

## Down-regulation of circadian clock gene period 2 in uterine endometrial stromal cells of pregnant rats during decidualization

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DOWN REGULATION OF CIRCADIAN CLOCK GENE *PERIOD 2* IN THE UTERUS  
ENDOMETRIAL STROMAL CELLS OF PREGNANT RATS DURING DECIDUALIZATION

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## Abstract

Circadian rhythms are modulated in a variety of peripheral tissues including the uterus, in which the endometrial stromal cells (UESCs) undergo proliferation and differentiation (decidualization) during gestation in rodents. Here we focused on circadian rhythms in the UESCs during implantation and decidualization. As revealed by analyses of cultured UESCs from pregnant *Per2* promoter-*dLuc* transgenic rats, the *Per2* oscillation was observed with approximately 24 h per cycle in response to dexamethasone. The *Per2* oscillation was enhanced in the UESCs during implantation, whereas they were attenuated during decidualization. *In vivo* studies showed that the PER2 protein in the uteri displayed a peak at ZT 4 (day 4.50 of gestation) and a trough at ZT 12 (day 4.83), indicating its circadian rhythms. Conversely, no significant circadian rhythms of the PER2 protein were observed during decidualization. Fluorescent immunohistochemical studies also supported circadian rhythms of the PER2 protein in its intracellular distribution. In accordance with *Per2*-mRNA expression, the circadian rhythms of vascular endothelial growth factor (*Vegf*) gene expression, having several E-box or E-box-like sites at the upstream of the transcription start site, were observed during implantation, showing a peak at ZT 0 and a trough at ZT 12. In contrast, the *Vegf*-mRNA expression displayed no circadian rhythms during decidualization. Collectively, the present results prove that

the *Per2* oscillation is down regulated in UESCs during decidualization. It is strongly suggested that cellular differentiation in UESCs interferes with circadian clockwork.

Key words: circadian rhythms; uterus; decidualization; implantation; *Per2*; *Vegf*

## INTRODUCTION

Circadian rhythms are primarily synchronized with environmental time by the twenty-four hour period of light-dark cycle. In mammals, the master clock in the suprachiasmatic nucleus (SCN) coordinates the subsidiary oscillators in the majority of peripheral tissues (Reppert and Weaver, 2001; Schibler and Sassone-Corsi, 2002; Yamamoto et al., 2004). At molecular level, the pacemaker system is composed of transcriptional and translational core feedback loops. The CLOCK and BMAL1, associated as heterodimers, bind to the E-box enhancer element 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; Reppert and Weaver, 2001; Ueda et al., 2005). Posttranslational processes involving the phosphorylation and degradation of proteins regulate nuclear import and export and are crucial for sustaining approximately a 24-h duration (Lee et al., 2001; Harms et al., 2004). On the other hand, autonomic circadian oscillators are functional in a variety of peripheral tissues including the heart, liver, kidney, lung, spleen, adrenal gland, skeletal muscles (Yamamoto et al., 2004; Lemos et al., 2006), and uterus (Nakamura et al., 2005; Dolatshad et al., 2006; He et al., 2007a). In contrast, thymocytes, testicular Leydig cells, and ovarian immature granulosa cells lack circadian rhythms (Morse et al., 2003; Alvarez et al., 2003; Alvarez and Sehgal,

2005; He et al., 2007b, c).

The peripheral circadian oscillator plays an essential role in synchronizing local physiology to operate in a circadian manner via regulation of the expression of clock-controlled genes (Storch et al., 2002). These physiological processes mainly include hormonal secretion and metabolic pathways like gluconeogenesis (Lemos et al., 2006), lipogenesis (Wijnen and Young, 2006), and bile acid homeostasis (Ma et al., 2009). Cell lines could also display several cycles of oscillations of the clock genes when treated with dexamethasone (DXM), a serum shock or other potential stimuli (Balsalobre et al., 2000a,b). Cellular differentiation may cause the suspension of the cyclic expression of clock genes (Alvarez and Sehgal, 2005; He et al., 2007b). However, currently little is known about whether circadian clockwork could be affected by changes of cellular physiological state.

