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Heparin-Binding Epidermal Growth Factor and Its Receptors Mediate Decidualization and Potentiate Survival of Human Endometrial Stromal Cells

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Abstract

Heparin-binding epidermal growth factor (HB-EGF) has pleiotropic biological functions in many tissues, including those of the female reproductive tract. It facilitates embryo development and mediates implantation and is thought to have a function in endometrial receptivity and maturation. The mature HB-EGF molecule manifests its activity as either a soluble factor (sol-HB-EGF) or a transmembrane precursor (tm-HB-EGF) and can bind two receptors, EGFR and ErbB4/HER4. In this study, we identify factors that modulate expression of HB-EGF, EGFR, and ErbB4 in endometrial stromal cells *in vitro*. We demonstrate that levels of sol- and tm-HB-EGF, EGFR, and ErbB4 are increased by cAMP, a potent inducer of decidualization of the endometrial stroma. We also show that production of sol- and tm-HB-EGF is differentially modulated by TNFα and TGFβ. Our data suggest that HB-EGF has a function in endometrial maturation in mediating decidualization and attenuating TNFα- and TGFβ-induced apoptosis of endometrial stromal cells.

Abbreviations

bFGF, Basic fibroblast growth factor; 8-Br-cAMP, 8-bromoadenosine-cAMP; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF; HRP, horseradish peroxidase; IGFBP, IGF binding protein; PDGF, platelet-derived growth factor; PRL, prolactin; sol, soluble factor; tm, transmembrane precursor

THE HUMAN ENDOMETRIUM undergoes extensive regeneration and maturation during the menstrual cycle in response to steroid hormones and in preparation for embryo implantation. Implantation occurs within a short window of time in the midsecretory stage of the cycle (d 20–24) (reviewed in Ref. 1) and requires the synchronization of embryonic development and the acquisition by the endometrium of a phenotype that is receptive to embryo implantation. Endometrial maturation involves stromal decidualization, the process of growth and differentiation that results in the transformation of precursor stromal cells into decidual cells. Decidualization begins around the blood vessels of the midsecretory phase endometrium and amplifies and extends throughout the stroma in response to the continued influence of estradiol and progesterone during implantation (2).

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The regulation of endometrial function involves a complex hierarchy of extracellular signaling cues including steroid hormones, growth factors and cytokines, and the extracellular matrix, the molecular details of which are poorly understood (reviewed in Ref. 3). Heparin-binding epidermal growth factor (HB-EGF) is one of the increasing numbers of growth factors that are now recognized as having a significant function in reproduction (reviewed in Ref. 4). It is a member of the epidermal growth factor (EGF) family of ligands that can be expressed as biologically active soluble (sol) and transmembrane (tm) forms (5) and can bind two receptors, EGFR and ErbB4 (HER4) (6). Studies in the mouse demonstrate a function for HB-EGF in blastocyst adhesion and development (7, 8). In the human endometrium, HB-EGF mRNA is expressed throughout the menstrual cycle, reaching maximal levels just before the implantation window (9), and levels of HB-EGF protein increase in uterine glands, stroma, endothelial cells, and the luminal surface of the endometrium during the time of implantation (9–11). We previously demonstrated that sol-HB-EGF improves preimplantation human embryo development (12) and that immobilized HB-EGF (tm-HB-EGF) mediates human blastocyst attachment (10). Previously reported data from this laboratory also indicate that HB-EGF acts as a mitogenic factor for human endometrial stromal cells (13). Thus soland tm-HB-EGF may be involved in mediating implantation of the human embryo as well as endometrial regeneration.

Endometrial decidualization is essential for successful implantation. It is initiated during the midsecretory stage of the menstrual cycle, when transformation of endometrial stromal cells into predecidual cells occurs, followed by a decidual response if pregnancy ensues. The decidua is both permissive for trophoblast invasion and at the same time impedes invasive trophoblast by forming a physical barrier (reviewed in Ref. 14). Decidualization of endometrial stromal cells can be induced *in vitro* by progesterone and estradiol mediated by cAMP (15–17), by other ligands that are coupled to cAMP signaling (18) and cAMP analogs alone (19).

