Involvement of insulin-like growth factor-binding protein-related protein 1 in decidualization of human endometrial stromal cells

Masahiko Kutsukake, Ryosuke Ishihara, Mikihiro Yoshie, Hiroshi Kogo and Kazuhiro Tamura

Department of Endocrine Pharmacology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji-shi, Tokyo 192-0392, Japan

1Correspondence address. Tel/Fax: +81-426-76-4536; E-mail: hiro@ps.toyaku.ac.jp

Uterine decidualization is crucial for successful implantation and the establishment of pregnancy. In the present study, the expression of insulin-like growth factor-binding protein (IGFBP)-related protein 1 (IGFBP-rP1) in the human uterus and endometrial stromal cells (ESCs) and its physiological significance in decidualization were examined. IGFBP-rP1 protein was localized in the glandular epithelium and stromal cells, and blood vessels in the endometrium. Cultured stromal cells expressed IGFBP-rP1 and secreted it into the medium. IGFBP-rP1 was localized mostly in the cytoplasm near the nucleus. Knocking down the endogenous IGFBP-rP1 expression in stromal cells, by a small interfering (si)RNA, diminished the expression of prolactin and IGFBP-1 which serve as decidual markers. These results suggest that IGFBP-rP1 may play a role in decidualization of ESCs.

Keywords: IGFBP-rP1; uterus; decidualization; endometrial stromal cells

Introduction

Successful implantation, decidualization and placenta formation are required for the establishment of a normal pregnancy. Ovarian steroids induce cyclic changes in the human endometrium during the menstrual cycle. Endometrial stromal cells (ESCs) differentiate into decidual cells in the mid and the late secretory phases, regardless of whether pregnancy has been established. The decidual tissue restricts excessive trophoblast invasion and provides the basis for placenta formation (King, 2000). Decidual cells start secreting prolactin (PRL) and insulin-like growth factor (IGF)-binding protein (IGFBP)-1 as their differentiation progresses (Tseng and Mazella, 2002). Consequently, enhanced IGFBP-1 and PRL expression is used as a marker of ESC decidualization.

IGF is known to play a role in the reproductive system and is important for the placenta that follows successful implantation and fetal growth (Nayak and Giudice, 2003). It has been suggested that the success of a pregnancy in various species may involve the regulation of IGF activity by IGFBPs (Han et al., 1999; Nayak and Giudice, 2003; Watthes et al., 1998). All IGFBPs (IGFBP-1 to -6) have been found to be expressed in the uterus of humans (Han et al., 1996), with IGFBP-1, -2, -4 and -6 being abundant in decidual cells. In particular, IGFBP-1 is the main IGFBP in human epithelial and decidualized stromal cells and is believed to be associated with stromal decidualization in primates (Irwin et al., 1999).

Recently, the IGFBPs that exhibit a low affinity for IGF have been re-classified as IGFBP-related proteins (IGFBP-rP) (Collet and Candy, 1998). One of these is IGFBP-related protein 1 (IGFBP-rP1), which is also called mac25 or IGFBP-7. The gene encoding this protein was originally identified as a gene that shows reduced mRNA expression in meningioma cell lines as compared with normal cells (Murphy et al., 1993). It shares high homology with the IGFBPs and binds with IGF-I and insulin, but its binding affinity for IGF-I is lower than those of IGFBP-1 to -6 (Yamanaka et al., 1997). Like the other IGFBPs, IGFBP-rP1 was recently found to be expressed in the reproductive tissues of many species, including the bovine corpus luteum (Casey et al., 2004), the porcine ovary (Wandji et al., 2000), and the human uterus (Dominguez et al., 2003; Kim et al., 2000) and testis (Degeorges et al., 2000). Moreover, we have found that IGFBP-rP1 is highly expressed in ESCs during the peri-implantation period of the rat. These observations suggest that IGFBP-rP1 may be involved in endometrial function in rats (Tamura et al., 2004).

Interestingly, it has been demonstrated in humans that IGFBP-rP1 mRNA expression is up-regulated during the mid to late secretory phases and that it may be involved in endometrial receptivity (Dominguez et al., 2003). The finding that IGFBP-rP1 mRNA expression is up-regulated during the mid to late secretory phases is particularly interesting since ESCs differentiate into decidual cells during this period. In this study, we have used an in vitro decidualization model to extend these observations and show that IGFBP-rP1
participates in the decidualization of human ESCs, although the mechanism involved remains unknown.

