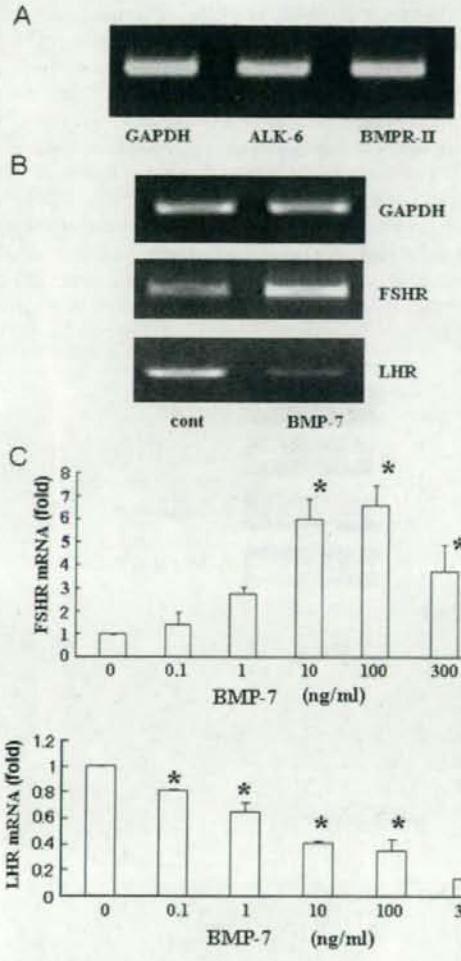
FIGURE 1



Shi. BMP-7 increases FSH receptor. Fertil Steril 2009.

FIGURE 1 Continued

Expression of bone morphogenetic protein 7 (BMP-7) receptor (BMPR) in human granulosa cells and effect of BMP-7 on FSH receptor (FSHR) mRNA and LH receptor (LHR) mRNA expression. (**A**) The receptor for BMP-7, activin receptor–like kinase (ALK) 6, and BMPR-II in human luteinized granulosa cells (LGCs) were examined with regular polymerase chain reaction (PCR). (**B**) LGCs were cultured with or without BMP-7 (100 ng/mL) for 24 h followed by regular PCR for GAPDH, FSHR, and LHR. (**C**) LGCs were cultured with the indicated concentrations of BMP-7 for 24 h. For the quantification of FSHR and LHR mRNA, real-time PCR analysis was performed and the expression levels of FSHR and LHR mRNA standardized by GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. *Significant difference at $P<.05$ (vs. control).

a time course study, BMP-7 (100 ng/mL) increased FSHR mRNA levels after 8 h, and the maximal induction of FSHR mRNA expression occurred after 24 h of treatment (Fig. 2). The specificity of BMP-7 was confirmed by the finding that BMP-7–induced FSHR mRNA expression was completely inhibited by an antibody for BMP-7, but not by the control antibody (data not shown).

Induction of FSHR mRNA Expression by BMP-7 was not Via Production of Activins

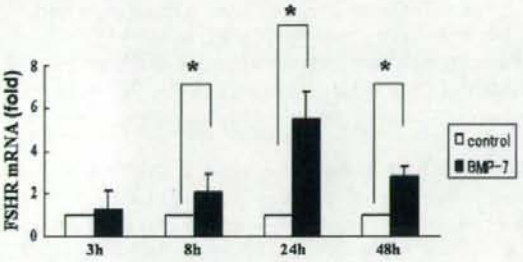
Activins are known to be a strong inducer of FSHR mRNA expression (9). Therefore, we first examined the mRNA expression levels of inhibin and activin subunits in GCs stimulated with BMP-7. As shown in Fig 3, BMP-7 increased the mRNA levels of inhibin/activin-βA and inhibin/activin-βB, but had no effect on inhibin-α mRNA expression. Based on this finding, we hypothesized that after induction of mRNA for inhibin/activin-β subunits by BMP-7, new activin protein was synthesized, and that this new protein was responsible for inducing FSHR mRNA expression. To determine whether activin or BMP-7 is responsible for the increase in FSHR mRNA, we used SB-431542, an ALK-4, -5, and -7 inhibitor (23) which can selectively inhibit ALK-4, the receptor of activins, but has no effect on ALK-6, the receptor for BMP-7 (12). The LGCs were cultured with BMP-7 (100 ng/mL) or activin-A (100 ng/mL) in the presence or absence of SB-431542 (10 μmol/L). As shown in Figure 4A, SB-431542 significantly suppressed the stimulatory effect of activin-A on FSHR mRNA, whereas SB-431542 had no effect on the up-regulation of FSHR mRNA induced by BMP-7. This finding suggests that although BMP-7 can induce mRNA expression of activin subunits, the BMP-7–induced increase in FSHR mRNA is not mediated by activins. Interestingly, combination of BMP-7 and activin-A did not have an additive effect to increase FSHR mRNA expression, implying some redundancy between the pathways under BMP-7 and activin-A (Fig. 4B).

BMP-7 Treatment Increases Functional FSHR

Cyclic AMP is a well recognized second messenger for activated FSHR. To assess whether FSHR mRNA induction by BMP-7 results in an increase in functional FSHR, LGCs were pretreated with or without BMP-7 (100 ng/mL) for 24 h, and subsequently cultured with 0.1 mmol/L IBMX in the presence or absence of FSH (0.5 IU/mL) for 2 h. As expected, FSH significantly increased cAMP production by LGCs

FIGURE 2

Effect of bone morphogenetic protein 7 (BMP-7) on FSH receptor (FSHR) mRNA expression. Human luteinized granulosa cells (LGCs) were cultured with BMP-7 (100 ng/mL) for different time intervals (3–48 h). Total RNA was then extracted from the LGCs and subjected to real-time polymerase chain reaction to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Solid and open bars represent the relative mRNA levels obtained from the culture in the presence or absence, respectively, of BMP-7. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the each control. *Significant difference at $P<.05$ (vs. control).



