ZOOLOGICAL SCIENCE **26**: 131–138 (2009) © 2009 Zoological Society of Japan

# Estradiol, Progesterone, and Transforming Growth Factor α Regulate Insulin-Like Growth Factor Binding Protein-3 (IGFBP3) Expression in Mouse Endometrial Cells

**Tetsuya Maekawa, Sakae Takeuchi, Munetoshi Kanayama and Sumio Takahashi***

*Department of Biology, Graduate School of Natural Science and Technology, Okayama University, Tsushima, Okayama 700-8530, Japan*

**Insulin-like growth factor 1 (IGF1) is involved in the proliferation of mouse and rat endometrial cells in a paracrine or autocrine manner. Insulin-like growth factor binding protein-3 (IGFBP3) modulates actions of IGFs directly or indirectly. The present study aimed to determine whether IGFBP3 is involved in the regulation of proliferation of mouse endometrial cells. Mouse endometrial epithelial cells and stromal cells were isolated, and cultured in a serum free medium. IGF1 stimulated DNA synthesis by endometrial epithelial and stromal cells, and IGFBP3 inhibited IGF1-induced DNA synthesis. Estradiol-17β (E2) decreased the *Igfbp3* mRNA level in endometrial stromal cells, whereas it increased the *Igf1* mRNA level. Transforming growth factor α (TGFα) significantly decreased IGFBP3 expression at both the mRNA and secreted protein levels in endometrial stromal cells. Progesterone (P4) did not affect the E2-induced down-regulation of *Igfbp3* mRNA expression in endometrial stromal cells, although P4 alone increased *Igfbp3* mRNA levels. The present findings suggest that in mouse endometrial stromal cells E2 enhances IGF1 action through enhancement of IGF1 synthesis and reduction of IGFBP3 synthesis, and that TGFα affects IGF1 actions through modulation of IGFBP3 levels.**

**Key words:** IGFBP3, IGF1, estrogen, mouse, uterus

## INTRODUCTION

The mammalian endometrium consists of epithelial and stromal cells. The proliferation of endometrial cells varies with cyclic changes of ovarian steroid hormone levels during the estrous cycle, and is controlled by ovarian steroid hormones. Estrogen stimulates the proliferation of endometrial epithelial cells, while simultaneous treatment with estrogen and progestin stimulates the proliferation of uterine stromal cells in adult mice (Huet-Hudson et al., 1989). The steroid hormone-induced proliferation of endometrial cells is thought to be mediated by growth factors produced in endometrial stromal cells (Tomooka et al., 1986; DiAugustine et al., 1988; Beck and Garner, 1992; Das et al., 1994; Cooke et al., 1997). Insulin-like growth factor 1 (IGF1) is expressed in all components of the mouse uterus and its expression is regulated by estrogen (Murphy et al., 1987; Ghahary et al., 1990; Kapur et al., 1992; Ohtsuki et al., 2007). Moreover, IGF1 promotes the proliferation of endometrial cells (Shiraga et al., 1997; Sato et al., 2002; Inoue et al., 2005; Zhu et al., 2007). These findings suggest that IGF1 is one of factors involved in the regulation of endometrial cell proliferation.

The insulin-like growth factor binding protein (IGFBP) family is composed of six subtypes, of which IGFBP3 is known to be the most abundantly expressed in many tissues. IGFBP3 modulates actions of IGF1 by forming an IGF1-IGFBP3 complex, resulting in the blockage of IGF1 actions or the prolongation of the half-life of IGF1 (Hwa et al., 1999; Duan and Xu, 2005). IGFBP3 also directly inhibited cell proliferation by binding to an IGFBP3 receptor in an IGF-independent signaling pathway (Mochizuki et al., 2006). Thus, IGFBP3 is likely to modulate actions of IGFs directly and indirectly in various tissues. The IGFBP3 receptor has been identified as a low-density lipoprotein receptor-related protein 1 (LRP1), which is also called transforming growth factor-β receptor type V (Leal et al., 1997; Danielpour and Song, 2006). *Lrp1* mRNA expression in the mouse uterus was studied to clarify the involvement of IGFBP3 receptors on the regulation of IGFBP3 actions within the endometrium.

IGFBP3 is expressed in the mouse uterus, and expression levels change during estrous cycle (Girvigian et al., 1994; Molnar and Murphy, 1994; Tang et al., 1994). These findings suggest that uterine IGFBP3 production is partly regulated by ovarian steroid hormones. The present study was aimed at clarifying the role of IGFBP3 in the proliferation of endometrial cells and the effect of estradiol-17β (E2) on IGFBP3 production in endometrial cells. Primary cultures

* Corresponding author. Phone: +81-86-251-7866;
Fax : +81-86-251-7876;
E-mail: stakaha@cc.okayama-u.ac.jp
doi:10.2108/zsj.26.131

of mouse endometrial epithelial and stromal cells were used in the present study.

In recent studies, epidermal growth factor (EGF) suppressed IGFBP3 expression in human cervical epithelial cells and esophageal epithelial cells (Hembree et al., 1994; Takaoka et al., 2006). Furthermore, excess expression of heparin-binding epidermal growth factor (HB-EGF) lowered IGFBP3 expression (Provenzano et al., 2005). These findings suggest that the production of IGFBP3 is partly regulated by members of the EGF family. Transforming growth factor α (TGFα), a member of the EGF family, is detected in the mouse uterus during the peri-implantation period (Tamada et al., 1991). In addition, TGFα expression is regulated by estrogen (Nelson et al., 1992). These observations led to the idea that TGFα is involved in the regulation of IGFBP3 expression in the mouse uterus. In the present study, we investigated the effect of TGFα on IGFBP3 expression in endometrial cells.

## MATERIALS AND METHODS

### Animals

Immature (21 to 23 days old) and adult (8 weeks old) female mice of the ICR strain were used in the present study. They were housed in a temperature-controlled animal room and were fed a commercial diet of CA-1 (CLEA Japan, Osaka) and tap water. All animal care and experiments were conducted in accordance with the Guidelines for Animal Experimentation of Okayama University, Japan.