Mice lacking the *Clock* gene display abnormal estrus cycles and are infertile (Low-Zeddies and Takahashi, 2001; Miller et al., 2004). Furthermore, implantation fails in *Bmal1* deficient mice, due to impaired steroidogenesis (Ratajczak et al., 2009), and mutations of *Per1* and *Per2* in mice display reproductive deficits in the middle-aged mutant females (Pilorz and Steinlechner, 2008). Several recent studies have demonstrated that circadian clock genes are rhythmically expressed in the uterus (Lemos et al., 2006; Nakamura et al., 2005; Dolatshad et al., 2006; Hirata et al., 2009; Akiyama et al., 2010). Chronic treatment with estradiol disrupts the

circadian rhythms of *Per1* and *Per2* expression in the liver, kidney and uterus (Lemos et al., 2006). In contrast, progesterone plays a pivotal role in the circadian clock gene *Per2* of the uterus endometrial stromal cells (UESCs) through transcriptional and translational feedback loops of the clockwork system (Hirata et al., 2009). In the uterus composed of heterogeneous cell types, ovarian steroids regulate the proliferation and differentiation of UESCs (Psychoyos, 1986; Yoshinaga, 1988; Carson et al., 2000). In rodents, the UESCs undergo proliferation and differentiation into decidual cells in response to ovarian steroids and blastocyst implantation at the early stage of pregnancy (Clarke and Sutherland, 1990; Zhang et al., 1994; Dey et al., 2004). This process ultimately results in the formation of the placenta.

Here we focus on the circadian oscillator in the UESCs during the stages of implantation and decidualization. In addition to Western blots, fluorescent immunohistochemistry and quantitative real-time PCR, the real-time monitoring system of *Per2* gene expression was employed to evaluate the circadian oscillations using transgenic rats constructed with mouse *Per2* promoter-destabilized luciferase (*Per2-dLuc*) reporter gene (Ueda et al., 2002). In the current study, we prove that the *Per2* expression is down regulated in the UESCs during decidualization and influences the expression of vascular endothelial growth factor (*Vegf*) gene.

## **MATERIALS AND METHODS**

## **Animals**

Mouse *Per2* promoter region (chr1 (-): 93289505-93293019 on the Build 36 assembly by NCBI and the Mouse Genome Sequencing Consortium), which is sufficient for circadian oscillation, was fused to a *dLuc* reporter gene (Ueda et al., 2002). *Per2-dLuc* transgenic rats were generated in accordance with the method described in the patent publication number WO/2002/081682 (Y.S. New Technology Institute, Utsunomiya, Japan). Transgenic and wild type rats were maintained under 12-h light and 12-h dark (ZT0: 8:00a.m. light on; ZT12: 20:00p.m. light off) with water and food *ad libitum*. Adult females were mated with fertile males, and 12:00 p.m. on the day of finding spermatozoa in the vaginal smear was designated as day 0.5 of gestation. All the experiments were performed under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, and Law No. 105 and Notification No. 6 of the Government of Japan.

## **Preparation and Culture of the UESCs**

The UESCs were isolated from *Per2-dLuc* transgenic rats on day 4.50 and day 6.50 of gestation as reported previously (He et al., 2007b; Oozono et al., 2008; Matsumoto et al., 2009). The uterine lumens were filled with PBS containing 0.1% collagenase and incubated at 37.8°C for 1 h in a shaking water bath. The dissociated cells were washed and seeded at the density of  $2 \times 10^5$  cells/dish. The culture medium was



replaced at 15 min after cell seeding to remove epithelial cells. Cells were cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C for 3 days.