Recent studies revealed that HB-EGF mRNA expression in isolated endometrial stromal and epithelial cells is under the control of estrogen and progesterone (20). However, little is known about the molecular regulators of HB-EGF protein or about HB-EGF receptor function in maturation of the human endometrium. In this study, we examine the effect of 8-bromoadenosine-cAMP (8-Br-cAMP), a broad spectrum inducer of decidualization, and that of specific growth factors known to be involved in endometrial function, on the expression of HB-EGF, EGFR, and ErbB4 in cultured human endometrial stromal cells. We present data showing that HB-EGF and its receptors have a function in decidualization of the human endometrium and promoting survival of endometrial stromal cells undergoing apoptosis in response to TNFα or TGFβ.

Materials and Methods

Tissue samples

Endometrial tissue samples were obtained with informed consent and in accordance with the requirements of the Central Oxford Research Ethics Committee from patients aged 20–46 yr undergoing sterilization or hysterectomy for benign indications, who had a regular 26- to 33-d menstrual cycle and who had received no hormonal medication in the preceding 3 months. The cycle stage of the endometrium was assessed according to criteria of Noyes *et al.* (21). Tissue samples were processed for stromal cell culture or immunohistochemistry as described below.

Cell culture and detection of sol- and tm-HB-EGF by ELISA

The isolation and culture of endometrial stromal cells was performed as described previously (13). Cells between passages 2 and 6 were seeded into 24-well plates, grown to confluence, serum starved overnight, and then stimulated for 48 h with 10 ng/ml TNFα, basic fibroblast growth factor (bFGF), EGF, TGFα, platelet-derived growth factor (PDGF), IL-11, or 1 ng/ml TGFβ (all from R&D Systems Europe, Ltd., Abingdon, UK). Cell-conditioned medium from replicate wells was processed for measurement of sol-HB-EGF as described below, and tm-HB-EGF in the remaining cell layer was shed by incubation in 1 μM phorbol-12-myristate-13 acetate (Sigma Ltd., Poole, UK) (22) in fresh media for 40 min at 37 C. The supernatant was removed and processed for measurement of HB-EGF as described below. Measurement of total (sol- + tm-) HB-EGF in replicate wells was performed by addition of phorbol-12-myristate-13 acetate to the cultures and incubation as described above. The supernatants were then processed for ELISA.

ELISA 96-well plates were coated for 18–24 h with 4 μg/ml goat antihuman HB-EGF polyclonal antibodies (AF-259-NA, R&D Systems). Nonspecific binding sites were blocked with 1% BSA in PBS. Cell-conditioned media or recombinant human HB-EGF standards (0–500 pg/ml, R&D Systems) were added to the wells and incubated for 24–48 h at 4 C. Captured HB-EGF was detected with biotin-conjugated goat antihuman HB-EGF polyclonal antibodies (BAF 259, R&D Systems) at 125 ng/ml for 2 h at room temperature followed by streptavidin conjugated with horseradish peroxidase (HRP) (dilution 1/1000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). HRP activity was detected with the use of Blue substrate (Neogen Europe, Ltd., Ayr, UK). The sensitivity of the assay was 15 pg/ml. Cells were lysed in 50 mM NaOH, and the total amount of cell protein was measured with Coomassie reagent (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's instructions. The amount of HB-EGF measured by ELISA was expressed per microgram total cell protein.

8-Br-cAMP induction of decidualization of stromal cells

Confluent stromal cells in 24-well plates were treated with 0.5 mM. 8-Br-cAMP (Sigma) in serum-free DMEM/F12 supplemented with either 25 μg/ml anti-HB-EGF antibodies (AF-259-NA, R&D Systems) or 2 and 10 μg/ml CRM197 (Sigma) for 7 d. The culture supernatants were collected and prolactin (PRL) and IGF binding protein (IGFBP)-1 were measured with the use of a PRL Immulite kit (DPC Ltd., UK), and DuoSet IGFBP-1 ELISA kit (R&D Systems), respectively. Levels of sol- and tm-HB-EGF were measured by ELISA as described above. The total amount of cell protein was prepared and measured with Coomassie reagent as described above, and the concentration of PRL, IGFBP-1, and HB-EGF were expressed per microgram total cell protein.

