IGF-1 Gene Expression Is Differentially Regulated by Estrogen Receptors α and β in Mouse Endometrial Stromal Cells and Ovarian Granulosa Cells

Yuki OGO1), Shusuke TANIUCHI1), Fumiya OJIMA1), Sayo HAYASHI1), Itsuo MURAKAMI1), Yuka SAITO1), Sakae TAKEUCHI1), Toshiyuki KUDO2) and Sumio TAKAHASHI1)

1)Department of Biology, Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan
2)School of Pharmacy, Shujitsu University, Okayama 703-8516, Japan

Abstract. Insulin-like growth factor 1 (IGF-1) is involved in regulations of reproductive functions in rats and mice. IGF-1 expression is regulated by estrogen in several reproductive organs including the uterus and ovary. Two types of estrogen receptor (ERα and ERβ) are expressed in mouse uteri and ovaries, and it is unclear whether they differently mediate IGF-1 gene transcription. To clarify the roles of ERα and ERβ, mouse endometrial stromal cells and ovarian granulosa cells were treated with ligands specific for individual estrogen receptors. In endometrial stromal cells, propyl-pyrazole-triol (PPT; ERα-selective agonist) increased Igf1 mRNA expression, which was suppressed by methyl-piperidino-pyrazole (MPP, ERα-selective antagonist), while diarylpropionitrile (DPN, ERβ-potency selective agonist) increased Igf1 mRNA expression, which was inhibited by MPP but not by 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-α]pyrimidin-3-yl]phenol (PHTPP; ERβ antagonist). PHTPP enhanced the DPN-induced increase in Igf1 mRNA expression. In ovarian granulosa cells, E2 and DPN decreased Igf1 mRNA expression, whereas PPT did not affect Igf1 mRNA levels. In these cells, PHTPP inhibited the DPN-induced decrease in Igf1 mRNA expression. These results suggest that ERα facilitates Igf1 transcription, whereas ERβ appears to inhibit Igf1 gene transcription in mouse endometrial stromal cells and ovarian granulosa cells.

Key words: ERα, ERβ, Insulin-like growth factor 1, Mouse

IGF-1 is involved in regulations of female reproductive functions in rats and mice. In the uterus, the synthesis of IGF-1 is stimulated by E2 [10–12]. IGF-1 promotes the proliferation of endometrial epithelial cells [13–15]. These findings indicate that IGF-1 is one of the growth factors that regulate the proliferation of endometrial epithelial cells. ERα is reported to be necessary for the IGF-1 signaling cascade controlling endometrial epithelial cell proliferation [16, 17]. It was recently reported that ERα–DNA interaction is necessary for E2-mediated regulation of Igf1 transcription in the mouse uterus [18]. On the other hand, Weihua et al. reported the elevation of IGF-1 gene expression in the uterus of ERβ knockout mice, but quantitative analysis of IGF-1 gene expression was not performed in their study [19].

In the ovary, IGF-1 is expressed in granulosa cells in growing and healthy follicles [20, 21] and is required for the proliferation of granulosa cells in early folliculogenesis [22, 23]. Also, IGF-1 augments expression of FSH receptors in preovulatory granulosa cells and regulates responsiveness to FSH in Graafian follicles [24]. In atretic follicles, IGF-1 is not detected. Thus, IGF-1 plays essential roles in the entry of follicles into FSH-dependent stages of follicular development.

IGF-1 expression in the ovary is thought to be regulated by estrogen [25], but it is still not clear how estrogen affects IGF-1 expression in granulosa cells. In mouse granulosa cells, ERβ is more abundant than ERα [26–30]. Hence, it is necessary to clarify which type of ERs is involved in IGF-1 expression in granulosa cells.
The present study was aimed at clarifying the role of ERα and ERβ in the regulation of IGF-1 gene transcription using mouse endometrial stromal cells and ovarian granulosa cells. Using specific agonists as well as antagonists for ERα and ERβ, we analyzed the expression of Igf1 mRNA in primary endometrial stromal cells and ovarian granulosa cells. Mammalian IGFI-1 genes consist of six exons [31–33]. Exons 1 and 2 are the leader exons, and alternative use of these leader exons generates two types of IGF-1 transcripts (class 1 and class 2) [34, 35]. These two types of IGF-1 transcripts have been detected in the mouse uterus and ovary [12]. We determined mainly the expression of class 1 Igf1 transcripts to uncover the role of ERs in the activity of each promoter, since class 1 Igf1 mRNA expression responds more to estrogen than class 2 Igf1 mRNA expression [12].