### Cell culture

Uterine epithelial and stromal cells were isolated according to methods used in previous studies (Ross et al., 1993; Shiraga et al., 1997; Komatsu et al., 2003). Isolated uterine epithelial cells were seeded on collagen-coated culture wells at a density of about $5 \times 10^4$ cells/cm$^2$. Isolated stromal cells separated from epithelial cells were seeded on poly-L-lysine coated culture wells at a density of about $6 \times 10^4$ cells/cm$^2$. Stromal cells were first cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium without phenol red (DME/F12, Sigma Aldrich, St. Louis, MO, USA) containing 2% dextran-coated charcoal-treated fetal bovine serum. After a 1 day pre-culture, the cells were cultured in serum-free DME/F12 supplemented with BSA (1 g/l), hydrocortisone (100 μg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathormone (200 ng/l), sodium selenite (5 μg/l), and insulin (100 μg/l). The plates were incubated at 37°C in an atmosphere of 5% $CO_2$, and the medium was changed every 2 days.

E2 and progesterone (P4) (Sigma-Aldrich) were initially dissolved in ethanol to yield 10 μM stock solutions (0.03% and 0.01% ethanol, respectively). Working solutions of E2 and P4 were made by diluting the stock solutions with culture medium. AG1478 (Biomol International, Plymouth Meeting, PA, USA) was initially dissolved in dimethyl sulfoxide (DMSO) (10 μM; 0.01% DMSO) and later diluted with culture medium.

### Determination of DNA synthesis

DNA synthesis was assayed by immunochemical detection of 5'-bromo-2'-deoxyuridine (BrdU) incorporation into the cell nucleus by using a Cell Proliferation ELISA, BrdU Kit (Roche Diagnostics, Mannheim, Germany). Cultured cells were treated with 100 ng/ml recombinant human IGF1 (Amersham Pharmacia Biotech, Uppsala, Sweden) with or without recombinant mouse IGFBP3 (R&D Systems, Minneapolis, MN, USA) for 16 or 48 hr. These cells were exposed to 100 μM BrdU for the last 4 hr. The cells were then fixed and incubated with a peroxidase-conjugated anti-BrdU monoclonal antibody from mouse-mouse hybrid cells (Anti-BrdU-POD). After washing, tetramethyl-benzidine was added as a substrate. The peroxidase reaction was stopped with 0.2 M sulfuric acid, and the absorbance at 450 nm was measured with a micro-plate reader (Bio-Rad, Hercules, CA, USA).

### cDNA synthesis and PCR amplification

Total RNA was prepared from cultured endometrial cells and the whole uterus by using a single-step method (Chomczynski and Sacchi, 1987), and was transcribed by using Thermoscript Reverse Transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions. Primers purchased from Invitrogen are listed in Table 1. PCR was performed with Takara Taq (Takara Bio, Otsu, Japan) and a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Branchburg, NJ, USA). PCR conditions were: 20 sec at 94°C; an appropriate number of cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec; and 10 min at 72°C. A 10-μl aliquot of each reaction was electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet rays. Signal intensities were measured with the Kodak 1D Imaging System (Eastman Kodak, CT, USA). The amount of each target mRNA was normalized to the amount of ribosomal protein L19 (*Rpl19*) mRNA.

**Table 1.** Sequences of primers used in RT-PCR.

| Name | Sequence | Length (bp) |
|---|---|---|
| *Igf1* | | |
| Forward | GTCGTCTTCACACCTCTTCTACCT | 135 |
| Reverse | TAAAAGCCCCTCGGTCCACACAC | |
| *Igf1r* | | |
| Forward | TTCTTCTATGTCCCCGCCAAA | 354 |
| Reverse | AGCCTCGTTTACCGTCTTGAT | |
| *Igfbp3* | | |
| Forward | TAAGCACCTACCTCCCCTCCCAACCT | 346 |
| Reverse | TTTGGGATGTGGACGCCTCTGGGACT | |
| *Lrp1* | | |
| Forward | GGGGACAATGACTGCCAAGAC | 415 |
| Reverse | CATCACACTCTTCCTTGGGCT | |
| *Tgfa* | | |
| Forward | AGCCAGAAGAAGCAAGCCATCACT | 502 |
| Reverse | CTCATTCTCGGTGTGGGTTAGCAA | |
| *Rpl19* | | |
| Forward | GAAATCGCCAATGCCAACTC | 406 |
| Reverse | TCTTAGACCTGCGAGCCTCA | |

### Western blot analysis of IGFBP3

Culture media (400 μl) were concentrated by lyophilization. The concentrated media were subjected to 12.5% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked for 1 hr with 1% bovine serum albumin (BSA) dissolved in Tris-buffered saline containing 0.1% Tween-20, and were then incubated sequentially with goat anti-mouse IGFBP3 antibody (1:1,000, R&D Systems, MN, USA) and horseradish peroxidase conjugated anti-goat secondary antibody (1:5,000, Santa Cruz Biotechnology, CA, USA). Peroxidase activity was detected by using luminol (Sigma Aldrich) and a hydrogen peroxide reaction. Recombinant mouse IGFBP3 (R&D Systems) was used as a positive control.

### Statistical analysis

Data were expressed as the mean±SEM. Differences among groups were analyzed by analysis of variance followed by Tukey's test.

## RESULTS

### *Igfbp3, Igf1*, and *Igf1r* mRNA levels in the mouse uterus during the estrous cycle

*Igfbp3*, *Igf1*, and *Igf1r* mRNA levels in the mouse uterus were analyzed at 24-hr intervals during the estrous cycle by using semi-quantitative RT-PCR. *Igfbp3* and *Igf1r* mRNA levels were highest at diestrus 1 during the 4-day estrous cycle. In contrast, *Igf1* mRNA levels were highest at proestrus (Fig. 1).