Tissue staining

Sections from tissue samples obtained from 13 patients (eight proliferative and five secretory stages of the menstrual cycle) were prepared as described previously (23). Sections were incubated in 10 μg/ml primary antibodies (mouse anti-ErbB4, clone HFR1) (24) for 1 h at room temperature, and chromogenic detection with the use of HRP was performed as described elsewhere (23). Control staining was performed with the same antibodies previously preincubated with the antigen peptide.

Detection of EGFR and ErbB4 by fluorescence-activated cell sorter analysis

Stromal cells were stimulated for 7 d in serum-free media with 8-Br-cAMP, as described above, fixed in ethanol, and permeabilized in 0.1% saponin (Sigma) and 2% rabbit serum in PBS as described previously (13). Receptors were detected by incubation in 5 μg/ml mouse

antihuman EGFR (36481A, BD Biosciences PharMingen, San Diego, CA) or 20 μg/ml goat antihuman ErbB4 (sc-283, Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1 h at 0 C, followed by goat antimouse IgG conjugated with R-phycoerythrin (R0480, DakoCytomation, Denmark) or fluorescein-conjugated rabbit antigoat IgG (F-2016, Sigma) according to the manufacturer's instructions. Mouse and goat IgG were used as corresponding negative controls. Fluorescence was detected in an Epics Altra flow cytometer (Beckman Coulter, Fullerton, CA). Between 10,000 and 20,000 events were collected for each antibody combination.

Cell survival assays

Stromal cells were plated on glass coverslips in 24-well plates and grown to confluence. TNFα (10 ng/ml) and TGFβ (1 ng/ml) were added in serum-free DMEM with or without 2 or 10 μg/ml CRM197 (Sigma). Cells were incubated for 4–7 d. Live (green) and apoptotic (orange) cells were distinguished after addition of 4 μg/ml acridine orange (Sigma) and 4 μg/ml ethidium bromide (Sigma) in PBS at room temperature.

Statistical analyses

The data were analyzed by ANOVA and/or paired student *t* test. Differences were considered significant at $P < 0.05$.

Results

Inhibition of HB-EGF reduces the decidualization capacity of 8-Br-cAMP-induced endometrial stromal cells

It has been shown previously that levels of HB-EGF are elevated in secretory, compared with proliferative, endometrium (10, 11). We therefore analyzed levels of HB-EGF secreted by endometrial stromal cells during 8-Br-cAMP-induced decidualization (19). Decidual transformation of stromal cells was confirmed by the detection of PRL in the conditioned medium (Fig. 1A). The levels of tm-HB-EGF were modestly elevated in response to 8-Br-cAMP (Fig. 1B), whereas there was a significantly higher (3-fold) increase in levels of sol-HB-EGF, compared with the untreated control.

We examined the possible function of 8-Br-cAMP-induced endometrial HB-EGF as a modulator of decidualization by blocking the action of HB-EGF. Decidualization of stromal cells was induced with 8-Br-cAMP in the presence of either HB-EGF neutralizing antibodies or CRM197, the diphtheria toxin-based HB-EGF inhibitor (Fig. 2). We observed that 8-Br-cAMP-induced PRL (Fig. 2A) and IGFBP-1 (Fig. 2B) levels were significantly inhibited by HB-EGF neutralizing antibodies and 10 μg/ml CRM197.

