The progesterone-responsive gene *14-3-3 τ* enhances the transcriptional activity of progesterone receptor in uterine cells

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Abstract

Members of the 14-3-3 family are intracellular dimeric phosphoserine-binding proteins that can associate with and modulate the activities of many proteins. In our efforts to isolate the genes regulated by progesterone (P₄) using suppressive subtractive hybridization, we previously found that *14-3-3 τ* is one of the genes upregulated by P₄. In this study, we demonstrated by quantitative RT-PCR (qRT-PCR), western blot analyses, and immunohistochemistry that 14-3-3 τ mRNA and protein levels were increased in the rat uterus after P₄ treatment. Furthermore, qRT-PCR indicated that P₄ increased *14-3-3 τ* mRNA levels in human endometrial epithelial cells and endometrial stromal cells (ESCs). Western blot and qRT-PCR analyses revealed that *in vitro* decidualization using cAMP and medroxyprogesterone 17-acetate increased levels of 14-3-3 τ mRNA and protein in ESCs. We have shown by qRT-PCR and western blot analyses that P₄ increased the mRNA and protein levels of 14-3-3 τ in Ishikawa cells that stably express P₄ receptor-B (PR-B). Immunocytochemistry revealed that 14-3-3 τ colocalizes with PR and translocates from the cytoplasm to the nucleus in response to P₄. Moreover, by luciferase reporter assay, we demonstrated that 14-3-3 τ enhances the transcriptional activity of PR-B. Taken together, we propose that *14-3-3 τ* is a P₄-responsive gene in uterine cells that modulates P₄ signaling.

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Introduction

The endometrium undergoes cyclic changes controlled by the ovarian steroid hormones estrogen and progesterone (P₄). During the proliferative phase, estrogen secreted from the granulosa cells in the ovary stimulates endometrial proliferation. After ovulation, estrogen and P₄ are secreted by the ovary to facilitate endometrial decidualization. Estrogen and P₄ actions are mediated by their specific receptors, namely, estrogen receptor and P₄ receptor (PR) respectively. P₄ also plays an important role in implantation, and PR is a member of the nuclear receptor superfamily that regulates the transcription of an array of responsive genes (Mangelsdorf *et al.* 1995). PR is detected as two distinct proteins, namely, PR-A and PR-B, which possess different molecular weights. In humans, the molecular weight of the PR-A protein is 94 kDa while that of PR-B is 120 kDa. These proteins are translated from distinct

mRNA subgroups that are transcribed from a single gene under the control of separate A and B promoters (Kastner *et al.* 1990, Giangrande & McDonnell 1999, Richer *et al.* 2002, Leonhardt *et al.* 2003). PRs and their cofactors are expressed in both the rat uterus and the human endometrium and are involved in the regulation of their responsive genes (Vienomen *et al.* 2004). Some P₄-responsive genes have been reported, such as calcitonin (Zhu *et al.* 1998), corticotrophin-releasing hormone (Makrigiannakis *et al.* 1995), insulin-like growth factor-binding protein-1 (IGFBP1; Gao *et al.* 1999), heparin-binding epidermal growth factor-like growth factor (Zhang *et al.* 1994), thrombospondin-1 (Iruela-Arispe *et al.* 1996), and FK506-binding protein 51 (FKBP51; Hubler *et al.* 2003) – these genes are related to uterine differentiation in normal female reproduction.

Several investigators have identified the genes regulated by P₄ in the uterus using cDNA microarray

analyses (Cheon *et al.* 2002, Jeong *et al.* 2005). Recently, gene-targeting technology created PR-A knockout (PR-A KO) mice that display reproductive anomalies, including the inability to ovulate, endometrial hyperplasia and inflammation, and defects in embryo implantation (Lydon *et al.* 1995, Conneely *et al.* 2001). Female infertility in PR-A KO mice is associated with defective embryo implantation and lack of decidualization of uterine stromal cells in response to P₄ (Mulac-Jericevic *et al.* 2000).

