Up-regulation of *Per1* expression by estradiol and progesterone in the rat uterus

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Abstract

It has been established that estrogen can alter circadian rhythms in behavior and endocrine physiology in rodents. The uterus is a reproductive organ that is critically dependent on regulation by ovarian steroids. Here, we examined the expression of *Per1* in different compartments of the uterus, and explored whether the ovarian steroids could regulate *Per1* expression employing ovariectomized rat uterus. RT-PCR analysis showed that *Per1* was cyclically expressed in the uterus. As revealed by *in situ* hybridization, the staining intensity of *Per1* mRNA was stronger at ZT 8 than at ZT 0 in the uterine luminal epithelium (LE), stroma (S), and myometrium (M) compartments, but was not changed in the glandular epithelium (GE). Both *in situ* hybridization and immunofluorescence analyses revealed that

estradiol (E₂) administration induced high expression of *Per1* in the LE, GE, and M, and less expression in the S compartment. Progesterone (P₄) treatment resulted in an obvious enhancement of *Per1* expression in the LE, GE, and S, but unchanged in the M compartment. Furthermore, the E₂- and P₄-activated *Per1* expression was significantly repressed by their respective antagonists, ICI182 780 and RU486. These findings were further supported by RT-PCR analysis of *Per1* expression in cultured uterine stromal cells. Collectively, the present data indicate that E₂ and P₄ might be involved in modification of circadian rhythm via direct regulation of the expression of clock genes.

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Introduction

Circadian rhythm is generated by genetically determined biological clock, and is prominently entrained by cues from the 24-h light:darkness cycle (Dunlap 1999, Reppert & Weaver 2001). In mammals, the central clock is located in the suprachiasmatic nucleus of the hypothalamus (Reppert & Weaver 2001). Circadian rhythms also exist in a variety of peripheral tissues, including the heart, liver, kidney, lung, spleen, skeletal muscles (Yamamoto *et al.* 2004), and the uterus (Dolatshad *et al.* 2006). At the molecular level, the clock system is composed of interlocked transcriptional and translational feedback loops. The CLOCK and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1), associated as heterodimers, bind to the E-box enhancer and positively drive the expression of *Period* genes (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* genes (*Cry1* and *Cry2*), whose proteins, in turn, form multimeric complexes and feed back to repress the transactivation by CLOCK/BMAL1 in the nucleus (Shearman *et al.* 2000, Ueda *et al.* 2005).

The peripheral clocks are synchronized by the central pacemaker through complex interaction of neural, humoral, and behavioral cues (Damiola *et al.* 2000, Schibler & Sassone-Corsi 2002). So far, the identification of resetting cues and their regulatory effects on clockwork are mostly

based on molecular biological studies in cultured cell lines *in vitro*. A variety of factors have been unraveled having the potential to reset circadian rhythms in peripheral cells, such as cAMP analog, forskolin, phorbol-12-myristate-13-acetate, endothelin, prostaglandin estradiol (E₂), fibroblast growth factor, calcium ionophores, retinoic acid, glucocorticoids, and parathyroid hormone (Akashi & Nishida 2000, Balsalobre *et al.* 2000a,b, Tsuchiya *et al.* 2005, Hinoi *et al.* 2006, Nakahata *et al.* 2006, Shirai *et al.* 2006). All these factors could induce an acute activation of *Per1* expression, and thus synchronize or reset the circadian rhythms in cultured cells. For instance, glucocorticoids could efficiently activate *Per1* expression and synchronizes circadian oscillations of clock genes in cultured cells, and could also phase shift the circadian rhythms in peripheral tissues *in vivo* (Balsalobre *et al.* 2000b).

Interestingly, it has long been established that ovarian steroids have significant modulatory effects on the circadian rhythms and sleep patterns. It has been demonstrated that estrogen administration alters the circadian rhythms in behavior and endocrine physiology in rodents (Morin *et al.* 1977, Thomas & Armstrong 1989). Recently, it was found that the phase of estrous cycle has a profound effect on the oscillation of PER2 protein within the brain (Perrin *et al.* 2006). It was further reported that chronic treatment with E₂

drastically alters the circadian rhythms of *Per1* and *Per2* expression in the liver, kidney, and uterus (Nakamura *et al.* 2005). In contrast, progesterone (P_4) was found to reduce the locomotor activity, but not the period of rhythms (Axelson *et al.* 1986, Labyak & Lee 1995). Despite the previous evidence, very little is currently known about the molecular mechanism underlying the sex steroidal effects on circadian clockwork. We hypothesize that the ovarian steroids probably modulate circadian rhythms through direct regulation of the expression of clock genes like *Per1*.

