Journal of Reproduction and Development, Vol. 60, No 5, 2014

—Original Article—

REV-ERBα Inhibits the *PTGS2* Expression in Bovine Uterus Endometrium Stromal and Epithelial Cells Exposed to Ovarian Steroids

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Abstract. The nuclear receptor REV-ERBα (encoded by *NR1D1*) has a critical role in metabolism and physiology as well as circadian rhythm. Here, we investigated the possible contribution of clock genes including *NR1D1* to the secretion of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) from bovine uterine stromal (USCs) and epithelial cells (UECs) by modulating the expression of *PTGS2*. The circadian oscillation of clock genes in the cells was weak compared with that reported in rodents, but the expression of *BMAL1*, *PER1*, and *NR1D1* was changed temporally by treatment with ovarian steroids. Significant expression of clock genes including *NR1D1* was detected in USCs exposed to progesterone. *NR1D1* was also significantly expressed in UECs exposed to estradiol. The expression of *PTGS2* was suppressed in USCs exposed to progesterone, while the expression was initially suppressed in UECs exposed to estradiol and then increased after long-term exposure to estradiol. *BMAL1* knockdown with specific siRNA caused a significant decrease in the transcript levels of *NR1D1* and *PTGS2* in USCs, but not in UECs. The production of $PGF_2\alpha$ also decreased in USCs after *BMAL1* knockdown, while its level did not significantly change in UECs. The transcript level of *PTGS2* was increased by treatment with the antagonist of REV-ERBα in both cell types, but the agonist was ineffective. In these two cell types treated with the agonist or antagonist, the $PGF_2\alpha$ production coincided well with the *PTGS2* expression. Collectively, these results indicate that REV-ERBα plays an inhibitory role in the expression of *PTGS2* in both bovine USCs and UECs treated with ovarian steroids.

Key words: Bovine uterus endometrium cells, Clock genes, Prostaglandin $F_2\alpha$, *PTGS2* gene, REV-ERBα

(**J. Reprod. Dev. 60:** 362–370, 2014)

In cows and sheep, luteolysis is induced by prostaglandin $F_2\alpha$ ($PGF_2\alpha$), which is secreted in a pulsatile mode from the uterine endometrium during the late luteal phase to the follicular phase. Progesterone (P_4), estradiol (E_2), and oxytocin have been regarded as the critical factors regulating the secretion of $PGF_2\alpha$ from the endometrium. It has been demonstrated that oxytocin promotes the secretion of $PGF_2\alpha$ as a pulse generator of its secretion in the endometrium [1, 2]. However, the critical roles of P_4 and E_2 in the secretion of $PGF_2\alpha$ are still unclear [3, 4]. It is generally accepted that ovarian steroids modulate the sensitivity of the endometrium to oxytocin by regulating the expression of the oxytocin receptor [5–7]. Conversely, several studies objected to the role of oxytocin in luteolysis [8–10], and it was proposed that oxytocin is not essential for $PGF_2\alpha$ secretion [3, 11]. Therefore, existence of another regulator(s) of the $PGF_2\alpha$ secretion in the endometrium was postulated [4], but no regulator has not been identified.

Prostaglandin G/H synthetase (PTGS) is the key rate-limiting enzyme converting arachidonic acid into PGG2 and PGH2, which are the precursors for $PGF_2\alpha$ and other metabolites. PTGS has two isoforms, PTGS1 and PTGS2. In the bovine endometrium, $PGF_2\alpha$ is synthesized mostly by PTGS2 [12]. The promoter region of the bovine *PTGS2* gene contains the E-box element and REV-ERBα/RORα response element (RORE), which are the circadian clock-controlled *cis*-regulatory elements.

The cellular clock components CLOCK and BMAL1 bind to the E-box enhancer and induce expression of the nuclear receptor REV-ERBα (encoded by *NR1D1*), resulting in repression of transcription of *BMAL1* through direct binding to RORE located in the *BMAL1* promoter [13]. In addition to regulating each other to sustain oscillations, the core clock proteins also entrain the rhythmic expression of numerous genes through binding to the E-box, RORE, and D-box at their promoters, which have been called clock-controlled genes (CCGs) and found to comprise a large family. The peripheral oscillators control the expression of downstream CCGs that are expressed in tissue-specific relationships. REV-ERBα has a critical role in the regulation of metabolism and physiology as well as circadian rhythms [14]. The cellular level of heme, identified as a physiological ligand for REV-ERBα [15], oscillates in a circadian manner [16]. SR8278 recently became available as a synthetic antagonist of REV-ERBα [17]. Based on recent studies, we raised the possibility that the *PTGS2* gene is a downstream CCG in bovine uterus endometrial cells.

We reported that circadian rhythmicity is weak in the rat uterus luminal epithelium as revealed by immunohistochemistry, although a strong immunostaining signal of PER2 protein is detected in the epithelial cell layer compared with that in the stromal cell layer [18]. Since regulation of the circadian clockwork may be different between stromal cells and epithelial cells, the two cell types for the

Received: April 1, 2014
Accepted: June 6, 2014
Published online in J-STAGE: July 10, 2014

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circadian clockwork need to be analyzed separately. In the present study, we extended our recent investigations to explore the possible contribution of the circadian clockwork to the secretion of $PGF_2\alpha$ from the bovine endometrium by modulating the expression of the *PTGS2* gene.

Materials and Methods

Isolation and culture of bovine USCs and UECs

Bovine uteri were collected from a slaughterhouse and transferred to laboratory on ice. The two cell types were isolated from the uteri of cows showing the luteal stage at days 11 to 17 as determined by ovarian morphology [19]. The caruncles were physically dissected from the endometrium of the bovine uterus. Tissue pieces were treated with 0.1% collagenase (Wako, Tokyo, Japan) at 37 C for 30 min and cultured for 1 week in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% charcoal-stripped FBS (Biological Industries, Kibbutz Beit Haemek, Israel) with 1× antibiotic-antimycotic mixed solution (AA; Nacalai Tesque, Kyoto, Japan). USCs and UECs migrated from the tissue pieces and separately proliferated in a monolayer (Supplementary Fig. 1: on-line only) [20]. USCs and UECs in primary culture were separated with trypsin-EDTA. USCs and UECs were peeled with 0.05% and 0.25% trypsin-EDTA, respectively. Both cell types separated (4.0×10^5 cells) were seeded on a 35-mm collagen-coated dish (Iwaki, Tokyo, Japan) with 2 ml DMEM/F12 supplemented with 1×AA. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 C for 48 h prior to other treatments.

