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—Original Article—

Inhibitory effect of insulin-like growth factor-binding protein-7 (IGFBP7) on *in vitro* angiogenesis of vascular endothelial cells in the rat corpus luteum

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Abstract. Angiogenesis in the developing corpus luteum (CL) is a prerequisite for establishment and maintenance of an early pregnancy. To explore the physiological significance of insulin-like growth factor-binding protein-7 (IGFBP7) in the developing CL, the effects of IGFBP7 on vascular endothelial growth factor (VEGFA)- and luteinizing hormone (LH)-induced *in vitro* tube formation were tested using isolated luteal microvascular endothelial cells (LECs). Capillary-like tube formation of LECs and their proliferation were stimulated by both VEGFA and LH. IGFBP7 treatment suppressed VEGFA- or LH-induced tube formation. The proliferation and migration of LECs, and phosphorylation of mitogen-activated protein kinase kinase and extracellular signal-regulated kinase 1/2 were inhibited by IGFBP7. Furthermore, IGFBP7 attenuated VEGFA-enhanced cyclooxygenase (COX)-2 mRNA expression and prostaglandin E_2 secretion. These findings suggest the possibility that luteal IGFBP7 secretion may suppress the stimulatory effect of VEGFA on angiogenesis in the early CL.

Key words: Angiogenesis, Corpus luteum (CL), Insulin-like growth factor-binding protein-7 (IGFBP7), Vascular endothelial growth factor (VEGFA)

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Granulosa cells and theca cells rapidly change into luteal cells, which produce high levels of progesterone and estradiol upon stimulation by a large amount of luteinizing hormone (LH), namely, the LH surge that causes ovulation. The mature corpus luteum (CL) is a highly vascularized small organ and the acquirement of luteal function requires the formation of new capillary vessels. The development of luteal vasculature is probably governed by several angiogenic and anti-angiogenic factors derived from luteal cells or endothelial cells of the newly formed CL [1, 2]. LH and human chorionic gonadotropin (hCG) may play a key role in the vascular physiology of reproductive organs during the early stage of pregnancy [3–5]. Vascular endothelial growth factor A (VEGFA) has a fundamental role in luteal vascularization [6]. In turn, thrombospondin-1, angiostatin, and vasohibin are novel angiogenesis inhibitors and play pivotal roles in the coordinated regulation of angiogenesis along with angiogenic factors such as VEGFA and prostaglandin E_2 (PGE_2) [7].

Insulin-like growth factor (IGF)-binding protein (IGFBP) 7 is a secreted protein that regulates various cellular functions including proliferation and adhesion [8, 9]. IGFBP7 is also known as IGFBP-related protein (IGFBP-rP) 1, mac25, angiomodulin (AGM), tumor-derived adhesion factor (TAF), and prostacyclin-stimulating factor (PSF). Although it belongs to a group of IGFBP-rPs, its affinity for IGF-1 is typically lower than that of IGFBP1 to IGFBP6. IGFBP7 binds insulin with high affinity. IGFBP7 also has actions that are independent of insulin and IGF binding [9]. It is expressed in endothelial cells and accumulates in capillary-like tubes of human vascular endothelial cells *in vitro* [10]. A recent study revealed that IGFBP7 is a novel component of endothelial cell-specific Weibel-Palade bodies and binds to ultra-large von Willebrand factor strings released by endothelial cells [11]. Exogenous IGFBP7 inhibits VEGFA-stimulated *in vitro* angiogenesis and proliferation in primate vascular endothelial cells [12, 13]. Therefore, in the current study, we determined the effects of LH and VEGFA on *in vitro* tube formation using luteal microvascular endothelial cells (LECs) isolated from the early CL, and further examined the impact of IGFBP7 on *in vitro* angiogenesis.

Materials and Methods

Reagents

MCDB131 medium, endothelial cell growth supplement (ECGS), and $CoCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IGFBP7 and VEGFA were obtained from R&D Systems (Minneapolis, MN, USA). Ovine LH (NIDDK, oLH26, AFP-5551B) was provided by Prof AF Parlow of the National Hormone and Pituitary Program, Harbor/UCLA Medical Center. Growth factor-reduced Matrigel (BD Biosciences, MA, USA) was gelatinated at 37 C in a CO_2 incubator for 30 min prior to the Matrigel assay. WST-8 reagent (Cell Counting Kit, Dojindo, Tokyo, Japan) was used to evaluate the proliferation of LECs. Poly(A)$^+$ RNA was isolated using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). The Phospho- ERK1/2

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(phospho-extracellular signal-regulated kinase 1/2) Pathway Kit and an MEK1/2 (anti-mitogen-activated protein kinase kinase 1/2 antibody) were purchased from Cell Signaling Technology (Beverly, MA, USA).