The uterus is one of the principal targets of estrogen and P_4 . It is composed of heterogeneous cell types including luminal and glandular epithelial cells, stromal cells, and muscle layers. In the adult uterus, E_2 stimulates the proliferation of epithelial cells (Quarmby & Korach 1984), whereas P_4 inhibits E_2 -induced hyperplasia of the epithelial compartments (Martin *et al.* 1973). In addition, P_4 acts to regulate the proliferation and differentiation of uterine stromal cells (Rider 2002). Herein, we employed the rat uterus as the model system to testify our hypothesis. The results showed that both ovarian steroids could up-regulate the expression of *Per1* gene, which sheds some light on how the steroids are involved in the modulation of circadian rhythms.

Materials and Methods

Reagents

E_2 , P_4 , mifepristone (RU486), and ICI182 780 were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), and 100× penicillin–streptomycin solution (PS) were obtained from GIBCO. Collagenase (type I) was purchased from Invitrogen. pGEM-T Easy Vector, T7, and SP6 RNA Polymerases were purchased from Promega. DIG RNA labeling kit, anti-DIG antibody, nitroblue tetrazolium salt (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Roche Diagnostics. Anti-PER1 polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO, USA), and Texas Red-conjugated goat F(ab')₂ anti-rabbit IgG (H+L) was from Leinco Technologies (St Louis, MO, USA). All the other chemicals used were of reagent grade and obtained from commercial sources.

Animals and treatments

Wistar rats were maintained under 12 h light:12 h darkness cycles (light between 0800 and 2000 h) with water and food available *ad libitum*. The female rats at 7 weeks of age were bilaterally ovariectomized (OVX) under ether anesthesia, and allowed for 1 week to recover from surgery. OVX rats were then subjected to hormonal treatments. In the first treatment, at ZT 0 (zeitgeber time, 0800 h), the rats were injected subcutaneously with a single dose of sesame oil (vehicle), E_2

(50 µg/kg), or P_4 (10 mg/kg). All hormones were given in 100 µl sesame oil. All OVX rats were killed at ZT 0 on the following day, and uteri were collected for histochemical examinations. All procedures were conducted in accordance with the Guideline for Animal Experiment in the Faculty of Medicine, Kyushu University and The Law (No. 105) and Notification (No. 6) of the Government.

Cell culture

Female rats at 3 months of age were mated with fertile males, and the day of finding a copulatory plug or sperm in the vaginal smear was designated as day 1 of pregnancy. Pregnant rats were killed on day 5 of gestation, and uteri were removed for isolation of uterine stromal cells (USCs). The uterine lumens, after flushing with PBS for several times, were filled with PBS containing 0.1% (w/v) collagenase and 1× PS. The closed uterine horns were incubated in a shaking water bath at 37 °C for 1 h. The dissociated cell suspensions were pooled, and the uteri were longitudinally cut open and pipetted many times to release more USCs. The harvested cells were washed thrice with fresh DMEM/F12, and seeded onto 35 mm collagen-coated dishes at a density of 2×10^5 cells/dish with 2 ml of culture medium (phenol red-free DMEM/F12 supplemented with 10% charcoal-treated FBS and 1× PS). The medium was changed 15 min after cell seeding to remove epithelial cells. Cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The USCs were positively immunostained for vimentin but negatively for cytokeratin. The staining result revealed a purity of ~95%.