Separated USCs and UECs were rinsed with PBS followed by blocking with blocking solution (2% goat serum in PBS) for 30 min at room temperature. These cells were then incubated for 18 h at 4 C with an anti-vimentin polyclonal antibody (1:200; Nichirei Bioscience, Tokyo, Japan) and an anti-cytokeratin monoclonal antibody (1:200; Sigma-Aldrich, St. Louis, MO, USA) diluted in blocking buffer. Goat serum was substituted for the primary antibody as a negative control. After washing several times with PBS, they were incubated with secondary antibodies (Santa Cruz Biotechnology, CA, USA) and DAPI (Sigma-Aldrich) diluted in blocking solution (1:250) for 1 h at room temperature. Immunostaining was detected under a fluorescence microscope (Nikon, Japan). The USCs were positively immunostained for vimentin (a marker protein of stromal cells) but negatively for cytokeratin (a marker of epithelial cells) (Supplementary Fig. 1). The UECs were strongly immunostained for cytokeratin, as reported previously [20].

Treatment with steroid hormones

USCs and UECs cultured for 2 days were treated with 100 nM P_4 (Sigma-Aldrich) and 100 nM E_2 (Sigma-Aldrich) dissolved in DMSO that was diluted in a serum-free medium with a final DMSO concentration < 0.1%, respectively, for 12 h in DMEM/F12 supplemented with 1× Insulin-Transferrin-Selenium (ITS: Life Technologies, Grand Island, NY, USA), 1× AA and 0.1% bovine serum albumin (BSA; Sigma-Aldrich). After treatment with P_4 or E_2, each cell was washed with culture medium and synchronized with 10 μM forskolin (Sigma-Aldrich) for 2 h in DMEM/F12 supplemented with 1× ITS, 1× AA and 0.1% BSA. Then, USCs and UECs were further cultured with 100 nM P_4 and 100 nM E_2 in DMEM/F12 supplemented with 1× ITS, 1× AA and 0.1% BSA, respectively, and subjected to each experiment.

Real-time monitoring of mouse Per1 promoter activity

The –1884/–102-bp region upstream of the translation start codon of mouse *Per1* was fused to the luciferase gene in the pGL3-Basic vector (Promega, *Per1-Luc* vector) [21]. The upstream region includes three E-box sites (–146 to –151, –509 to –514, and –1255 to –1260) and a cAMP response element (CRE, –1725 to –1732). The *Per1-Luc* vector (1.0 μg/dish) was transfected into cultured rat and bovine USCs using Hillymax (Wako). Rat USCs were prepared from uteri at the diestrus stage according to a previous report [18]. These cells were maintained in serum-free DMEM/F12 supplemented with 0.1 mM luciferin (Wako), 0.1% BSA, 1% ITS, 1×AA and 100 nM P_4 after synchronization for 2 h with 10 μM forskolin. Luciferase activity was chronologically monitored at 37 C with a Kronos Dio AB-2550 luminometer (ATTO, Tokyo, Japan) interfaced with a computer for continuous data acquisition [21].

BMAL1-specific siRNA transfection

The sequence targeting the *BMAL1* mRNA and non-silencing RNA for the bovine was purchased from Sigma-Aldrich. The scrambled sequence for the *BMAL1* siRNA was used as a control. The sequences of RNA oligos used are listed in Supplementary Table 1 (on-line only). USCs and UECs were separately seeded on 35-mm collagen-coated dishes with 2 ml DMEM/F12 supplemented with 1× AA, 1× ITS, and 0.1% BSA. After 24 h in culture, the medium was removed, and the *BMAL1*-specific siRNA and non-silencing RNA diluted in Opti-MEM were transfected into cells using Lipofectamine® RNAiMAX reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. Both the *BMAL1*-specific siRNA and non-silencing RNA were used at a final concentration of 100 nM. The cells were maintained with transfection medium for an additional 24 h. The medium was replaced with a medium supplemented with 1× AA, 1× ITS, and 0.1% BSA. Then, USCs and UECs were cultured with 100 nM P_4 and 100 nM E_2, respectively, for 12 h and synchronized with forskolin.

Treatment with heme and SR8278

USCs and UECs were treated with 50 μM heme (Sigma-Aldrich) or 10 μM SR8278 (Sigma-Aldrich) dissolved in DMSO in the presence of steroid hormones after synchronization with forskolin. As a control, each cell was treated with 0.1% DMSO instead of heme and SR8278.

RNA extraction and RT-qPCR

Cultured cells were harvested at indicated time points, and total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase (Qiagen). The cDNAs were generated by RT with Oligo $(dT)_{15}$ and Random Primers using a GoTaq® 2-Step RT-qPCR System (Promega, Madison, WI, USA). The primer sets used for the RT-qPCR are listed in Supplementary Table 2 (on-line only). All primer pairs were designed to span introns to prevent amplification of products from genomic DNA. RT-qPCR was performed in a 50-μl