LEC culture

All experimental protocols using rats were reviewed and approved by the Institutional Animal Care Committees at the Tokyo University of Pharmacy and Life Sciences and were in compliance with institutional guidelines for experimental animal care (approval number #F8-0801). Highly luteinized ovaries were collected from immature female Wistar-Imamichi rats (3 weeks old, Imamichi Institute for Animal Reproduction, Ibaraki, Japan) treated with equine chorionic gonadotropin (50 IU, Asuka Pharmaceutical, Tokyo) and hCG (25 IU, Asuka Pharmaceutical). Whole dispersed cells obtained from the ovaries of 3 rats were subjected to the centrifugation procedure for endothelial cell enrichment with the gradient Percoll solution in each experiment [14]. Isolated LECs (Cell Systems, Kirkland, WA, USA) were grown on a 0.1% gelatin-coated dish in MCDB131 medium containing 10% fetal bovine serum (FBS; JRH Biosciences, ACSL, Lenexa, MD, USA), ECGS (50 μg/ml), and antibiotics. The percentage of CD31, an endothelial marker in isolated LECs was 89.3% (average). Experiments were performed using cells between the third and sixth passages.

Cell proliferation and migration assay

Changes in cell number after IGFBP7 treatment were monitored using a proliferation bioassay, namely, the WST-8 assay. LECs (1×10^4 cells) were seeded in 48-well culture plates in serum-free MCDB131 medium lacking ECGS and were treated with IGFBP7 in the presence or absence of VEGFA (10 ng/ml) for 24 h prior to the evaluation of cell proliferation. LEC migration was monitored using a Transwell system (Chemotaxicell; Kurabo, Osaka, Japan) equipped with polycarbonate filters with an 8 μm pore size. Cells resuspended in basal media containing 2% FBS were loaded onto the upper compartment, which was coated with Matrigel. LECs (2×10^4 cells) were incubated for 24 h with IGFBP7 in the presence or absence of VEGFA or LH in a Chemotaxicell. The remaining cells in the upper chamber were removed using a cotton stick. Migrated cells on the opposite side of the upper chamber were counted following staining with DAPI. Three independent sets of experiments were performed in triplicate, and the results are expressed as the percentage of the number of control cells.

In vitro endothelial tube formation on Matrigel

LECs were resuspended at a density of 1×10^4 cells/ml in serum-free MCDB131 medium lacking ECGS and then seeded onto gelatinated Matrigel. Cells were treated for 24 h with various concentrations of IGFBP7 in the presence or absence of VEGFA or LH. Culture plates were photographed at ×10 original magnification to analyze the formation of tubular structures. Tubular formation was determined by measuring the total tube lengths of tube-like cells in three randomly selected fields using the KSW-500U software (Kurabo, Tokyo, Japan), as previously reported [12]. The data were expressed as the percentage of the tube length in control samples. These experiments were repeated at least three times.

RT-PCR analysis of VEGFA, fibroblast growth factor (FGF)-2, cyclooxygenase (COX)-2, and IGFBP7

Subconfluent LECs were prepared in a 6-well culture plate in serum-free MCDB131 medium and treated for 2 h with various concentrations of IGFBP7. Poly $(A)^+$ RNA (0.1 μg) was subjected to RT-PCR using specific primer pairs and a One Step RNA PCR Kit (AMV, TaKaRa), according to the manufacturer's instructions. RT-PCR for VEGFA, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as previously described [14]. In addition, the mRNA levels of FGF-2 were examined using specific primers (sense, 5'-GGATCCCAAGCGGCTCTACTG -3'; antisense, 5'-GAAGGGTTTGACAAGATTGAC-3'). The PCR protocol was as follows: 27 cycles at 95 C for 45 sec, 53 C for 45 sec and 72 C for 45 sec. The predicted lengths of the fragments were 511 bp. GAPDH served as an internal control. The PCR products were separated in a 1.5% agarose gel containing ethidium bromide and were visualized under ultraviolet transillumination.