Materials and Methods

Preparation of ESCs

Endometrial tissues from the proliferative phase were used for stromal cell isolation. Human endometrial tissue samples were obtained from premenopausal women, aged 29–35 years, undergoing a hysterectomy for benign indications. Endometrial samples were diagnosed as being in the proliferative phase by histological examination according to the standard histological criteria (Noyes et al., 1950) and referring to the patients’ menstrual history and serum hormonal levels. Sample collection was conducted with the informed consent of the patients in accordance with the requirements of the Clinical Research Ethics Committee of the Tokyo Medical University Hospital (Shinjuku, Tokyo).

The preparation of ESCs was performed as described previously (Tamura et al., 2006, 2007). Briefly, the minced tissue samples were digested in calcium-and magnesium-free Hank’s solution (CMF-Hanks) containing 0.25% collagenase (Type 1A, Sigma, St. Louis, MO) and then strained through a 38 μm stainless steel sieve (Sanpo, Tokyo) to remove undigested tissue and mucous material. To remove glandular cells, the filtrates were passed through a 38 μm sieve (Sanpo) which stromal cells can pass. The passed solutions were centrifuged and resuspended in a small volume of CMF-Hanks. The ESCs were cultured in DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) containing charcoal-dextran-treated 10% (v/v) fetal bovine serum (10% stripped FBS, Hyclone, South Logan, UT, USA) and antibiotics (100 μg/ml penicillin, 10 μg/ml streptomycin, 100 μg/ml gentamicin and 2.5 μg/ml fungizone; Invitrogen). To induce decidualization, the cells were cultured in 12-well culture plates with DMEM/F-12 supplemented with 10% stripped FBS, Hyclone, South Logan, UT, USA) and antibiotics (100 μg/ml penicillin, 10 μg/ml streptomycin, 100 μg/ml gentamicin and 2.5 μg/ml fungizone; Invitrogen). To induce decidualization, the cells were cultured in 12-well culture plates with DMEM/F-12 medium containing 1% FBS and antibiotics, supplemented with 1 mM Ns, 2′-O-dibutyryl adenosine 3′, 5′-cyclic monophosphate (db-cAMP) or both 1 mM progesterone and 10 nM 17β-estradiol (P/E).

Immunofluorescent staining in tissue sections and cultured ESCs

To examine the immunohistochemical localization of IGFBP-rP1 in endometrial tissues, human tissue arrays (Normal endometrium, n = 59) were purchased from Super Bio Chips Laboratories (Seoul, South Korea). The human tissue array slides specify the phase of the menstrual cycle (proliferative or secretory) for each sample. Tissues were fixed in 4% parafomaldehyde (PFA) before being processed and embedded in paraffin wax following standard procedures (Tamura et al., 2004). De-waxed tissue sections were incubated with PBS containing 0.1% Triton X-100 at room temperature (R/T) for 5 min. The slides were blocked for 2 h with 3% normal goat serum (NGS) in PBS and incubated for 1 h with 5 μg/ml monoclonal anti-human IGFBP-rP1 antibody (R&D Systems Inc., Minneapolis, MN, USA) in PBS containing 1% NGS. As a negative control, the serial sections were incubated with PBS containing 0.1% Tween 20 and 0.1% bovine serum albumin (BSA), the slides were incubated with Alexa Fluor 594-labeled goat anti-mouse IgG (5 μg/ml) in PBS containing 1% NGS. Nuclear counter staining was performed using 300 nM 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen). The slides were mounted and sealed using SlowFade gold antifade reagent (Invitrogen). The difference in IGFBP-rP1 protein expression was evaluated based upon the intensity of the endometrial staining in the proliferative and the secretory phases of the menstrual cycle.