To isolate the genes regulated by P₄ in the rat uterus, we previously performed suppressive subtractive hybridization (Ohno *et al.* 2008), and 14-3-3 τ was identified as one of the genes that were upregulated in the rat uterus after P₄ treatment. The 14-3-3 proteins form a family of highly conserved molecules that are expressed in a wide range of organisms and tissues (Obsilová *et al.* 2008). There are nine isoforms (α , β , γ , δ , ϵ , η , σ , τ , and ζ) in vertebrates, and additional isoforms exist in yeasts, plants, amphibians, and invertebrates (Aitken 2006). All these proteins have a molecular mass of ~30 kDa and exist as homodimers or heterodimers. Furthermore, 14-3-3 proteins regulate various cellular activities by binding to phosphorylated signaling molecules, including Raf-1, Cbl, Bad, Cdc25C, and IGF1 receptor. In addition, 14-3-3 proteins have been shown to enhance the transcriptional activity of glucocorticoid receptor (GR) and androgen receptor (AR) (Wakui *et al.* 1997, Haendler *et al.* 2001, Zilliacus *et al.* 2001, Kim *et al.* 2005, Quayle & Sadar 2007, Titus *et al.* 2009). Moreover, Liang *et al.* (2006) demonstrated that aldosterone induced increased levels of 14-3-3 β mRNA and protein in mouse cortical collecting duct epithelium. In this study, we examined the expression and regulation of 14-3-3 τ in the rat uterus, cultured human endometrial cells, and human endometrium. Furthermore, we analyzed the effect of 14-3-3 τ on PR-B transcriptional activity and the mechanism underlying the change induced by 14-3-3 τ in the transcriptional activity of PR-B.

Materials and methods

Animals and treatment

In this study, we used 7-week-old female Sprague Dawley rats (Japan SLC, Hamamatsu, Japan). All animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals. Our research was approved by the Research Ethics committee of the University of Tokyo. The rats were administered the hormone or vehicle 2 weeks after being ovariectomized. The ovariectomized rats were s.c. injected with 20 μ g 17 β -estradiol (E₂) or 2 mg P₄ (Sigma) dissolved in corn oil. The control rats

were administered only corn oil. The E₂-treated and P₄-treated rats were killed, and each organ was collected at the appropriate time after E₂ and P₄ injections respectively. After excising the uterus, it was rapidly frozen and stored at -80 °C until use.

RNA isolation and cDNA preparation

The total RNA was isolated from the rat tissues using the ToTALLY RNA kit (Ambion, Austin, TX, USA), following the manufacturer's instruction. The RNA concentration was determined by NanoDrop (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from 500 ng of total RNA by RT using PrimeScript Reverse Transcriptase (TaKaRa, Tokyo, Japan), following the manufacturer's instruction.

Materials

DMEM/F-12, OPTI-MEM I reduced serum medium 1 $\times$, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies, Inc. Charcoal-dextran-treated FBS was purchased from Hyclone (Logan, UT, USA). E₂, P₄, 8-bromoadenosine cAMP sodium salt (8-Br-cAMP), and medroxyprogesterone 17-acetate (MPA) were from Sigma-Aldrich Corp. The luciferase assay kit was obtained from Promega Corp. The primary antibodies used for western blot analyses in this study were polyclonal anti-14-3-3 τ (Santa Cruz Biotechnology), monoclonal anti- β -actin (Sigma), anti-HA (Santa Cruz Biotechnology), and monoclonal anti-FLAG (Sigma). The secondary antibodies used in this study were anti-rabbit and anti-mouse from Santa Cruz Biotechnology, and the fluorescent secondary antibodies were anti-mouse labeled with Alexa Fluor 488 and anti-rabbit labeled with Alexa Fluor 594 from Invitrogen.

Cell culture

Human endometrial adenocarcinoma Ishikawa cells (purchased from ATCC, Rockville, MD, USA) were routinely cultured at 37 °C in 5% CO₂ atmosphere in phenol red-containing DMEM/F-12, 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The HEK293 cells stably expressing PR-B were routinely cultured under the same conditions as the human endometrial adenocarcinoma Ishikawa cells.