Levels of ErbB4 are increased in secretory stage endometrial stroma

Having established a requirement for HB-EGF for the expression of decidualization markers by endometrial stromal cells, we investigated the expression profile of the HB-EGF-specific receptor, ErbB4, in human endometrial tissue. The expression of EGFR in the human endometrium was previously reported to undergo cyclical changes (25). Increased expression of ErbB4 in endometrial glands was also demonstrated previously in the secretory stage of the cycle (25, 26). We performed a detailed immunohistochemical analysis of ErbB4 localization throughout the cycle (Fig. 3). Our data reveal that during the proliferative stage of the cycle, ErbB4 is found only in the stroma of the basalis layer of the endometrium (Fig. 3A). During the secretory stage, increased levels of ErbB4 were observed in both the basalis and functionalis layers in the stroma and glandular epithelium (Fig. 3B). High magnification revealed positive staining on the apical, lateral, and basal membranes of epithelium and intense staining on the membrane of stromal cells (Fig. 3C). Sections

incubated with anti-ErbB4 antibodies that had been preadsorbed with the peptide antigen were negative (Fig. 3D).

EGFR and ErbB4 are elevated in 8-Br-cAMP-induced decidualization of endometrial stromal cells

The expression pattern of EGFR and ErbB4 in endometrial tissue suggests that regulation of both receptors is under the influence of steroid hormones and that they may have some function in decidualization. We therefore measured the levels of EGFR and ErbB4 on the surface of endometrial stromal cells during 8-Br-cAMP-induced decidualization (Fig. 4). The levels of both EGFR (Fig. 4, A and B) and ErbB4 (Fig. 4, C and D) increased in response to 8-Br-cAMP approximately 2-fold and approximately 3-fold, respectively.

TNFα and TGFβ differentially modulate sol- and tm-HB-EGF production by endometrial stromal cells

Signaling by a number of different hormones and growth factors is mediated by 8-Br-cAMP. To dissect further the molecular hierarchy of the growth factors involved in secretory stage endometrial function, we analyzed the effect of TNFα, TGFβ, bFGF, EGF, TGFα, PDGF, and IL-11, growth factors relevant to endometrial function or previously shown to modulate HB-EGF in other systems, on HB-EGF expression (Fig. 5). The assessment of total (sol- + tm-) HB-EGF expression revealed that TNFα and TGFβ significantly increased HB-EGF expression, compared with the untreated control, whereas bFGF, EGF, TGFα, PDGF, and IL-11 did not significantly alter levels of HB-EGF (Fig. 5A). We further analyzed the effect of TNFα and TGFβ on the expression of sol- *vs.* tm-HB-EGF. Treatment with both TNFα and TGFβ resulted in increased levels of sol-HB-EGF. Levels of tm-HB-EGF were increased in response to TGFβ but not TNFα (Fig. 5B).

Inhibition of HB-EGF activity decreases survival of stromal cells exposed to TNFα/TGFβ treatment

Because both TNFα and TGFβ have been shown to induce apoptosis of some cell types, we explored further the possible function of TNFα- and TGFβ-induced HB-EGF in the context of the human endometrium (Fig. 6). Stromal cells were stimulated with TNFα or TGFβ in the presence or absence of the HB-EGF-specific inhibitor CRM197. We observed that TNFα-and TGFβ-treated cells survived in the presence of CRM197 for a much shorter period (2–4 d) than TNFα- or TGFβ-treated cells without the inhibitor (>4 d) (Fig. 6). Notably, stromal cells exposed to TGFβ were more sensitive to the low concentrations of CRM197 than cells exposed to TNFα.

Discussion

There is persuasive evidence to suggest that HB-EGF and its receptors have an important function in the endometrium. Here we dissected further the function of HB-EGF signaling in decidualization and endometrial cell survival, processes that are particularly relevant to secretory stage endometrium. We demonstrate that: 1) levels of both sol- and tm-HB-EGF, EGFR, and ErbB4 increase in response to 8-Br-cAMP and that HB-EGF mediates decidualization of endometrial stromal cells; 2) production of sol- and tm-HB-EGF by endometrial stromal cells are differentially regulated by TNFα and TGFβ; and 3) HB-EGF potentiates the survival of stromal cells exposed to apoptotic factors TNFα and TGFβ.

