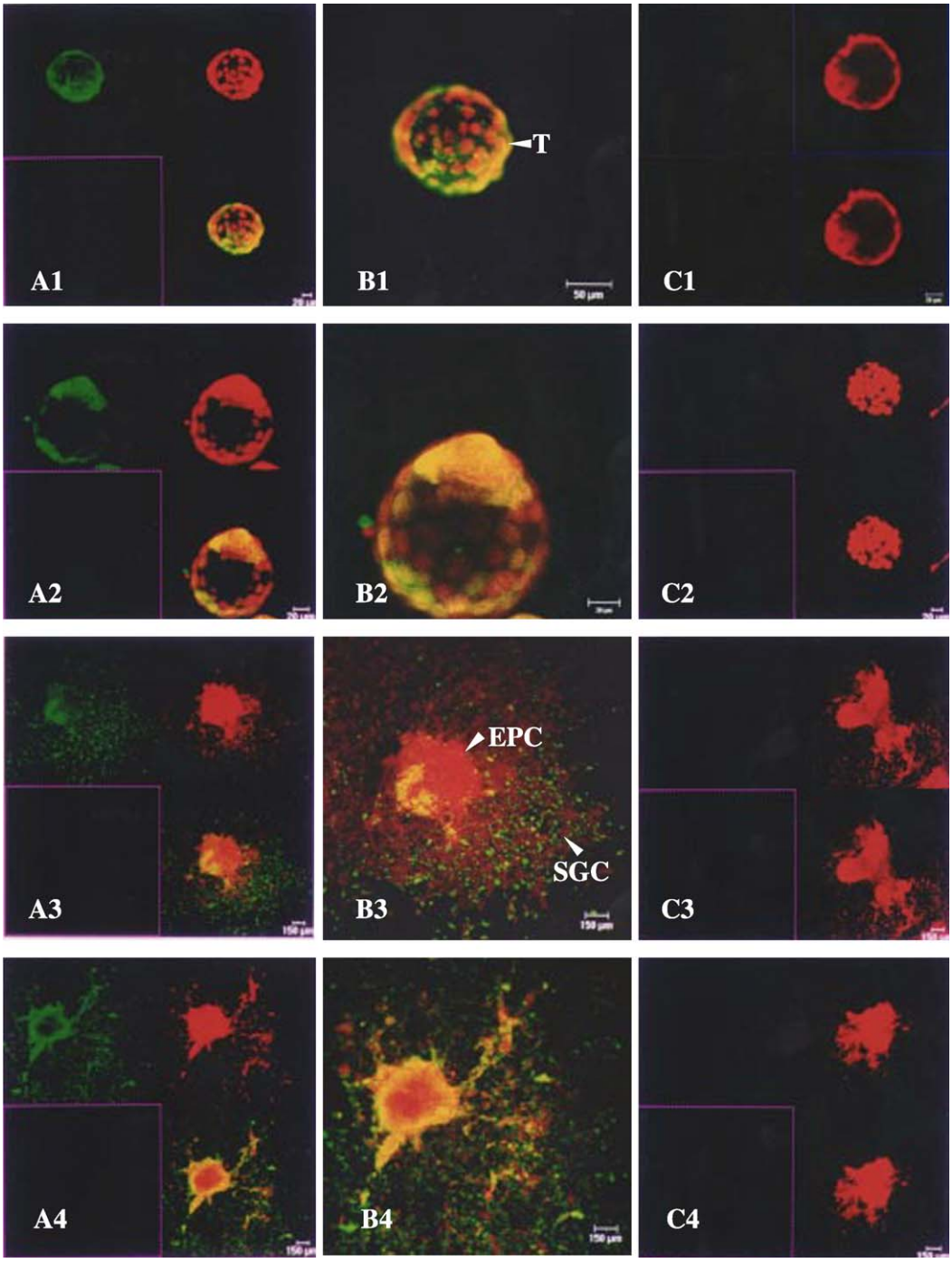