Total RNA extraction and RT-PCR

Female rats at 9–10 weeks of age at diestrus were killed at 4-h intervals over a daily cycle. Two uterine horns of each rat were collected, with one horn used for total RNA extraction and the other for *in situ* hybridization. In addition, cultured USCs were harvested at the indicated times and subjected to total RNA isolation. The RNA concentration was determined using 260/280 u.v. spectrophotometry (Pharmacia Biotech), and RNA integrity was checked by agarose gel electrophoresis. One microgram of total RNA was utilized for cDNA synthesis in 20 µl mixture using Moloney–murine leukemia virus (M-MLV) reverse transcriptase (Amersham Biosciences) and oligo-dT primer according to the manufacturer's protocol. The expression levels of β -actin and *Per1* were examined by PCR using the following primers: β -actin (NM_031144), forward: 5'-TTG CGC TCA GGA GGA GCA AT-3' (445–464), reverse: 5'-ATC ATG TTT GAG ACC TTC AA-3' (1069–1088); *Per1* (XM_340822), forward: 5'-AGT ACG CGC TGG CCT GTG TCA A-3' (689–710), reverse: 5'-GGC GCT TCA TAA CCG GAG TGG AT-3' (1222–1244). PCR was performed in a 10 µl mixture containing 1× PCR buffer, 0.25 U AmpiTaq-Gold enzyme (Applied Biosystems, Tokyo, Japan), 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.2 µM of each primer, and 1 µl of 1:5 diluted cDNA as a template. Semiquantitative RT-PCR was

performed to measure the expression levels of *Per1* and β -*actin* (internal control). The following cycling conditions were applied after an initial denaturation step (95 °C for 10 min), denaturation at 95 °C for 50 s, annealing at 50 °C for 50 s, and extension at 72 °C for 50 s. The 30 cycles of PCR for both *Per1* and β -*actin* were currently used within the linear range of amplification based on the amplification curves. The PCR products were subjected to 2% agarose gel electrophoresis. The bands, stained by ethidium bromide, were visualized by u.v. fluorescence. Densitometric intensity was quantified by Scion Image (NIH software).

In situ hybridization

A 556 bp (689–1244) fragment of PCR product of *Per1* (cDNA from USC5) was purified from agarose gel and cloned into pGEM-T Easy Vector. Sense and antisense probes were transcribed *in vitro* from linearized plasmids containing T7 or SP6 polymerase sites using a DIG labeling kit. Uteri collected from normal and OVX rats were cut into 6–8 mm pieces, and embedded in OCT compound for immediate freezing on a bed over liquid nitrogen. The tissues were cryosectioned at 8 μ m thickness, and mounted on silane-coated slides. After fixation (4% paraformaldehyde, pH 7.4) and acetylation (0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0)), each tissue was covered with 200 μ l hybridization buffer and prehybridized in a humidified chamber at 42 °C for 2 h. The hybridization buffer consists of 4 $\times$ (SSC), 50% (v/v) formamide, 100 mg/ml dextran sulfate, 1 $\times$ Denhardt's solution, 10 mg/ml salmon sperm DNA, and 0.5 mg/ml tRNA. Hybridization was carried out in the hybridization buffer containing 1 μ g/ml denatured (80 °C for 10 min) antisense or sense RNA probe at 42 °C for 16 h, with the buffer covered with parafilm protecting from evaporation. After hybridization, the sections were first rinsed in 5 $\times$ SSC, and then washed twice in 50% (v/v) formamide/2 $\times$ SSC at 42 °C for 30 min, 2 $\times$ SSC and 0.2 $\times$ SSC at 42 °C each for 30 min. After washing with buffer 1 (0.1 M Tris (pH 7.6) and 0.15 M NaCl) for 10 min at room temperature (RT), the slides were incubated in buffer 2 (1.5% (w/v) blocking reagent in buffer 1) at RT for 2 h. Immunological examination of the hybridized probe was performed in a humidified chamber with anti-DIG antibody conjugated with alkaline phosphatase (1:500 dilution in buffer 2) overnight at 4 °C. Slides were washed twice in buffer 1 for 15 min at RT and equilibrated in buffer 3 (0.1 M Tris (pH 9.5), 0.15 M NaCl, and 50 mM MgCl₂) for 5 min. For color development, slides were incubated in a NBT/BCIP solution at RT for 2–4 h. The reaction was stopped with Tris–EDTA (0.1 M Tris–HCl, 0.01 M EDTA (pH 8.0)) for 15 min, and the slides were cover slipped for signal visualization.

Immunofluorescence analyses

Uteri were collected from vehicle or hormone-treated OVX rats and frozen as outlined previously. Air-dried tissue sections

of 8 μ m thickness were fixed for 5 min in acetone at –20 °C. Non-specific binding was blocked using 2% (v/v) goat serum in PBS (blocking buffer) for 30 min. Sections were incubated for 12–18 h at 4 °C with rabbit anti-mPER1 polyclonal antibody diluted in blocking buffer (1:500). Rabbit serum was substituted for the primary antibody as negative control. After washing with PBS, they were incubated with the second antibody for 1 h at RT. Sections were subsequently washed in PBS and mounted with Mount-Quick Aqueous (Daido Sangyo, Japan). Immunostaining was detected under a fluorescence microscope (Nikon, Japan).