Western blot analysis

Subconfluent LECs were treated with IGFBP7 in serum-free MCDB131 medium lacking ECGS in the presence or absence of VEGFA and/or IGFBP7. Cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology). Samples (5–10 μg protein) were subjected to SDS-PAGE and transferred to a polyvinylidene membrane. The membranes were incubated with an anti-hypoxia-inducible factor-1α (HIF-1α) antibody (1:1000, Sigma), an anti-phospho-c-Raf antibody, an anti-phospho-MEK1/2 antibody, an anti-phospho-p42/44 mitogen-activated protein kinase (MAPK) (ERK1/2) antibody (1:1000) from a Phospho-ERK1/2 Pathway Kit, or an anti-MEK1/2 antibody (1:1000). After the detection of target proteins, the same membranes were reprobed with a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma-Aldrich). Goat anti-rabbit or anti-mouse IgG (Vector Lab., Burlingame, CA, USA) (0.5 μg/ml) conjugated with horseradish peroxidase served as the secondary antibody for each analysis. Signals were detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Wellesley, MA, USA). All blotting experiments were repeated at least twice and representative data are shown.

Measurement of PGE_2 in culture media

Cells were cultured in 24-well tissue culture plates and treated with IGFBP7 in the presence or absence of VEGFA for 24 h. The levels of PGE_2 in the culture media were determined using a PGE_2 Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI, USA), as described previously [15]. Three independent sets of experiments were performed in triplicate.

Statistical analysis

Values represent means ± SEM. Significance was assessed using the Tukey-Kramer multiple comparisons test. A P values <0.05 was considered to be significant.