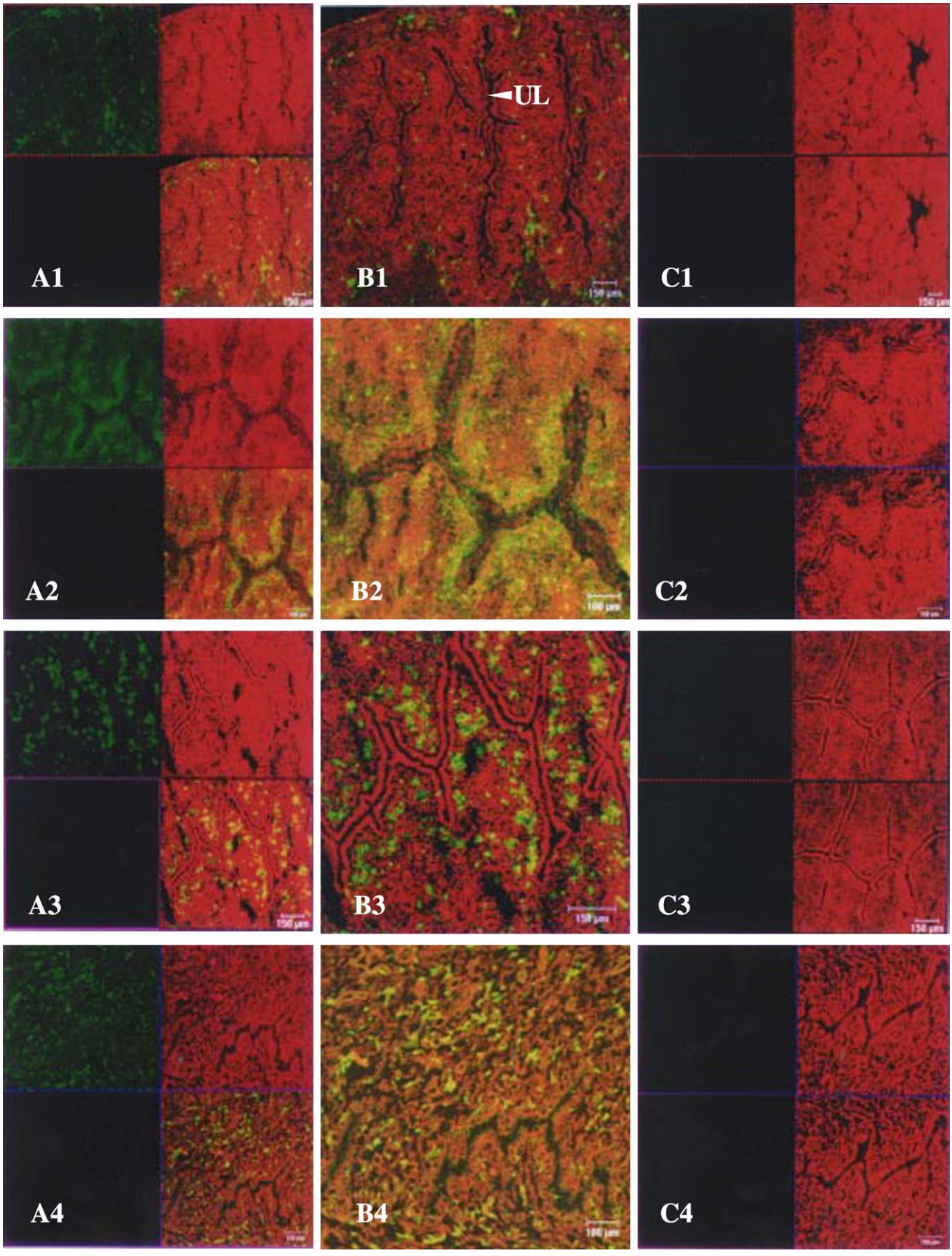