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(Fig. 5). Conversely, BMP-7 pretreatment had no effect on the production of cAMP by LGCs. However, BMP-7 pretreatment significantly enhanced the FSH-induced increase in cAMP levels in LGCs compared with nonpretreatment.

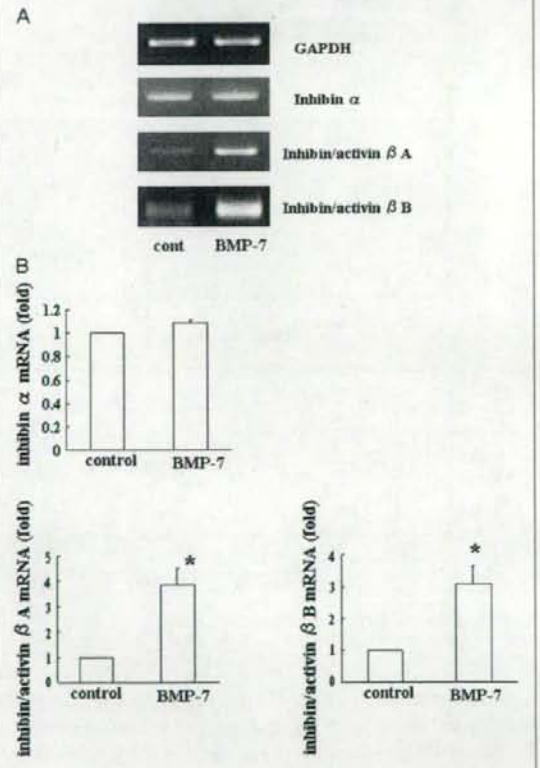
DISCUSSION

The BMP family members are important for folliculogenesis in many species, and there is a growing recognition that BMPs contribute to folliculogenesis by inhibiting luteinization of granulosa cells (12). In the present study, we found that BMP-7 induced FSH receptor (FSHR) mRNA expression in human granulosa cells, suggesting that BMP-7 may contribute to increasing FSH sensitivity of granulosa cells, thus promoting folliculogenesis. Our finding is consistent with that of Lee et al. (18), who, using mouse neonatal ovary, reported that BMP-7 increased FSHR mRNA. On the other hand, BMP-7 treatment inhibited expression of mRNA for LHR, a key factor required by granulosa cells to undergo luteinization (24). Furthermore, we found that the FSHR mRNA induced by BMP-7 resulted in an increase in functional FSHR, as indicated by the finding that FSH stimulated the production of cAMP in BMP-7–primed GCs compared with the control cells.

In the ovary, activins are recognized as important factors in the induction and maintenance of FSHR (7). Our observation

FIGURE 3

Effect of bone morphogenetic protein 7 (BMP-7) on the expression of inhibin/activin-α, inhibin/activin-βA, and inhibin/activin-βB mRNA. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) for 24 h. Total RNA was then extracted and subjected to (**A**) regular and (**B**) real-time polymerase chain reaction to determine the mRNA levels of inhibin/activin-α, inhibin/activin-βA, and inhibin/activin-βB. The expression levels of indicated mRNAs were standardized by GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. *Significant difference at $P<0.05$ (vs. control).



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that BMP-7 increased mRNA levels of not only FSHR but also inhibin/activin-β subunits (Figs. 1 and 3) led us to examine the possibility that the increase in FSHR mRNA might be mediated by an increase in activin protein synthesis. However, SB-431542, an inhibitor of activins but not BMP-7 signaling (23), failed to suppress BMP-7–induced FSHR mRNA expression (Fig. 4A). A possible explanation for this result

FIGURE 4

(**A**) Effect of SB-431542 on activin-A or bone morphogenetic protein 7 (BMP-7)–induced FSH receptor (FSHR) mRNA expression. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) or activin-A (100 ng/mL) in the presence or absence of SB-431542 (10 μmol/L), a selective inhibitor of activin receptor–like kinase 4, 5, and 7, for 24 h. Total RNA was then extracted from the LGCs and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. Bars with different letters indicate a significant difference at $P<.05$. *Significant difference at $P<.05$. NS = not significant. (**B**) LGCs were cultured with BMP-7 (100 ng/mL) and/or activin-A (100 ng/mL) for 24 h, followed by real-time PCR to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. Bars with different letters indicate a significant difference at $P\leq.05$ (b vs. c: $P\leq.02$; c vs. d: $P\leq.01$).