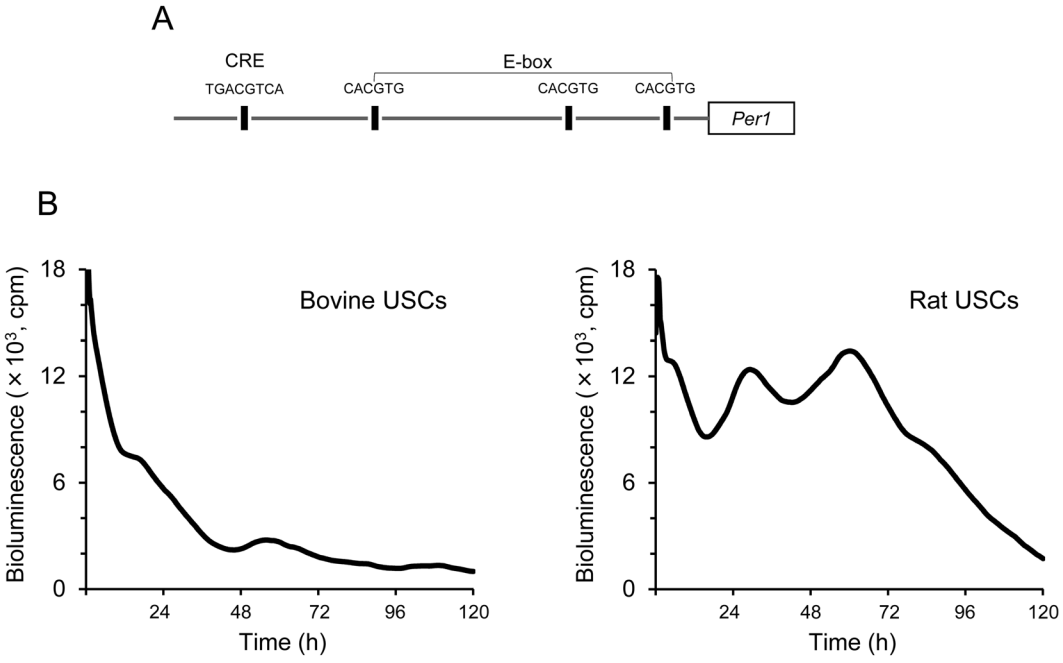

Fig. 1. Generation of bioluminescence oscillations by rat and bovine USCs transfected with pGL3 vector containing the mouse *Per1* promoter region after synchronization with forskolin. (A) Schematic of the pGL3 vector containing the mouse *Per1* promoter region (upper). Black bars indicate the location of E-box motifs (–146 to –151, –509 to –514, and –1255 to –1260) and a cyclic-AMP response element (CRE, –1725 to –1732). (B) Bioluminescence activity was induced in bovine and rat USCs transfected with 1 μg of the constructed vector by synchronization with forskolin. Bioluminescence was monitored in real time in serum-free medium DMEM/F12 supplemented with 0.1 mM luciferin, 0.1% BSA, 1% ITS, 1×AA and 100 nM P_4. Each value represents the means of three independent determinations.

volume containing a 20-ng cDNA sample in GoTaq® qPCR Master Mix and 250 nM specific primers with an Mx3000P Real-time qPCR System (Agilent Technologies, Santa Clara, CA, USA) using the parameters described in our previous report [22]. The relative quantification of gene expression was analyzed from the measured threshold cycles (Ct) using the comparative cycle threshold (ΔCt) method [18]. The ΔCt for each sample was normalized to the average level of the constitutively expressed housekeeping gene *GAPDH*. Gene expression was then normalized to the level of the gene of interest in the control samples.

$PGF_2\alpha$ assay

Culture supernatants were collected at 48 h after synchronization. Then the $PGF_2\alpha$ contents were measured using EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The intra-assay and inter-assay variabilities were < 10%.

Data analysis and statistics

Data are expressed as the means ± SEM of at least three independent experiments, each performed with duplicate samples. The statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison test or the Student's *t* test, as indicated using the SigmaPlot software (Ver. 12.0; Systat Software, San Jose, CA, USA). Differences were considered significant at $P<0.05$ or less. Rhythmicity in gene expression was determined by the single Cosinor method using Timing Series Single 6.3 (Expert Soft Tech.) [23].

Results

Bioluminescence activity in rat and bovine USCs transfected with the mouse Per1-Luc vector

To investigate whether the cellular clockwork functions in bovine uterus cells, we first analyzed mouse *Per1* promoter activity as an indicator of the clockwork. There are three E-box sites and one CRE in the mouse *Per1* promoter region (Fig. 1A). The rat USCs exposed to P_4 were used as a positive control, in which a robust circadian clockwork has been confirmed [18, 24]. Real-time monitoring of bioluminescence clearly revealed that there were clear differences in oscillation profiles between rat and bovine USCs. Rat USCs displayed a sharp peak until approximately 30 min after synchronization. Thereafter, the promoter activity in rat USCs showed stable oscillation for 5 days. Conversely, bovine USCs displayed high activity immediately after synchronization, and then the activity in bovine USCs decreased and showed only several small peaks (Fig. 1B).

Expression of core clock genes in bovine USCs

We next analyzed the temporal changes of the clock gene transcript levels over the course of 48 h using bovine USCs. After synchronization with forskolin, the clock genes *PER1* and *NR1D1* displayed no significant expression in the absence of P_4 (Fig. 2). *BMAL1* only showed significantly high expression at 6 h ($P<0.05$). However, these clock gene transcripts showed no diurnal rhythms. P_4 was added to the culture medium, because the stroma is comprised