### IGFBP3 inhibited IGF1-induced DNA synthesis in endometrial cells

Endometrial epithelial and stromal cells were treated with recombinant mouse IGFBP3 with or without IGF1 (100 ng/ml) to study effect of IGFBP3 on DNA synthesis. Based on a preliminary study, we measured DNA synthesis by detecting BrdU uptake 48 hr after IGF1/IGFBP3 treatment in endometrial epithelial cells, or 16 hr in stromal cells (Shiraga et al., 1997; Inoue et al., 2005). IGF1 stimulated DNA synthesis in both endometrial epithelial and stromal cells, and IGFBP3 inhibited this stimulatory effect of IGF1 in a dose-dependent manner (Fig. 2).

### E2 and TGFα suppressed IGFBP3 expression in endometrial stromal cells

*Igf1*, *Igfbp3*, *Igf1r*, and *Lrp1* mRNA levels in endometrial stromal cells were analyzed by using semi-quantitative RT-PCR. E2 treatment increased *Igf1* mRNA levels after 24 hr, whereas I*gfbp3* mRNA levels decreased. E2 had no effect on *Lrp1* or *Igf1r* mRNA levels (Fig. 3).

The effect of TGFα on *Igfbp3* mRNA levels in endometrial cells were studied, because *Tgfa* and *Egfr* mRNAs are both expressed in mouse endometrial cells, and EGF family growth factors are involved in IGFBP3 production in various tissues and cells. E2 significantly stimulated *Tgfa* mRNA expression in endometrial stromal cells (Fig. 4A). In endometrial stromal cells, TGFα reduced *Igfbp3* mRNA expression in a dose-dependent manner, but did not affect *Lrp1* mRNA levels (Fig. 4B).

Endometrial stromal cells were treated with E2 or TGFα, and the cellular *Igfbp3* mRNA levels (Fig. 5A) and IGFBP3 protein levels in the culture media were analyzed by western blotting (Fig. 5B). Recombinant mouse IGFBP3 was used as a positive control, and two immunoreactive bands of 35 kDa

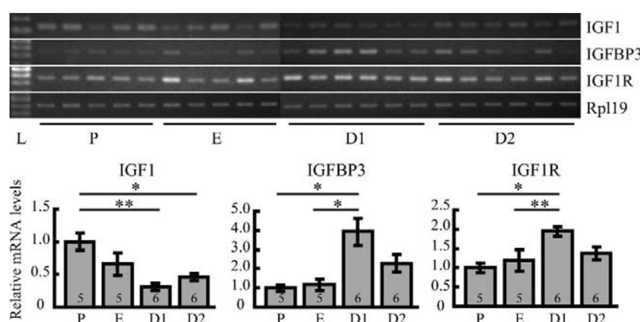



**Fig. 1.** Expression of *Igfbp3*, *Igf1*, and *Igf1r* mRNA in the uterus during the estrous cycle. Uteri were obtained from adult female mice at each stage of the estrous cycle. The upper panel shows ethidium bromide-stained electrophoretic patterns of PCR products. Band intensities were measured, and normalized according to the *Rpl19* mRNA levels. L, 100-bp DNA ladder; P, proestrus; E, estrus; D1, 1st day of diestrus; D2, 2nd day of diestrus. In the histograms, each column represents a mean±SEM. The data are shown as relative values compared to that at proestrus. The number in each column indicates the number mice used. *, p<0.01; **, p<0.05, significantly different between stages of the estrous cycle.

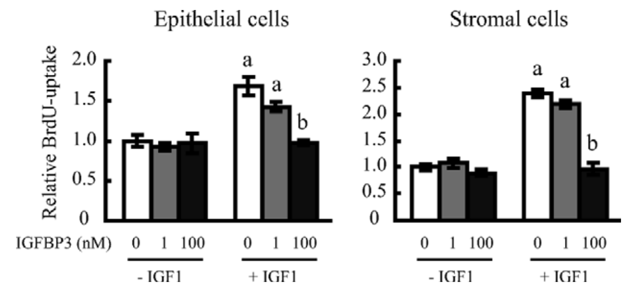
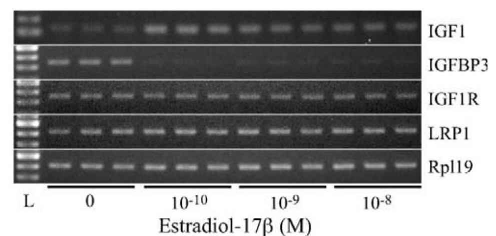



**Fig. 2.** Effect of IGFBP3 on DNA synthesis in cultured endometrial cells. Endometrial cells were treated with IGFBP3 (0, 10, 100 mM) in the presence (100 ng/ml) or absence of IGF1 for 48 hr (epithelial cells) or 16 hr (stromal cells). BrdU (100 µM) was added for the last 4 hr of IGF1 treatment. BrdU incorporation into the nucleus was analyzed as DNA synthesis. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. a, p<0.05, significantly different from corresponding control (–IGF1); b, p<0.05, significantly different from corresponding IGF1-treated group (+IGF1).



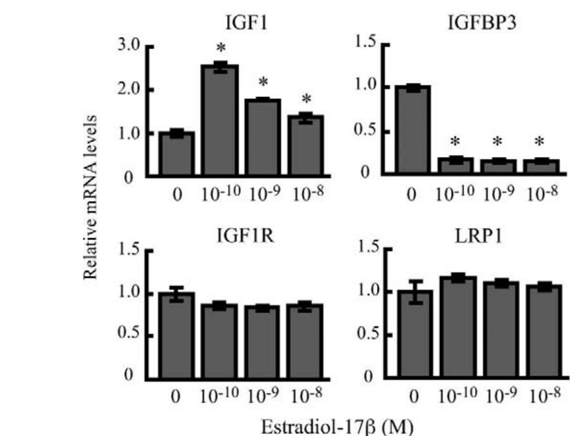




**Fig. 3.** Effect of E2 on *Igf1*, *Igfbp3*, *Igf1r*, and *Lrp1* mRNA expression in endometrial stromal cells. *Igf1*, *Igfbp3*, *Igf1r*, and *Lrp1* mRNA levels were analyzed by semi-quantitative RT-PCR. Endometrial stromal cells were treated with E2 (0, $10^{-10}$, $10^{-9}$, $10^{-8}$ M) for 24 hr. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. L, 100-bp DNA ladder; *, p<0.05, significantly different from control.