Fig. 3. Localization of EGF and EGFR proteins in the mouse blastocyst and in vitro cultured EPC. EGF and EGFR were localized immunohistologically using a FITC-conjugated secondary antibody (green). Nuclei were labeled by propidium iodide (PI, red). FITC and PI are shown separately and then overlaid (A1–A4). B1–B4 are the overlain pictures at a higher magnification. C1–C4 are the corresponding controls for each group. A1–B1–C1 and A2–B2–C2 show EGF and EGFR localizations in the mouse blastocyst trophoblast, respectively. A3–B3–C3 and A4–B4–C4 show EGF and EGFR localizations in the mouse EPC in vitro culture.

[4–6]. In order to elucidate EGF action, we studied the localization of EGF protein in the peri-implantation uterus. From days 1 to 4 of pregnancy, EGF distribution shifted from the uterine epithelium to the stroma, correlating with the proliferation activity of endometrial cells at the pre-implantation stage [1]. These proliferation and differentiation activities are crucial for the competence of uterine receptivity.

At the implantation stage, however, distribution of EGF in the uterus differed greatly from that at the pre-implantation stage. On D6–D8, EGF protein accumulates at the mesometrial pole of uterus, where eventually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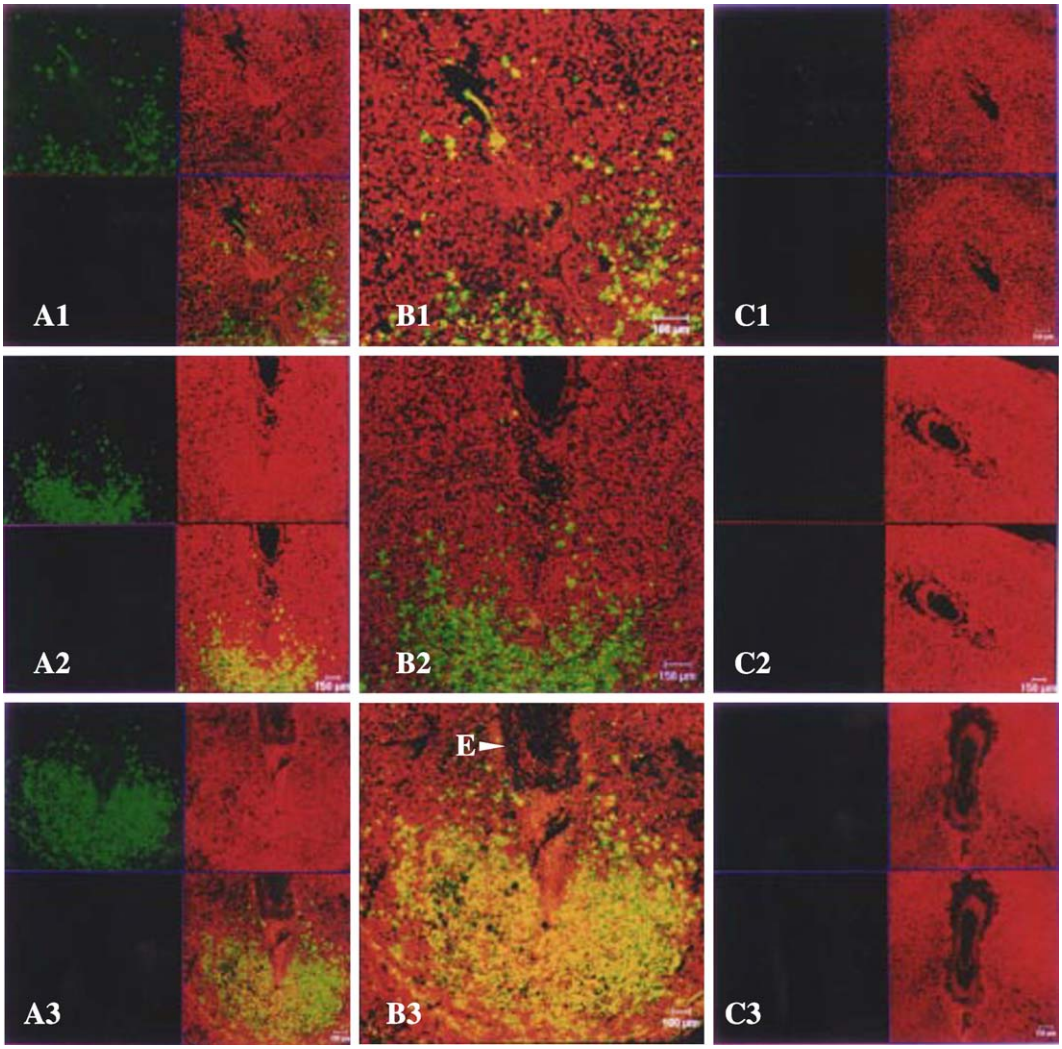