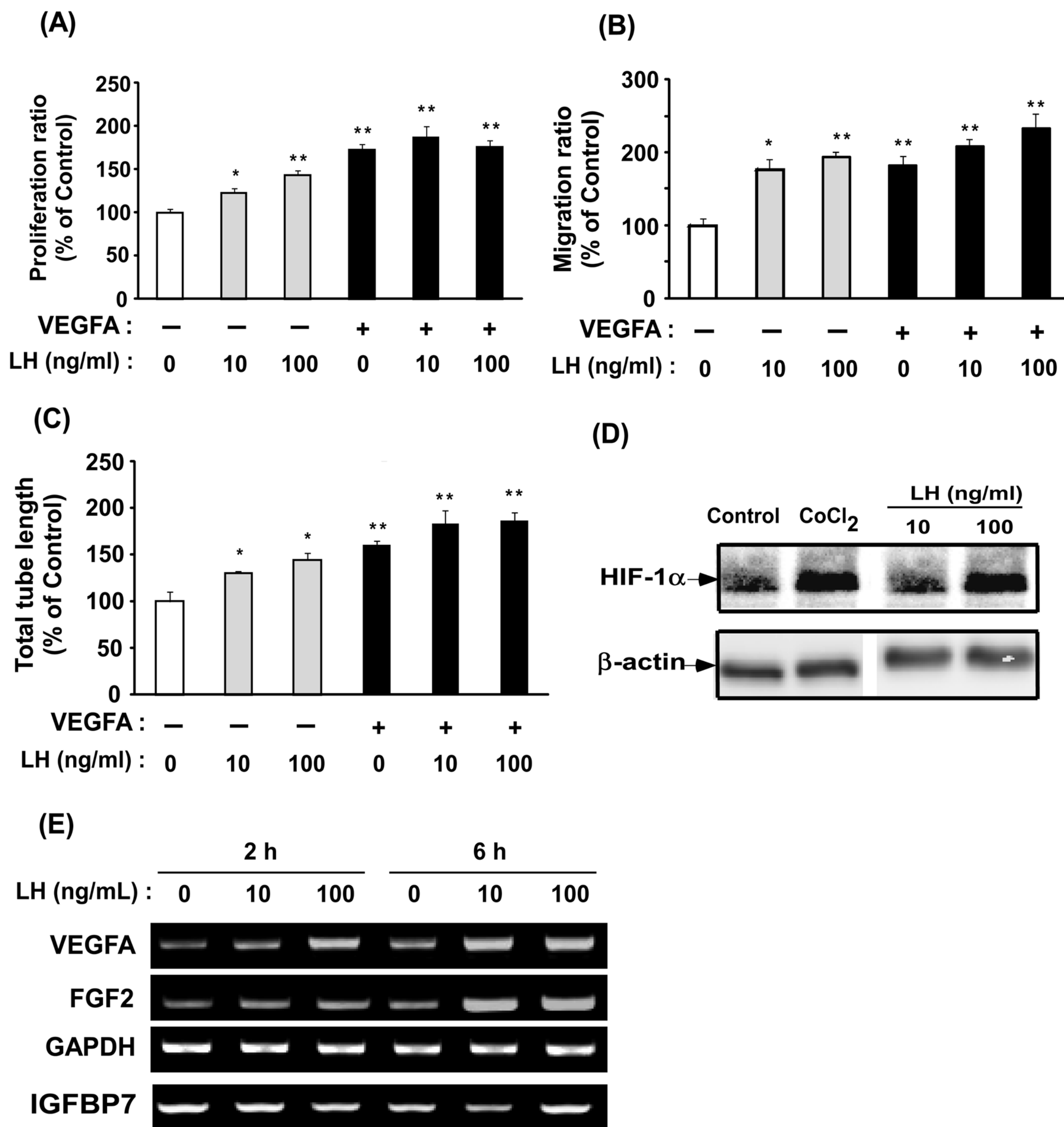

Fig. 1. Angiogenic response to vascular endothelial growth factor A (VEGFA) and/or luteinizing hormone (LH) in luteal microvascular endothelial cells (LECs). (A) Effect of VEGFA (10 ng/ml) and/or LH treatment on proliferation of LECs. The number of cells was evaluated using a WST-8 assay. Each value represents the mean ± SEM. *$P<0.05$ *vs.* control; **$P<0.01$ *vs.* control (untreated cells; VEGFA: –/LH 0). (B) Effect of VEGFA and/or LH on migration of LECs. Migrated cells were counted following staining with DAPI. *$P<0.05$ *vs.* control; **$P<0.01$ *vs.* control (C) Effect of VEGFA and/or LH on tubular formation of LECs. LECs seeded on Matrigel were treated with LH in the presence or absence of VEGFA (10 ng/ml). The data are expressed as the percentage of the tube length in control samples. *$P<0.05$ *vs.* control; **$P<0.01$ *vs.* control. (D) Effect of LH on hypoxia-inducible factor (HIF)-1α expression in LECs. Cells were incubated with $CoCl_2$ (0.5 mM) or LH (10, 100 ng/ml). Cell lysates were subjected to Western blot analysis for HIF-1α and representative images are shown. (E) Effect of LH on VEGFA and fibroblast growth factor 2 (FGF-2) mRNA expression in LECs. LECs were incubated with LH for 2 or 6 h, and RNA was subjected to semiquantitative RT-PCR for VEGFA, FGF-2, IGFBP7 and GAPDH. The PCR products were photographed under ultraviolet transillumination.

Results

The effects of LH on the proliferation, migration, and tube formation of LECs

Treatment with LH or VEGFA significantly increased the proliferation (Fig. 1A), migration (Fig. 1B), and total tube length (Fig. 1C) of LECs; however, there were no additive effects upon treatment with both LH and VEGFA. LH treatment (100 ng/ml) increased the HIF-1α protein level, which was similar to treatment with $CoCl_2$, a chemical inducer of HIF-1 (Fig. 1D). Furthermore, the mRNA

expression of VEGFA and FGF-2 was enhanced after 6 h of LH treatment (Fig. 1E). There was no change in the IGFBP7 mRNA level in LH-treated cells.

The effects of IGFBP7 on VEGFA-induced angiogenesis and activation of MAPK signaling in LECs

The effects of IGFBP7 on VEGFA- and LH-induced angiogenesis of LECs *in vitro* were examined (Fig. 2). Cells were treated with IGFBP7 in the presence of VEGFA or LH in the *in vitro* Matrigel assay. IGFBP7 treatment significantly decreased VEGFA- and LH-induced tube formation of LECs (Fig. 2A and Fig. 2B and E, respectively). Furthermore, IGFBP7 treatment (160 ng/ml) completely blocked VEGFA-stimulated LEC proliferation (Fig. 2C) and migration (Fig. 2D), although IGFBP7 treatment (10 or 160 ng/ml) did not influence baseline LEC proliferation or migration. Pretreatment with IGFBP7 for 6 h also reduced LEC proliferation induced by VEGFA (Fig. 3), suggesting that the interference with VEGFA-triggered intracellular signaling caused by IGFBP7 may have potent effects in vascular endothelial cells. Activation of MAPKs was evaluated in the presence or absence of VEGFA (Fig. 4). VEGFA treatment increased the levels of phosphorylated c-Raf, MEK, and ERK1/2; however, VEGFA-stimulated phosphorylation of these proteins was suppressed by treatment with IGFBP7. Treatment with VEGFA and/or IGFBP7 did not affect the total level of MEK1/2 or β-actin.