### **Real-Time Monitoring of Circadian Oscillations**

The UESCs cultured were treated for 2 h at 37°C with 100 nM DXM dissolved in absolute ethanol that was diluted in a serum-free medium with final ethanol concentration of below 0.01%. Upon the onset of bioluminescent measurement (time 0 h), the cultured cells were given in replaced fresh medium supplemented with 5% charcoal-treated FBS, 1% PS, 15 mM HEPES and 0.1 mM luciferin. Monitoring of bioluminescence was performed for 4 days at 37°C using Kronos AB-2500 interfaced to computer for continuous data acquisition as previously described (He et al., 2007b, c; Hirata et al., 2009).

### **Western Blot**

The pieces of uterine tissues (ca. 0.1 g) were weighed and homogenized in an ice-cold buffer containing 50 mM Tris-HCl (pH 6.8). Aliquots of the proteins (30 µg) were mixed with 5x electrophoresis Laemmli sample buffer and then treated at 100°C for 3 min. SDS-PAGE was performed on 10% polyacrylamide gels and the proteins were electrotransferred onto Immobilon-PVDF (Millipore). Membranes were blocked in 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 0.05% Tween 20

with 10% nonfat dry milk and incubated at 4°C with primary antibodies, polyclonal anti-human PER2 antibody (H-90) (1:1000, Santa Cruz Biotechnology) and monoclonal anti- $\alpha$ -tubulin antibody DM1A (1:3000, Sigma) and then with the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:1000, Santa Cruz Biotechnology) and goat anti-mouse IgG antibody (1:1500) to detect each protein, respectively. After being washed several times, membranes were incubated with LumiGLO (Cell Signaling Technology) and exposed to Fuji Medical X-ray film (Fuji Film, Tokyo, Japan) to visualize the bound proteins.

### **Fluorescent Immunohistochemistry**

The pieces of uterine tissues were embedded in an OCT compound and frozen immediately in liquid nitrogen. Air-dried tissue sections of 5- $\mu$ 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 48°C with rabbit anti-PER2 polyclonal antibody (H-90) diluted in blocking buffer (1:250). Rabbit serum was substituted for the primary antibody as the 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, Tokyo, Japan). Immunostaining was detected under a fluorescence microscope (Nikon, Tokyo, Japan).

### **Quantitative Real-Time PCR**

The pieces of uterine tissues (ca. 0.1 g) were homogenized in 1 ml of Sepasol-RNA I Super (Nacalai Tesque, Tokyo, Japan) for 2 min in ice with a homogenizer. An aliquot of 0.8 ml homogenates was taken and transferred into a small tube and then total RNA was isolated according to the manufacture's protocol. The RNA concentration was determined using 260/280 UV spectrophotometry (Pharmacia Biotech, UK), and RNA integrity was checked by agarose gel electrophoresis. One microgram of total RNA were reverse transcribed in 20  $\mu$ l of mixture using MMLV High Performance Reverse Transcriptase (Epicentre Biotechnologies) and Oligo-dT primer according to the manufacture's protocol. Primer sets used for real-time PCR were listed in Table 1. PCR was performed with a 1:15 dilution of cDNA samples in Master SYBR Green I mixture (Roche Diagnostics) with specific primers (0.25  $\mu$ M final of each primer) using Mx3000P Real-time QPCR System (Stratagene). Relative quantification of *Per2* mRNA and *Vegf* mRNA levels was performed using the comparative cycle threshold ( $\Delta$ Ct) method. The  $\Delta$ Ct for each sample was normalized to *Gapdh* and expressed as relative to ZT 0 of pregnant day 4.33.

### **Statistical Analyses**

Data were expressed as means  $\pm$  SEM of at least three separate experiments, each

performed with triplicate samples. One-way ANOVA was used to determine significant differences between groups followed by Student's *t* test or Duncan's test using the SigmaPlot (Ver. 11.2, Systat Software, Inc. San Jose, CA, USA). *P* values of <0.05 was considered statistically significant.