Statistical analyses

The quantification of *in situ* hybridization and immunofluorescent staining intensity were performed as previously reported (Han *et al.* 2007). A box was drawn through each compartment in a random orientation, and the pixel value of each box was measured by NIH Scion Image. A mean value was obtained from the determination of three boxes for each slide section. The values in all figures were shown as means $\pm$ s.d. of three independent sections from different samples. The non-specific staining intensity with sense RNA probe was used to normalize *Per1* mRNA staining signal and the fluorescence intensity after incubation with normal rabbit serum to standardize PER1 protein intensity. The RT-PCR results were similarly expressed as means $\pm$ s.d. of three independent experiments on different RNA sets. The differences between them were evaluated using Student's *t*-test following one-way ANOVA. A *P* value <0.05 was considered significant.

Results

Per1 expression in different compartments of the diestrous uterus

The RT-PCR analysis showed rhythmic changes of *Per1* mRNA level in the rat uteri at the diestrous stage (Fig. 1). The trough and peak of *Per1* expression appeared around ZT 0 and ZT 8 respectively. The transcript level of *Per1* was then analyzed by *in situ* hybridization in different compartments of the uterus at ZT 0 and ZT 8. The expression of *Per1* mRNA can be readily detected in both the endometrium and the myometrium (M) at ZT 0 (Fig. 2A–C) and ZT 8 (Fig. 2D–F). Strong signals were observed in the luminal epithelium (LE) and glandular epithelium (GE), as compared with that in the stroma (S). The staining intensity of *Per1* mRNA was approximately twofold higher at ZT 8 (Fig. 2D, E and H) than at ZT 0 (Fig. 2A, B and H) in the S, LE, and M compartments. However, no obvious change of *Per1* expression was found in the GE compartment (Fig. 2C, F, and H). No specific products were observed when using the sense riboprobes (Fig. 2G).

Figure 1 Cyclic expression of *Per1* mRNA in the uterus. Adult female rats at diestrus were killed at 4-h intervals over a daily cycle (ZT 0, 0800 h). Total RNA was extracted and subjected to RT-PCR analysis of *Per1* transcript levels, which were normalized to β -actin values (the maximal, set as 100). Data are presented as mean $\pm$ of three independent experiments.

Effects of E_2 and P_4 on *Per1* expression in different uterine compartments

To examine the effects of E_2 and P_4 on the expression of *Per1* in the uterus, OVX rats were treated with steroid hormones at ZT 0 and uteri were collected 24 h later. *In situ* hybridization analysis showed that the E_2 -primed uteri exhibited much stronger staining in the LE (ca 3.1-fold), GE (ca 2.8-fold), and M (ca 3.2-fold), while less intense signal in the S (ca 2.0-fold) compartment (Fig. 3C, D, and G), as compared with the control uteri (Fig. 3A, B and G). P_4 administration resulted in intense staining in the S (ca 3.3-fold), LE (ca 2.4-fold), and GE (ca 2.1-fold) compartments, but not in the M (ca 1.2-fold; Fig. 3E–G). Immunofluorescent staining for PER1 protein was also examined in the oil-, E_2 -, and P_4 -administrated uteri. In the control uteri, weak staining of PER1 was observed in the S compartment, and relatively stronger staining in the LE and GE (Fig. 4A and B). With E_2 treatment (Fig. 4C, D, and G), PER1 protein was more intensively expressed in the S (ca 2.2-fold), LE (ca 3.2-fold), and GE (ca 2.1-fold) as compared with the counterparts of the control uteri. Treatment with P_4 also resulted in stronger immunostaining of PER1 in the S (ca 2.6-fold), LE (ca 2.9-fold), and GE (ca 2.9-fold) compartments (Fig. 4E, F and G).