To investigate the expression of IGFBP-rP1 in cultured cells, cells were seeded onto poly-l-lysine-coated cover slips (Asahi Techno Glass Co., Tokyo, Japan) placed in 24-well culture dishes, cultured for 24 h in DMEM/F-12 medium containing 10% stripped FBS without antibiotics and treated for 24 h with or without IGFBP-rP1 siRNA (20 pmol/well). The cells were further incubated for 48 h with db-cAMP or P/E, fixed in 4% PFA, permeabilized with 0.1% Triton X-100 (Lara-Lemus et al. 2006) and then incubated with the monoclonal anti-human IGFBP-rP1 antibody as described above. In order to detect the distribution of IGFBP-rP1 on the surface of endometrial cells, cells that had not been permeabilized with 0.1% Triton X-100 were also processed for immunocytochemistry as described above.

Semi-quantitative RT–PCR and quantitative real-time RT–PCR analysis

Poly(A)⁺ RNA was extracted from cultured ESCs using the QuickPrep micro mRNA purification kit (GE Healthcare UK Limited, Buckinghamshire, UK) and RNA concentration was determined by measuring the A260/A280 absorbance ratio with an Ultraspec 3000 (GE Healthcare).

Quantitative changes in mRNA expression of IGFBP-1, PRL, IGFBP-rP1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were examined by real-time RT–PCR analysis using the iScript™ One-Step RT–PCR kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA). The following specific primers were used: IGFBP-rP1; sense, 5′-GGCCCGAAGAGCC TGAG-3′, antisense, 5′-TCATATTCTCCAGATCCCTCTCA-3′, the product size: 74 bp; IGFBP-1; sense, 5′-AATGTTATTATATCAGCGA CAG-3′, antisense, 5′-GGTACGACCGCCAGAAGT-3′, the product size: 73 bp; PRL; sense, 5′-AAAGGACTGCGATGAGAG-3′, antisense, 5′-GGGTCTGAGGGTCACCTG-3′, the product size: 127 bp; G3PDH, sense, 5′-AGCCACATGCTGCAGACA-3′, antisense, 5′-GCCAATACGCAAATCC-3′, the product size: 66 bp. The PCR involved 40 cycles of 95°C for 10 s, 60°C for 30 s. PCRs were run using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) and iQ5 Optical system software was used to collect the data and calculate the threshold cycle (Ct). The expression of each mRNA was normalized with G3PDH and analyzed by the comparative Ct method.

The levels of IGFBP-rP1, IGFBP-1 and PRL mRNAs were also examined using a One step RNA PCR kit (TaKaRa, Shiga, Japan). 0.1 μg of RNA and the specific primers (IGFBP-rP1; sense, 5′-CATTCTGAATGTCC TGGTGCCCGA-3′, antisense, 5′-GAGGGTTTATAGCTCGCCACCTC C-3′, the product size: 351 bp; IGFBP-1; sense, 5′-TGTTGCGAGAGGCA GGAGGCC-3′, antisense, 5′-AGGGATCTCTTCTTCCATCCA-3′, the product size: 378 bp; PRL; sense, 5′-GACAGAGACACCAAGAAGATG CCAAATA-3′, antisense, 5′-GCCAATGGAAGCTATACAGGACCGT CTC-3′, the product size: 875 bp; G3PDH; sense, 5′-ACACAGTCCCTG CCATCAC-3′, antisense, 5′-TCCACACCTGTTGCTGTGA-3′, the product size: 452 bp). Each PCR involved 28 cycles of 94°C for 45 s, 63°C for 45 s, 72°C for 1 min for IGFBP-rP1 and G3PDH, 94°C for 45 s, 56°C for 45 s, 72°C for 1 min for IGFBP-1 and 94°C for 45 s, 52°C for 45 s, 72°C for 1 min for PRL. The amplification products were separated in a 1.2% agarose gel containing 1 μg/ml ethidium bromide and the PCR products were photographed under UV transillumination.

siRNA transfection

An IGFBP-rP1-specific siRNA was synthesized by Darmaco Inc. (siGEN-OME SMARTpool reagent; Chicago, IL, USA). This siRNA is a mixture of four types of siRNA. Another siRNA was obtained from Nippon EGT Co., Ltd (Toyama, Japan. sense, 5′-GGCACACUGGAAGUCCUdTdT-3′; antisense, 5′-AGUCAUCUCCAGUUGCCUdTdT-3′). Similar results were obtained using these two siRNAs from different companies. The irrelevant siRNA (sc-37007, Santa Cruz biotechnology Inc., CA, USA) served as a control treatment. siRNA transfection (20 pmol) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Stromal cells at 40–50% confluency were treated with the siRNA for 24 h in 24-well culture dishes. Inhibition of IGFBP-rP1 mRNA expression by each siRNA was observed within 24 h and the knock-down was maintained for at least 96 h after the medium was changed (data not shown). Cells were stimulated with db-cAMP or P/E 24 h after the siRNA treatment. Recombinant human IGFBP-rP1 (R&D Systems Inc.) was added to the medium as specified for different experiments.