## **RESULTS**

### **Per2-dLuc Circadian Oscillation in the UESCs of Pregnant Rats during the Stages of Implantation and Decidualization**

The UESCs were prepared from pregnant *Per2-dLuc* transgenic rats during the stages of implantation (day 4.50, ZT 4) and decidualization (day 6.50, ZT 4). After culture of the UESCs for 3 days, the cells were treated with DXM for 2 h, and subjected to real-time monitoring of *Per2-dLuc* oscillation. The *Per2* circadian oscillation was generated by DXM. The oscillations in both the UESCs were driving with approximately 24 h per cycle, albeit with a continuous decreasing of amplitude. The first peak times of *Per2-dLuc* oscillation were not significantly different between the UESCs on day 4.50 and day 6.50 of gestation, showing approximately 32 h (Table 2). The times of second and third peaks were mostly identical. However, the oscillatory intensity was distinct between both the UESCs (Fig. 1a). After exposure to DXM, the circadian oscillation was greatly enhanced in the UESCs on day 4.50 of gestation during 96-h monitoring. In contrast, in the UESCs on day 6.50 of gestation, the

oscillatory intensity was attenuated. The bioluminescence values of the first and second peaks were significantly different between the UESCs on day 4.50 and day 6.50 of gestation ( $p<0.05$ ) (Fig. 1b).

### **Circadian Rhythm of the PER2 Protein in the Uterine Tissues during the Implantation Stage**

Western blot analysis was performed to investigate circadian rhythm of the PER2 protein in the uterine tissues of wild type rats during the implantation stage of gestation (day 4.33 to day 5.16) (Fig. 2a). The N-terminus-targeted antibody detected a major band with approximate 70 kDa, although it was not similar to a size of the predicted full length in the PER2. A peak level of PER2 protein was observed at ZT 0 to ZT 4 (day 4.34-4.50 of gestation), and a trough was at ZT 12 (day 4.83) (ZT 0 versus ZT 12,  $p<0.05$ ). Then, the intracellular distribution of PER2 protein was investigated by fluorescent immunohistochemistry in different compartments of the uteri at ZT 4 and ZT 12. A strong immunostaining signal was detected in the luminal epithelium, as compared to that in the stromal cell layer (Fig. 2b). In particular, the cytoplasm of luminal epithelium displayed a strong signal at both ZT 4 and ZT 12, suggesting a continuous expression of the PER2 protein. On the other hand, in the stromal layer, immunostaining signals were observed in both the nuclei and cytoplasm at ZT 4, whereas signals were observed predominantly in the nuclei at

ZT12 (Fig. 2b). Consequently, fluorescent immunohistochemical studies may support circadian rhythm of the PER2 protein at least in the stromal cell layer as revealed by Western blot analyses, showing that the PER2 protein is greatly expressed at ZT 4, whereas its expression is decreased at ZT12.

### **Circadian Rhythm of the PER2 Protein in the Uterine Tissues at the Decidualization Stage**

Circadian rhythm of the PER2 protein was also investigated in the uterine tissues of wild type rats during the initial stage of decidualization (day 6.33 to day 7.16) by Western blot. In contrast to the implantation stage, no significant rhythm of the PER2 protein were found during the decidualization stage ( $p>0.05$ ) (Fig. 3a). As revealed by fluorescent immunohistochemistry, the cytoplasm of luminal epithelium also displayed a strong signal at both ZT 4 and ZT 12. As compared to the implantation stage, immunostaining signals were observed predominantly in the nuclei of stromal cell layers at both ZT4 and ZT12 (Fig. 3b). The finding that little PER2 immunostaining signals were detected in the cytoplasm may indicate low expression of the PER2 protein during the decidualization stage.