Figure 2 Representative photomicrographs of *in situ* hybridization analyses for *Per1* mRNA in diestrous uteri. The uterine samples were obtained at ZT 0 (A–C) and ZT 8 (D–F). (C) and (F) show the insert sections of (A) and (D) at higher magnification respectively. Dark shades show the staining of *Per1* mRNA. No signal was obtained in the uteri stained with sense probe (G). (H) shows the fold changes of staining intensity of *Per1* in different uterine compartments at ZT 8 relative to those at ZT 0 (set at 1). Data are presented as mean $\pm$ s.d. of three independent sections. *Statistical significance ($P < 0.05$), ZT 8 versus ZT 0. Samples from three individual rats were analyzed for each group, and at least three sections were examined by histochemistry. S, stroma; LE, luminal epithelium; GE, glandular epithelium; M, myometrium. Scale bars, 100 μ m.

Figure 3 Representative photomicrographs of *in situ* hybridization analyses for *Per1* mRNA in the uteri treated with E₂ or P₄. At ZT 0, the OVX rats were injected subcutaneously with 100 µl oil-containing vehicle (A and B), 50 µg/kg E₂ (C and D), or 10 mg/kg P₄ (E and F), and uteri were removed after 24 h. (G) shows the fold changes of *Per1* staining intensity in different compartments of the E₂- and P₄-administrated uteri relative to the control (set at 1). Data are presented as mean ± s.d. of three independent sections.

*Statistical significance ($P < 0.05$) compared with the control. Samples from three individual rats were analyzed for each group, and at least three sections were examined by histochemistry. S, stroma; LE, luminal epithelium; GE, glandular epithelium; M, myometrium. Scale bars, 100 µm.

No staining was observed in the negative control (data not shown). Taken together, immunohistochemical observations of *Per1* expression in response to E₂ or P₄ were similar to the results revealed by *in situ* hybridization.

Repression of E₂- and P₄-regulated *Per1* expression by respective antagonist treatments

The antagonists for estrogen receptor (ER) and progesterone receptor (PR) were utilized to evaluate whether the stimulatory effects of E₂ and P₄ on *Per1* expression are mediated via their respective receptors. The antagonists were injected at ZT 0, and the steroids were given after 2 h.

Figure 4 Representative photomicrographs of immunofluorescent analyses for PER1 protein in the uteri treated with E₂ or P₄. At ZT 0, the OVX rats were injected subcutaneously with 100 µl oil-containing vehicle (A and B), 50 µg/kg E₂ (C and D), or 10 mg/kg P₄ (E and F), and uteri were removed after 24 h. (G) shows the fold changes of *Per1* staining intensity in different compartments of the E₂- and P₄-administrated uteri relative to the control (set at 1). Data are presented as mean ± s.d. of three independent sections. *Statistical significance ($P < 0.05$) compared with the control. Samples from three individual rats were analyzed for each group, and at least three sections were examined by histochemistry. S, stroma; LE, luminal epithelium; GE, glandular epithelium. Scale bars, 100 µm.

Pretreatment with ICI182 780 (ER antagonist) caused a significant decrease in the staining intensity of *Per1* mRNA in all the examined compartments, the LE (ca 70%), GE (ca 60%), S (ca 60%), and M (ca 50%; Fig. 5C, D and G), as compared with the control administrated E₂ alone (Fig. 5A, B and G). On the other hand, the uteri treated with P₄ alone exhibited strong staining of *Per1* in the LE, GE, and S compartments (Fig. 5E), whereas RU486 (PR antagonist) pretreatment remarkably inhibited *Per1* expression in the GE

Figure 5 Representative photomicrographs of *in situ* hybridization analyses for *Per1* mRNA in the uteri treated with E_2 , ICI182 780 + E_2 , P_4 , or RU486 + P_4 . At ZT 0, the OVX rats were injected with 100 μ l oil-containing vehicle, 3 mg/kg ICI182 780 (ER antagonist), or 30 mg/kg RU486 (PR antagonist), after 2 h, followed by injection with 50 μ g/kg E_2 or 10 mg/kg P_4 . The uteri were collected at ZT 0 the following day. (A) and (B) E_2 , (C) and (D) ICI182 780 + E_2 , (E) P_4 , and (F) RU486 + P_4 . (G) and (H) show the percentage repressions of *Per1* staining intensity in different compartments of antagonist-pretreated uteri relative to the control (set at 100%). Data are presented as mean $\pm$ s.d. of three independent sections. *Statistical significance ($P < 0.05$) compared with the control. Samples from three individual rats were analyzed for each group, and at least three sections were examined by histochemistry. S, stroma; LE, luminal epithelium; GE, glandular epithelium; M, Myometrium. Scale bars, 100 μ m.

and S by ~ 60 and 70% respectively (Fig. 5F and H). The intensity of *Per1* staining in the LE also showed $\sim 50\%$ reduction (Fig. 5F and H). These findings suggest that ER and PR might mediate E_2 - and P_4 -regulated *Per1* expression in the uterus respectively.