Western blot analysis

An aliquot of 15 μl of conditioned media and 5 μg of cell lysate from cultured ESCs were separated on 10–20% gradient SDS-polyacrylamide gels (Daiichi Pure Chemical, Tokyo, Japan) and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Immobloktm (Dainippon Sumitomo Pharma Co. Ltd, Tokyo, Japan) and then incubated with monoclonal human anti-IGFBP-rP1 antibody (2 μg/ml, R&D Systems Inc.), or anti-β-actin antibody (1:1000, Sigma-Aldrich, St Louis, MO, USA) in Tris-buffered saline containing 0.1% Tween 20.
(TBST). The membranes were subsequently washed in TBST and incubated with anti-mouse IgG or anti-rabbit IgG antibody conjugated with horse-radish peroxidase (Vector Lab. Inc., Burlingame, CA, USA). The specific bands were detected using Western lighting chemiluminescence reagent (PerkinElmer Inc., Wellesley, MA, USA) and BioMax Light Film (Kodak, Rochester, NY, USA).

**ELISA of IGFBP-rP1 and IGFBP-1**

IGFBP-rP1 levels in the culture medium and cell lysates were measured using a sandwich ELISA composed of a combination of polyclonal anti-IGFBP-rP1 antibodies and monoclonal anti-IGFBP-rP1 antibodies (R&D Systems Inc.) that we have recently developed. The polyclonal anti-IGFBP-rP1 antibody was diluted to 1 μg/ml in PBS and incubated overnight at R/T in a poly-L-lysine-coated 96-well microtiter plate. The wells were washed with PBS containing 0.1% Tween 20 (PBST) and blocked with 3% BSA in PBS at R/T for 1 h. The wells were then washed with PBST and incubated with a sample or a standard recombinant IGFBP-rP1 solution (0.24, 0.97, 3.9, 15.6 and 62.5 ng/ml) diluted with PBS containing 0.1% BSA for 2 h at R/T. The wells were washed with PBST and 1 μg/ml monoclonal anti-IGFBP-rP1 antibody in PBS containing 0.1% BSA was added and the plates were incubated for 2 h at R/T. The plates were washed with PBST and then incubated for 1 h at R/T with 1 μg/ml peroxidase-conjugated goat anti-mouse IgG antibody (Vector Lab. Inc.). After another wash with PBST, 100 μl of substrate reagent (R&D Systems Inc.) was added, and the chromogenic reaction was allowed to proceed for 10 min at R/T. After this, 50 μl of 2 N HCl was added to each well to stop the reaction, and the plate was subsequently read at 450 nm in a microtiter plate reader (SAFIRE, Wako Pure Chemical, Osaka, Japan). The inter- and intra-assay coefficients of variation were 19.9 and 14.0%, respectively. The levels of IGFBP-rP1 for each sample were normalized to cellular DNA content or protein. DNA extraction was performed using the method described by Gross-Belard et al. (1972) and the amount of DNA present was determined using an Ultraspec 3000 (GE Healthcare). The concentration of IGFBP-1 in conditioned medium was measured using the human IGFBP-1 Duoset kit (R&D Systems Inc.) according to the manufacturer’s protocol. The values of IGFBP-1 for each sample were normalized to cellular DNA content.