### **Circadian Rhythms of Per2 and Vegf Transcripts in the Uterine Tissues during Implantation and Decidualization**

Circadian rhythm of the *Per2*-mRNA expression was investigated in the uterine tissues of wild type rats using real-time qPCR during the stages of implantation (day 4.33 to day 5.16) and initial decidualization (day 6.33 to day 7.16). During the implantation stage, a peak and a trough of *Per2*-mRNA expression appeared at ZT 0 (day 4.33) and ZT 8-12 (day 4.67 to day 4.83), respectively (ZT 0 versus ZT 8-12,  $p < 0.05$ ) (Fig. 4a). After the trough, the transcripts increased but not significantly different at between ZT 12 (day 4.83) and ZT 20 (day 5.16) (ZT 12 versus ZT 20,  $p > 0.05$ ). The peak and trough of *Per2*-mRNA expression were seen approximately 4 h prior to those of PER2 protein levels. In contrast, during the decidualization stage, *Per2*-mRNA expression was markedly reduced and displayed no circadian rhythm. Furthermore, we investigated circadian rhythm of the *Vegf* gene expression that has several E-box or E-box-like sites at the upstream of the transcription start site. In accordance with *Per2*-mRNA expression, in the uterine tissues during the implantation stage, circadian rhythm of *Vegf*-mRNA expression was observed, showing a peak time at ZT 0 and a trough at ZT 12 (ZT 0 versus ZT 12,  $p < 0.05$ ) (Fig. 4b). In contrast, the *Vegf*-mRNA expression displayed no circadian rhythm during the initial stage of decidualization.

## DISCUSSION

Circadian core clock genes such as *Per1* and *Per2* are rhythmically expressed in the

uterus (Johnson et al., 2002; Horard et al., 2004; Nakamura et al., 2005; Dolatshad et al., 2006; He et al., 2007a; Hirata et al., 2009; Akiyama et al., 2010). Dolatshad and coworkers (2006) analyzed temporal expression pattern of the *Per2*-mRNA in the uterus of estrous mice, and showed its robust daily variation with a peak around early dark periods. However, little is known about whether the circadian clockwork is modulated during gestation. In rodents and humans, the UESCs undergo proliferation and differentiation into decidual cells, and the placenta is ultimately formed. We previously described the possibility that cellular differentiation interferes with the circadian clockwork in differentiating cells (He et al., 2007b). The UESCs isolated from pregnant rats on day 4.50 of gestation are proliferative in culture. *In vitro* decidualization of the UESCs induced by medroxyprogesterone acetate plus 2-O-dibutyryl adenosine 3':5'-cAMP results in disappearance of *Per2-dLuc* oscillation. In the current study, the UESCs prepared from the *Per2-dLuc* transgenic rats were employed to analyze circadian oscillation on day 4.50 and day 6.50 of gestation. On day 4.50, treatment of the UESCs with DXM caused generation of circadian *Per2-dLuc* oscillation, indicating that glucocorticoid response *cis*-elements in the rat *Per2* promoter are functional and transactivate the transcription of *Per2* gene (Travnickova-Bendova et al., 2002; Yamamoto et al., 2004; He et al., 2007a). Although the first peak of *Per2-dLuc* oscillation in the stromal cells was delayed for approximately 8 h as compared with ovarian cells (He et al., 2007b), the circadian



oscillation was driving with approximately 24 h per cycle at least during the stage of implantation. Interestingly, the circadian oscillation was attenuated in the decidualizing cells only 2 days after implantation. However, the time of each peak of *Per2-dLuc* oscillation was mostly identical between the UESCs on day 4.50 and day 6.50 of gestation. In consequent, the present data support that normal circadian clockwork is disturbed during cellular differentiation.

It was investigated whether the *in vitro* circadian oscillation of *Per2-dLuc* results from the *in vivo* circadian rhythms of *Per2*-mRNA and its protein. In Western blotting analysis with the N-terminus-targeted antibody, we identified a major band of PER2 protein with approximate 70 kDa. However, the predicted size of PER2 is 140 kDa. Several studies reported that small sizes of PER1 and PER2 with 55-70 kDa were recognized in the liver, brain, lung and pancreas (Chilov et al. 2001; Muhlbauer et al. 2004; Bendová & Sumová 2006). Various molecular sizes may result from different organs, N- or C-terminus targeted antibodies used, and different protocols of protein isolation (Lee et al. 2001; Chilov et al. 2001; Akashi et al. 2002; Hastings et al. 2003). In addition, it is possible that PER protein isoforms are generated from splicing variants of *Per* genes (Taruscio et al. 2000). In the present study, a 70-kDa PER2 protein was analyzed for investigation of circadian rhythms. The circadian rhythms of *Per2*-mRNA and its protein were observed in the uteri of pregnant rats during the stage of implantation. Conversely, no circadian rhythm of the *Per2* gene was