E₂ and *P₄* regulation of *Per1* expression in cultured USCs

The regulation of *Per1* expression by E_2 and P_4 was confirmed in cultured USCs. The USCs reached confluence after 3-day culture, was further cultured in the presence of vehicle (ethanol), E_2 (10 nM), or P_4 (100 nM), and collected for RT-PCR analysis at 6 and 24 h. E_2 significantly though slightly activated *Per1* expression at 6 h, but not at 24 h (Fig. 6A). In contrast, P_4 treatment resulted in enhanced *Per1* transcript level at both 6 and 24 h (Fig. 6B). Furthermore, we examined whether RU486 could antagonize the P_4 -activated *Per1* expression in cultured USCs. Cells were treated with vehicle or 10 μ M RU486 for 2 h, and then cultured in the

presence of P_4 or P_4 + RU486. After 6 h, the cells were harvested for analysis of *Per1* mRNA level. The expression of *Per1* was significantly inhibited in the RU486-treated cells relative to the control cells exposed to P_4 alone (Fig. 6C).

Discussion

The uterus, a highly dynamic tissue, undergoes profound remodeling, implicating cyclic proliferation, differentiation, and differential gene expression dependent on the levels of circulating sex steroid hormones. Recent studies have demonstrated that circadian clock genes are rhythmically expressed in the uterus (Nakamura *et al.* 2005, Dolatshad *et al.* 2006) as well as other peripheral tissues (Yamamoto *et al.* 2004). However, their findings were based on the mRNA analyses prepared from the entire uterine tissue using RT-PCR or northern blotting methods. In the present study, we investigated the expression of *Per1*, a core clock gene, in

Figure 6 Stimulatory effects of E₂ and P₄ on *Per1* expression in cultured USC. The 3-day cultured USC were further cultured in fresh medium containing vehicle (A–C), 10 nM E₂ (A), 100 nM P₄ (B and C), or 100 nM P₄ + 10 μ M RU486 (RU) (C). The cells were harvested at the indicated times for RT-PCR analysis of *Per1* expression. *Per1* transcript levels were normalized to β -actin values and expressed as arbitrary units relative to the vehicle control (set as 1). Data are presented as mean $\pm$ s.d. of three independent experiments. Different letters are significant ($P < 0.05$).

different compartments of the uterus with *in situ* hybridization. As expected, our results showed that the staining intensity of *Per1* mRNA was obviously higher at ZT 8 than at ZT 0 in the LE, S, and M compartments of the diestrous uterus, which was consistent with the rhythm of *Per1* as revealed by RT-PCR. Surprisingly, the staining signal was not apparently changed at ZT 0 and ZT 8 in the GE compartment, suggesting the lack of normal clockwork in control of uterine glandular physiology. It has been previously suggested that cellular differentiation process is responsible for the absence of circadian rhythms in the testis and thymus tissues (Alvarez *et al.* 2003, Alvarez & Sehgal 2005). Whether or not cellular differentiation contributes to the constant expression of *Per1* gene in glandular epithelial cells remains to be explored.

More importantly, the present study demonstrated that the expression of *Per1* increased in the rat uterus after treatment with E₂ and P₄. It is well known that, at ZT 0, the transcription of *Per1* is at the lowest level in almost all peripheral tissues and is less likely activated by the CLOCK/BMAL1 heterodimers (Yamamoto *et al.* 2004). Hence, all the uteri were collected at ZT 0 to evaluate the steroidal effects on *Per1* expression. The analysis by *in situ* hybridization indicates that E₂ administration causes higher expression of *Per1* in the uterine LE, GE, and M, but to a lesser extent in the S compartment. The promotion of *Per1* expression by E₂ was further confirmed by immunohistochemical studies. In addition, the finding that ICI182 780 suppressed E₂-activated