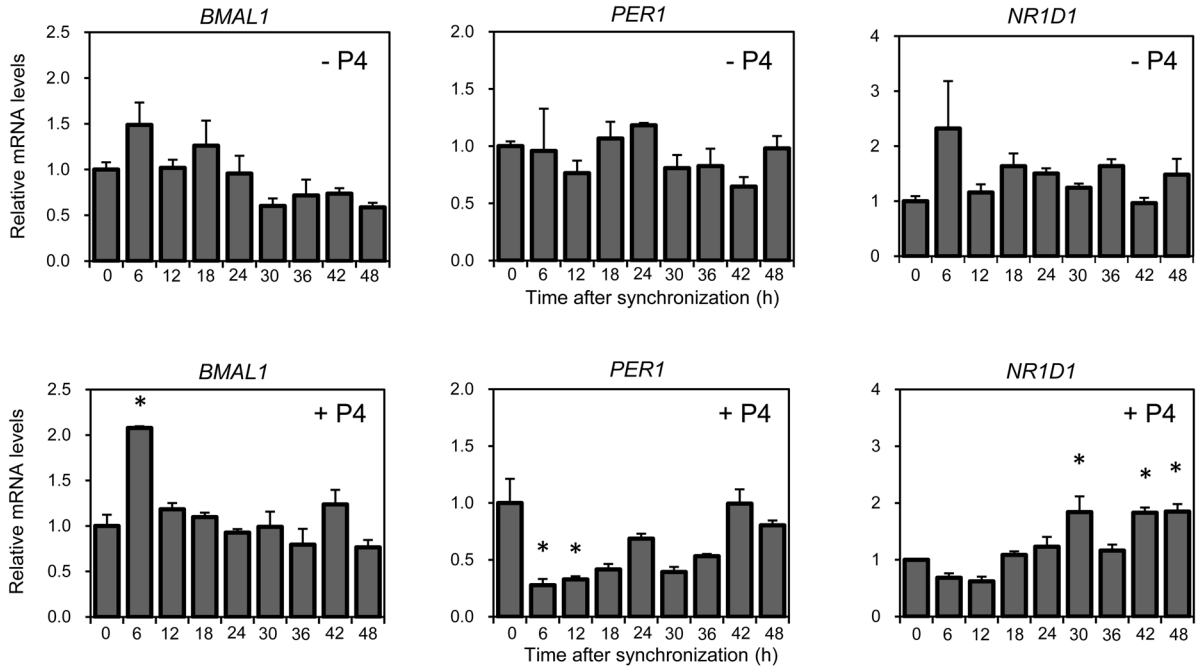

Fig. 2. Expression profiles of core clock gene transcripts over the course of 48 h in bovine USCs. After synchronization with forskolin, total RNA samples were collected at 6 h interval from cells cultured with (bottom) or without (upper) the presence of P_4. RT-qPCR analyses of transcript levels were performed using their specific primers. The relative transcript level was normalized to *GAPDH* and expressed relative to the first time point (0 h). Each value represents the mean ± SEM of three independent experiments. The statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison tests. * $P<0.05$ *vs.* 0 h.

of progesterone-targeting cells and is fitted to the luteal stage. In the presence of 100 nM P_4, significant expression of three clock genes was detected ($P<0.01$) (Fig. 2). Several peaks of clock gene transcripts were observed until 48 h, but their diurnal rhythms were not significant, except in the case of *PER1* (Cosinor, P=0.0006). The relative expression of *NR1D1* was high in the presence of P_4, especially at 24 to 48 h ($P<0.05$).

Expression of core clock genes in bovine UECs

We also analyzed the temporal changes in the clock gene transcripts in UECs. After synchronization, the clock genes *PER1* and *NR1D1* displayed no significant expression in the absence of E_2 (Fig. 3), but *BMAL1* displayed significant expression ($P<0.05$) and diurnal rhythms (Cosinor, P=0.0013). E_2 was also added to the culture medium, because the epithelium is comprised of estrogen-targeting cells. In the presence of 100 nM E_2, *NR1D1* displayed a significant expression and peaked at around 30 h ($P<0.05$) (Fig. 3). *PER1* also showed significant expression ($P<0.01$). However, the diurnal rhythms of *PER1* and *NR1D1* were not significant, except in the case of *BMAL1* (Cosinor, P=0.0365).

Expression of PTGS2 in bovine USCs and UECs

The expression level of *PTGS2* was investigated in bovine USCs and UECs. After synchronization, *PTGS2* displayed significant expression in both cell types. As shown in Fig. 4, however, different responses of the two cell types to steroid hormones were observed. In the USCs exposed to P_4, *PTGS2* displayed significantly high expression and peaked at 6 to 12 h ($P<0.01$) (Fig. 4A). In addition, the diurnal rhythm of the *PTGS2* transcript was significant (Cosinor, P=0.0009). The expression level of *PTGS2* was low at 30 to 48 h in the presence of P_4, whereas it was high in the absence of P_4. In the UECs exposed to E_2, *PTGS2* also showed significant expression and peaked at 48 h ($P<0.01$) (Fig. 4B). However, the diurnal rhythm of the *PTGS2* transcript was not significant. The expression of *PTGS2* was suppressed until 30 h in the presence of E_2 compared with in the absence of E_2. After long exposure to E_2 (48 h), the *PTGS2* transcript level was dramatically increased ($P<0.01$).

Effect of BMAL1 knockdown on the expression of PTGS2 in bovine USCs and UECs

We used *BMAL1*-specific siRNA to investigate whether the *PTGS2* expression is controlled under BMAL1 transcriptional regulation in the two bovine cell types. BMAL1 associated with CLOCK or NPAS2 promotes the transcription of genes such as *NR1D1* through binding to the E-box at the promoter region. The transfection of *BMAL1*-specific siRNA caused a significant decrease in the *BMAL1* transcript level of both the USCs ($P<0.01$) and the UECs ($P<0.05$) (Fig. 5). Concomitantly, the *NR1D1* transcript level was significantly decreased in the USCs ($P<0.01$), while it did not change in the UECs. The *PTGS2* transcript level was also significantly decreased in the USCs ($P<0.05$). Conversely, the UECs displayed no downregulation of the *PTGS2* transcript after *BMAL1* knockdown.