Isolation and culture of endometrial stromal cells and treatments

The endometrial tissues were obtained from women undergoing hysterectomies for benign diseases unrelated to endometrial pathology. All patients had regular menstrual cycles, and none had received hormonal

treatment for at least three cycles before the surgery. The specimens were annotated according to the criteria as described (Noyes *et al.* 1950) and the menstrual history of the patients. The experimental procedures were approved by the Research Ethics Committee of the University of Tokyo, and all patients gave written consent for tissue collection. The cultured endometrial stromal cells (ESCs) were separated and maintained in a monolayer culture, as described previously (Koga *et al.* 2001). Briefly, the endometrial tissue was minced into small pieces and digested by incubation in DMEM/F-12 containing 0.25% type I collagenase (Sigma) and deoxyribonuclease I (60 U/ml; Invitrogen). The dispersed endometrial cells were separated into the ESC and the endometrial epithelial cells (EECs) by filtration through a cell strainer with 40 μ m nylon pores (Becton Dickinson and Co., Franklin Lakes, NJ, USA). The filtered ESCs were separated, centrifuged, and plated onto 100 mm culture dishes containing phenol red-free DMEM/F-12 and 10% charcoal dextran-treated FBS. At the first passage, the cells were plated in six-well plates (Becton Dickinson) at a density of 6×10^5 cells per well. The EECs were separated, centrifuged, and plated onto 24-well plates (Becton Dickinson) containing phenol red-free DMEM/F-12 with 10% charcoal dextran-treated FBS at a density of 1×10^5 cells per well.

The decidualization model in ESCs

cAMP and/or MPA can induce the decidualization of ESCs (Gellersen & Brosens 2003). The decidualization of ESCs was confirmed by the expression of *IGFBP1* and prolactin (*PRL*) mRNA extracted from ESC by quantitative RT-PCR (qRT-PCR) analysis. The ESCs were treated with 0.5 mM 8-Br-cAMP and/or 10^{-6} M MPA for 3 days as the decidualization stimulus. Subsequently, the RNA and protein extracts were collected for qRT-PCR and western blot analyses.

RT-PCR

PCR amplification was performed in a total volume of 50 μ l containing 5 pmol of the forward and reverse primers of 14-3-3 τ and *GAPDH*, 10 nmol of each dNTP, $1 \times$ Taq buffer, and 2.5 U Taq polymerase (TaKaRa, Otsu, Shiga, Japan). The following PCR conditions were used: 50 °C for 2 min, 95 °C for 10 min, then 45 cycles of 20 s at 95 °C, and 1 min annealing/extension at 60 °C. All PCR products were electrophoresed on 2.5% agarose gels. Primer sequences are available on request.

qRT-PCR analyses

cDNA was quantified by qRT-PCR (10 min at 50 °C, 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C,

and 1 min at 60 °C) using the SYBR Green PCR master mix (Applied Biosystems) and the ABI 7000 Fast Real-Time PCR system (Applied Biosystems). The relative difference in the amounts of PCR products was evaluated using an internal control (β -actin and 18S rRNA). Primer sequences are available on request.

Immunoprecipitation and western blotting

The cells were lysed in Nonidet P-40 lysis buffer (Zhu *et al.* 2008) containing an EDTA-free protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The protein concentrations were determined using the BCA protein assay reagent kit (Thermo Scientific, Rockford, IL, USA). For immunoprecipitation of FLAG-PR-B, equal amounts of cellular proteins were immunoprecipitated with anti-FLAG M2-agarose beads. The blots were incubated with the primary antibodies (anti-14-3-3 τ , 1:1000; anti- β -actin, 1:3000; anti-HA, 1:2000; and anti-FLAG, 1:1000). After washing with Tris-buffered saline containing Tween 20 (TBS-T) buffer three times for 10 min each, the blots were incubated with the HRP-conjugated secondary antibodies (anti-rabbit, 1:10 000, and anti-mouse, 1:10 000).

Immunohistochemistry of the rat uterus

Immunohistochemical procedures involving the rat uterus were performed using paraffin-embedded sections. The sections were each dipped twice into xylene, 100% ethanol, and 95% ethanol. After blocking with 3% H₂O₂ in methanol and 10% goat serum, the sections were incubated with the anti-14-3-3 τ antibody and normal rabbit IgG (control) at room temperature overnight and then with the biotinylated secondary antibody (anti-rabbit IgG) for 10 min at room temperature. The samples were then incubated with streptavidin conjugated with HRP (Histofine SAB-PO kit; Nichirei, Inc., Tokyo, Japan) for 5 min at room temperature and visualized using a peroxidase substrate kit with 3,3'-diaminobenzidine (Histofine SAB-PO kit; DAB substrate kit; Nichirei, Inc.).