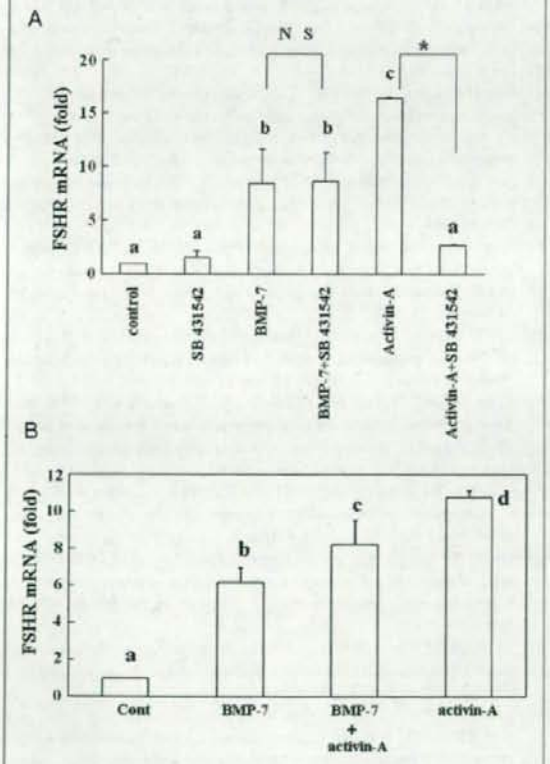

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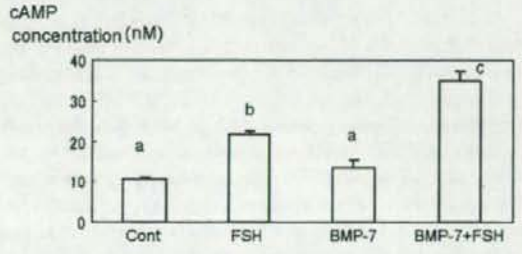
FIGURE 5

Effect of bone morphogenetic protein 7 (BMP-7) and FSH on cyclic adenosine monophosphate (cAMP) concentration. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) for 24 h. The cells were then cultured with 0.1 mmol/L IBMX in the presence or absence of FSH (0.5 IU/mL) for 2 h. The cAMP concentration in the supernatant was measured using a cAMP EIA kit. Data from one representative experiment out of three separate experiments was shown. Results shown are mean ± SD values from quadruplet experimental wells. Bars with different letters indicate a significant difference at $P<.01$ (a vs. b: $P\leq.01$; a vs. c: $P\leq.001$; b vs. c: $P\leq.01$).



Shi. BMP-7 increases FSH receptor. Fertil Steril 2009.

could be that BMP-7–induced inhibin/activin-β subunits are preferentially recruited to produce inhibin, dimerizing with an inhibin-α subunit that is known to be abundantly expressed in the mature GCs used in this study (25). Thus, it is speculated that BMP-7 and activin-A act on different receptors to increase FSHR mRNA expression. Interestingly, however, combination of BMP-7 and activin-A did not have an additive effect to increase FSHR mRNA expression. Bone morphogenetic protein 7 and activin-A may use redundant signaling pathways downstream of the point affected by SB431542.

Administration of FSH reagent is a standard method for infertility treatment, but many patients are unresponsive to this therapy. Thus, induction of FSHR in growing follicles would be a desirable therapy for infertility patients. Activins have the potential to serve this purpose, because they are recognized as a strong inducer of FSHR. However, because the majority of circulating activins can be bound and inactivated by follistatin (26–28), it appears to be difficult to stimulate ovarian follicles with exogenously administered activins. On the other hand, the relative affinity of follistatin for BMP-7 is less than 1% compared with activins, and the effect of BMP-7 on SMAD phosphorylation in granulosa cells is not reduced in the presence of high doses of follistatin (15). Therefore, BMP-7 would be more suitable than activins for therapeutic use. Notably, the possibility of BMP-7 administration as the new treatment for renal disease has been evaluated by many laboratories (29).

We also found that BMP-7 suppressed LHR expression. Growth and differentiation factor 9, another transforming growth factor (TGF) β superfamily member, also suppresses LHR expression in GCs (30). The present findings suggest that in human GCs, BMP-7 stimulation and inhibition of FSHR and LHR mRNA expression, respectively, may play a role in the course of follicle growth and maturation, in which the increased FSH sensitivity results in induction of folliculogenesis and the decrease in LH sensitivity results in inhibition of ovulation and luteinization. Pangas et al. (24) reported that in an ovarian conditional mouse knockout of *Smad4*, which is a common SMAD for TGF-β superfamily signaling, GCs undergo premature luteinization and express lower levels of FSHR and higher levels of LHR compared with control. Given that BMP-7 also uses the SMAD signaling pathway, the present data appear to be consistent with the description of the *Smad4* knockout mouse.

Abir et al. (17) reported that BMP-7 mRNA is detected only in theca cells of the human ovary, whereas BMP-7 protein is detected in oocytes, GCs, and theca cells. This discrepancy might be due to the cross-reactivity of BMP-7 antibody for other homologous proteins, such as BMP-6. In the present experiment, BMP-7 mRNA was not detected by PCR in granulosa cells, whereas BMP-6 mRNA was amplified abundantly (data not shown). These findings suggest that in human ovaries, as in sheep and mouse (12), BMP-7 expression is primarily localized to the theca cells.

Cultured LGCs used in the present study may not represent the stages of growing follicles. However, our findings that FSHR mRNA levels are clearly up-regulated by BMP-7 in human LGCs are new and open new insights into our understanding of FSHR regulation in the human ovary.

In summary, the present study demonstrated that BMP-7 increased the expression of FSHR mRNA in human GCs, while decreasing LHR mRNA expression. The effect of BMP-7 on FSHR mRNA expression was found to be independent of the effect of activins on FSHR expression. These findings indicate that BMP-7 may play a role in follicular maturation while inhibiting ovulation and luteinization in human ovary, and they identify BMP-7 as a potential treatment for human infertility in patients with a low response to FSH reagent.