**Materials and Methods**

**Animals**

Immature (21–23 days old) female ICR mice (CLEA Japan, Osaka, Japan) were used in the present study. All animal care and experiments were approved by the Institutional Animal Care and Use Committee at Okayama University (OKU-2012304), and were conducted in accordance with the Guidelines for Animals Experimentation of Okayama University, Japan.

**Endometrial stromal cell culture**

Endometrial stromal cells were isolated from 21- to 23-day-old mice using previously described methods [13, 36, 37]. Isolated endometrial stromal cells separated from epithelial cells were seeded in poly-L-lysine-coated culture wells at a density of 6.2 × 10⁴ cells/cm². Endometrial stromal cells were first cultured in a 1:1 mixture of phenol red-free DMEM and Ham’s F-12 medium (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) containing 2% dextran-coated charcoal-treated fetal bovine serum (DC-FBS; v/v, Life Technologies, Grand Island, NY, USA). After pre-culture for 1 day, the medium was switched to serum-free DMEM/F12 supplemented with BSA (1 g/l), hydrocortisone (100 µg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathyroid hormone (200 ng/l), sodium selenite (5 µg/l) and insulin (100 µg/l) (all from Sigma-Aldrich). The plates were incubated at 37 C in an atmosphere of 5% CO₂ and treated with the indicated hormones/compounds 2 days later.

**Isolation and culture of ovarian granulosa cells**

Ovaries from 21- to 23-day-old mice were dissected free of connective tissues and collected in DMEM/F12 containing 0.3% BSA. The ovaries were punctured with a 27-gauge needle, and a mixture of granulosa cells and oocytes was filtered through cell strainers under ultraviolet light. The isolated granulosa cells but not the oocytes to pass through. After being centrifuged at 500 × g for 5 min at 4 C, the isolated granulosa cells were seeded in culture wells at a density of 1.3 × 10⁶ cells/cm². The granulosa cells were first cultured in DMEM/F12 containing 10% DC-FBS. After pre-culture for 1 day, the medium was switched to serum-free DMEM/F12 (phenol red-free). The plates were incubated at 37 C in an atmosphere of 5% CO₂, and treated with the indicated hormones/compounds 2 days later.

**Cell line culture**

Human endometrial adenocarcinoma cells (HEC-1-A cells) were obtained from the Health Science Research Resources Bank (Sennan, Osaka, Japan) and were maintained in DMEM (Sigma-Aldrich) containing 10% FBS at 37 C under 5% CO₂. To measure luciferase activity, the cells were grown in phenol red-free DMEM/F12 containing 10% DC-FBS. Cells were seeded into 48-well plates at 0.7 × 10⁴ cells/well and cultured for 24 h before transfection.

**Estrogen agonist and antagonist treatment**

Endometrial stromal cells and granulosa cells were treated with propyl-pyrazole-triol (PPT), a ERα-selective agonist [38–40]; diarylpropionitrile (DPN), a ERβ-potency selective agonist [41–43]; methyl-piperidino-pyrazole (MPP), a selective ERα antagonist [42, 44, 45]; or 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazol-1,5-α] pyrimidin-3-yl]phenol (PHTPP), a selective ERβ antagonist [46].

E2 (Sigma-Aldrich) and PHTPP (Tocris Bio, Ellisville, MO, USA) were initially dissolved in sterile ethanol. PPT (Sigma-Aldrich), MPP (Sigma-Aldrich) and DPN (Tocris Bioscience) were initially dissolved in dimethyl sulfoxide (DMSO). Before use, the compounds were diluted in serum-free culture medium. The final concentrations of ethanol or DMSO were < 0.01%.

**RNA preparation and cDNA synthesis**

Total RNA was prepared from cultured endometrial stromal cells and ovarian granulosa cells using TRIzol reagent (Bioline, London, UK). Total RNA was reverse transcribed using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) with random hexamers according to the manufacturer’s instructions.