Generation of Ishikawa cells stably expressing FLAG-PR-B

The PR-negative Ishikawa cells were transfected with human FLAG-PR-B cDNA or the empty vector pcDNA3 by FuGENE HD reagents (Roche Diagnostics) and then selected using G418 sulfate (700 μ g/ml; Wako, Osaka, Japan). The G418-resistant cells were selected, and several independent clones were isolated. To validate the expression of exogenous human FLAG-PR-B, the cells were analyzed using qRT-PCR and western blots. We obtained Ishikawa cells stably expressing

PR-B (Ishikawa PR-B #1, Ishikawa PR-B #2) and possessing the empty pcDNA3 vector (Ishikawa-vector #1, Ishikawa-vector #2).

Immunocytochemistry

The cells (Ishikawa PR-B #1, Ishikawa PR-B #2, Ishikawa-vector #1, and Ishikawa-vector #2) were plated onto culture coverslips at the bottom of 24-well plates and covered with DMEM supplemented with 10% charcoal dextran-treated FBS. The cells were then treated with ethanol or 10^{-7} M P_4 for 24 h. After treatment with hormones, the cells were rinsed once with ice-cold PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and finally fixed with ice-cold methanol for 10 min at -30°C . The cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. The cells were then washed twice with PBS and blocked with 1% BSA in PBS for 1 h before incubating with the primary antibody (anti-14-3-3 τ and anti-FLAG) in 1% BSA-PBS for an additional hour. The cells were washed three times with PBS and incubated with the secondary antibodies (Alexa Fluor 488 conjugated with goat anti-mouse antibody and Alexa Fluor 594 conjugated with goat anti-rabbit antibody). The cells were washed once with PBS for nuclear staining. The culture coverslips were inverted and mounted using a DAPI Fluoromount G (Southern Biotech, Birmingham, AL, USA). The digital confocal images were collected using a fluorescence microscope equipped with Fluoview FV10i (Olympus, Tokyo, Japan).

Cell culture and transient transfection for the reporter assay

PR-negative endometrial adenocarcinoma Ishikawa cells were maintained in OPTI-MEM I reduced serum medium supplemented with 10% charcoal dextran-treated heat-inactivated FBS at 37°C under 5% CO_2 atmosphere. The Ishikawa cells were seeded onto 24-well plates at 2×10^4 cells/well and grown in the same medium for 24 h. The medium was replaced with OPTI-MEM I reduced serum medium and then transiently transfected with 50 ng of the MMTV-luciferase reporter construct, 20 ng of the pRL reporter construct, 50 ng of the HA-PR-B expression vector, and 50 ng of the FLAG-14-3-3 τ expression vector using FuGENE HD reagents (Roche Diagnostics). After 24 h, the serum-starved cells were treated with ethanol or 10^{-7} M P_4 for 24 h and harvested. The luciferase assays were performed using the luciferase assay system (Promega Corp.). The cells were extracted with 120 μl /well reporter lysis buffer, and the supernatants were collected and assayed using a Mithras LB 940 multimode reader (Berthold, Calmbacher, Germany).

Figure 1 Expression and distribution of 14-3-3 τ in the rat uterus. Distribution of 14-3-3 τ mRNA in rat tissues as analyzed by RT-PCR using specific primers for 14-3-3 τ and GAPDH (A). 14-3-3 τ mRNA was detected in various rat organs, including the uterus. PCR products derived from GAPDH mRNA were used as the control. Localization of the 14-3-3 τ protein in the rat uterus by immunohistochemistry (B). The uterus was removed from ovariectomized rats at the indicated times after P_4 injection (2 mg/rat). Sections were stained with anti-14-3-3 τ antibody. Scale bars indicate 100 μm . LE, luminal epithelium; GE, glandular epithelium; St, stroma. The 14-3-3 τ protein was induced in the rat uterus by P_4 (2 mg/rat) (C). However, the 14-3-3 τ protein was not induced in the rat uterus by E_2 (20 μg /rat) (D). The uterus was removed from ovariectomized rats at the indicated times after P_4 injection. Expression of the 14-3-3 τ protein was induced by P_4 . No significant change was detected in the induction of 14-3-3 τ mRNA expression following treatment with estrogen. Statistical differences among groups were obtained using Student's *t*-tests, $n=3$; * $P<0.05$. Error bars, s.e.m.; NS, not significant; WCEs, whole cell extracts.