The involvement of HB-EGF, EGFR, and ErbB4 in decidualization and endometrial maturation is suggested by a number of observations. First, the local application of HB-EGF-soaked beads promotes decidualization in the mouse uterus (27). Second, HB-EGF is a potent stimulator of PRL gene expression in mouse pituitary cell lines (28). Third, the

expression of HB-EGF mRNA in endometrial cells *in vitro* is regulated by estrogen and progesterone (20). Fourth, we have recently shown that exogenous HB-EGF stimulates expression of IL-11, a cytokine known to play a role in the decidualization process in mice (29), in cultures of human endometrial stromal cells derived from the secretory phase of the menstrual cycle (30). Finally, EGFR and ErbB4 are present in secretory-stage endometrium (25, 26, 31) and EGFR increases in endometrial stromal cells during *in vivo* and *in vitro* decidualization (32, 33).

Here we present further evidence for the role of HB-EGF in decidualization. Decidualization *in vivo* occurs in response to steroid hormones, and hormonal control of gene expression in the endometrium is mediated by cAMP signaling (34–36). The increase in levels of sol- and tm-HB-EGF we observe during 8-Br-cAMP-induced decidualization of stromal cells is therefore consistent with other reports describing progesterone and estradiol regulation of HB-EGF mRNA expression *in vitro* (20); the pattern of HB-EGF expression *in vivo* in stromal cells; and the pattern of HB-EGF expression *in vivo* (9, 11, 13). We further demonstrate that HB-EGF plays a role in the decidualization of human endometrial stromal cells. In these experiments the use of two different inhibitors of HB-EGF activity, the diphtheria toxin analog CRM197 (37) and neutralizing HB-EGF antibodies, results in decreased levels of prolactin and IGFBP-1.

The production of HB-EGF in other cell types is regulated by various factors in addition to steroid hormones (38–40). Here we attempt to identify known EGFs and cytokines mediating HB-EGF expression in human endometrial stromal cells. The growth factors bFGF, EGF, TGFα, PDGF, and IL-11 do not modulate production of HB-EGF in these cells. We have shown previously that HB-EGF modulates levels of IL-11 secreted by endometrial stromal cells (30), and these new data thus confirm that HB-EGF is upstream of IL-11 in the signaling cascade resulting in decidualization.

Of the growth factors we tested, only TNFα and TGFβ significantly increase levels of HB-EGF. Both TNFα and TGFβ are regulated by steroid hormones and are involved in endometrial maturation, decidualization, and regeneration. In the human endometrium, TNFα mRNA and protein are present in the stroma from midproliferative to late-secretory stage tissue and in the decidua during the first trimester of pregnancy (41, 42), and TGFβ mRNA is also elevated in late-secretory stage, and decidualized, endometrium (43, 44). Levels of TNFα mRNA also increase in progesterone-induced decidualized endometrial stromal cells (45). In the absence of implantation, TNFα is believed to induce apoptosis leading to endometrial shedding (46). Recently we demonstrated that TNFα increases the mitogenic function of HB-EGF in stromal cells, suggesting the involvement of these factors in endometrial regeneration (13). Like TNFα, TGFβ has been implicated in endometrial stromal cell apoptosis and exerts growth-regulatory effects in both epithelial and stromal cells (47, 48).

The present study demonstrates the differential regulation of sol- and tm-HB-EGF by TNFα and TGFβ in that both induce the production of sol-HB-EGF, whereas levels of tm-HB-EGF are elevated in response to TGFβ but not TNFα. Further studies will be required to identify mechanisms underlying these observed differences in the effects of TGFβ and TNFα on the production of different forms of HB-EGF. Two possibilities are that there is differential modulation of either tm-HB-EGF synthesis and/or sol-HB-EGF shedding, the latter in turn being regulated by availability of matrix metalloproteases and tissue inhibitors of matrix metalloproteases involved in shedding of sol-HB-EGF from tm-HB-EGF (49).