**RT-PCR**

PCR was performed using Blend Taq (Toyobo, Tokyo, Japan) and a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Branchburg, NJ, USA). The primers used in the present study were designed, and summarized in Table 1. The PCR conditions were as follows: 2 min 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 a final step at 72 C for 10 min. Aliquots (9 µl) of each reaction were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

**Real-time PCR**

Real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio) with an ABI PRISM 7300 Real Time PCR System (Applied Biosystems). The PCR program was as follows: initial denaturation at 95 C for 10 sec, 40 cycles of 95 C for 5 sec and 60 C for 31 sec and melting-curve analysis (95 C for 15 sec, 60 C for 1 min, 95 C for 15 sec and 60 C for 15 sec). Melting-curve analysis was conducted to confirm the absence of primer dimers. The primers are summarized in Table 1. A standard curve was generated by serial dilution of a preparation of total cDNA. The mRNA expression of each target gene was normalized for the mRNA expression of ribosomal protein L19 (Rpl19).

**Plasmid construction**

The expression vector for human ERα (pSG5-hERα) was con-
ERα-potency selective agonist. MPP (10 –6 M) inhibited ERα activity that DPN binds to ERβ and ERα, although DPN is thought to be a ERβ-expressing cells (Fig. 1A and C). These results indicate increased luciferase activity in ERα-expressing C). By contrast, DPN increased luciferase activity in ERα-expressing cells, but not in ERβ-expressing cells (Fig. 1A and ERα-expressing cells, but not in ERβ-expressing cells (Fig. 1A and C). PPT increased luciferase activity in increased luciferase activity above the basal levels in the presence of ERα or ERβ (Fig. 1A and C). By contrast, DPN increased luciferase activity in ERα-expressing cells and ERβ-expressing cells (Fig. 1A and C). These results indicate that DPN binds to ERβ and ERα, although DPN is thought to be a ERβ-potency selective agonist. MPP (10 –6 M) inhibited ERα activity induced by E2, but not ERβ activity stimulated by E2 (Fig. 1B and D). MPP alone (10 –7 and 10 –6 M) inhibited ERα activity. PHTPP

Protein extraction and Western blotting analysis

Endometrial stromal cells and ovarian granulosa cells were grown in 10-cm dishes. After washing with ice-cold PBS, the cells were lysed in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then scraped into a microfuge tube. The cell lysate was passed through a 26-gauge needle, and sonicated twice for 5 min each. After being centrifuged at 9,000 × g for 5 min at 4 C, the protein concentration of the supernatant was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). To detect the proteins, cell lysates (5–60 µg) were separated by 10% SDS-PAGE and transferred to Protran nitrocellulose membranes (0.45 µm pore size; Whatman, Dassel, Germany). The membranes were blocked for 1 h with 1% dried milk for ERα and ERβ) dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature. After washing once in TBS-T for 10 min at room temperature, the membranes were incubated overnight at 4 C with primary antibodies against ERα (1:2,000; H184; Santa Cruz) and ERβ (1:500; H150; Santa Cruz). The anti-ERα and anti-ERβ antibodies were diluted in TBS-T containing 1% dried milk. After incubation with the primary antibody, the membranes were washed twice in TBS-T and then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; Amersham Biosciences, Little Chalfont, UK) in TBS-T for 1 h at room temperature. After washing three times in TBS-T, the protein bands were visualized using an enhanced chemiluminescence detection system (ECL Prime; GE Healthcare, Little Chalfont, UK) and a Lumino-image analyzer (LAS-4000mini; Fujifilm, Tokyo, Japan).

Statistics

Data are expressed as means ± SEM, and they were analyzed by ANOVA followed by Dunnett’s test. Differences were considered significant at P < 0.05.