The effects of exogenous IGFBP7 on VEGFA-stimulated COX-2 expression in LECs

The effects of IGFBP7 on COX-2 and VEGFA expression (Fig. 5A) and PGE_2 secretion (Fig. 5B) were examined. Treatment with IGFBP7 alone (160 ng/ml) increased the COX-2 mRNA level, but not the VEGFA mRNA level. Treatment with VEGFA alone increased the mRNA levels of both COX-2 and VEGFA, whereas this was inhibited by co-treatment with IGFBP7. The amount of PGE_2 released into the culture media was increased by VEGFA treatment, whereas co-treatment with IGFBP-7 reduced this secretion to the baseline level.

Discussion

LH plays essential roles in the formation and maintenance of the CL through the LH/hCG receptor. LH stimulates angiogenesis in the ovary [5] and promotes the production of angiogenic factors in luteinized steroidogenic cells [16]. HIF-1α expression induced by LH plays a role in the regulation of VEGFA expression in human luteinized granulosa cells, and the nuclear HIF-1α level in granulosa-lutein cells is the highest during luteal formation [17]. Reduced oxygen levels permit the accumulation of HIF-1α protein in the cytoplasm, and HIF-1α subsequently translocates to the nucleus, engages HIF-1β, and forms the HIF-1 complex that initiates VEGF transcription and mRNA stabilization. Both HIF proteins were localized to the nucleus of luteal cells in the early luteal phase [18]. The LH/hCG receptor is abundantly expressed in endothelial cells of the uterus during the luteal phase and hCG increases the migration and capillary formation of endothelial cells [19]. A direct angiogenic effect of hCG on endothelial cells has been demonstrated in several *in vivo* and *in vitro* experimental models [17, 19–21]. Our present data from LECs indicate that LH stimulates *in vitro* angiogenesis in part through LH-mediated angiogenic factor expression. However, the *in vivo* angiogenic effect of LH is probably exerted by an indirect effect via a paracrine loop involving enhanced secretion of VEGF by steroidogenic luteal cells as well as LECs and a direct angiogenic effect of LH via interaction with the LH receptor in LECs.

IGFBP7 is ubiquitously expressed in normal tissues and implicated in diverse physiological process including proliferation, adhesion, senescence, and apoptosis [8, 9]. In addition, IGFBP7 is abundantly expressed in reproductive organs including blood vessels in the preovulatory follicular wall [22], granulosa cells of antral follicles [23], the CL [23, 24], and the endometrium [25, 26]. Several studies, including ours, have suggested that IGFBP7 functions as a physiological regulator of uterine receptivity [25, 26] and follicular steroidogenesis [23]. In addition, endothelial cells express IGFBP7 mRNA and IGFBP7 is highly expressed in blood vessels of various human cancer tissues [12, 27, 28], suggesting the significance of this protein in vascular physiology and pathology. It was recently shown that VEGFA-induced IGFBP7 binds to integrin αvβ3 and increases the vascular permeability of cancer vasculature [28]. These data suggest that IGFBP7 in blood vessels of tumors leads to a unique tumor vasculature with characteristics different from those of healthy vascular tissue. Furthermore, IGFBP7 may be a novel tumor suppressor gene in various malignancies [8, 28–30]. However, the physiological role of IGFBP7 in normal vascular function remains unknown. In the present study, to gain further insight into the mechanisms that regulate angiogenesis in the newly formed CL, the effects of IGFBP7 on angiogenesis-related responses were examined using isolated LECs.

Vascular tube formation is required for endothelial cell proliferation and migration, and VEGFA stimulates a MAPK cascade through the VEGFA receptor to trigger these responses. IGFBP7 inhibited VEGFA-induced proliferation and migration of LECs, as well as the phosphorylation of c-Raf, MEK, and ERK1/2 in these cells. These results are consistent with previous studies showing that IGFBP7 suppresses VEGFA-stimulated tube formation in human umbilical vein endothelial cells (HUVECs) [12] and rhesus macaque retinal endothelial cells [13]. Our data clearly showed that exogenous IGFBP7 blocks both LH- and VEGFA-stimulated tube formation in LECs isolated from gonadotropin-primed immature rats. However, the impact of IGFBP7 on endothelial cells seems to be altered with a context- and dose-dependent manner, because there are contradictory reports showing angiogenic effect [28, 31] and anti-angiogenic effects [12, 13]. A recent study [11] suggested that IGFBP7 is specifically sorted to Weibel-Palade bodies (WPBs) which are known to play roles in coagulation and inflammation. IGFBP7 released from WPBs might cause a rapid increase in local levels of IGFBP7 allowing for a spatially and temporally restricted regulation of angiogenesis. It has been shown that IGFBP7 directly binds to the IGF-I receptor and blocks its activation, suggesting that IGFBP7 is a component of the physiological mechanism directed to preserve the IGF-I receptor integrity during differentiation or maintenance of normal tissues [32]. Phan *et al.* [16] reported that hCG inhibits expression of IGFBP7 in human granulosa cells. Although this led us to speculate that LH/hCG enhances luteal angiogenesis by diminishing the expression of an anti-angiogenic factor, namely, IGFBP7, in luteinized granulosa