Statistical analyses

Data from three independent experiments are presented. The JMP software (SAS Institute, Inc., Cary, NC, USA) was used to analyze the data using Student's *t*-tests. *P* values <0.05 were considered statistically significant. Values are presented as the mean $\pm$ S.E.M.

Results

14-3-3 τ mRNA was detected in the rat uterus

To examine the distribution of 14-3-3 τ mRNA in rat tissues, we performed RT-PCR using specific primers for 14-3-3 τ (Fig. 1A, upper lane) and *GAPDH* (Fig. 1A, lower lane). These results revealed that 14-3-3 τ mRNA was present in various organs, including the uterus (Fig. 1A).

Localization of the 14-3-3 τ protein in the rat uterus by immunohistochemistry

We performed immunohistochemistry to examine the localization of the 14-3-3 τ protein in the rat uterus (Fig. 1B). The 14-3-3 τ protein was detected in the stroma at 12 h and in the glandular epithelium at 24 h after the administration of P₄ (Fig. 1B). Normal rabbit IgG was used as the negative control (Fig. 1B, left panel).

P₄ increased the levels of 14-3-3 τ mRNA and protein in the rat uterus

We investigated the regulation of 14-3-3 τ mRNA and protein by P₄ in the rat uterus (Fig. 1C and D). The ovariectomized rats were s.c. administered 20 μ g/rat of E₂ or 2 mg/rat of P₄. The amount of 14-3-3 τ mRNA in the P₄-treated rats at 12 and 24 h was significantly higher than that at 0 h ($^{***}P < 0.005$; Fig. 1C). In addition, the amount of 14-3-3 τ protein in the P₄-treated rats at 12 and 24 h was significantly higher than that at 0 h ($^{*}P < 0.05$; Fig. 1C). Estrogen did not significantly affect the induction of 14-3-3 τ mRNA (Fig. 1D).

P₄ increased the levels of 14-3-3 τ mRNA in cultured EECs and ESCs

We investigated the regulation of 14-3-3 τ by P₄ in EECs (Fig. 2A) and ESCs (Fig. 2B). qRT-PCR analyses revealed that 14-3-3 τ mRNA expression in both cell types was significantly higher at 12 h after treatment with P₄.

cAMP and MPA increased 14-3-3 τ mRNA and protein levels in cultured ESCs

We also analyzed the regulation of 14-3-3 τ by the *in vitro* decidualization model. The ESCs were treated with

Figure 2 Expression of 14-3-3 τ in EECs and ESCs following stimulation by P₄ or decidualization with 8-Br-MPA and cAMP. 14-3-3 τ mRNA was induced by P₄ (10⁻⁷ M) in EECs (A) and ESCs (B). 14-3-3 τ mRNA and protein expression was induced by *in vitro* decidualization with cAMP (0.5 mM) and 8-Br-MPA (10⁻⁶ M) for 3 days (C). Prolactin (PRL) (D) and IGFBP1 (E) mRNA were induced by *in vitro* decidualization for 3 days. Statistical differences among groups were obtained using Student's *t*-tests, *n*=3; $^{*}P < 0.05$; $^{***}P < 0.005$. Error bars, S.E.M.; NS, not significant.

0.5 mM 8-Br-cAMP and/or 10⁻⁶ M MPA. qRT-PCR and western blot analyses demonstrated that 14-3-3 τ mRNA and protein levels were significantly higher at 3 days after treatment with 8-Br-cAMP and MPA (Fig. 2C). In addition, PRL and IGFBP1 mRNA expression levels were significantly higher than the control, after treatment with 8-Br-cAMP and/or MPA (Fig. 2D and E).