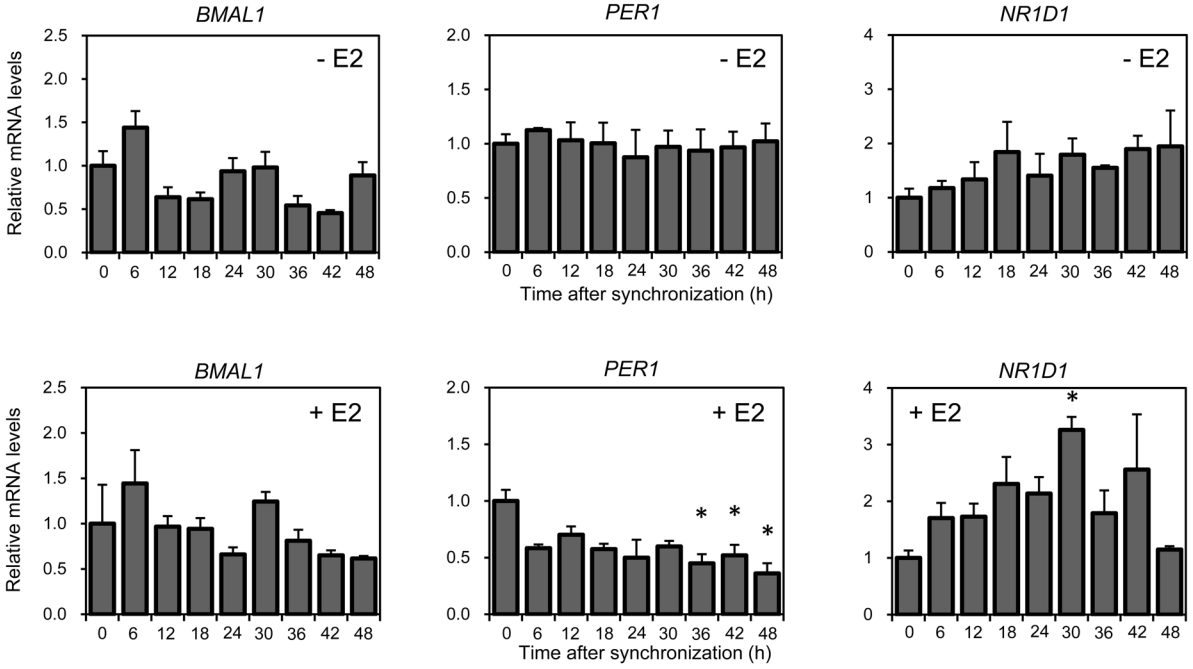

Fig. 3. Expression profiles of core clock gene transcripts over the course of 48 h in bovine UECs. After synchronization with forskolin, total RNA samples were collected at 6 h interval from cells cultured with (bottom) or without (upper) the presence of estradiol. RT-qPCR analyses of transcript levels were performed using their specific primers. The relative transcript level was normalized to *GAPDH* and expressed as relative to the first time point (0 h). Each value represents the mean ± SEM of three independent experiments. The statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison tests. * P<0.05 *vs.* 0 h.

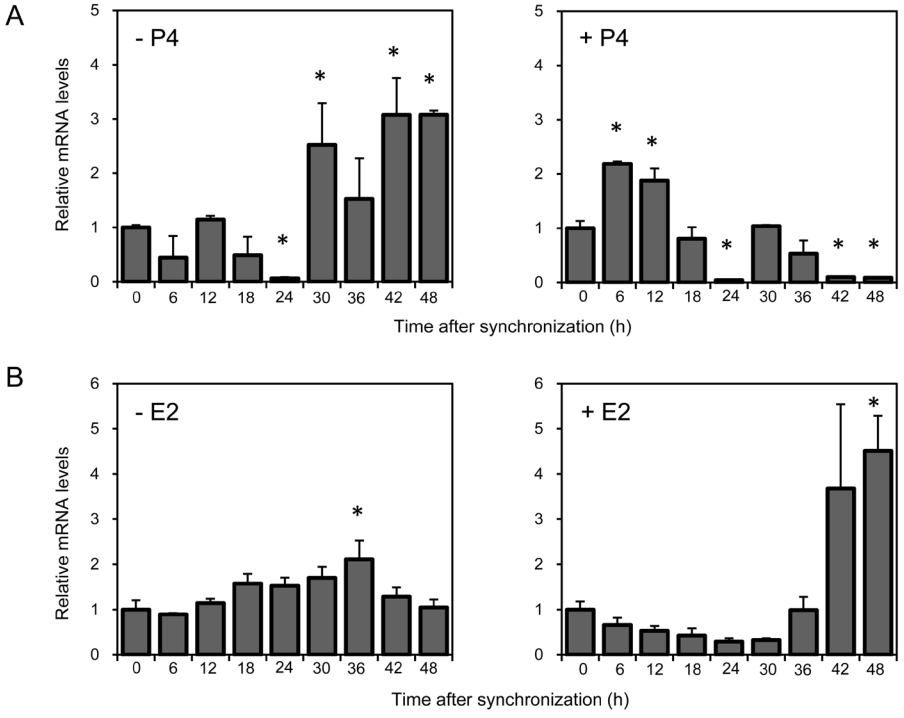

Fig. 4. Expression of the *PTGS2* gene in bovine USCs and UECs. After synchronization with forskolin, total RNA samples were collected at 6 h interval from USCs (A) and UECs (B) cultured with or without the presence of ovarian steroids. RT-qPCR analyses of transcript levels were performed using their specific primers. The relative transcript level was normalized to *GAPDH* and expressed as relative to the first time point (0 h). Each value represents the mean ± SEM of three independent experiments. The statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison tests. * P<0.05 *vs.* 0 h.