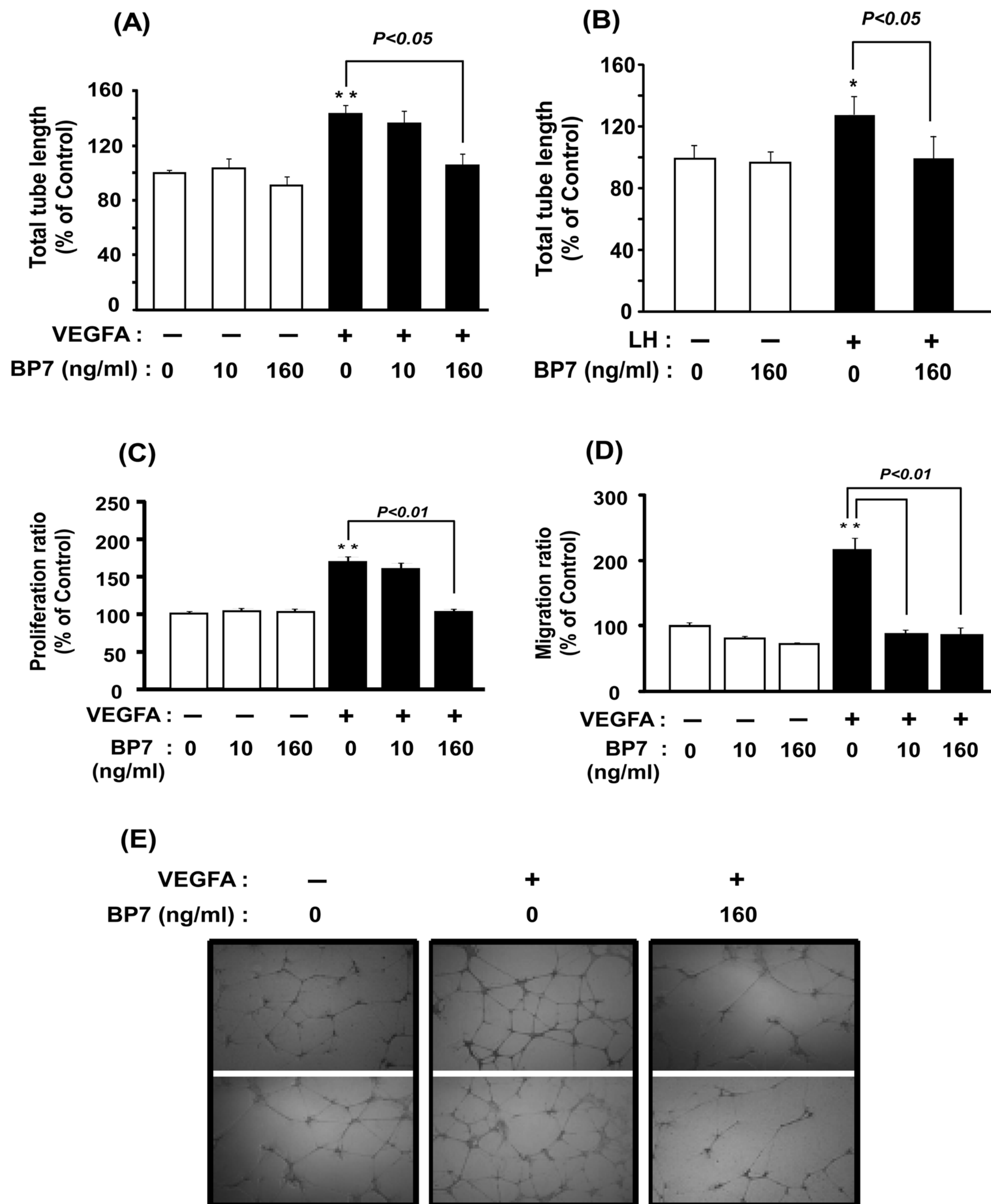

Fig. 2. Effect of IGFBP7 on VEGFA-induced *in vitro* angiogenesis in LECs. (A, B) LECs were treated with IGFBP7 (10 or 160 ng/ml) in the presence or absence of VEGFA (10 ng/ml) (A) or LH (100 ng/ml) (B). Tubular formation was determined, and the data were expressed as the percentage of the tube length in control samples. Each value is the mean ± SEM. *P<0.05, **P<0.01. (C) LECs were incubated with IGFBP7 in the presence or absence of VEGFA (10 ng/ml). The number of cells was evaluated by a WST-8 assay. **P<0.01 *vs.* control. (D) Migrated LECs were counted after cells were incubated with IGFBP7 in the presence or absence of VEGFA (10 ng/ml) in a Chemotaxicell. **P<0.01 *vs.* control (untreated cells). (E) Representative pictures in (A) showing the effect of IGFBP7 on VEGF-stimulated tubular formation. BP7: IGFBP7.

cells, we could not detect the inhibitory effect of LH on IGFBP7 expression in LECs.

COX-2, a rate-limiting enzyme in the arachidonic acids cascade, plays an essential role in the biosynthesis of PGE2, and inhibition of COX-2 suppresses neovascularization [33, 34]. COX-2 expression is elevated in premalignant and malignant tumors and associated with