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Interleukin (IL)-1β Stimulates Migration and Survival of First-Trimester Villous Cytotrophoblast Cells through Endometrial Epithelial Cell-Derived IL-8

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IL-1, secreted by human embryos and trophoblast cells, is important for successful implantation and pregnancy. We previously reported that IL-1β induced IL-8 production in human endometrial stromal cells (ESCs) and that induction was regulated by substances implicated in implantation. In the present study using human primary cells in culture, we measured IL-1β-induced production of IL-8 from endometrial epithelial cells (EECs) and ESCs and examined effects of the endometrium-derived IL-8 on migration and number of first-trimester villous cytotrophoblast cells (vCTs). Both basal and IL-1β-induced IL-8 levels of cell supernatants were much higher in EECs than ESCs. Addition of IL-1β to EECs increased the chemotactic activity of the supernatants to vCTs, and this effect was suppressed by immunoneutralization with anti-IL-8 antibody. Supernatants of IL-1β-stimulated EECs yielded significantly higher number of vCTs compared with those of untreated EECs, and the effect was inhibited by IL-8 antibody. These findings suggest that IL-1 promotes implantation by stimulating EECs to produce IL-8, which subsequently induces migration of vCTs and contributes to survival of vCTs. (***Endocrinology*** **150: 350–356, 2009)**

Migration and survival of trophoblast cells are essential for establishing a pregnancy. These phenomena are controlled elaborately by various substances that are secreted from the embryo, the endometrium, or both during the implantation process (1–5). In particular, molecular cross talk between the embryo and endometrium is indispensable for their coordinated development, which is required for successful implantation (6, 7).

IL-1 is a typical cytokine that affects the implantation process at the interface between the embryo and endometrium (8–10). In view of the findings that IL-1 increases secretion of prostaglandin E2 and leukemia inhibitory factor (LIF) and expression of the integrin β3-subunit in human endometrium (11–13), IL-1 may be one of the first signals that the blastocyst exerts on the endometrium. The notion is also supported by evidence of IL-1 expression in human embryo and trophoblast as described below. It has been shown that successful implantation after *in vitro* fertilization is correlated positively with high concentrations of IL-1α and IL-1β, two active ligands of IL-1, in the embryos' culture media (14, 15). Immunohistochemical studies have demonstrated the localization of IL-1β in first-trimester villous cytotrophoblast cells (vCTs) (16, 17). In addition, an *in vivo* study in mice has shown that administration of IL-1 receptor antagonist significantly reduces the number of implanted embryos (18).

With the aim of gaining a better understanding of the interaction between the human endometrium and trophoblast, a recent study identified the gene expression profile of endometrial stromal cells (ESCs) cocultured with first-trimester trophoblast explants (19). The study revealed that one of the most up-regulated genes was chemokine IL-8, which was up-regulated more than 300-fold. In another study, IL-8 from decidual natural killer cells promoted invasion of first-trimester extravillous trophoblast cells (20). These findings imply that endometrial IL-8 is involved in the process of implantation under the stimulus of trophoblast.

Endometrial IL-8 acts as not only a chemoattractant of leukocytes but also an autocrine growth factor (21). IL-8 acts on cells through IL-8 chemokine receptors CXCR-1 and CXCR2, which are expressed in endometrium (22). In contrast to the roles

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Abbreviations: CCK-8, Cell Counting Kit-8; CXCR, chemokine receptor; DNase I, deoxyribonuclease I; EEC, endometrial epithelial cell; EEC-SN, supernatant of EEC; ESC, endometrial stromal cell; ESC-SN, supernatant of ESC; F12, Ham's F12 medium; FBS, fetal bovine serum; HPF, high-power field; IL-8Ab, IL-8 antibody; LIF, leukemia inhibitory factor; mIgG, mouse IgG1; PF, peritoneal fluid; PL, peritoneal leukocyte; vCT, villous cytotrophoblast cell.

of IL-8 on endometrium, its chemotactic and proliferative effects on first-trimester vCTs still remain obscure. As for expression of the receptors, a study has shown that CXCR2 is expressed in primary first-trimester trophoblast cells and a trophoblast cell line BeWo cells but has not examined the CXCR1 expression (23).

We reported previously that IL-1 induces IL-8 production in human ESCs and the induction is modulated by substances that are implicated in implantation (24, 25). Combined with the above findings, we hypothesized that the development of trophoblast in early pregnancy is affected by endometrial IL-8, which is up-regulated by embryo-derived IL-1. To address this issue, we first examined IL-1β-induced production of IL-8 from human endometrial epithelial cells (EECs) and ESCs. We then assessed effects of the culture media from EECs stimulated by IL-1β on migration and number of human first-trimester vCTs.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics, and magnesium sulfate ($MgSO_4$) were purchased from Sigma (St. Louis, MO). DMEM and Ham's F12 medium, DMEM/F12, 0.25% trypsin and 0.25% trypsin/EDTA, were from Life Technologies (Rockville, MD). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT). Ficoll-Paque Plus (1.077 g/ml) was from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal antihuman IL-8 antibody (IL-8Ab), human recombinant IL-1β and human recombinant IL-8 were from R&D Systems (Minneapolis, MN). Isotype control mouse IgG1 (mIgG) and mouse monoclonal antibodies to human pan-cytokeratin (AE1/AE3), human vimentin, and human CD45 were from Dako (Glostrup, Denmark). Mouse monoclonal antibody to human cytokeratin 7 was from AbD Serotec (Oxford, UK). Deoxyribonuclease I (DNase I) was from Takara (Tokyo, Japan).