Per1 expression indicates that the regulation of *Per1* expression is at least partly through the ER-mediated pathway. Therefore, the differences in *Per1* expression among the compartments might be due to differential expression of ER. Actually, expression of ER mRNA or protein is higher in the LE, GE, and M compartments than in the S compartment at 24 h after E₂ administration (Nephew *et al.* 2000). This pattern of ER distribution, to some extent, supports our findings of *Per1* expression profile in the uterus in response to E₂ treatment. RT-PCR analysis revealed that *Per1* expression was slightly augmented by E₂ in cultured USC at 6 h, but not at 24 h. The different observations between *in vitro* and *in vivo* may attribute to distinct environmental context. On the other hand, P₄ efficiently activated the expression of *Per1* in the entire endometrium via the mediation of PR. The PR-mediated up-regulation of *Per1* expression by P₄ was further supported by the findings in cultured USC *in vitro*. However, no obvious enhancement of *Per1* transcription by P₄ was observed in the M compartment, although the myometrial cells of OVX rat uterus are also positive to PR (Tibbetts *et al.* 1998). The distinct responsiveness of *Per1* expression to P₄ in the endometrium and myometrium (M) may also be due to their differential microenvironmental context.

E₂ and P₄ could bind to their respective ER and PR, which, in turn, bind to specific response elements (ERE and PRE) in the promoters of target genes, thereby regulating

gene transcription (Bagchi *et al.* 1988, Anderson & Gorski 2000, Edwards 2005). Therefore, our present findings suggest that the activated expression of *Per1* by ovarian steroids might result from the direct binding of ER or PR to some potential element(s) in *Per1* promoter. Indeed, as stated in the previous report (Nakamura *et al.* 2005), although there is no consensus ERE (5'-GGTCANNNTGACC-3'), some ERE-half sites (5'-GGTCA-3' or 5'-TGACC-3') are located in the 5' flanking region of *Per1* promoter (GenBank accession number, AB030818). Additionally, a PRE-half element (5'-TGTTACT-3') is located in the first intron of *Per1* gene within +868/+874 (+1, the transcription start site). It remains to be elucidated whether ER and PR promote *Per1* transcription by direct binding to their respective putative element(s) in *Per1* promoter, or via yet unknown indirect pathways.

The present finding of the pattern of *Per1* expression under the regulation by the steroids is attractive. As is well known, the E-box element in *Per1* promoter is essential for binding by CLOCK/BMAL1 and generation of circadian rhythms (Darlington *et al.* 1998, Travnickova-Bendova *et al.* 2002). In addition, other two important up-regulatory elements, the cAMP response element (CRE) and glucocorticoid response element (GRE), have been clearly defined by *in vitro* studies. In response to various resetting cues (Balsalobre *et al.* 2000b, Tsuchiya *et al.* 2005, Yamamoto *et al.* 2005, Hinoi *et al.* 2006), the CRE-binding protein (CREB) or glucocorticoid receptor (GR) was activated, which, in turn, transactivates the transcription of *Per1* in the nucleus by binding to the CRE or GRE respectively (Travnickova-Bendova *et al.* 2002, Yamamoto *et al.* 2005, He *et al.* 2007). It is important to note that both CREB and GR act to induce an acute and robust enhancement of *Per1* transcription with a peak at 1 h. In contrast, estrogen and P₄ tend to induce a chronic up-regulation of *Per1* expression. This finding suggests that there may exist other pathway(s) involved in regulating clock gene expression and resetting or modulating the circadian rhythms *in vivo* by the humoral cues. *In vitro* studies using clock gene transgenic cells (He *et al.* 2007,) will help to provide direct evidence of whether E₂ or P₄ could serve to reset the circadian clockwork.

In conclusion, the present study demonstrates the existence of circadian rhythms in the uterine LE, S, and M compartments, but not obviously in the GE. The finding of up-regulation of *Per1* expression by E₂ may contribute to understand how ER functions to phase shift the circadian rhythms of clock gene expression and to alter the behavior and endocrine rhythms (Morin *et al.* 1977, Thomas & Armstrong 1989, Nakamura *et al.* 2005, Perrin *et al.* 2006). We also find that P₄ efficiently activates the expression of *Per1* gene in the uterus, which indicates that P₄ might also have profound effects on circadian clockwork. However, although *Per1* gene is subjected to the regulation by both E₂ and P₄, it is less likely that *Per1* directly exerts physiological functions but rather indirectly regulates uterine physiology via modulation of circadian clockwork. Furthermore, comprehensive studies

on clock gene expression in P₄-responsive tissues are required to further understand the potential actions of P₄ on the modulation of circadian rhythms.

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