decreased cancer patient survival. [35]. VEGFA stimulates COX-2 mRNA expression in HUVECs [36, 37], as well as membrane-associated PGE synthase and COX-2 mRNA expression in rat ovarian cells [14]. The pro-angiogenic effects of COX-2 may be primarily mediated by several eicosanoids, namely, PGE_2, TXA_2, and PGI_2 [38]. Thus, VEGFA-induced PGE_2 may directly promote

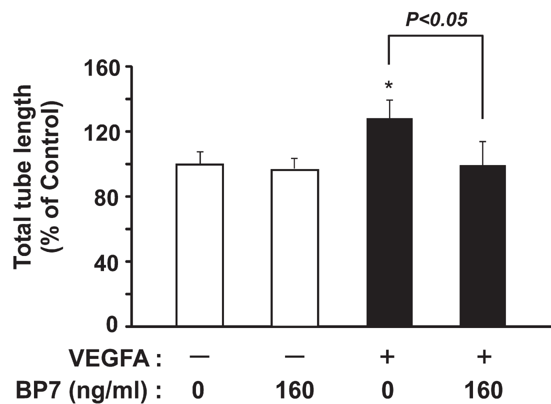

Fig. 3. Effects of IGFBP7 pretreatment on VEGFA-stimulated tubular formation in LECs. LECs were preincubated for 6 h with IGFBP7 (160 ng/ml), and cells seeded on Matrigel were cultured in the presence or absence of VEGFA (10 ng/ml). BP7: IGFBP7

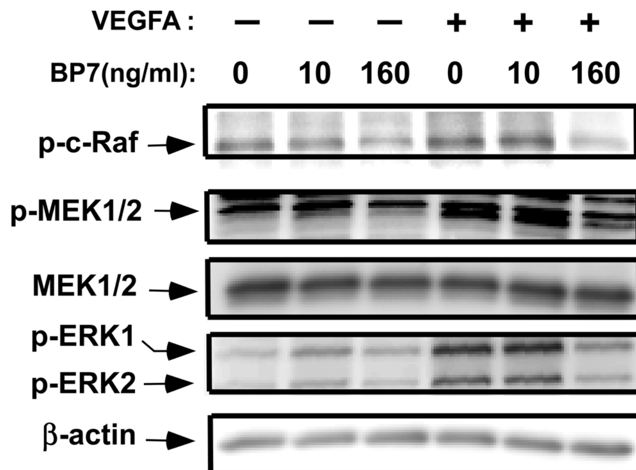

Fig. 4. Effects of IGFBP7 on VEGFA-induced phosphorylation of mitogen-activated protein kinases in LECs. LECs were treated with VEGFA (10 ng/ml) for 20 min following incubation with IGFBP7 (10 or 160 ng/ml) for 24 h. Cell lysate was subjected to Western blot analysis using the following primary antibodies: phospho-c-Raf (Ser338), phospho-mitogen-activated protein kinase kinase (MEK)1/2 (Ser217/221), phospho-ERK1/2 (Th202/Tyr204), and MEK1/2. Representative blots are shown. β-actin served as a loading control. BP7: IGFBP7.