P₄ increased 14-3-3 τ mRNA and protein expression in the Ishikawa cells stably expressing PR-B

We analyzed the expression of the PR-B protein by immunoprecipitation and western blot analyses using the anti-FLAG antibody for FLAG-PR-B in the Ishikawa cells stably expressing PR-B (Fig. 3A). Although there were no significant increases in 14-3-3 τ and FKBP51

Figure 3 Expression of 14-3-3 τ in Ishikawa cells stably expressing PR-B following treatment with P₄. The expression of the FLAG-PR-B protein in Ishikawa cells stably expressing PR-B was confirmed by immunoprecipitation and western blotting. The protein from HEK293 cells stably expressing PR-B was used as a positive control (A). The Ishikawa-vector and Ishikawa PR-B cells were treated with P₄ (10⁻⁷ M). The amounts of 14-3-3 τ and FKBP51 mRNA were analyzed by qRT-PCR (B). Ishikawa cells stably expressing PR-B (PR-B #1, PR-B #2) were treated with P₄ (10⁻⁷ M) (C). The levels of 14-3-3 τ protein were analyzed by western blot. Statistical differences among groups were obtained using Student's *t*-tests, *n*=3; **P*<0.05. Error bars, S.E.M.; NS, not significant.

mRNA levels in the Ishikawa vector transfectant cells (Ishikawa-vector #1, Ishikawa-vector #2), the 14-3-3 τ and FKBP51 mRNA levels in the Ishikawa cells stably expressing PR-B (Ishikawa-PR-B #1, Ishikawa-PR-B #2) were significantly upregulated 12 h after the P₄ treatment (Fig. 3B). Notably, P₄ increased the levels of the 14-3-3 τ protein in the Ishikawa cells stably expressing PR-B (Fig. 3C).

Immunocytochemistry analyses revealed translocation of 14-3-3 τ from the cytoplasm to the nucleus in response to P₄ by interacting with PR-B

We conducted immunocytochemical analyses to elucidate the subcellular localization of 14-3-3 τ and PR-B in the Ishikawa cells stably expressing PR-B and the Ishikawa cells transfected with pcDNA3 (Fig. 4). The cells were treated with ethanol or 10⁻⁷ M P₄ for 24 h. Although 14-3-3 τ was predominantly expressed in the cytoplasm in the absence of P₄ (Fig. 4C) in the Ishikawa cells stably expressing PR-B, 14-3-3 τ translocated from the cytoplasm to the nucleus in response to ligand stimulation (Fig. 4D). Conversely, 14-3-3 τ in the Ishikawa-vector cells did not translocate in response to ligand stimulation (Fig. 4A and B). In addition, PR-B was primarily expressed in the cytoplasm without P₄ stimulation (Fig. 4C) and translocated to the nucleus after ligand treatment (Fig. 4D). We obtained the same results with another Ishikawa cell line stably expressing PR-B (data not shown). Thus, colocalization of 14-3-3 τ and PR-B was detected in the absence and presence of the ligand (Fig. 4C and D).

14-3-3 τ enhances the transcriptional activity of PR-B

We assessed whether 14-3-3 τ was associated with the transcriptional activity of PR-B. Ishikawa cells were transiently transfected with the MMTV-Luc reporter vector, PR-B expression vector, 14-3-3 τ expression vector, and empty vector. Then, they were incubated in the absence or presence of P₄. In the absence of P₄, overexpression of 14-3-3 τ enhanced the transcriptional activity of PR-B by 1.7-fold compared with the cells that were not transfected with the 14-3-3 τ expression vector. Furthermore, in the presence of P₄, overexpression of 14-3-3 τ enhanced the transcriptional activity of PR-B by 2.5-fold compared with the cells that were not transfected with the 14-3-3 τ expression vector (Fig. 5A). These data indicate that 14-3-3 τ is a positive regulator of PR-B transcriptional activity. In these experiments, the expression of each protein (HA-PR-B and FLAG-14-3-3 τ) was confirmed by western blot analyses (Fig. 5B).