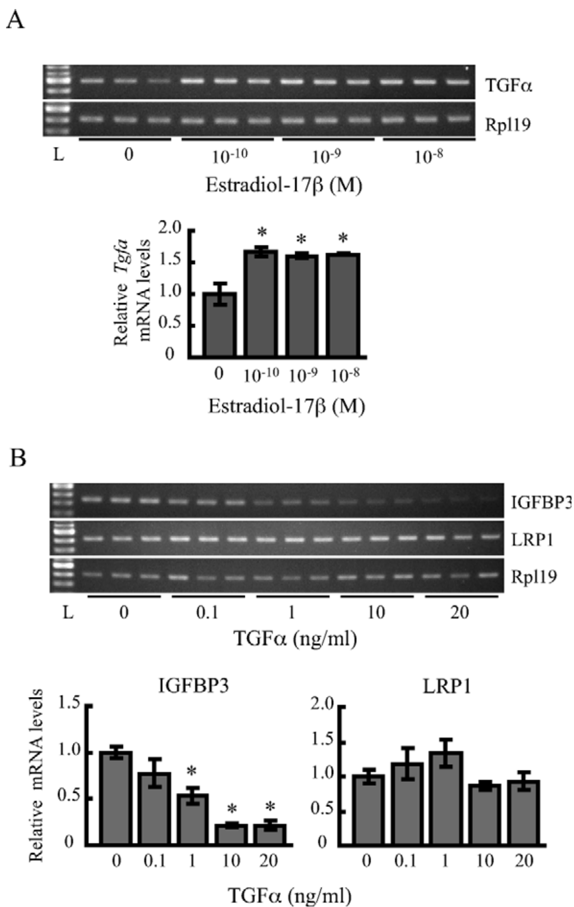



**Fig. 4.** *Tgfa* mRNA expression, and effects of TGFα on *Igfbp3* and *Lrp1* mRNA expression in endometrial stromal cells. **(A)** The effect of E2 on *Tgfa* mRNA expression was analyzed by semi-quantitative RT-PCR. Endometrial stromal cells were treated with E2 (0, $10^{-10}$, $10^{-9}$, $10^{-8}$ M) for 24 hr. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. *, p<0.05, significantly different from corresponding control; L, 100-bp DNA ladder. **(B)** The effect of TGFα on *Igfbp3* and *Lrp1* mRNA expressions was analyzed by semi-quantitative RT-PCR. Endometrial stromal cells were treated with TGFα (0, 0.1, 1, 10, 20 ng/ml) for 24 hr. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. *, p<0.05, significantly different from corresponding control.

and 40 kDa were detected. In the culture media of endometrial stromal cells, two immunoreactive bands were also detected (37 and 42 kDa). After 48 hr, both E2 and TGFα decreased the *Igfbp3* mRNA and secreted IGFBP3 protein levels.

### AG1478 inhibited the TGFα effect on *Igfbp3* mRNA expression, but not the effect of E2

Endometrial stromal cells were treated with AG1478, an inhibitor of the tyrosine kinase of the EGFR, in the presence of TGFα or E2, to examine the TGFα signaling pathway involving EGFR. AG1478 nullified the inhibitory effect of TGFα on *Igfbp3* mRNA expression, but did not restore the E2-induced suppression of *Igfbp3* mRNA expression (Fig. 6).

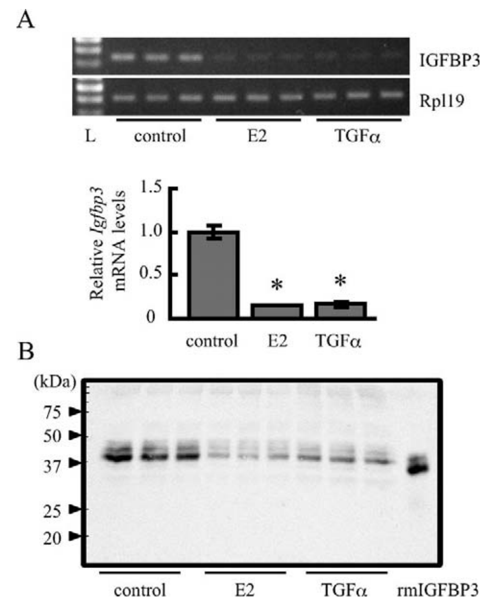



**Fig. 5.** Effects of E2 and TGFα on *Igfbp3* mRNA and IGFBP3 expression in endometrial stromal cells. Endometrial stromal cells were treated with E2 ($10^{-9}$ M) or TGFα (10 ng/ml). At 48 hr, conditioned media (400 μl) were harvested and lyophilized. **(A)** *Igfbp3* mRNA levels were analyzed by semi-quantitative RT-PCR. L, 100-bp DNA ladder. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. *, p<0.05, significantly different from control. **(B)** IGFBP3 expression was analyzed by western blotting using anti-mouse IGFBP3 antibody. Extracts of concentrated media of endometrial stromal cells (400 μl) and rmIGFBP3 (15 ng) were subjected to SDS-PAGE in a 12.5% gel under reducing conditions; rmIGFBP3 served as a positive control. The positions of molecular weight markers are shown on the left.

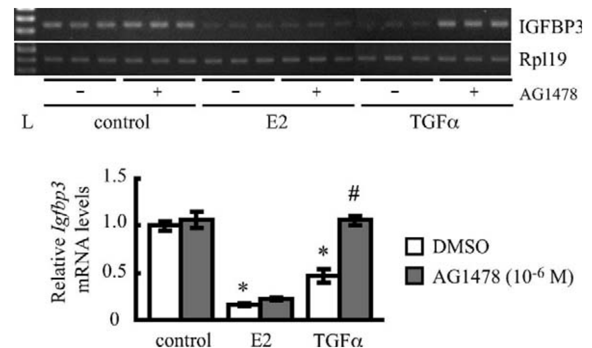



**Fig. 6.** Effect of AG1478 on *Igfbp3* mRNA expression in endometrial stromal cells. Endometrial stromal cells were treated with AG1478 ($10^{-6}$ M) in the presence of E2 ($10^{-9}$ M) or TGFα (10 ng/ml) for 24 hr. *Igfbp3* mRNA levels were analyzed by semi-quantitative RT-PCR. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. L, 100-bp DNA ladder; *, p<0.05, significantly different from corresponding control of vehicle (DMSO) treatment; #, p<0.05, significantly different from DMSO treatment.