Tissue sources

Endometrial tissues were obtained from a total of 20 women (43.7 ± 4.2 yr, mean ± SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroids without endometrial pathologies. Although the relatively high reproductive age range of the subjects and the myometrium pathology results may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue in healthy young women. All subjects had regular menstrual cycles and had not received hormone therapy for at least 6 months before surgery. Placental tissues between 5 and 8 wk of gestation (7.4 ± 6.7 wk) were obtained from a total of 25 women (29.6 ± 6.7 yr) undergoing elective terminations of pregnancy for isolation of first-trimester vCTs. Peritoneal leukocytes (PLs) were used as a positive control in the experiment of RT-PCR. To obtain PL, peritoneal fluid (PF) with PL was obtained from a woman with endometriosis undergoing laparoscopy. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before starting any manipulative procedures. The Institutional Review Board of the University of Tokyo approved this study, and written informed consent for use of the tissue samples was obtained from each woman. The tissues were collected under sterile conditions and were processed for primary cell cultures.

Isolation, purification, and culture of EECs, ESCs, first-trimester vCTs, and PLs

The isolation and culture of human EECs and ESCs was carried out as described previously (2, 26). Endometrial tissues were minced and incubated in DMEM/F12 containing 0.25% type I collagenase and 15 U/ml DNase I for 60 min at 37 C. The resulting dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (Becton Dickinson, Lincoln Park, NJ). Intact endometrial epithelial glands were retained by the strainer, whereas dispersed ESCs passed through the strainer into the filtrate. ESCs in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 5% FBS and antibiotics. ESCs were then plated in a 100-mm culture plate and incubated at 37 C in a humidified 5% CO_2-95% air atmosphere. At the first passage, ESCs were plated at a density of 2×10^5 cells/well into 12-well culture plates for experimental use. Cells enriched with endometrial epithelial glands were collected by backwashing the strainer with DMEM/F12, plated in a 100-mm plate, and incubated at 37 C for 60 min to allow any contaminating stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment to culture plates. EECs at a density of 2×10^5 cells/well in 12-well culture plates were used for the experiments.

The isolation and culture of first-trimester vCTs were performed as described previously (2). Briefly, the first-trimester placental tissues were washed in PBS, and the soft villous material was cut away from connective tissue and vessels. The washed tissue was incubated in sterile PBS containing 1 mM $MgSO_4$, 0.125% trypsin, and 30 U/ml DNase I for 30 min at 37 C with mild stirring. The suspension was then filtered through a 100-μm nylon cell strainer, and the cells were centrifuged at $200 \times g$ for 5 min to obtain a cell pellet, which was resuspended in DMEM/F12 with 5% FBS. The cell suspension was layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 15 min. The cells recovered from the interface were washed with PBS and resuspended in DMEM/F12. The remaining leukocytes were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblast cells. The cells were washed with PBS, the medium was changed to DMEM/F12 with 10% FBS, and the cells were placed in type IV collagen-coated plates (BD Biosciences, Bedford, MA) and incubated at 37 C in a humidified 5% CO_2-95% air atmosphere. The cells were plated at a density of 2×10^4 cells/well in 96-well plates for cell number assays, and at a density of 4×10^5 cells/well in 6-well plates for RT-PCR and *in vitro* migration assay. After incubation for 24 h, the cells were ready for experimental use.

PLs were collected as previously described (27). PF containing peritoneal leukocytes was centrifuged at $200 \times g$ for 5 min, and the supernatant removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 30 min. PLs were recovered from the interface.

We confirmed the purity of EECs, ESCs, vCTs, and PLs by immunocytochemistry as we described previously (2). The purity of EEC preparations was greater than 95%, as judged by positive cellular staining for pan-cytokeratin (a marker for epithelial cells), negative cellular staining for vimentin (a marker for stromal cells), and CD45 (a marker for leukocytes). The purity of ESC preparations was greater than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for pan-cytokeratin and CD45. The purity of vCT preparations was greater than 90%, as judged by positive cellular staining for cytokeratin 7 (a marker for trophoblast cells) and negative staining for vimentin and CD45. The purity of PLs was greater than 90%, as judged by positive cellular staining for CD45.

Treatment of endometrial cell cultures

When ESCs and EECs approached confluence, the complete media were removed and replaced with fresh media and antibiotics, and the cells were cultured for 24 h. The wells were then replenished with FBS-free media containing different concentrations of IL-1β (0, 0.1, 1, and 10 ng/ml) and incubated for an additional 24 h. After IL-1β treatment, the cell supernatants of ESCs and EECs (ESC-SN and EEC-SN, respectively) were collected, centrifuged and stored at −80 C for subsequent analysis.

Measurement of IL-8 in endometrial cell supernatants

Concentrations of IL-8 in endometrial cell culture media were measured using human IL-8 ELISA kit (R&D Systems) following the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan microplate reader (ASYS Hitech GmbH, Eugendorf, Austria). The total amount of IL-8 in the culture medium was calculated from the liquid

volume and the concentration of IL-8. After collection of the culture media, the cultured cells were homogenized, and the amount of total protein in the homogenized cells was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Data of IL-8 content in the culture media were normalized against the total protein content of the cell lysates.