Results

ER ligand activities of PPT, DPN, MPP and PHTPP

The ER ligand activities of PPT, DPN, MPP and PHTPP were examined using an estrogen-responsive reporter gene plasmid ((ERE)3-Luc) in HEC-1-A cells. HEC-1-A cells were transfected with (ERE)3-Luc and expression plasmids for ERα or ERβ. E2 increased luciferase activity above the basal levels in the presence of ERα or ERβ (Fig. 1A and C). PPT increased luciferase activity in ERα-expressing cells, but not in ERβ-expressing cells (Fig. 1A and C). By contrast, DPN increased luciferase activity in ERα-expressing cells and ERβ-expressing cells (Fig. 1A and C). These results indicate that DPN binds to ERβ and ERα, although DPN is thought to be a ERβ-potency selective agonist. MPP (10 –6 M) inhibited ERα activity induced by E2, but not ERβ activity stimulated by E2 (Fig. 1B and D). MPP alone (10 –7 and 10 –6 M) inhibited ERα activity. PHTPP

Table 1. Primer sequence used for RT-PCR and Real-time PCR

| Gene | Sequence (5′–3′) | Length (bp) |
|------|-----------------|-------------|
| RT-PCR |                 |             |
| Krt19 | Forward GTGTCTGATGGCTGCCTGCT | 539 |
|       | Reverse CTCAGGATCTGGCTAGGTCG |             |
| Vim   | Forward GGCCGAGGAATGGTAGAACGTC | 320 |
|       | Reverse GGGCCATCTAAACATTTGGAGCAG |             |
| Erα   | Forward CTAATTCGACAATCGACGC | 347 |
|       | Reverse GTGCTTCAATCTCCCTCCTCTC |             |
| Erβ   | Forward GGTTGCTGGCTCTGTGAAGGATGT | 670 |
|       | Reverse CCCTGCCGAGGAGCAGTCTCAA |             |
| RpL19 | Forward GAAAATCGCCAATCGCCAATCTC | 406 |
|       | Reverse TGAGCTCGCAGTGCTAAGA |             |
| Real-time PCR |               |             |
| class1 Igf | Forward GCAAGCTTCTGAACTCAATTTTAA | 89 |
|         | Reverse GTGAAAGGAGTGTGAAGAGCACATTG |             |
| class2 Igf | Forward CACCCTGTCCTAAGCTGTTATGTTTC | 143 |
|         | Reverse GAGAGAGGAGTGTGAAGAGCACATTG |             |
| RpL19  | Forward CCGCACGCAATGAGTGATC | 60 |
|         | Reverse CGCACGCGAGGACACTAGA |             |

The expression vector pSG5-hERβ was constructed by inserting cDNA encoding human ERβ (530 bp) into the multiple cloning region of the EcoRI site of pSG5. The expression vector pSG5-hERβ was constructed by inserting cDNA encoding human ERα into the SacI/Hind III site of pGL4.12 (Promega, Madison, WI, USA). The phRL-TK vector (Promega) was used as an internal control in the reporter assay.

HEC-1-A cells, seeded in 48-well plates, were transfected with a mixture of 0.2 µg of (ERE)3-Luc reporter plasmid, 1 ng of phRL-TK vector as an internal control and 50 ng of the ERα or ERβ expression vector, using 0.5 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in DMEM/F12 supplemented with insulin-transferrin-selenium (Life Technologies). Twenty-four hours after transfection, the cells were placed in fresh medium containing ER agonists and antagonists. Reporter gene activity was assayed 24 h after ligand treatment. For the reporter assay, endometrial stromal cells were plated onto 24-well plates and grown in DMEM/F12 containing 10% DC-FBS for 2 days before transfection. After 1 day of culture, the medium was switched to serum-free DMEM/F12.

Luciferase reporter gene activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA) according to the manufacturer’s instructions. Luciferase activity was normalized to the Renilla luciferase activity (phRL-TK vector) of each sample.