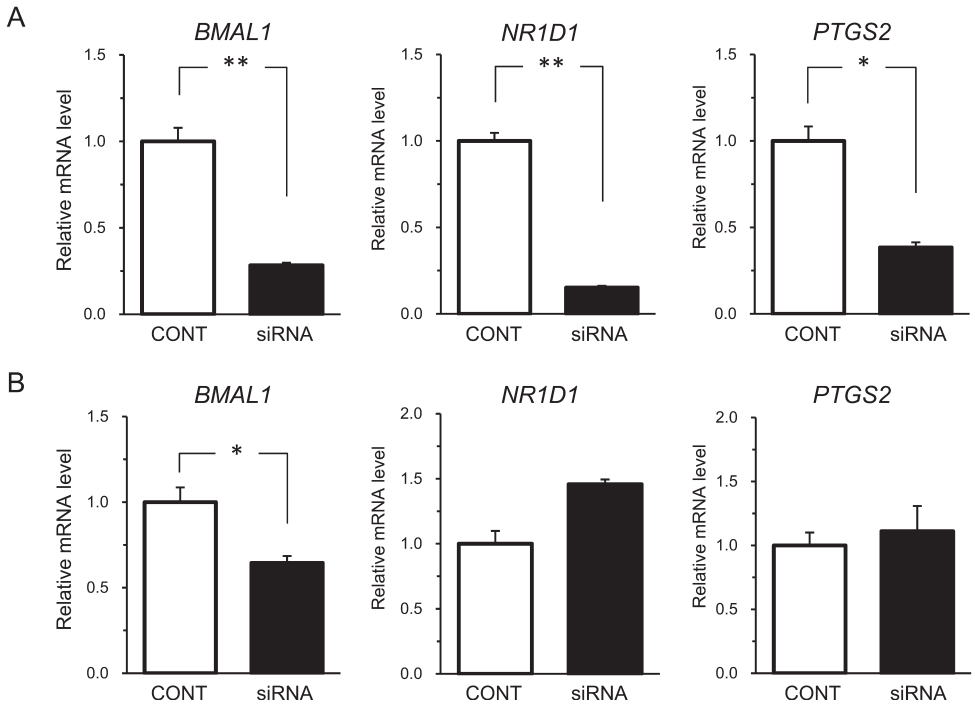

Fig. 5. Expression of the *BMAL1*, *NR1D1* and *PTGS2* gene transcripts in bovine USCs and UECs transfected with *BMAL1*-specific siRNA or non-silencing RNA. USCs (A) and UECs (B) were separately treated with *BMAL1*-specific siRNA (siRNA) or non-silencing RNA (CONT) according to the indicated protocols. The cells were then synchronized with forskolin. Total RNA samples were collected at 30 h for the *BMAL1* transcript and 48 h for the *NR1D1* and *PTGS2* transcripts after synchronization. RT-qPCR analyses of transcript levels were performed using their specific primers. The relative transcript level was normalized to *GAPDH* and expressed relative to the non-silencing RNA group. Each value represents the means ± SEM of three independent determinations. The statistical analyses were performed by one-way ANOVA with the Student's *t* test. ** P<0.01; * P<0.05.

Effects of heme and SR8278 on the PTGS2 expression in bovine USCs and UECs

To further investigate the regulation of *PTGS2* expression, we treated bovine USCs and UECs with the agonist (heme) or antagonist (SR8278) of REV-ERBα. As shown in Fig. 6, the *Ptgs2* transcript level was dramatically increased by SR8278 in both cell types. Conversely, treatment with heme did not alter the expression. During treatment with heme or SR8278, different transcript levels of the clock genes *BMAL1* and *NR1D1* were observed. The *NR1D1* transcript level was greatly increased by SR8278 in the USCs but not in the UECs. However, the *BMAL1* transcript level was not changed by SR8278 in the USCs and UECs, probably due to the absence of the REV-ERBα action. Treatment with heme increased the *NR1D1* transcript levels in both cell types, although their increases were very small. Treatment with heme also increased the *BMAL1* transcript level in the USCs.

Production of $PGF_2\alpha$ by bovine USCs and UECs

To further test whether the *PTGS2* expression is regulated by BMAL1 and/or REV-ERBα, we determined the production of $PGF_2\alpha$ in culture media after treatment with *BMAL1*-specific siRNA and the agonist or antagonist of REV-ERBα. As shown in Fig. 7A, the level of $PGF_2\alpha$ significantly decreased in USCs after the transfection of *BMAL1*-specific siRNA (P<0.05), and this was coincident with the decreased transcript level of *PTGS2*. Conversely, the level of $PGF_2\alpha$ did not significantly change in UECs transfected with *BMAL1*-specific siRNA, in which the transcript level of *PTGS2* remained unchanged. In both cell types treated with heme or SR8278, the production of $PGF_2\alpha$ was well reflected by the expression of *PTGS2*. As shown in Fig. 7B, the level of $PGF_2\alpha$ increased approximately twofold in the presence of SR8278. Conversely, treatment with heme caused no significant changes in the $PGF_2\alpha$ level.

Discussion

The regulation of $PGF_2\alpha$ production in the bovine uterus endometrium during the estrus cycle remains poorly understood, although P_4, E_2 and oxytocin are well known as the regulatory hormones [3, 4, 11]. In the present study, we focused on control of the cellular circadian clockwork related to $PGF_2\alpha$ production in bovine USCs and UECs. We demonstrated that the nuclear receptor REV-ERBα plays an inhibitory role in $PGF_2\alpha$ secretion, which is mediated through direct inhibition of *PTGS2* expression in both cell types. We also showed that BMAL1 promotes $PGF_2\alpha$ secretion as a heterodimer with CLOCK, which is mediated through the transactivation of the *PTGS2* expression in USCs. The secretion of $PGF_2\alpha$ may be balanced by the inhibitory or stimulatory transcriptional regulation of REV-ERBα and BMAL1/CLOCK, respectively.

Circadian clockwork systems generate cellular rhythms in physiological functions via identified transcriptional and posttranscriptional regulatory processes. The cellular clock components CLOCK and