In vitro migration assay

In vitro migration assay was performed in 24-well plates containing Transwell permeable supports with an 8-μm polycarbonate membrane (Costar, Cambridge, MA) for vCTs as previously described (2). EEC-SNs that had or had not been stimulated by 0.1, 1, and 10 ng/ml IL-1β for 24 h [IL-1β (0.1, 1, 10 ng/ml) EEC-SN and control EEC-SN, respectively] or FBS-free medium with or without IL-8 (100 pg/ml) was added to the lower chambers. In the experiments using the neutralizing antibody, control EEC-SN and EEC-SN that had been stimulated by 1 ng/ml IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of IL-8Ab or isotype control mIgG and plated in the lower chambers. Cultured vCTs were plated at a density of 2×10^5 cells/well in the upper chamber of the Transwell membranes, which contained 100 μl FBS-free DMEM/F12, and were incubated for 72 h at 37 C in a 5% CO_2 atmosphere. After the incubation, the upper surface of the membranes was gently cleansed with a cotton swab. Then the cells that had migrated through the pores were fixed with acetone/methanol and stained with hematoxylin and eosin. The filter was gently cut from the chamber, and the migrated cells were counted from the underside of the filter. The number of vCTs that migrated across the filters was counted in 10 randomly selected high-power fields (HPFs) per filter under the light microscope.

Measurement of vCT number

To measure the cell number, we used the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) as we previously described (28).

The cultured medium was removed from vCTs and replaced with fresh medium supplemented with antibiotics 24 h before treatment. Cultured vCTs were treated with control and IL-1β (0.1, 1, 10 ng/ml) EEC-SN or FBS-free medium with or without 100 pg/ml IL-8. In the experiments using neutralizing antibody, control and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml IL-8Ab or isotype control mIgG before the treatment of vCTs was started. After 72 h incubation, CCK-8 assay was performed.

In the CCK-8 assay, CCK-8 solutions containing the tetrazolium salt WST-8 were added and incubated at 37 C for an additional 2 h. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measuring the OD at 450 nm in the DigiScan microplate reader (ASYS Hitech).

RT-PCR of *CXCR1* and *CXCR2* mRNA

RT-PCR was performed as reported previously (29). Total RNA was extracted from vCTs and PLs using the RNAeasy minikit (QIAGEN, Hilden, Germany). Reverse transcription was performed using ReverTra Ace-α (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a total volume of 20 μl, and cDNA was amplified using oligonucleotide primers based on the human *CXCR1* and *CXCR2* sequences. PCR was performed using the ReverTra Dash kit (Toyobo) according to the manufacturer's instructions. *CXCR1* primers (sense, 5′-TACTGTTGGACACACCTGGC-3′; antisense, 5′-TAGACATCAGTGACGGAGCG-3′) were designed to amplify a 294-bp fragment. *CXCR2* primers (sense, 5′-CACAGTGAAGACATCGGTGG-3′; antisense, 5′-AGGGATTCTGGTTCACATGG-3′) were designed to amplify a 246-bp fragment. The expression levels of *CXCR1* and *CXCR2* mRNA were normalized to *GAPDH* mRNA, which was used as an internal control and as a loading control. Human *GAPDH* primers (Toyobo) were designed to amplify a 452-bp fragment. The PCR conditions for *CXCR1* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. The PCR conditions for *CXCR2* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. PCR products were purified using the QIAEX II gel extraction kit (QIAGEN), and their sequence identities confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were evaluated using ANOVA with *post hoc* analysis using Fisher's protected least-significance difference test. $P < 0.05$ were accepted as significant.

Results

Both basal and IL-1β-induced IL-8 levels of culture media are higher in EECs than ESCs

Both ESCs and EECs have been reported to produce IL-8 in response to IL-1 (24, 25, 30). IL-1 has two bioactive ligands, IL-1α and IL-1β, possessed of similar biological effects (31). Therefore, in the present study, we used IL-1β as a representative of IL-1 ligands, and compared IL-8 production in response to IL-1β between ESCs and EECs. As shown in Fig. 1, both the basal IL-8 levels and the IL-1β-induced IL-8 levels were higher in EEC-SN than in ESC-SN. The basal IL-8 level of EEC-SN was 72-fold higher than that of ESC-SN. The IL-8 level of media in EECs stimulated by 1 ng/ml IL-1β was 5 times higher than that of ESCs. Based on these results, we chose to use the EEC-SN in all subsequent experiments to evaluate the effects of endometrial IL-8 on first trimester vCT.

Migration of first-trimester vCTs is stimulated by EEC-SN treated with IL-1β via IL-8

To study the chemotactic effects of EEC-derived IL-8 on first-trimester vCTs, *in vitro* migration assay was performed. As illustrated in Fig. 2A, more vCTs migrated to IL-1β (0.1, 1, 10 ng/ml) EEC-SN than control EEC-SN. The effect appeared to be maximal at 1 ng/ml. Recombinant IL-8 also promoted the chemotaxis of

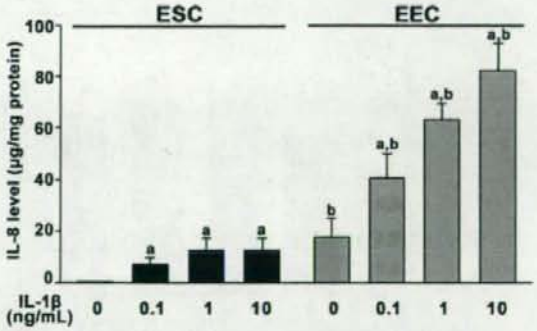

FIG. 1. IL-1β-stimulated IL-8 secretion from ESCs and EECs. Human ESCs (n = 10) and EECs (n = 10) were cultured in FBS-free media with different doses of IL-1β for 24 h. The supernatants were collected and assayed for IL-8 by ELISA. Values are normalized to the total protein content of the cell lysate. The values (total IL-8 content of the supernatants/total protein content of the cell extract, micrograms per milligram protein) are presented as the mean ± SEM of the combined data of separate experiments using different ESC and EEC preparations. a, $P < 0.05$ when compared with the same cell type without IL-1β stimulation; b, $P < 0.05$ when compared with ESCs stimulated by the same dose of IL-1β.