We have shown previously that sol- and tm-HB-EGF both act as mitogenic factors for endometrial stromal cells (13). However, it is also possible that they have discrete functions

in the human endometrium, perhaps dictated by their differential interaction with specific receptors. For example, sol-HB-EGF has been shown to function as a chemotactic factor for cells expressing ErbB4 (50). It is thus possible that TNFα/TGFβ-regulated sol-HB-EGF functions as a chemotactic factor for the human blastocyst as it invades the stroma. Because TGFβ has been implicated in trophoblast anchoring and invasion (51, 52), it is also possible that this is achieved via up-regulation of tm-HB-EGF expression by TGFβ in the later stages of blastocyst implantation.

We demonstrate that CRM197 accelerates apoptosis of stromal cells exposed to TNFα and TGFβ, both of which promote apoptosis of endometrial cells (46, 47). These data suggest that sol- and tm-HB-EGF function as cytoprotective agents for endometrial cells, as has been observed for other cell systems (53), indicating one of the generic functions of this growth factor.

The regulation of EGFR and ErbB4 presents an additional level of control of HB-EGF function. Our data confirm previous observations of increased expression of ErbB4 in endometrial glands during the secretory stage of the menstrual cycle (26). We further demonstrate a gradient of ErbB4 levels in the stroma of proliferative-stage endometrium, being highest in the basalis and lowest in the functionalis, and abundant expression throughout the stroma and epithelium in secretory-stage tissue. This pattern of expression implies that the expression of ErbB4, like EGFR and HB-EGF, is subject to hormonal control. In this study we have analyzed the effect of 8-Br-cAMP on the expression of EGFR and ErbB4 during decidualization. Our data confirm that cAMP increases EGFR, as previously observed by others (32, 45), and demonstrate that cAMP also stimulates the expression of ErbB4 in cultured stromal cells. These data suggest that both receptors may contribute to stromal cell decidualization, although their functions might be distinct. Indeed, functional differences between EGFR and ErbB4 in the human endometrium are implied by our previous observation that tyrosine phosphorylation of EGFR, but not ErbB4, occurs in response to both sol- and tm-HB-EGF (13).

In conclusion, in this study we have identified regulatory mechanisms that are involved in the production of sol- and tm-HB-EGF and HB-EGF receptors EGFR and ErbB4 in human endometrial stromal cells. Our findings suggest functions for HB-EGF-receptor interactions in the human endometrium during the secretory stage of the cycle, notably decidualization and cell survival. We suggest that the HB-EGF ligand-receptor interactions have potentially overlapping and distinct functions in the human endometrium. The regulatory pathways of HB-EGF production we highlight here provide insight into potential local mechanisms for the control of activity of HB-EGF and other ligands of the EGF family.

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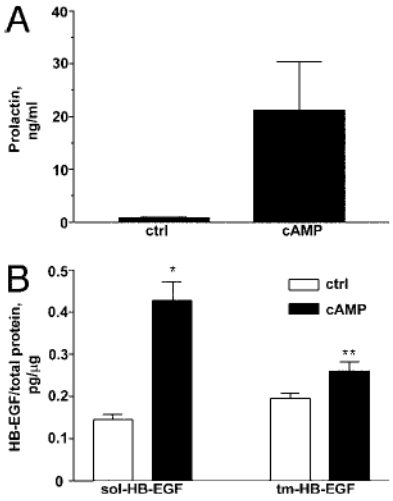

Fig. 1.

8-Br-cAMP modulates production of HB-EGF by endometrial stromal cells. Stromal cells were induced with 8-Br-cAMP for 7 d. Graphs represent collated data from three independent experiments with different cell lines showing levels of PRL (A), sol- and tm-HB-EGF secreted into the medium or present on the cell surface (B). Significant differences between control (ctrl) and 8-Br-cAMP treatment are indicated by * ($P < 0.0001$) and ** ($P < 0.05$).