Table 1. Primer sequence used for RT-PCR and Real-time PCR

| Gene | Sequence (5′–3′) | Length (bp) |
|------|-----------------|-------------|
| RT-PCR |                 |             |
| Krt19 | Forward GTGTCTGATGGCTGCCTGCT | 539 |
|       | Reverse CTCAGGATCTGGCTAGGTCG |             |
| Vim   | Forward GGCCGAGGAATGGTAGAACGTC | 320 |
|       | Reverse GGGCCATCTAAACATTTGGAGCAG |             |
| Erα   | Forward CTAATTCGACAATCGACGC | 347 |
|       | Reverse GTGCTTCAATCTCCCTCCTCTC |             |
| Erβ   | Forward GGTTGCTGGCTCTGTGAAGGATGT | 670 |
|       | Reverse CCCTGCCGAGGAGCAGTCTCAA |             |
| RpL19 | Forward GAAAATCGCCAATCGCCAATCTC | 406 |
|       | Reverse TGAGCTCGCAGTGCTAAGA |             |
| Real-time PCR |               |             |
| class1 Igf | Forward GCAAGCTTCTGAACTCAATTTTAA | 89 |
|         | Reverse GTGAAAGGAGTGTGAAGAGCACATTG |             |
| class2 Igf | Forward CACCCTGTCCTAAGCTGTTATGTTTC | 143 |
|         | Reverse GAGAGAGGAGTGTGAAGAGCACATTG |             |
| RpL19  | Forward CCGCACGCAATGAGTGATC | 60 |
|         | Reverse CGCACGCGAGGACACTAGA |             |
Expression of estrogen receptors in cultured endometrial stromal cells

RT-PCR analyses of ER mRNAs and Western blots of ERs were performed using cultured endometrial cells. In the cultured cells, vimentin mRNA (a marker for stromal cells, *Fln*) was detected, but cytokeratin 19 mRNA (a marker for epithelial cells, *Krt19*) was not detected (Fig. 2A), showing that cultured cells mostly consisted of endometrial stroma cells. In our previous study, immunocytochemical analysis already revealed that the cultured cells expressed vimentin but not cytokeratin [37]. The mRNAs and protein products of *ERα* and *ERβ* were detected in the cultured endometrial stromal cells (Fig. 2B and C).

Expression of estrogen receptors in cultured granulosa cells

RT-PCR analyses of ER mRNAs and Western blots of ERs were performed using cultured granulosa cells. Dissociated ovarian cells obtained from immature mouse ovaries, expressed FSH receptor and aromatase mRNA expression (data not shown), indicating that the cultured cells consisted of granulosa cells. The mRNAs and protein products of *ERα* and *ERβ* were detected in the cultured granulosa cells (Fig. 4A and B).

Effects of E2 on class 1 and class 2 *Igf1* mRNA expression in cultured endometrial stromal cells

To determine whether *Igf1* mRNA expression was regulated by E2 in our culture system, we measured *Igf1* mRNA levels by real-time RT-PCR. E2 (10^{-6} M) significantly increased the mRNA levels of class 1 and class 2 *Igf1* in cultured endometrial stromal cells (Fig. 2D). Class 1 *Igf1* mRNA increased more in response to E2 treatment than class 2 *Igf1* mRNA expression. These results indicate that the cultured endometrial stromal cells were responsive to estrogen in our culture system.

Effects of PPT and MPP on class 1 *Igf1* mRNA expression in cultured endometrial stromal cells

To clarify the role of ERs on *Igf1* mRNA expression, we determined the effects of PPT and MPP on *Igf1* mRNA expression in cultured endometrial stromal cells. The cultured endometrial stromal cells were treated with PPT for 24 h. PPT increased class 1 *Igf1* mRNA expression in a dose-dependent manner (Fig. 3A). Based on these data, PPT was administered at the concentration of 10^{-7} M in the following studies. MPP (10^{-6} M) significantly inhibited the PPT-induced and E2-induced increase in *Igf1* mRNA expression (Fig. 3B and E). MPP alone decreased class 1 *Igf1* mRNA expression as observed in the ERE-mediated reporter gene analysis.

Effects of DPN, PHTPP and MPP on class 1 *Igf1* mRNA expression in cultured endometrial stromal cells

To clarify the role of ERβ on *Igf1* mRNA expression, we examined the effects of DPN, PHTPP and MPP on *Igf1* mRNA expression in cultured endometrial stromal cells. Treatment with DPN for 24 h increased class 1 *Igf1* mRNA expression in a dose-dependent manner (Fig. 3C). Based on these data, DPN was used at the concentration of 10^{-8} M in the following studies. A high dose of PHTPP (10^{-6} M) slightly increased class 1 *Igf1* mRNA expression, and facilitated the DPN-induced increase in class 1 *Igf1* mRNA expression (Fig. 3D). By contrast, MPP decreased class 1 *Igf1* mRNA expression, and decreased DPN-induced class 1 *Igf1* mRNA expression.