**Nh2 bioassay for PRL**

PRL concentrations in conditioned media were measured using the Nh2 lymphocyte bioassay as described (Zinger et al., 2003). Briefly, Nh2 cells were maintained in RPMI-1640 medium containing 10% heat-inactivated horse serum, 10% FBS, 5 μM 2-mercaptoethanol and antibiotics. One day before the assay, cells were cultured in a starvation medium (RPMI-1640 medium containing 10% stripped FBS, 1% FBS and 2-mercaptoethanol). Cells were then seeded in 96-well plates (10,000 cells/well) in treatment medium (RPMI-1640 medium containing 10% heat-inactivated horse serum and 2-mercaptoethanol) and incubated for 3 days with either human PRL standard, or with medium aliquots from ESCs (diluted 1:40, in triplicate). Nb2 cell number was determined by the WST-1 assay as above. The amount of PRL was calculated from the standard curve and is expressed as ng PRL/μg cellular DNA/well. To demonstrate that the Nb2 cell proliferation was due to PRL in the conditioned medium, Nb2 cells were co-incubated with anti-rat PRL antibody.

**Statistics**

All experiments were repeated at least twice. The results of the ELISA and realtime RT–PCR analysis are presented as mean ± SD and examined by one-way ANOVA with Fisher's protected least significant difference test. The immunofluorescent staining was analyzed using the Mann–Whitney test. Differences were considered to be significant when P-values were less than 0.05.

**Results**

**Immunolocalization of IGFBP-rP1 in the human endometrium**

Human endometrial tissue array plates were subjected to immunofluorescent staining in order to determine the distribution of IGFBP-rP1 in the human endometrium. The images in Fig. 1A–F are representative data from 59 samples in the array tissue. The negative control was performed on a serial section from A, C and E using mouse IgG instead of anti-IGFBP-rP1 antibody (the primary antibody) and the secondary antibody were used. Alexa594 (conjugated with secondary antibody, red) and DAPI (nuclear counter stain, blue) served as the fluorescent materials. s, stroma; ge, glandular epithelium; le, luminal epithelium; bv, blood vessel. Original magnification ×100 (A–D), ×400 (E and F).

Negative control staining was performed on the serial sections A, C and E, where the mouse IgG instead of anti-IGFBP-rP1 antibody (the primary antibody) and the secondary antibody were used. Alexa594 (conjugated with secondary antibody, red) and DAPI (nuclear counter stain, blue) served as the fluorescent materials. s, stroma; ge, glandular epithelium; le, luminal epithelium; bv, blood vessel. Original magnification ×100 (A–D), ×400 (E and F).

**Figure 1**: Immunolocalization of IGFBP-rP1 in human endometrial tissues

**Endometrial tissues in the proliferative phase** (A and B) and the late secretory phase (C–F) were subjected to immunofluorescence staining. (B, D and F) Intense signals were detected in glandular epithelial cells in the late secretory phase (Fig. 1C and E). Weak signals were detected in stromal cells and blood vessels. In the proliferative phase, weak signals were detected in the stromal cells as well as the glandular and luminal epithelium (Fig. 1A). Thus, immunoreactive signals in glandular epithelial cells in the proliferative phase were weaker than those in the secretory phase. The intensity of staining of all sections in the array was evaluated (Table 1). Strong staining was observed in six tissues in the secretory phase, but there were no sections with strong signals in proliferative tissues. Moderate staining was obtained in nine sections in the secretory phase and in two sections in the proliferative phase. Overall, the intensity of staining in secretory tissues was significantly stronger than that of proliferative tissues.

**Secretion and expression of IGFBP-rP1 in ESCs in vitro**

Since we had confirmed by immunohistochemistry that IGFBP-rP1 protein is expressed by human ESCs (Fig. 1), we examined whether cultured ESCs secrete IGFBP-rP1 and how this secretion changed during in vitro decidualization (Fig. 2). Western blot analysis of the conditioned medium indicated the presence of IGFBP-rP1 protein within 6 h of ESCs incubation and revealed that this protein gradually accumulated over the 24 h incubation period. By quantitative measurement of IGFBP-rP1 after the 24 h incubation period, the medium and cell lysates were found to contain 4.14 ng/μg cultured cellular DNA and 36.8 pg/μg total protein, respectively; this did not change with further incubation time. Furthermore, treatment with the decidual stimulus 1 mM db-cAMP did not significantly alter the secretion or cellular content of IGFBP-rP1 (Fig. 2B).
mRNA inhibited ESC decidualization, but that secreted IGFBP-rP1 in ESC decidualization, we performed an IGFBP-rP1 replacement experiment (conditions b and c in Fig. 3A). IGFBP-rP1 siRNA treatment decreased the db-cAMP-induced mRNA expression of both IGFBP-1 and PRL (Fig. 3B and C). In the replacement experiments, IGFBP-rP1 added to the medium either before (c) or upon (b) db-cAMP treatment affected neither IGFBP-1 nor PRL mRNA expression. Furthermore, the analysis of the protein levels of the conditioned media taken at the end of the experiments revealed that the IGFBP-rP1 siRNA treatment reduced IGFBP-1 levels as well as PRL levels in the media (Fig. 3C). Treatment of recombinant IGFBP-rP1 had no effect on this pattern. These experiments indicated that the knock-down of IGFBP-rP1 mRNA inhibited ESC decidualization, but that secreted IGFBP-rP1 does not appear to play a role in ESC decidualization. These results were confirmed using a second IGFBP-rP1 specific siRNA (data not shown).