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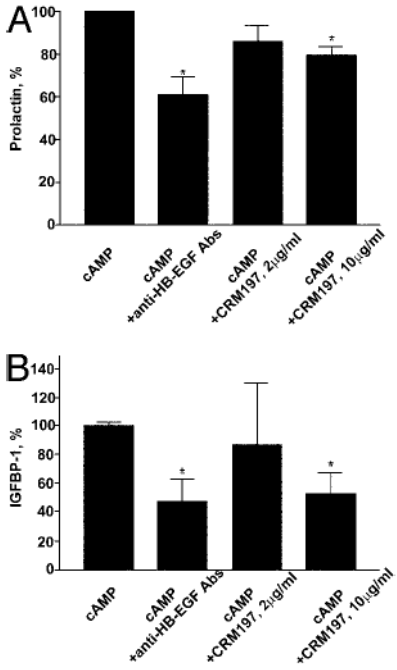

Fig. 2.

HB-EGF neutralization reduces production of PRL and IGFBP-1 by endometrial stromal cells induced to decidualize with 8-Br-cAMP. A, Inhibition of 8-Br-cAMP-induced PRL secretion. *Bars* represent collated data from at least four independent experiments with four different cell lines. B, Inhibition of 8-Br-cAMP-induced IGFBP-1 secretion. *Bars* represent collated data from three independent experiments with three different cell lines. The data are represented as percent inhibition with Br-9-cAMP-treated control representing 100%. Significant inhibition is indicated by * ($P < 0.005$).

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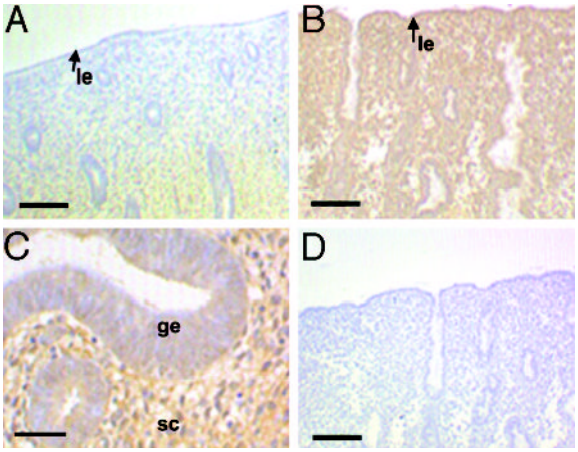

Fig. 3.

Expression of ErbB4 in human endometrium during the menstrual cycle. Tissue sections derived from proliferative (A) and secretory (B and C) endometrium were stained with anti-ErbB4 antibodies. Lumenal edge (*arrow*, le), glandular epithelium (ge), and stromal cells (sc) are indicated. D, Control staining was performed with antibodies preincubated with the appropriate control peptide. *Scale bars*, 50 μm (A, B and D) and 125 μm (C).

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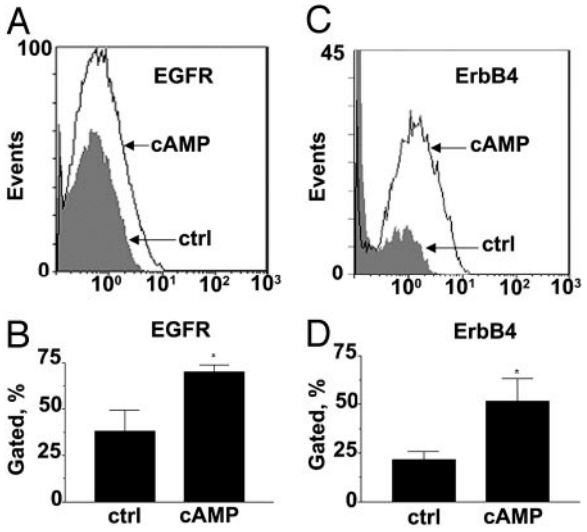

Fig. 4.