### *Igfbp3* mRNA expression was not decreased by E2 or TGFα in endometrial epithelial cells

Endometrial epithelial cells were treated with E2 or TGFα, and their cellular *Igfbp3* mRNA levels were analyzed after 24 hr. E2 increased *Igfbp3* mRNA in a dose-dependent

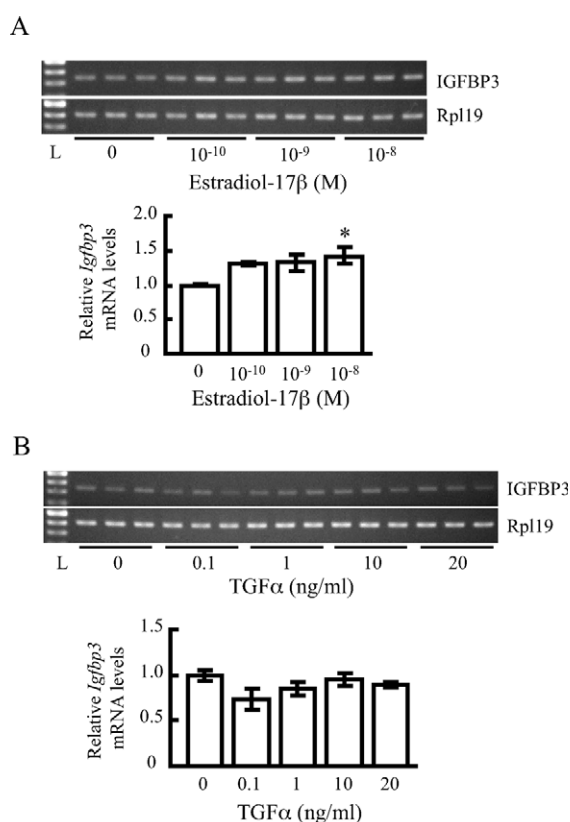



**Fig. 7.** Effects of **(A)** Estradiol-17β (E2) and **(B)** TGFα on *Igfbp3* mRNA expression in endometrial epithelial cells. Endometrial epithelial cells were treated with E2 (0, $10^{-10}$, $10^{-9}$, $10^{-8}$ M) or TGFα (0, 0.1, 1, 10, 20 ng/ml) for 24 hr. *Igfbp3* mRNA levels were analyzed by semi-quantitative RT-PCR. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. L, 100-bp DNA ladder; *, p<0.05, significantly different from control.

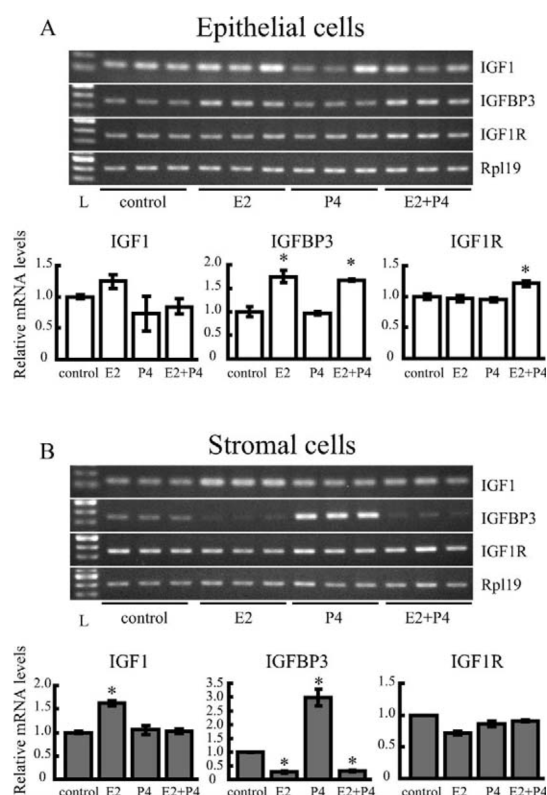



**Fig. 8.** Effects of E2 and/or P4 on *Igf1*, *Igfbp3*, and *Igf1r* mRNA expression in endometrial cells. Endometrial epithelial cells **(A)** and stromal cells **(B)** were treated with E2 ($10^{-9}$ M) and/or P4 ($10^{-7}$ M) for 24 hr *Igf1*, *Igfbp3*, and *Igf1r* mRNA levels were analyzed by semi-quantitative RT-PCR. Each column represents the mean±SEM of triplicate wells. Experiments were replicated three times. L, 100-bp DNA ladder; *, p<0.05, significantly different from control.

manner (Fig. 7A), but TGFα had no effect on *Igfbp3* mRNA expression (Fig. 7B).

### P4 increased *Igfbp3* mRNA levels in endometrial stromal cells, but not in endometrial epithelial cells

Endometrial epithelial and stromal cells were treated with E2 ($10^{-9}$ M) and/or P4 ($10^{-7}$ M), and harvested 24 hr after the treatment to study the involvement of P4 in *Igfbp3* mRNA expression. In endometrial epithelial cells, P4 increased *Igfbp3* and *Igf1r* mRNA levels in the presence of E2, and E2 alone increased *Igfbp3* mRNA levels, as already shown (Fig. 8A). In endometrial stromal cells, P4 increased *Igfbp3* mRNA levels. E2 increased *Igf1* mRNA levels and decreased *Igfbp3* mRNA levels, but P4 did not nullify the E2-induced decrease in *Igfbp3* mRNA levels (Fig. 8B).