### Localizational of IGFBP-rP1 in ESCs

The cellular localization of IGFBP-rP1 was examined in cultured ESCs by immunofluorescent staining. The IGFBP-rP1 signals were localized mostly in the cytoplasm near the nucleus (Fig. 4, control). The levels of intracellular IGFBP-rP1 were reduced by IGFBP-rP1 siRNA treatment. db-cAMP treatment had no effect on the intracellular localization of IGFBP-rP1 (comparison among 0.1% Triton X-100-treated groups).

### Effect of IGFBP-rP1 knock-down on the in vitro decidualization of ESCs

When human ESCs decidualize in response to treatment with P/E or db-cAMP, their expression of PRL and IGFBP-1 mRNA is enhanced (Kasahara et al., 2001). To explore whether stromal IGFBP-rP1 expression is involved in the *in vitro* decidualization of human ESCs, we knocked down IGFBP-rP1 expression in ESCs prior to their decidualization (Fig. 3). To further investigate the role of secreted IGFBP-rP1 in ESC decidualization, we performed an IGFBP-rP1 replacement experiment (conditions b and c in Fig. 3A).

**Effect of IGFBP-rP1 knock-down on the in vitro decidualization induced by physiological decidual stimulus**

Cells treated with IGFBP-rP1 siRNA before treatment with ovarian steroids exhibited markedly decreased mRNA expression of both IGFBP-rP1 and PRL (Fig. 5A). To examine this notion further, exogenous recombinant IGFBP-rP1 was added to the decidualizing ESC cells (Fig. 5B). While P/E enhanced PRL mRNA expression (as observed in Fig. 5A), recombinant IGFBP-rP1 at 100–800 ng/ml did not significantly change PRL mRNA levels in either the absence or presence of P/E.

### Discussion

It has been reported that IGFBP-rP1 is expressed in the human uterus and that its mRNA levels are up-regulated in stromal cells during the receptive phase of the menstrual cycle (Dominguez et al., 2003). However, the role this protein plays in stromal differentiation has not been investigated to date. In this study, we sought to clarify the physiological significance of the IGFBP-rP1, i.e. expressed in human endometrium. In particular, we focussed on the role of IGFBP-rP1 expression in ESCs, in our preliminary observation by semi-quantitative RT–PCR analysis using endometrial tissues, IGFBP-rP1 mRNA expression was detected in the endometrium throughout all stages of the menstrual cycle, but it was expressed at its highest levels during the late secretory stage. This is essentially consistent with the above observations by Dominguez et al. (2003). Immunohistochemical analysis revealed that IGFBP-rP1 protein was significantly expressed in glandular epithelial cells and weakly expressed in stromal cells, especially in the secretory phase. Cultured ESCs secreted a large amount of IGFBP-rP1 into the media as shown in Fig. 2; these levels were similar to those detected when the glandular epithelium cell line (EM-1), which is established by Kyo et al. (2003), was cultured (data not shown). To determine whether IGFBP-rP1 participates in ESC differentiation in the secretory phase, we examined the effect of IGFBP-rP1-specific siRNA and recombinant IGFBP-rP1 in a decidualization model using cultured human ESCs. ESC decidualization can be induced by treatment with db-cAMP or ovarian steroids (Kasahara et al., 2001; Tang et al., 1993), and this differentiation process is associated with increased PRL and IGFBP-1 expression and secretion that serve as decidualization markers. In IGFBP-rP1 siRNA-treated cells, neither decidual marker was fully induced despite treatment with db-cAMP nor ovarian steroids. This suggests that IGFBP-rP1 may be necessary for the initiation of decidualization. We next assessed the effect of replacing IGFBP-rP1 in the medium of siRNA-treated ESCs using excess amounts of recombinant IGFBP-rP1. However, treatment of recombinant IGFBP-rP1 failed to recover the mRNA expression and protein secretion of the decidualization marker to the levels seen in the control siRNA-treated cells. These results suggest that even