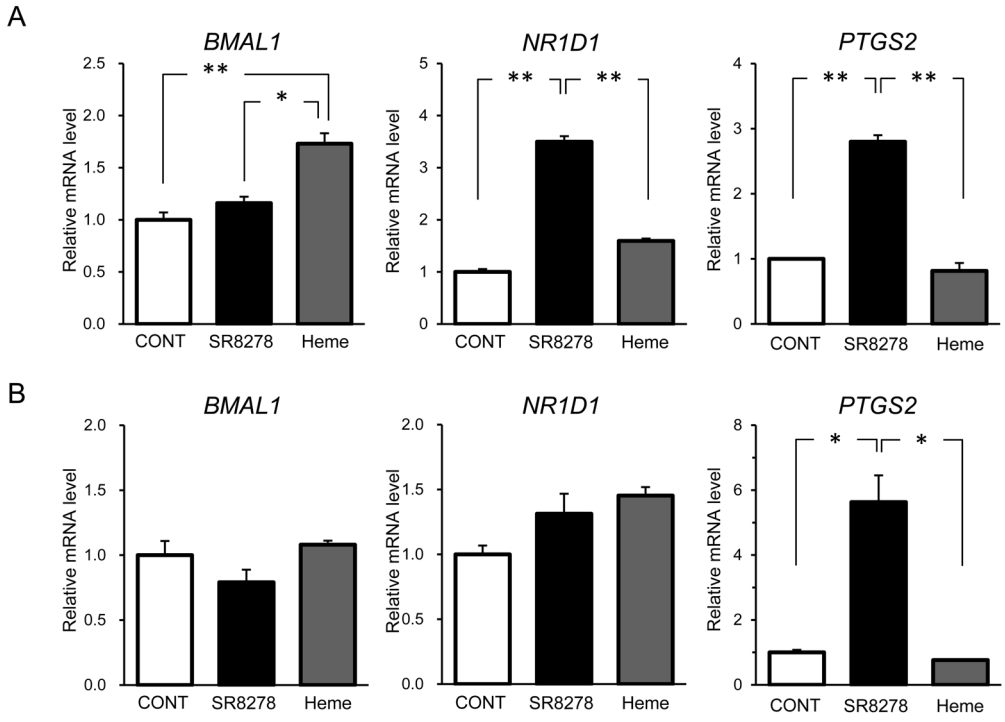

Fig. 6. Expression of the *PTGS2* gene transcript in bovine USCs and UECs treated with the agonist or antagonist of REV-ERBα. USCs (A) and UECs (B) were separately treated with the agonist (heme) or antagonist (SR8278) of REV-ERBα according to the indicated protocols. Cells were then synchronized with forskolin. Total RNA samples were collected at 30 h for the *BMAL1* transcript and 48 h for the *NR1D1* and *PTGS2* transcripts after synchronization. RT-qPCR analyses of transcript levels were performed using their specific primers. The relative transcript level was normalized to *GAPDH* and expressed as relative to the CONT group. Each value represents the means ± SEM of three independent determinations. The statistical analyses were performed by one-way ANOVA with the Student's *t* test. ** $P<0.01$; * $P<0.05$.

BMAL1 bind to the E-box enhancer and positively drive the expression of the *Period* genes (*Per1-3*) and the *Cryptochrome* genes (*Cry1-2*). In turn, PER and CRY proteins heterodimerize and undergo phosphorylation. The PER-CRY complexes translocate to the nucleus and repress the activity of CLOCK-BMAL1 heterodimers [25, 26]. Further adding to the complexity, CLOCK-BMAL1 heterodimers induce expression of the nuclear receptor REV-ERBα (encoded by *NR1D1*), resulting in repression of the transcription of *BMAL1* through direct binding to RORE located in the *BMAL1* promoter [13]. The conservative transcriptional feedback loop resides in the ovine circadian clock [27], and the circadian expression of the core clock genes *BMAL1*, *PER2*, and *CRY1* is generated in the ovine liver [28]. It was thus expected that clock genes would also be driven in oscillatory patterns in bovine uterus endometrial cells, which would be similar to murine cells [18, 29]. Our present results showed that the circadian clockwork machinery functions in bovine USCs exposed to P_4, while it was weak compared to that in rat (Fig. 1B). We also showed that the bovine *PER1* transcript level exhibited a significant diurnal rhythm in bovine USCs exposed to P_4 (Fig. 2). However, comprehensively, the diurnal rhythms of the clock gene expression were not significant in bovine USCs and UECs in the presence or absence of steroid hormones, except for *PER1* and *BMAL1* under some conditions (Figs. 2 and 3). Therefore, it is supposed that the circadian rhythm of clock genes, at least, that we observed may be weak in both USCs and UECs.

In regard to the oscillation, the superiority of clock genes and strength of circadian oscillation in the uterus may depend on cell types and physiological states. An immunohistochemical analysis revealed that the PER2 protein expression is constitutive in epithelial cells but not in the stromal cells [23]. In addition, the circadian rhythm of clock genes was reported to be significant for *Cry1* and not for *Per1*, *Per2* or *Bmal1* in the mouse uterus during the late stage of pregnancy [30]. Moreover, there may be differences among animal species. Most of the clock genes displayed no significant rhythms in bovine lymphocytes [31], unlike murine lymphocytes [32].

In the present study, the diurnal rhythms of clock genes were not particularly significant, but steroid hormones differentially affected the temporal changes in clock gene expression in both the USCs and UECs. In the USCs, the transcript levels of *NR1D1* increased, especially at 24 to 48 h after synchronization in the presence of P_4 (Fig. 2). In the UECs, the transcript levels of *NR1D1* and *PER1* changed significantly in the presence of E_2 (Fig. 4). As revealed by chromatin immunoprecipitation followed by a massive parallel sequencing (ChIP-seq) analysis coupled with microarrays in the mouse uterus, progesterone receptor binding sites are abundant near the coding regions of clock genes (*Clock*, *Npas2*, *Cry1*, *Per1*, and *Nr1d2*) [33]. The phase of *Per2* oscillation in the uterus from *Per2*::*Luc* knock-in mice is affected by E_2, and the *Per2* oscillation in the uterus during the mouse estrus cycle is modulated by fluctuating E_2 and P_4 [29, 34]. These findings, taken together with our present

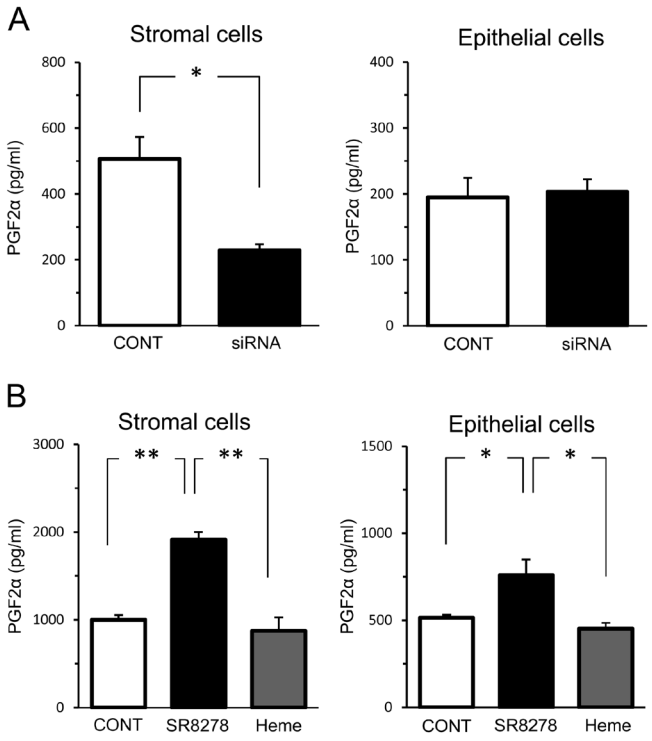

Fig. 7. Production of $PGF_2\alpha$ by bovine USCs and UECs treated with *BMAL1*-specific siRNA and the agonist or the antagonist of REV-ERBα. USCs and UECs were treated with *BMAL1*-specific siRNA (A) and the agonist or antagonist of REV-ERBα (B) as described in Figs. 4 and 5. The culture media were collected at 48 h after synchronization with forskolin and assayed for $PGF_2\alpha$. Each value represents the means ± SEM of three independent determinations. The statistical analyses were performed by one-way ANOVA with the Student's *t* test. ** $P<0.01$; * $P<0.05$.

data, indicate that the expression of clock genes affected by steroid hormones may alter the expression of clock-controlled genes and modulate physiological functions in endometrial cells.