## DISCUSSION

IGFBPs are known to modulate actions of IGF1 and IGF2 in various tissues, and IGFBP3 has been widely shown to inhibit cell proliferation (Huynh et al., 2002; Mishra and Murphy, 2004; Strange et al., 2004; Duan and Xu, 2005). The present study demonstrated, for the first time, that IGFBP3 exerts an inhibitory effect on the proliferation of mouse endometrial cells in the presence of IGF1. IGF1 stimulates DNA synthesis in endometrial epithelial and stromal cells (Shiraga et al., 1997; Sato et al., 2002; Inoue et al., 2005; Zhu et al., 2007). IGFBP3 treatment nullified this stimulatory effect of IGF1 on endometrial cells, but IGFBP3 alone did not affect DNA synthesis in endometrial stromal cells. Although the binding of IGFBP3 to IGF1 in our culture system for mouse endometrial cells was not ascertained in the present study, IGFBP3 released from endometrial cells probably modulates IGF1 actions in an autocrine or paracrine manner.

The anti-proliferative effects of IGFBPs, including IGFBP3, occur through IGF-independent mechanisms as well as IGF-dependent ones (Huang et al., 2003; Cavaille et al., 2006; Mochizuki et al., 2006). *Lrp1* mRNA was detected in endometrial epithelial and stromal cells, but IGFBP3 alone did not affect DNA synthesis in the endometrial cells. These results suggest that the IGF-independent anti-proliferative action of IGFBP3 did not function, at least in our culture system for mouse endometrial cells. It is not clear what actions of IGFBP3 are regulated through Lrp1, the IGFBP3 receptor expressed in endometrial cells.

E2 decreased IGFBP3 expression in endometrial stromal cells, which is consistent with previous studies of uteri in several species (Molnar and Murphy, 1994;

Kleinman et al., 1995; Liu et al., 1997; Peterson et al., 1998). The E2-induced decrease in *Igfbp3* mRNA levels was inhibited by pure antiestrogen ICI182,780 (data not shown). The mechanism by which estrogen regulates Igfbp3 expression is not clear. The promoter of rat *Igfbp3* has been characterized, and contains two contiguous sequences showing 80% similarity with the consensus estrogen response element (ERE) (Albiston et al., 1995). The 5'-flanking region of mouse *Igfbp3*, the putative promoter, also contains an ERE-like sequence. Functional analysis of the promoter of mouse *Igfbp3* is needed.

*Igf1* mRNA levels changed during the estrous cycle, which was consistent with our previous report (Ohtsuki et al., 2007). Estrogen stimulates *Igf1* transcription (Murphy et al., 1987; Ghahary et al., 1990; Kapur et al., 1992; Zhou et al., 1994; Ohtsuki et al., 2005), and high levels of circulating estrogen at proestrus induced high *Igf1* mRNA levels. *Igf1r* mRNA levels also changed during the estrous cycle; they were high at diestrus and low during proestrus and estrus, suggesting that the responsiveness of endometrial cells to IGF1 depends upon the stage of the estrous cycle, and possibly upon levels of estrogen and progestin. Since estrogen induced *Igf1r* mRNA expression only in endometrial epithelial cells, *Igf1r* mRNA expression may be regulated in tissue-specific manner.

*Igfbp3* mRNA levels were highest at diestrus and low during proestrus and estrus. This reduction in *Igfbp3* mRNA levels may have been caused by high circulating estrogen levels, since estrogen decreased *Igfbp3* mRNA levels in endometrial stromal cells. Importantly, the change in *Igfbp3* mRNA levels during the estrous cycle was in a pattern opposite to that of *Igf1* mRNA expression. IGF1 is regarded as one of the growth factors controlling the estrogen-induced proliferation of endometrial cells. IGF1 has been shown to stimulate DNA replication in both endometrial epithelial and stromal cells (Shiraga et al., 1997; Inoue et al., 2005). In the present study, the mitogenic action of IGF1 was depressed by high levels of IGFBP3. Therefore, estrogen probably stimulates IGF1 action in the mouse endometrium by stimulating *Igf1* transcription and inhibiting *Igfbp3* transcription. The correlation between the expression of *Igf1* and *Igfbp3* and the proliferation of endometrial cells during the estrous cycle remains to be studied.

Progestin is essential for the initiation of the cell cycle in endometrial stromal cells. P4 stimulates the proliferation of endometrial stromal cells in the presence of estrogen (Komatsu et al., 2003), although estrogen alone does not stimulate proliferation (Quarmby and Korach, 1984). P4 alone increased *Igfbp3* mRNA expression in endometrial stromal cells, while P4 had no effect on *Igfbp3* mRNA expression in endometrial epithelial cells. Furthermore, P4 did not affect the E2-induced down-regulation of IGFBP3 expression. These findings correspond well to in-vivo data showing that *Igfbp3* mRNA levels are high at diestrus 1 and 2, during which circulating levels of P4 are also high (Smith et al., 1975). Circulating levels of estrogen and progestin are higher at estrus. High levels of estrogen will reduce the effect of progestin on IGFBP3 expression. However, circulating levels of estrogen decrease from proestrus, and progestin is dominant during late estrus and diestrus. Therefore, progestin stimulates IGFBP3 production from endometrial stromal cells at diestrus.

*Igfbp3* mRNA was detected by in situ hybridization in the endometrial stromal cells juxtaposed to endometrial epithelial cells in the rat uterus, but was not detected in the endometrial epithelial cells (Girvigian et al., 1994). *Igfbp4* mRNA was detected in endometrial luminal epithelial cells. Interestingly, estrogen increased *Igfbp3* mRNA expression in endometrial epithelial cells, indicating that estrogen differentially regulates *Igfbp3* transcription in a tissue-specific manner.

The roles of IGFBP3, produced in endometrial epithelial cells, are not clear. The proliferation of endometrial epithelial cells was mainly regulated by growth factors produced in endometrial stromal cells, and levels of IGFBP3 secreted from endometrial epithelial cells may be low compared to those of endometrial stromal cells, even if estrogen stimulated *Igfbp3* transcription in the epithelial cells. Hence, epithelial IGFBP3 may not be involved in the estrogen-induced proliferation of endometrial epithelial cells.