Expression of EGFR and ErbB4 is elevated during 8-Br-cAMP-induced decidualization of endometrial stromal cells. Stromal cells were treated with 8-Br-cAMP for 7 d and subjected to fluorescence-activated cell sorter. A, Representative diagram of EGFR expression in 8-Br-cAMP-treated and untreated (ctrl) cells. B, Collated data from measurement of EGFR in three independent experiments with three different cell lines. C, Representative diagram of ErbB4 expression in 8-Br-cAMP-treated and untreated (ctrl) cells. D, Collated data from measurements of ErbB4 in five independent experiments with five different cell lines. Significant differences between control and 8-Br-cAMP-treated cells are indicated by * ($P < 0.05$).

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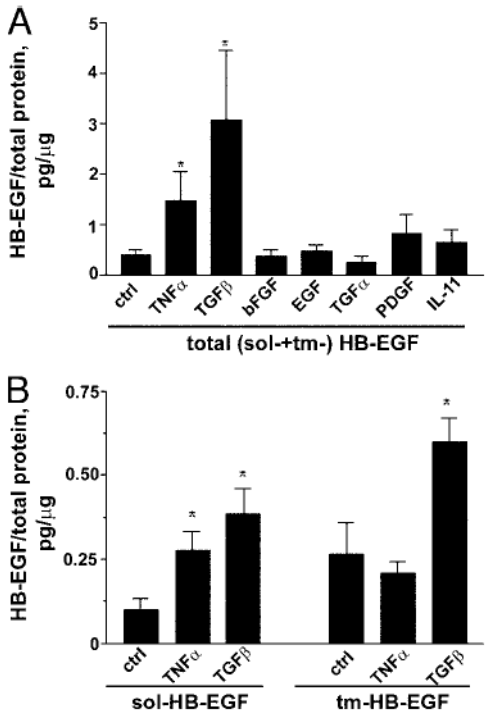

Fig. 5.

Modulation of HB-EGF production by endometrial stromal cells by growth factors. A, Levels of total (sol- + tm-) HB-EGF. *Bars* represent collated data from five (TNFα, TGFβ, bFGF, EGF, TGFα) or two (PDGF, IL-11) independent experiments with different cell lines. B, Levels of sol- and tm-HB-EGF. *Bars* represent collated data from five independent experiments with different cell lines. Significant differences between control (ctrl) and growth factor treatment are indicated by * ($P < 0.05$).

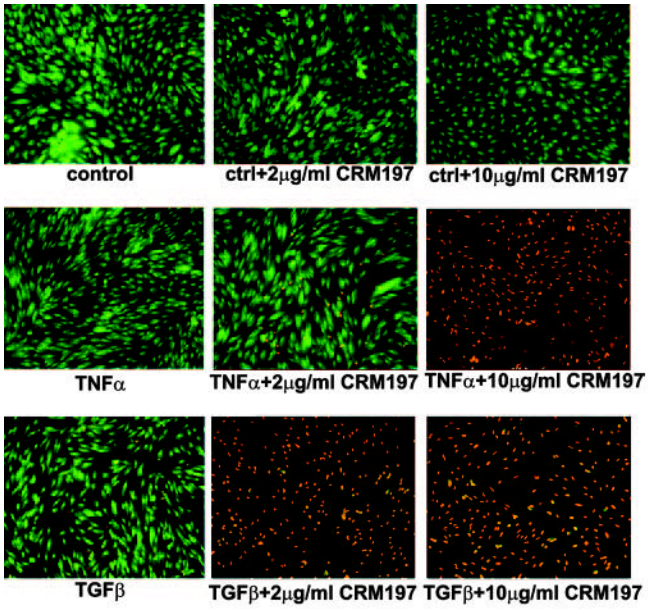

Fig. 6.

Inhibition of HB-EGF with CRM197 diminishes survival of endometrial stromal cells exposed to TNFα or TGFβ. Endometrial stromal cells cultured for 48 h in the absence or presence of TNFα or TGFβ, with or without CRM179 as indicated, were stained with acridine orange and ethidium bromide. Live cells are *green* and those undergoing cell death are *orange*. The results are representative of two independent experiments with two different cell lines.

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