Figure 4 Localization of 14-3-3 τ and PR-B in the absence or presence of P₄ by immunocytochemistry. Ishikawa cells expressing PR-B and Ishikawa-vector cells were treated with P₄ (10⁻⁷ M). Nuclei were counterstained by DAPI. Anti-14-3-3 τ and the anti-FLAG antibodies were used for immunocytochemistry; (A) and (C) ethanol; (B) and (D) P₄(+).

Discussion

In this study, we revealed the localization and regulation of 14-3-3 τ mRNA in the rat uterus. We also indicated that P₄ induced 14-3-3 τ mRNA and protein expression in the rat uterus. Immunohistochemistry analyses showed that the 14-3-3 τ protein was present in both the stroma and epithelium of the rat uterus after the administration of P₄. The results of the qRT-PCR and western blot analyses were in agreement with the immunohistochemistry and confirmed the induction of 14-3-3 τ by P₄. In addition, we showed that 14-3-3 τ was induced in the human EECs and ESCs at 12 h after the administration of P₄.

Decidualization occurs during the mid-secretory phase of the menstrual cycle and is indispensable for embryo implantation. Importantly, decidualization can be modeled by treatment with 8-Br-cAMP and MPA. We revealed that in ESCs, 14-3-3 τ mRNA and protein expression levels were increased for 3 days following administration of cAMP and MPA. Embryo implantation occurs during the 'implantation window,' which

is a relatively short time period (a few days in humans) during the mid-secretory phase of the menstrual cycle. Recently, epithelio-mesenchymal transition (EMT) in the trophoblast cells has been reported to be important for embryo implantation (Kalluri & Weinberg 2009). EMT is known to be activated by macrophage-stimulating protein (MSP) in collaboration with macrophage-stimulating receptor (MSTR, also known Ron) (Wang *et al.* 2004). Ron is known to express in the human ESCs (Matsuzaki *et al.* 2005). Ron in the trophoblast and trophoblast cells is essential for embryo implantation in the mouse (Muraoka *et al.* 1999, Hess *et al.* 2003). A previous study suggested that MSP-Ron-dependent phosphorylation and 14-3-3 association with Ron might be critically involved in human epidermal wound healing (Santoro *et al.* 2003). Thus, it is tempting to speculate that upregulation of 14-3-3 τ after a decidualization stimulus in the ESCs may influence embryo implantation as in the trophoblast and trophoblast cells.

To understand P₄ signaling in endometrial cells, we established Ishikawa cells stably expressing PR-B as

Figure 5 The effect of 14-3-3 τ on the transcriptional activity of PR-B analyzed by luciferase assay. 14-3-3 τ enhanced the transcriptional activity of PR-B (A). The FLAG-14-3-3 τ , HA-PR-B, MMTV-luc, and pRL expression vectors were transfected into PR-negative endometrial adenocarcinoma Ishikawa cells to analyze the effect of 14-3-3 τ on the transcriptional activity of PR-B in the absence and presence of P₄ (10⁻⁷ M). The expression of each protein (HA-PR-B and FLAG-14-3-3 τ) was confirmed by western blot (B). Statistical differences among groups were obtained using Student's *t*-tests, *n*=4; ****P*<0.005. Error bars, S.E.M.; NS, not significant.

a P₄-sensitive endometrial cell model together with Ishikawa cells transfected with the pcDNA3 vector. P₄ induced 14-3-3 τ mRNA and protein expression in the Ishikawa cells stably expressing PR-B, but not in the control cells, suggesting that 14-3-3 τ is a P₄-responsive gene. A previous study reported that aldosterone induced 14-3-3 β mRNA and protein expression in the mouse cortical collecting duct epithelium and that 14-3-3 β has aldosterone-responsive elements upstream of the promoter (Liang *et al.* 2006). A database search (Match-1.0 Public, gene-regulation.com, sponsored by BIOBASE) enabled the identification of ten P₄-responsive elements (PREs) within 3000 bp upstream of the gene, one PRE within 405 bp of intron 1 of the gene, and four PREs within 1000 bp downstream of the human 14-3-3 τ gene. The database search also revealed 18 PREs within 3000 bp upstream of the 14-3-3 τ gene, one PRE within 370 bp of intron 1 of the 14-3-3 τ gene, and six PREs within 1000 bp downstream of the rat