We previously showed that TGFα acts as a mitogenic factor in endometrial stromal cells (Komatsu et al., 2003). In the present study, we demonstrated for the first time that TGFα decreases IGFBP3 expression in mouse endometrial stromal cells. Members of the EGF family, such as EGF and HB-EGF, have been shown to reduce IGFBP3 expression in various cell types (Hembree et al., 1994; Wraight and Werther, 1995; Provenzano et al., 2005; Takaoka et al., 2006). In contrast, TGFα stimulates IGFBP3 synthesis in human mammary epithelial cells (Sivaprasad et al., 2004). Thus, TGFα exhibits opposite effects on the modulation of IGFBP3 expression, according to cell type.

In mouse endometrial stromal cells, TGFα induced inhibition of *Igfbp3* mRNA expression by way of EGFR signaling, because AG1478 blocked TGFα inhibition of *Igbp3* mRNA expression. In contrast, the E2-induced decrease in *Igfbp3* mRNA levels in endometrial stromal cells was not recovered by AG1478, indicating that EGFR signaling was not involved in E2 inhibition of *Igfbp3* mRNA expression, although E2 stimulated TGFα expression. These results suggest that E2 and TGFα act separately on *Igfbp3* expression.

The proliferation of endometrial cells is regulated by growth factors, including IGF1 produced in endometrial stromal cells (Bigsby and Cunha, 1986; Astrahantseff and Morris, 1994; Cooke et al., 1997; Pierro et al., 2001). IGF1 expression and IGFBP3 expression were conversely regulated by estrogen in endometrial stromal cells. Up-regulation of IGF1 expression and down-regulation of IGFBP3 expression by estrogen in the endometrial stromal cells was demonstrated, which suggests that estrogen enhances IGF1 action through the reduction of IGFBP3 expression at the transcription level. TGFα, whose production was stimulated by estrogen, decreased IGFBP3 production at the level of transcription, resulting in the enhancement of IGF1 action. These results suggest that estrogen suppresses IGFBP3 production in multiple ways, and stimulates the proliferation of endometrial cells through the enhancement of IGF1 action.

**ACKNOWLEDGMENTS**

This study was supported in part by a Grant-in-Aid-for Scientific

Research from the Japan Society for the Promotion of Science to S.T.

## REFERENCES

Albiston AL, Saffery R, Herington AC (1995) Cloning and characterization of the promoter for the rat insulin-like growth factor-binding protein-3 gene. Endocrinology 136: 696–704

Astrahantseff KN, Morris JE (1994) Estradiol-17β stimulates proliferation of uterine epithelial cells cultured with stromal cells but not cultured separately. In vitro Cell Dev Biol 30A: 769–776

Beck CA, Garner CW (1992) Stimulation of DNA synthesis in rat uterine cells by growth factors and uterine extracts. Mol Cell Endocrinol 84: 109–118

Bigsby RM, Cunha GR (1986) Estrogen stimulation of deoxyribonucleic acid synthesis in uterine epithelial cells which lack estrogen receptors. Endocrinology 119: 390–396

Cavaille F, Neau E, Vouters M, Bry-Gauillard H, Colombel A, Milliez J, Le Bouc Y (2006) IGFBP-1 inhibits EGF mitogenic activity in cultured endometrial stromal cells. Biochem Biophys Res Commun 345: 754–760

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR (1997) Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci USA 94: 6535–6540

Danielpour D, Song K (2006) Cross-talk between IGF-I and TGF-β signaling pathways. Cytokine Growth Factor Rev 17: 59–74

Das SK, Tsukamura H, Paria BC, Andrews GK, Dey SK (1994) Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. Endocrinology 134: 971–981

DiAugustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA, Teng CT (1988) Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. Endocrinology 122: 2355–2363

Duan C, Xu Q (2005) Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. Gen Comp Endocrinol 142: 44–52

Ghahary A, Chakrabarti S, Murphy LJ (1990) Localization of the sites of synthesis and action of insulin-like growth factor-I in the rat uterus. Mol Endocrinol 4: 191–195

Girvigian MR, Nakatani A, Ling N, Shimasaki S, Erickson GF (1994) Insulin-like growth factor binding proteins show distinct patterns of expression in the rat uterus. Biol Reprod 51: 296–302

Hembree JR, Agarwal C, Eckert RL (1994) Epidermal growth factor suppresses insulin-like growth factor binding protein 3 levels in human papillomavirus type 16-immortalized cervical epithelial cells and thereby potentiates the effects of insulin-like growth factor 1. Cancer Res 54: 3160–3166

Huang SS, Ling TY, Tseng WF, Huang YH, Tang FM, Leal SM, Huang JS (2003) Cellular growth inhibition by IGFBP-3 and TGF-β1 requires LRP-1. FASEB J 17: 2068–2081

Huet-Hudson YM, Andrews GK, Dey SK (1989) Cell type-specific localization of c-*myc* protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. Endocrinology 125: 1683–1690

Huynh H, Chow PK, Ooi LL, Soo KC (2002) A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth Differ 13: 115–122

Hwa V, Oh Y, Rosenfeld RG (1999) The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20: 761–787

Inoue A, Takeuchi S, Takahashi S (2005) Insulin-like growth factor-I stimulated DNA replication in mouse endometrial stromal cells. J Reprod Dev 51: 305–313

Kapur S, Tamada H, Dey SK, Andrews GK (1992) Expression of insulin-like growth factor-I (IGF-I) and its receptor in the peri-implantation mouse uterus, and cell-specific regulation of IGF-I gene expression by estradiol and progesterone. Biol Reprod 46: 208–219

Kleinman D, Karas M, Roberts CT, Jr., LeRoith D, Phillip M, Segev Y, Levy J, Sharoni Y (1995) Modulation of insulin-like growth factor I (IGF-I) receptors and membrane-associated IGF-binding proteins in endometrial cancer cells by estradiol. Endocrinology 136: 2531–2537

Komatsu N, Maekawa T, Takeuchi S, Takahashi S (2003) Epidermal growth factor and transforming growth factor-α stimulate the proliferation of mouse uterine stromal cells. Zool Sci 20: 639–645