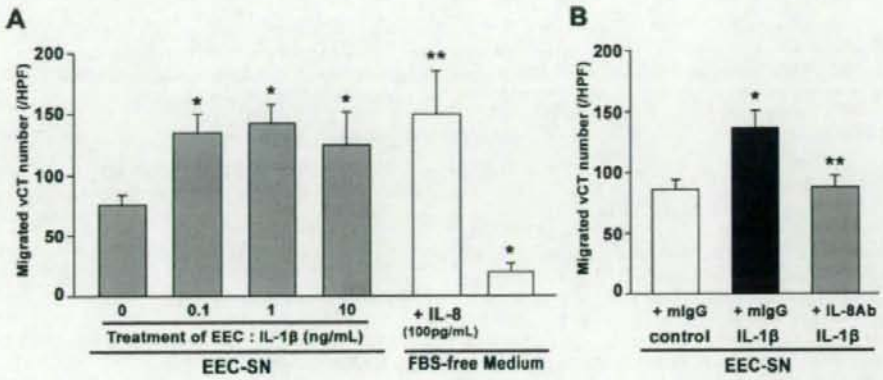

FIG. 2. Stimulatory effect of EEC-SN treated with IL-1β on migration of first-trimester vCTs through IL-8. *In vitro* migration assay was performed to determine whether migration of human first-trimester vCTs was affected by endometrial IL-8 expression. Cultured vCTs were plated at a density of 2×10^5 cells in the upper chambers of Transwell membranes for 72 h. After incubation, vCTs that had migrated through the pores were fixed and counted. The number of vCTs that had migrated across the filters was counted in 10 randomly selected HPFs per filter under the light microscope. Values represent the cell number per HPF. A, EEC-SN that had or had not been treated with IL-1β for 24 h (IL-1β EEC-SN and control EEC-SN, respectively) or FBS-free media with or without IL-8 were plated in the lower chambers. Values are the mean ± SEM of the combined data from six independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and EEC-SN that had been treated with 1 ng/ml of IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of anti-IL-8Ab or isotype control mIgG and plated in the lower chambers. Values are the mean ± SEM of the combined data from 10 independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

vCTs. In contrast, immunoneutralization with IL-8Ab abolished the chemotactic activity of IL-1β (1ng/ml) EEC-SN (Fig. 2B).

EEC-SN treated with IL-1β yielded higher number of first-trimester vCTs via IL-8

To study the effects exerted by EEC-derived IL-8 on number of first-trimester vCTs, we used CCK-8 assay. As illustrated in Fig. 3A, when compared with control EEC-SN, IL-1β (0.1, 1, 10 ng/ml) EEC-SN up-regulated the vCT number. Recombinant IL-8 also increased in the vCT number. The effects of IL-1β (1ng/ml) EEC-SN were eliminated by immunoneutralization with IL-8Ab (Fig. 3B).

CXCR1 and *CXCR2* mRNA are expressed in first-trimester vCTs

To examine the expression of IL-8 receptors in first-trimester vCTs, RT-PCR was performed using primers specific to *CXCR1* and *CXCR2* (receptors for IL-8). A sample of PLs was used as a positive control for IL-8 receptor-expressing cells (32–35). As demonstrated in Fig. 4, mRNAs of IL-8 re-

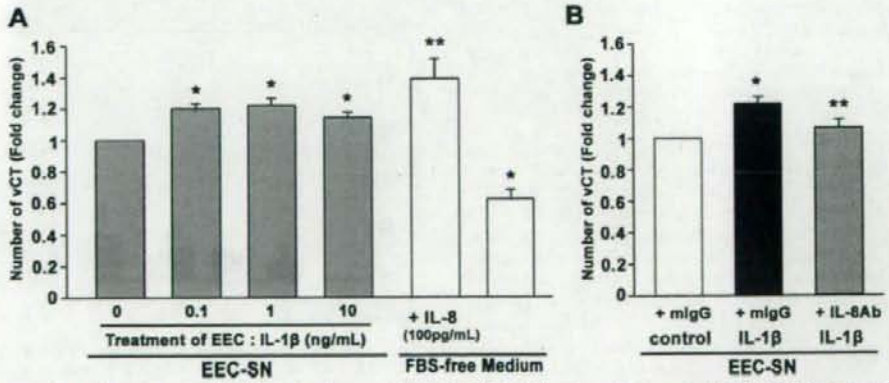

FIG. 3. Stimulatory effect of EEC-SN treated with IL-1β on increase in number of first-trimester vCTs through IL-8. The CCK-8 assay was performed to determine whether number of human first-trimester vCTs was affected by endometrial IL-8 expression. Before the assay, vCTs were treated for 72 h. A, vCTs were treated with control EEC-SN and IL-1β (0.1, 1, and 10 ng/ml) EEC-SN or FBS-free media with or without 100 pg/ml of IL-8. The values represent the ratio of the cell number relative to control EEC-SN. Values are the mean ± SEM of the combined data from seven independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml of IL-8Ab or mIgG. The values represent the ratio of the cell number relative to control EEC-SN with mIgG. Values are the mean ± SEM of the combined data from nine independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