14-3-3 τ gene. These data suggest a potential relationship and the regulatory mechanism of 14-3-3 with the steroid hormone receptor. Moreover, a previous study showed that 14-3-3 η has a cAMP response element sequence at the transcription initiation site (Muratake *et al.* 1996). In addition, a further database search demonstrated that the human 14-3-3 τ gene has seven cAMP response elements within 3000 bp upstream of the 14-3-3 τ gene and four cAMP response elements within 405 bp of intron 1 of the 14-3-3 τ gene.

Further immunocytochemistry analyses revealed that 14-3-3 τ translocated from the cytoplasm to the nucleus in accordance with PR-B following P₄ treatment, whereas it did not translocate in the Ishikawa-vector-transfected cells. These results were consistent with the previous finding that PR-B is expressed in the cytoplasm without P₄ stimulation and that it translocates to the nucleus after ligand treatment (Lim *et al.* 1999). It has also been reported that 14-3-3 τ translocates with the target protein human telomerase reverse transcriptase (hTERT) from the cytoplasm to the nucleus (Seimiya *et al.* 2000). Furthermore, there is evidence that 14-3-3 η translocates with the target protein TLX2 from the cytoplasm to the nucleus (Tang *et al.* 1998). Haendler *et al.* indicated that 14-3-3 η may maintain the cytoplasmic AR in the optimal configuration for ligand recognition or form a bridge with other proteins involved in the signaling pathway that cross talks with AR (Quayle & Sadar 2007). In addition to this, previous studies have indicated that 14-3-3 η increases GR stabilization via the inhibition of proteasome-dependent degradation, which consequently leads to increased GR transcriptional activity (Jeanelos *et al.* 2001, Zilliacus *et al.* 2001). The aforementioned relationship between 14-3-3 τ and PR-B may be applicable in this case. Despite this, we cannot completely rule out the possibility that 14-3-3 τ changes the localization of a corepressor from the nucleus to the cytoplasm in the cell signaling processes within 0–24 h of P₄ administration. 14-3-3 η has been proposed to bind to and export RIP140, a corepressor of GR, out of the nucleus, thus enhancing GR transcriptional activity (Zilliacus *et al.* 2001). Previous studies showed that FKHR (Foxo1a) and p27kip1 repress PR-B transcriptional activity in a dose-dependent manner (Zhao *et al.* 2001, Lange 2005). Other studies reported that phosphorylated Akt induces 14-3-3 τ to change the localization of FKHR and p27kip1 from the nucleus to the cytoplasm by direct interaction (Rena *et al.* 2001, Fujita *et al.* 2002).

We also analyzed the effect of 14-3-3 τ on the transcriptional activity of PR-B in the endometrial adenocarcinoma Ishikawa cells. The luciferase analyses revealed that 14-3-3 τ enhanced the transcriptional activity of PR-B in the absence and presence of P₄. To the best of our knowledge, this is the first study to show that 14-3-3 τ enhances the transcriptional activity

of PR-B. Previous studies have shown that certain isoforms of 14-3-3 enhance the transcriptional activity of some nuclear receptors. Indeed, 14-3-3 η (Zilliacus *et al.* 2001, Kim *et al.* 2005) and 14-3-3 σ (Quayle & Sadar 2007) have been shown to enhance the transcriptional activity of GR and AR.

In summary, we have shown that 14-3-3 τ is upregulated by treatment with P₄ in the rat uterus, human EECs and ESCs, and Ishikawa cells stably expressing PR-B and enhances transcriptional activity of PR-B in endometrial adenocarcinoma Ishikawa cells. We suggest that 14-3-3 τ , a P₄-responsive gene, modulates P₄ signaling in uterine cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M I, H H, and S I designed the research; M I, T U, M S, Y H, R T, F Z, M K, and H N performed the experiments; K H-I contributed new reagents/analytic tools; M I, T U, H H, and S I analyzed the data; H H, M M, T F, T Y, S K, S I, and Y T supervised the research; and M I, T U, H H, and S I wrote the paper.

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