Leal SM, Liu Q, Huang SS, Huang JS (1997) The type V transforming growth factor β receptor is the putative insulin-like growth factor-binding protein 3 receptor. J Biol Chem 272: 20572–20576

Liu HC, He ZY, Mele C, Damario M, Davis O, Rosenwaks Z (1997) Hormonal regulation of expression of messenger RNA encoding insulin-like growth factor binding proteins in human endometrial stromal cells cultured in vitro. Mol Hum Reprod 3: 21–26

Mishra S, Murphy LJ (2004) The effects of insulin-like growth factor binding protein-3 (IGFBP-3) on T47D breast cancer cells enriched for IGFBP-3 binding sites. Mol Cell Biochem 267: 83–89

Mochizuki T, Sakai K, Iwashita M (2006) Effects of insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) on endometrial cancer (HHUA) cell apoptosis and EGF stimulated cell proliferation in vitro. Growth Horm IGF Res 16: 202–210

Molnar P, Murphy LJ (1994) Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. J Mol Endocrinol 13: 59–67

Murphy LJ, Murphy LC, Friesen HG (1987) Estrogen induces insulin-like growth factor-I expression in the rat uterus. Mol Endocrinol 1: 445–450

Nelson KG, Takahashi T, Lee DC, Luetteke NC, Bossert NL, Ross K, Eitzman BE, McLachlan JA (1992) Transforming growth factor-α is a potential mediator of estrogen action in the mouse uterus. Endocrinology 131: 1657–1664

Ohtsuki T, Otsuki M, Murakami Y, Hirata K, Takeuchi S, Takahashi S (2007) Alternative leader-exon usage in mouse IGF-I mRNA variants: class 1 and class 2 IGF-I mRNAs. Zool Sci 24: 241–247

Ohtsuki T, Otsuki M, Murakami Y, Maekawa T, Yamamoto T, Akasaka K, Takeuchi S, Takahashi S (2005) Organ-specific and age-dependent expression of insulin-like growth factor-I (IGF-I) mRNA variants: IGF-IA and IB mRNAs in the mouse. Zool Sci 22: 1011–1021

Peterson AJ, Ledgard AM, Hodgkinson SC (1998) Oestrogen regulation of insulin-like growth factor binding protein-3 (IGFBP-3) and expression of IGFBP-3 messenger RNA in the ovine endometrium. Reprod Fertil Dev 10: 241–247

Pierro E, Minici F, Alesiani O, Miceli F, Proto C, Screpanti I, Mancuso S, Lanzone A (2001) Stromal-epithelial interactions modulate estrogen responsiveness in normal human endometrium. Biol Reprod 64: 831–838

Provenzano AP, Besner GE, James PF, Harding PA (2005) Heparin-binding EGF-like growth factor (HB-EGF) overexpression in transgenic mice downregulates insulin-like growth factor binding protein (IGFBP)-3 and -4 mRNA. Growth Factors 23: 19–31

Quarmby VE, Korach KS (1984) The influence of 17β-estradiol on patterns of cell division in the uterus. Endocrinology 114: 694–702

Ross AK, Nelson KG, Sakai Y, Fukamachi H, Burroughs CD,

McLachlan JA (1993) Isolation and culture of mouse uterine epithelial and stromal cells. In “Methods in Toxicology 3” Ed by JJ Heindel, RE Chapin, Academic Press, San Diego, pp 371–385

Sato T, Wang G, Hardy MP, Kurita T, Cunha GR, Cooke PS (2002) Role of systemic and local IGF-I in the effects of estrogen on growth and epithelial proliferation of mouse uterus. Endocrinology 143: 2673–2679

Shiraga M, Takahashi S, Miyake T, Takeuchi S, Fukamachi H (1997) Insulin-like growth factor-I stimulates proliferation of mouse uterine epithelial cells in primary culture. Proc Soc Exp Biol Med 215: 412–417

Sivaprasad U, Fleming J, Verma PS, Hogan KA, Desury G, Cohick WS (2004) Stimulation of insulin-like growth factor (IGF) binding protein-3 synthesis by IGF-I and transforming growth factor-$\alpha$ is mediated by both phosphatidylinositol-3 kinase and mitogen-activated protein kinase pathways in mammary epithelial cells. Endocrinology 145: 4213–4221

Smith MS, Freeman ME, Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96: 219–226

Strange KS, Wilkinson D, Edin G, Emerman JT (2004) Mitogenic properties of insulin-like growth factors I and II, insulin-like growth factor binding protein-3 and epidermal growth factor on human breast stromal cells in primary culture. Breast Cancer Res Treat 84: 77–84

Takaoka M, Smith CE, Mashiba MK, Okawa T, Andl CD, El-Deiry WS, Nakagawa H (2006) EGF-mediated regulation of IGFBP-3 determines esophageal epithelial cellular response to IGF-I. Am J Physiol 290: G404–G416

Tamada H, Das SK, Andrews GK, Dey SK (1991) Cell-type-specific expression of transforming growth factor-$\alpha$ in the mouse uterus during the peri-implantation period. Biol Reprod 45: 365–372

Tang XM, Rossi MJ, Masterson BJ, Chegini N (1994) Insulin-like growth factor I (IGF-I), IGF-I receptors, and IGF binding proteins 1-4 in human uterine tissue: tissue localization and IGF-I action in endometrial stromal and myometrial smooth muscle cells in vitro. Biol Reprod 50: 1113–1125

Tomooka Y, DiAugustine RP, McLachlan JA (1986) Proliferation of mouse uterine epithelial cells in vitro. Endocrinology 118: 1011–1018

Wraight CJ, Werther GA (1995) Insulin-like growth factor-I and epidermal growth factor regulate insulin-like growth factor binding protein-3 (IGFBP-3) in the human keratinocyte cell line HaCaT. J Invest Dermatol 105: 602–607

Zhou J, Dsupin BA, Giudice LC, Bondy CA (1994) Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. J Clin Endocrinol Metab 79: 1723–1734

Zhu L, Pollard JW (2007) Estradiol-17$\beta$ regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. Proc Natl Acad Sci USA 104: 15847–15851

(Received October 20, 2008 / Accepted November 16, 2008)