Fig. 5. Localization of EGF in the mouse implanting uteri (D6–D8). The green signal shows EGF or EGFR protein labeled with FITC-conjugated secondary antibody, while the red signal shows nuclei labeled by propidium iodide (PI). A1–A3 are dual fluorescence channels for FITC (top left) and PI (top right), which are overlaid into one picture below. B1–B3 are overlain pictures at a higher magnification. C1–C3 are the corresponding controls. A1–B1–C1, A2–B2–C2 and A3–B3–C3 show EGF localization in the pregnant mouse uterus on D6, D7 and D8, respectively. E, embryo.

the SGC will invade and form the placenta. The localization of EGF suggests a role in the regulation of SGC invasiveness and the establishment of the placenta. Evidence for this relationship is supported by work in EPC cultures.

Steroid hormones (E_2/P_4) control expression of ligands of the EGF family [4–6] and its receptors in the pregnant uterus [32]. This interaction results in the differentiation or proliferation of epithelial and stromal cells. Supporting evidence from Tan et al. [33] showed that the expression of estrogen receptor (ER) occurred in a temporal and cell type-specific manner. On D1 and D2 it was primarily localized in the luminal and glandular epithelia. On D3 and D4 the accumulation was localized primarily in stromal cells in addition to its presence in the epithelium. On days 6–8, the accumulation of ER-alpha mRNA was primarily localized in the secondary decidual zone with more intense localization in the subepithelial cells at the mesometrial pole. Our EGF distribution pattern here is surprisingly similar to the ER mRNA, indicating that EGF may be involved in the estrogen signalling pathway that

Fig. 4. Localization of EGF in the mouse pre-implantation uterus (D1–D4). The green signal denotes EGF or EGFR protein labeled by FITC-conjugated secondary antibody, while the red signal shows nuclei labeled by propidium iodide (PI). A1–A4 are dual fluorescence channels for FITC (top left) and PI (top right), which are overlaid into one picture below. B1–B4 are overlain pictures at a higher magnification. C1–C4 are the corresponding controls. A1–B1–C1, A2–B2–C2, A3–B3–C3 and A4–B4–C4 show EGF localization in the pregnant mouse uterus on D1, D2, D3 and D4, respectively. T, trophoblast; EPC, ectoplacental cone; SGC, secondary trophoblast giant cells; UL, uterine lumen.

regulates uterine cell proliferation and differentiation. Future work should be focused on detailing their relationship.

4. Materials and methods

4.1. Animals

Animals were fed and treated as described previously [26,34]. Adult (virginal; age five to six weeks) mice of the outbred Kunming white strain were purchased from the Experimental Animal Center of the Institute of Heredity Science, Chinese Academy of Sciences. The animals were fed at room temperature (about 25 °C) and with a constant photoperiod (light : dark cycle, 14 : 10 h). Food and water were freely available.

4.2. Intra-uterine injection

Each female mouse was caged with one male to mate naturally overnight. D1 of pregnancy was designated the next morning when a vaginal plug was formed. Pregnant mice on day 3 were injected with 1 mg EGF antibody (5 ml stock solution, 200 mg/ml, Santa Cruz) into the lumen of the left uterine horn adjacent to the ovary, while the right horn was injected with 1 mg goat IgG as control. On day 8, the treated animals were sacrificed to count the number of implanted embryos. A *t*-test was performed to analyze the significance of the difference.

4.3. Preparation and treatment of uterine tissues from pregnant mice

The whole uteri of naturally mated pregnant mice on days 1–8 were collected. Pre-implantation (D1–D4) uterine horns were cut into two to three pieces and frozen longitudinal sections were prepared to demonstrate variations of the uterine lumen. Cross-sections of implanted uteri (D6–D8) were prepared to include the invading embryo.