Expression of estrogen receptors in culture granulosa cells

RT-PCR analyses of ER mRNAs and Western blots of ERs were performed using cultured granulosa cells. Dissociated ovarian cells obtained from immature mouse ovaries, expressed FSH receptor and aromatase mRNA expression (data not shown), indicating that the cultured cells consisted of granulosa cells. The mRNAs and protein products of *ERα* and *ERβ* were detected in the cultured granulosa cells (Fig. 4A and B).

Effects of E2, PPT, DPN, MPP and PHTPP on class 1 *Igf1* mRNA expression in cultured ovarian granulosa cells

The granulosa cells were then treated with E2, PPT, DPN, MPP and PHTPP for 24 h. E2 and DPN both decreased class 1 *Igf1* mRNA expression, whereas no change occurred in cells treated with PPT (Fig. 4C). PHTPP significantly inhibited the decrease in *Igf1* mRNA expression induced by DPN, while MPP did not induce significant changes in *Igf1* mRNA expression.

**Discussion**

We investigated roles of ERα and ERβ in the regulation of *Igf1* transcription in the murine endometrial stromal cells and ovarian granulosa cells. The nuclear receptors ERα and ERβ are expressed in these cellular systems, with ERα being a major receptor in endometrial cells [27] and ERβ being a major receptor in granulosa cells [28, 29]. The present results clearly suggest that *Igf1* transcription in mouse endometrial stromal cells is regulated mainly through ERα, which is consistent with previous reports, and that *Igf1* transcription in granulosa cells was regulated through ERβ in an inhibitory manner. Thus, ERα and ERβ differentially regulate *Igf1* transcription depending upon the types of IGF-1-expressing cells.

The cultured endometrial stromal cells, obtained from immature mouse uteri, expressed the two ERs. E2 treatment significantly increased *Igf1* mRNA expressions in the endometrial cells. Ovarian granulosa cells also expressed the two ERs, and E2 treatment significantly decreased *Igf1* mRNA. These results altogether indicate that the cultured endometrial stromal cells and granulosa cells retained their functional properties in vitro. Hence, our primary culture system is suitable for analysis of the regulation of *Igf1* transcription. Accordingly, we used these two types of cells as an experimental model system in this study.

To determine the effects of selective ER agonists and antagonists, an estrogen-responsive reporter gene plasmid ((ERE)_5-Luc) was transfected into HEC-1-A cells together with ERα or ERβ expression vectors. The transiently transfected cells were then treated with PPT, DPN, MPP and PHTPP. PPT showed agonistic activity via ERα, and DPN was an ERβ-potency selective agonist, as PPT did not activate the luciferase gene via ERβ [38]. On the other hand, although it is thought that DPN is an ERβ-potency selective agonist, it actually showed agonistic activity via both ERα and ERβ, which was similar to that described in a previous report [42]. MPP and PHTPP prevented luciferase gene activation promoted by E2 via ERα and ERβ, respectively. Interestingly, MPP (10^{-7}, 10^{-6} M) appeared to decrease ERα activity, which is consistent with a previous report [42], and PHTPP (10^{-6} M) also appeared to decrease ERβ activity. The reasons for these effects are not clear, but these results may suggest that MPP and PHTPP bind to ERα and ERβ, respectively, and exert inverse agonist activities. From these results, it seems that MPP is highly selective for ERα,
whereas PHTPP is highly selective for ERβ [44, 46].