| Table 1: Comparison of the intensity of IGFBP-rP1 staining in human endometrial stromal cells during the proliferative and secretory phases |
|-----------------|----------------|----------------|----------------|
|                  | Intensity      |                |                |
| Proliferative    | −              | +              | ++             | +++            |
| Secretory        | 0              | 24             | 2              | 0              |

Immunofluorescence was performed on human endometrium tissue array slides. The tissue array slides contained 26 sections of endometrium in the proliferative phase and 33 in the secretory phase of the menstrual cycle. The intensity of the staining was classified into four degrees; strong (++++), moderate (+++), normal (+) or faint (−). Statistical analysis was performed by the Mann–Whitney test. *P < 0.01 versus proliferative.

**Figure 2: Expression and secretion of IGFBP-rP1 in cultured human endometrial stromal cells during *in vitro* decidualization**

Subconfluent endometrial stromal cells were cultured with (+) or without (−) 1 mM db-cAMP. Samples of the conditioned medium and cell lysate were harvested at the indicated timepoints. (A) The medium (left panel) and cell lysate (right panel) samples were subjected to western blot analysis for detecting IGFBP-rP1. Representative data from five independent experiments were shown. β-Actin was used as a loading control for the cell lysate. (B) The levels of IGFBP-rP1 in the medium and cell lysate samples (n = 3, each) were analyzed by ELISA. The values obtained for IGFBP-rP1 in the medium and those in cell lysates were normalized relative to the contents of cellular DNA and total protein, respectively.
though ESCs secrete relatively high amounts of IGFBP-rP1, secreted IGFBP-rP1 is not involved directly in the decidualization process of stromal cells. Intracellular replacement might recover the progression of decidualization. In this experiment, although significant differences in PRL levels were seen in IGFBP-rP1-treated cells between the db-cAMP-treated group pretreated with IGFBP-rP1 and the db-cAMP only treated group or the db-cAMP plus IGFBP-rP1-treated group, we could not find the reason. Interestingly, much higher expression of IGFBP-rP1 was detected in glandular epithelial cells than stromal cells; the functional significance of this protein in the glandular epithelium was not explored in this study.

Prostate cancer cells that stably overexpress IGFBP-rP1 exhibit characteristics of neuroendocrine differentiation (Wilson et al., 2001). IGFBP-rP1 protein is localized in the nuclei of Hs578T breast or M12 prostate cancer cells and interacts with a novel nuclear protein, NEDF/25.1. It has been suggested that interactions between non-secreted IGFBP-rP1 and NEDF/25.1 in the nucleus may promote the differentiation of prostate cancer cells into neuroendocrine cells (Wilson et al., 2001). However, our study in cultured human ESCs showed that IGFBP-rP1 was localized in the cytoplasm near the nucleus, but not in the nucleus itself. Moreover, since a decidual stimulus such as ovarian steroids did not change the localization of IGFBP-rP1 (data not shown), it appears that IGFBP-rP1 does not translocate from the cytoplasm to the nucleus during the process of decidualization. Results obtained from immunofluorescence staining support the possibility that non-secreted IGFBP-rP1 protein in the cytoplasm may participate in ESC differentiation. This notion is further reinforced by the fact that exogenous IGFBP-rP1 in the