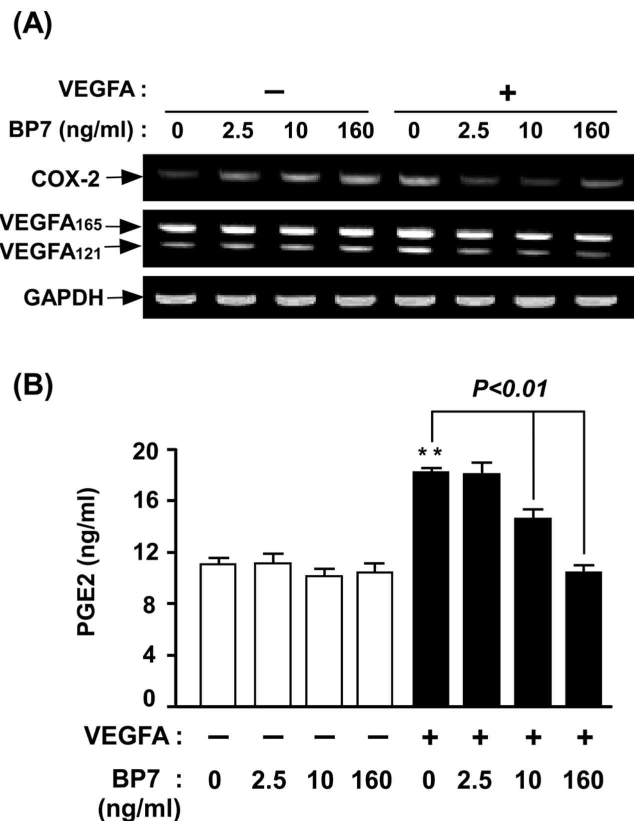

Fig. 5. Effects of IGFBP7 on VEGFA-stimulated cyclooxygenase-2 (COX-2) mRNA expression and prostaglandin E_2 (PGE_2) production in LECs. (A) LECs were incubated with IGFBP7 (2.5–160 ng/ml) for 2 h in the presence or absence of VEGFA (10 ng/ml). RNA was analyzed with semiquantitative RT-PCR for VEGFA and COX-2. (B) LECs were incubated for 24 h with IGFBP7 in the presence or absence of VEGFA (10 ng/ml). The culture medium was collected for the PGE_2 assay. The results are expressed as means ± SEM. *P<0.05 *vs.* control; **P<0.01 *vs.* control (untreated cells). BP7: IGFBP7.

angiogenesis in endothelial cells [14, 37]. Recent data suggest that PGE_2 directly stimulates tube formation in LECs mainly via the EP2 receptor and protein kinase A signaling [14]. Endogenous PGE_2 produced by luteinizing follicular cells and LECs may stimulate luteal angiogenesis. In this study, VEGFA- stimulated PGE_2 production and COX-2 expression in LECs were suppressed by IGFBP7 treatment. Thus, IGFBP7 inhibited VEGFA-induced angiogenic molecules. But, the levels of PGE2 in media did not necessarily correlate with COX-2 mRNA expression in the present study. The steady-state level of PGE2 is maintained by interplay between the biosynthetic pathway including COX and PGE2 synthases and the catabolic pathways involving 15-hydroxyprostaglandin dehydrogenase (15-PGDH). 15-PGDH catalyzes the rate-limiting step in the breakdown of PGE2, and 15-PGDH expression has been shown to be affected by PGE2 [39] and several growth factors including EGF (epidermal growth factor) and HGF (hepatocyte growth factor) [40]. IGFBP7 might, therefore, influence the catabolic pathway of PGE2. Furthermore, VEGFA induced $VEGFA_{121}$ mRNA expression and this effect was abolished by IGFBP7 treatment in LECs. Our previous study [15] showed that VEGFA stimulates PGE2 secretion and VEGF mRNA expression in steroidogenic luteal cells. The decrease in $VEGFA_{121}$ expression might be partially due to the reduction of the stimulatory effects of PGE2 secreted from LECs. A detailed examination would be needed to prove our hypothesis that endogenous IGFBP7 produced by LECs suppresses angiogenesis of the cells in an autocrine fashion.

In conclusion, the present study suggests that exogenous IGFBP7 inhibits *in vitro* angiogenesis of LECs, and we hypothesize that IGFBP7 secreted into the CL tissue may inhibit VEGFA-stimulated

angiogenesis in the luteinizing ovary after ovulation.

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