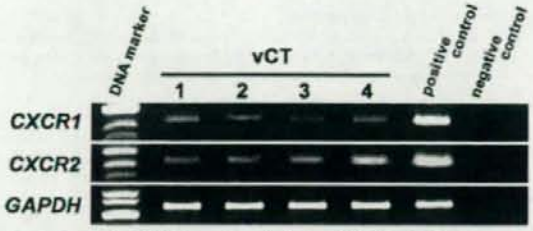

FIG. 4. Expression of IL-8 receptors *CXCR1 and CXCR2 mRNA in human first-trimester vCTs. Total RNA isolated from human first-trimester vCTs was reverse transcribed and amplified by PCR using primers for CXCR1 and CXCR2. Amplification of the internal control GAPDH was used to ensure RNA quality and as a loading control. The results are from four vCT samples (vCT 1–4) of different individuals. Gestational age of the vCT samples were; vCT1, 6 wk; vCT2, 6 wk, vCT3, 7 wk, vCT4, 8 wk. DNA marker, φX174/HinfI; positive control, peritoneal leukocytes; negative control, water without cDNA.*

ceptors *CXCR1* and *CXCR2* mRNA were expressed in first-trimester vCTs.

Discussion

In the present study, we demonstrated that IL-1β induced the production of IL-8 from endometrial cells and that endometrial cell-derived IL-8 promoted migration of first-trimester vCTs and yielded higher number of vCTs.

IL-1 is produced by human embryo and first-trimester vCTs (8, 9) and is thought to play a regulatory role in human embryo implantation. It has been reported previously that IL-1 up-regulates integrin-β3, a marker of uterine receptivity in endometrial epithelial cells (12). Furthermore, IL-1β can induce the expression of prostaglandins (36) and LIF (37), factors that are important for the implantation process (6), in human endometrial and decidual cells. Our findings that IL-1β stimulated migration of first-trimester vCTs and increased the cell number via endometrium-derived IL-8 suggest a novel function for IL-1β and extend the notion that IL-1β is a pivotal and multifunctional factor in human embryo implantation.

The CXC chemokine IL-8 participates in the migration of leukocytes such as neutrophils and T lymphocytes (20, 21, 38–41). As well as being able to encourage the accumulation of leukocytes, uterine IL-8 is thought to have unique roles in endometrial angiogenesis, apoptosis, proliferation, and differentiation (42). These events are crucial to preparing the endometrium for implantation. Combined with the promotive effects on migration of first-trimester vCTs and maintenance of the number of live cells in the present study, IL-1β-induced secretion of IL-8 may orchestrate the implantation process and therefore affect both the endometrium and trophoblast. As a note, because IL-8 is secreted by first-trimester extravillous trophoblast cell line HTR8 (43) and its secretion is enhanced by IL-1 in third-trimester trophoblast cells (44), IL-1 secreted by first-trimester vCTs may induce IL-8 secretion from vCTs themselves as well as endometrial cells. IL-1, therefore, might be involved in implantation via trophoblast-derived IL-8. However, in the present study, we did not observe any difference between cell-free IL-1β EEC-SN and FBS-free medium without any additions and between control EEC-SN with IL-8Ab and control EEC-SN with isotype mIgG on the migration and cell number of vCTs (data not shown). Further studies are warranted to elucidate the detail mechanism.

The current study demonstrated that both basal and IL-1β-induced IL-8 levels were much higher in EEC-SN than ESC-SN. The implication for this difference is not clearly understood at the moment. However, it is interesting to note that our recent study has demonstrated that CXCL11, which stimulates migration of first-trimester vCTs, is also inducible in epithelial cells but not stromal cells (2). It can therefore be speculated that like CXCL11, epithelium-derived IL-8 is involved in a relatively early-phase of implantation, assuming that the endometrial epithelium is an important component in initiating the molecular interactions between embryo and endometrium (2, 45).

Migration of first-trimester trophoblast cells needs to be a tightly regulated process for successful implantation and for circumventing undesirable complications of pregnancy such as poor fetal growth or preeclampsia. The chemokines CX3CL1, CCL14, CCL4, CXCL9, CXCL10, and CXCL11 promote the migration of trophoblast cells into the endometrium (1, 2). Furthermore, decidual natural killer cell-derived IL-8 promotes the invasion of first-trimester extravillous trophoblast cells into the decidua (20). Our findings are interesting in that trophoblast may control its own activity through cross talk with EECs. Collectively, the molecular mechanism that fine-tunes trophoblast migration is believed to work in a spatiotemporally specific manner.

A previous study demonstrated that the supernatants of first-trimester primary decidual cells inhibit the proliferation of trophoblast cell line BeWo cells, whereas those of BeWo cells and first-trimester primary trophoblast cells promote the proliferation of decidual cells (46). These findings indicate that a regulatory loop to control the growth of fetal and maternal cells exists at the fetomaternal interface. Thus, cross talk between trophoblast and endometrium seems to be important for maintenance of trophoblast cell numbers. The studies on the inhibitory role of IL-1β in the proliferation of trophoblast cell lines BeWo cells and JAR cells (47, 48) imply a possibility that IL-1β is one of the regulating factors in the growth of placenta. In light of our findings that IL-8 derived from IL-1β-stimulated EECs could yield a higher number of vCTs, it can be speculated that endometrial IL-8 may act as one of the survival factors for first-trimester vCTs and facilitate placental growth in early pregnancy. Thus, not only IL-1β but also subsequent IL-8 may participate in the regulatory loop of placental growth at the fetomaternal interface.

In summary, we have shown that IL-1β is able to induce secretion of IL-8 from EECs and that EEC-derived IL-8 is able to stimulate the migration of human first-trimester vCTs and yield a higher number of vCTs. These findings suggest that human vCTs may regulate their own status via IL-8 secreted by IL-1β-stimulated EECs to accomplish successful implantation.

Acknowledgments

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