Figure 3: Effect of IGFBP-rP1 knock-down and recombinant IGFBP-rP1 replacement on PRL and IGFBP-1 expression in endometrial stromal cells
(A) Depiction of the cell culture conditions employed. (a) ESCs were treated for 24 h with IGFBP-rP1 siRNA, further cultured for 24 h and stimulated with 1 mM db-cAMP. The cells and the media were collected 48 h later. (b) In addition to the condition of (a), recombinant human IGFBP-rP1 (400 ng/ml) was added to the culture medium with db-cAMP to examine the effect of IGFBP-rP1 replacement on ESC decidualization. (c) IGFBP-rP1 (400 ng/ml) was added to the culture medium after 24 h incubation of IGFBP-rP1 siRNA. (B and C) ESCs were cultured under the three conditions (a–c) as shown in (A). (B) The mRNA levels of IGFBP-1 and PRL analyzed by quantitative real-time RT-PCR. Each value is the mean ± SD from three cultures in representative experiments. Similar results were obtained in three independent experiments. cont., no db-cAMP treatment. *P < 0.01 versus control siRNA. (C) The levels of IGFBP-1 and PRL secreted in the medium. IGFBP-1 and PRL levels were assayed by ELISA and Nb2 bioassay, respectively. Each value is the mean ± SD from three cultures in representative experiments. cont., no db-cAMP treatment. *P < 0.01 versus control siRNA.
It also failed to recover the depressed expression of the decidual markers that were induced by IGFBP-rP1 siRNA. We confirmed that exogenous IGFBP-rP1 was not internalized into ESCs (data not shown). These observations also suggest that intracellular IGFBP-rP1, rather than secreted IGFBP-rP1, is important for stromal decidualization in humans. What is the role of this intracellular IGFBP-rP1 in ESCs? Superoxide dismutase (SOD) has been shown to be associated with decidualization in humans (Kajihara et al., 2006; Sugino et al., 2007). The blockage of manganese (Mn)-SOD activity causes oxidative stress-induced cell death in human ESCs, indicating that Mn-SOD protects ESCs from oxidative stress by scavenging superoxide radicals generated in the mitochondria. Plymate et al. (2003) have identified Mn-SOD as one of the downstream mediators that are responsible for IGFBP-rP1-induced suppression of tumor growth. Accordingly, IGFBP-rP1 might regulate Mn-SOD activity to modulate ESCs decidualization. IGFBP-rP1 binds to vascular endothelial cell growth factor (VEGF) (Usui et al., 2002) and CXCL10/IP10 (IP-10) (Nagakubo et al., 2003) which regulate angiogenesis. The IGFBP-rP1 signal was found in blood vessels of the endometrium in the secretory phase in the present study. IGFBP-rP1, which is secreted from blood vessels and/or glandular epithelial cells, might modulate VEGF- or IP-10-stimulated vascular formation by binding with these factors in the endometrium in an autocrine or paracrine manner.

In our previous study, IGFBP-rP1 mRNA was increased in ESCs close to the smooth muscle of the inter-implantation site during the time of implantation in rats (Tamura et al., 2004). When ESCs prepared from rat uteri in early pregnancy are treated with exogenous IGFBP-rP1, the cells are arrested in the G1 phase of the cell cycle. Moreover, exogenous IGFBP-rP1 treatment of rat myometrial preparations increases their prostacyclin secretion. Thus, IGFBP-rP1 clearly affects the uterine cell proliferation. IGFBP-rP1 protein secreted from ESCs probably acts directly on rat endometrial cells to change their characterization. The observations in rat ESCs contrast with those described in human ESCs. For example, exogenous IGFBP-rP1 does not interfere with the proliferation of human ESCs, but has been reported to interfere with the proliferation of rat ESCs. These observations led us to propose that secreted IGFBP-rP1 is more important to generate a proper uterine environment for establishing the decidualization in rats than in humans.

In conclusion, the current study has shown that IGFBP-rP1 is expressed in the endometrial stroma as well as in glandular epithelium during the secretory phase of the menstrual cycle. IGFBP-rP1 plays an important role in decidualization as its normal progression was blocked by the knock-down of intracellular IGFBP-rP1 in an in vitro model. These results suggest that the intracellular IGFBP-rP1 in ESCs may be involved in endometrial stromal differentiation.

Acknowledgements
The authors gratefully thank Prof. K. Isaka (Tokyo Medical University) for tissue collection.

Funding
This work was partially supported by a Grant-in-Aid for Scientific Research (16591689) to K.T. from the Japan Society for the Promotion of Science and the High-Tech Research Center Project for Private Universities: Ministry of Education, Culture, Sport, Science and Technology.
References


Submitted on June 21, 2007; accepted on August 3, 2007