Here we found that the transcript level of *PTGS2* significantly decreased with circadian oscillation in USCs during exposure to P_4 for 48 h, while its transcript level increased in UECs after exposure to E_2 for 48 h (Fig. 4). Consequently, we propose that the *PTGS2* expression in USCs is regulated by the circadian clockwork and P_4. It is possible that other clock genes that we did not analyze in Fig. 1 may cause circadian oscillation in the *PTGS2* expression in USCs. Conversely, the circadian clockwork in UECs may not affect the *PTGS2* expression. Incidentally, the transcript level of *PTGS2* significantly increased in the absence of P_4 and E_2 (Fig. 4), but the expression may result from the action of the forskolin used as a resetting factor for the clockwork. It is known that *PTGS2* expression is induced by cyclic AMP [35, 36].

BMAL1 transactivates the target genes by binding of the heterodimer with CLOCK to the E-box element (5'-CACGTG-3') on the promoter region of the responsive clock genes such as *PER*, *NR1D1*, *DBP* and *RORα* [26, 27]. There are functional E-box elements in the promoter region of the rat *PTGS2* gene [37], and our recent study demonstrated that *Bmal1* knockdown causes a significant decrease in the *Ptgs2* expression and PGE_2 production in rat ovarian granulosa cells [22]. In the promoter region of the bovine *PTGS2* gene, also there are also several E-box sites [38]. In the present study, *BMAL1* knockdown caused significant decreases in *PTGS2* expression and $PGF_2\alpha$ production in the USCs in the presence of P_4 (Figs. 5 and 7). Conversely, *BMAL1* knockdown had no significant effect on the *PTGS2* expression and $PGF_2\alpha$ production in the UECs in the presence of E_2 (Figs. 5 and 7). In the UECs, *BMAL1* knockdown also had no effect on *NR1D1* expression. This finding may indicate that BMAL1 transactivation in the *PTGS2* and *NR1D1* genes is weak in UECs exposed to E_2. Actually, it has been reported that E_2 disrupts the circadian expression of *Per1* and *Per2*, which are promoted by BMAL1, in the liver, kidney and uterus of rats [39]. In the present results, the synchronization of USCs exposed to P_4 caused a significant increase in *BMAL1* transcripts after 6 h, while the UECs exposed to E_2 displayed no significant increase in *BMAL1* transcripts (Figs. 2 and 3). These results suggest that the effect of *BMAL1* knockdown is weak in the E_2-treated UECs, probably through E_2 inhibition of the transcriptional activity of *BMAL1*. Taken together with the present data, these findings suggest that the expression of bovine *PTGS2* is controlled under BMAL1 or its responsive clock genes in USCs, but not UECs, in the presence of ovarian steroids.

Interestingly, when the transcript level of *NR1D1* was high in the USCs and UECs in the presence of P_4 or E_2, the transcript level of *PTGS2* was low. We therefore focused on the possible regulation of *PTGS2* expression by REV-ERBα. The bovine *PTGS2* gene has two ROREs in the promoter region within −3000 bp upstream from the transcriptional start site (AC_000173). We analyzed the expression of *PTGS2* using the antagonist (SR8278) and agonist (heme) of REV-ERBα. The agonist was ineffective, while the antagonist resulted in increased transcript levels of *PTGS2* in both cell types in the presence of P_4 or E_2 (Fig. 6). At least, these results indicate that REV-ERBα represses *PTGS2* gene expression.

Concomitantly, $PGF_2\alpha$ production completely coincided with the *PTGS2* transcript level (Fig. 7). It is also of interest that the antagonist clearly enhanced the *PTGS2* expression in the UECs, in which the circadian regulation of *PTGS2* was weak as revealed by *BMAL1* knockdown. In bovine UECs, therefore, *PTGS2* expression is dominantly controlled under REV-ERBα, but not the circadian clockwork, in the presence of E_2. Unlike UECs, however, bovine USCs are controlled under both REV-ERBα and the circadian clockwork. Our finding that treatment with the antagonist increased the transcript level of *NR1D1* in the USCs also supports the autoregulation of *NR1D1* expression as reported previously [40, 41]. However, it is reasonable that the antagonist had no significant effect on the transcript level of *BMAL1* in the USCs, which has been found to be repressed by REV-ERBα [13]. Unexpectedly, however, treatment with heme increased the *BMAL1* transcript level in the USCs in the present study, and the mechanism remains to be investigated.

In conclusion, the circadian oscillation of clock genes is partially weak in bovine USCs and UECs, but ovarian steroid hormones may exert differential influences on the expression of the clock genes and the *PTGS2* gene. *BMAL1* knockdown decreased both the *PTGS2* expression and $PGF_2\alpha$ production in USCs in the presence of P_4, indicating circadian regulation of the *PTGS2* expression. However,

the *PTGS2* expression in UECs is mostly independent of the circadian clockwork. The *PTGS2* expression was commonly suppressed in both cell types by REV-ERBα in the presence of steroid hormones. The present findings contribute to our understanding of the intercellular mechanisms underlying the *PTGS2* expression and $PGF_2\alpha$ production in bovine uterus endometrium cells.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS No. 22380152, 24658246) (to M-A Hattori). Keishiro Isayama was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (Grant No. 6117).

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