4.4. Collection of mouse blastocysts

Female mice were super-ovulated by pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) as previously described [26]. Following hCG injection, each female was caged with a male mouse overnight, and pregnant mice were selected according to the presence of the vaginal plug. Blastocysts were flushed from the uterus of day 4 pregnant mice with Hank's solution, rinsed in Ham's F-12 culture solution [Ham's F-12 media (Gibco) complementing 2.2 mM calcium lactate, 2.05 mM glutamine, 12.5 mM $NaHCO_3$, 400 IU/ml gentamycin sulfate] and transferred in droplets of preheated Ham's F-12 culture solution and incubated at 37 °C in a 95% CO_2 incubator. Blastocysts isolated from the zona pellucida were used as samples for immunostaining.

4.5. Separation and in vitro culture of mouse ectoplacental cone

The protocol of Zhang et al. was used [34]. Pregnant females on D8.5 were dissected and EPCs were separated from the implanting embryos with very fine forceps, and cultured in Ham's F-12 media in a 24-well plate. After 48 h when the SGC grew out around the attached EPC, we removed the culture media and fixed the samples for immunohistochemical staining.

4.6. Indirect immunofluorescence and laser scanning confocal microscopy

Frozen sections (10 mm) were fixed for 30 min in fresh 4% paraformaldehyde (PFA, Sigma) containing 0.2% Triton X-100. Samples were blocked for 45 min with 5% BSA at room temperature, then incubated in the primary antibody against mouse EGF (diluted 1 : 100 in PBS) at 4 °C overnight. After rinsing in PBS, the sections were incubated for 60 min in fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) diluted 1 : 100 in PBS at 37 °C and were rinsed several times with PBS. Nuclei were stained with 0.01 mg/ml propidium iodide (Sigma) for 15 min. Finally, the slides were rinsed in PBS to remove excess fluorescence agents, and viewed under laser scanning confocal microscope (Leica). Parallel samples of uterine sections as control were fixed in 4% PFA, incubated with goat IgG (also diluted in 1 : 100 in PBS) at 4 °C overnight.

4.7. Reverse transcription-polymerase chain reaction

Mouse blastocysts and peri-implantational uterus tissue were prepared as above. Total RNA was isolated by using Trizol reagent kit (Gibco), and reverse transcribed (RT) by oligo(dT) priming and AMV reverse transcriptase (Promega Corporation, Madison, WI). RT product (5 ml; approximately 20 ng RNA) was used for PCR amplification as below: 95 °C—5 min followed by 35 cycles of 94 °C—30 s, 53 °C—30 s, 72 °C—30 s. The 10 ml PCR products were visualized under ultraviolet light on a 1.5% agarose gel containing 1 mg/ml ethidium bromide. EGF sense primer: 5′-CTCGTTTCTCTTTCATCCTTTGCC-3′, antisense primer: 5′-TGTTCCATCTGGGTCAATCCG-3′, and the PCR fragment size is 449 bp. EGFR sense primer: 5′-GCTCCCCATATGTCTCCCGC-3′, antisense primer: 5′-GCCGCTCCCCCTTTTCCAGC-3′, the fragment size is 489 bp. Beta-actin was used as the inner control. PCR products were sequenced to confirm the fidelity.

4.8. Dot blot

The blastocysts were collected and uteri were isolated as before. After homogenization, the protein concentration in the tissue was calibrated by spectrometry. Tissue lysates from the blastocysts and the periimplantational uteri were boiled in the SDS/β-mercaptoethanol sample buffer and about 10 μg proteins were dotted onto the NC membrane. Blots were blocked in 10% non-fat milk for 1 h and incubated overnight at 4 °C with the primary antibodies, diluted 1 : 500 to a final concentration of 2 μg/ml in TTBS (30 mM Tris, pH 7.4; 150 mM NaCl; 0.1% Tween 20). After TTBS washing for 4 × 15 min, membrane was incubated for 1 h in goat anti-rabbit IgG (Santa Cruz company) diluted 1 : 500 in TTBS. Finally, membrane was washed with TTBS for 4 × 15 min and TBS 2 × 15 min and developing the blot signals in NBT/BCIP solution at room temperature for 15 min until the purple color became visible.

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