Next, we treated endometrial stromal cells with the same agonists and antagonists. In endometrial stromal cells, Igf1 mRNA expression was increased by PPT in a dose-dependent manner. This result led us to conclude that ERα mediates promotion of Igf1 transcription, because PPT is highly selective for ERα [38–40]. Additionally, the ERα-selective antagonist MPP decreased PPT-induced Igf1 mRNA expression, indicating that ERα is involved in estrogen-induced upregulation of Igf1 mRNA expression in endometrial stromal cells. These results are consistent with previous reports showing that ERα is required for the activation of IGF-1 receptors and IGF-1-induced proliferation of epithelial cells [16–18]. DPN, which activated ERα and ERβ, increased Igf1 mRNA expression in endometrial stromal cells, which was similar to the effects of PPT. To elucidate whether these effects of DPN were mediated by ERα or ERβ, we co-treated cells with ERα and ERβ antagonists. MPP, an ERα-selective antagonist, inhibited the DPN-induced increase in Igf1 mRNA expression, whereas PHTPP, an ERβ-selective antagonist, did not affect DPN-induced Igf1 mRNA expression. These results suggest that PHTPP increases Igf1 mRNA expression via ERα but not via ERβ. Treatment with PHTPP increased Igf1 mRNA expression, and promoted DPN-induced Igf1 mRNA expression. These findings suggest that, unlike ERα, ERβ may have an inhibitory role in the
regulation of Igf1 mRNA expression. Alternatively, ERβ may not directly affect Igf1 transcription but does compete with ERα, since PHTPP exerts its antagonistic action on ERβ without suppressing ERα activity. It was previously reported that Igf1 mRNA expression was increased in the uterus of ERβ knockout mice [19]. Hence, it is highly probable that this was due to the lack of ERβ, which may be able to suppress Igf1 transcription.

PHTPP was shown to have an inverse agonist activity on ERβ in the present and previous studies [42]. PHTPP alone increased Igf1 mRNA expression. Therefore, it seems reasonable that ligand-activated
ERβ is involved in inhibitory regulation of IGF-1 expression. It was previously reported that E2 does not elicit estrogenic responses in ERα-knockout mice, such as DNA synthesis in endometrial epithelial cells, even though ERβ is expressed in endometrial stromal cells [48]. Taken together, these results may also suggest the possibility that Igf1 expression is regulated by ERβ in an inhibitory manner in endometrial cells.

The regulatory mechanism of ovarian IGF-1 expression seems to be still unclear. Erb mRNA, which encodes ERβ, is primarily detected in the granulosa cells of the mouse ovary [27]. Therefore, to further analyze the involvement of ERβ in the regulation of Igf1 transcription, we used ovarian granulosa cells. Of note, E2 and DPN decreased Igf1 mRNA expression in the granulosa cells unlike their effects in endometrial stromal cells. In granulosa cells, the effects of DPN are most likely to be mediated by ERβ rather than ERα, because the ERα agonist PPT and MPP did not affect Igf1 mRNA expression. Furthermore, the ERβ antagonist PHTPP inhibited the effects of DPN. From these results, we think that ERβ has an inhibitory effect rather than no effect on Igf1 transcription in granulosa cells.

Hernandez et al. [25] reported that diethylstilbestrol increased Igf1 mRNA levels in the ovary of hypophysectomized rats. On the other hand, we found an inhibitory effect of estrogen on Igf1 mRNA expression in granulosa cells through ERβ using a culture system of granulosa cells. The reason for this discrepancy is not clear, but may be partly explained by the difference in the sampling methods (whole ovary or granulosa cells). If estrogens decrease IGF-1 expression thorough ERβ in developing follicles, the proliferation of granulosa cells and responsiveness to FSH in such follicles will be reduced or nullified. It is possible that estrogen regulates follicular development and functions in an autocrine and paracrine manner through the IGF-1 system.

In mouse ovaries, ERβ promoted the proliferation of granulosa cells in early folliculogenesis [49] and also follicular maturation from the early antral to preovulatory stages [50]. In Igf1 null mouse ovaries, the proliferation of granulosa cells was decreased, and E2 stimulated the proliferation of granulosa cells, although the increase was less compared with that of wild-type mice [23]. These findings suggest that estrogen directly stimulates the proliferation of granulosa cells, and that IGF-1 enhances the estrogen-induced proliferation of granulosa cells.

In conclusion, the present results led us to consider that the nuclear receptors ERα and ERβ differentially regulate Igf1 mRNA expression, as ERα promotes its expression and ERβ inhibits its expression. Further studies are needed to determine the precise role of ERβ in the regulation of Igf1 transcription.

Acknowledgments

The authors would like to thank Dr F Otsuka and Dr K Inagaki for their kind guidance on granulosa cell culture, and K Masuda for his help with endometrial stroma cell culture. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to Sumio Takahashi (Nos. 19370025, 22570066)

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