observed in the uteri during decidualization. Circadian rhythm of the PER2 protein also showed a peak around early light periods and a trough around the onset of dark periods. However, the peak time of PER2 protein was nearly 4 h behind as compared with its transcript. A previous report using estrous mice described a maximal expression of *Per2*-mRNA in the uteri around early dark periods (Dolatshad et al., 2006). We also observed a maximal expression of *Per1*-mRNA in the uteri of estrous rats at ZT 8 (unpublished data). It is noted that circadian clockwork is reversed during gestation. Fluorescent immunohistochemical studies supported circadian rhythm of the PER2 protein in the stromal cells of pregnant rat uteri, but not in the luminal epithelium. When the PER2 protein was highly expressed in the stromal cells on day 4.50 of gestation (ZT4), it distributed in both the nucleus and cytoplasm. However, at 8 h after the peak (ZT12), the PER2 protein mostly disappeared in the cytoplasm of stromal cells and existed predominantly in the nucleus, suggesting functional core feedback loops of circadian clockwork system. Conversely, on day 6.50 of gestation, the PER2 protein was observed predominantly in the nucleus at both ZT4 and ZT12. To our knowledge, no report is available regarding whether the circadian clock is involved in the regulation of uterine stromal decidualization, which is critical for successful pregnancy in rodents (Gu et al., 1994). The present finding suggests that the circadian oscillator in the endometrial stroma is impaired during decidualization.

Some clock-controlled genes are expressed in the rat oviduct rhythmically over 24 h (Kennaway et al., 2003). During the stage of implantation, dramatic changes are observed in the expression of hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and VEGF, angiogenesis, down regulation of anti-adhesion proteins such as mucin 1 (MUC1) and MUC4, and up regulation of adhesion proteins. Although these genes are modulated by changes in estradiol, progesterone, and oxygen tension, several of these genes are known to interact with the clock gene transcription factors. For example, BMAL1 can dimerise with HIF1 $\alpha$  and potentially bind to the hypoxia response elements in gene promoters and drive the transcription of target genes (Hogenesch et al., 1998). During the stage of decidualization, the UESCs undergo proliferation and differentiation into the decidual cells (Brosens et al., 2002). The decidual cells express many peptides including desmin (Glasser and Julian, 1986), IGF binding proteins (Zhou et al., 1994), tumor necrosis factor (Yelavarthi et al., 1991), and decidual PRL-related protein (Daly et al., 1983; Orwig et al., 1997). It is possible that several of these factors are under control of the circadian clockwork. In the current study, the *Vegf* gene was investigated, because there are at least 9 E-box and E-box-like sites within the -5000 upstream of transcription start site (NC\_005108). Circadian rhythm of the *Vegf*-mRNA was observed during the stage of implantation, whereas it was not seen during the stage of decidualization. The fact that circadian rhythm of the *Vegf* transcript was consistent with that of the *Per2* transcript strongly

suggests a clock regulation of the *Vegf* gene. The PER2 protein is reported to suppress the expression of heterodimers BMAL1/HIF1 $\alpha$ -induced *Vegf* gene (Koyanagi et al., 2003). It is possible that dysfunction of the circadian clockwork during the stage of decidualization is necessary to increase expression of the *Vegf* gene for formation of the placenta. Actually, level of the *Per2* transcript in the uterine tissues was markedly reduced at decidualization.

In conclusion, we characterized the *Per2* circadian oscillation in the uteri of pregnant rats, especially focusing on the stages of implantation and decidualization. We provided evidence that the circadian oscillation is actively driving during the implantation stage but not the decidualization stage. Changes of the circadian oscillation influence circadian expression of a clock-controlled gene such as *Vegf*. The present findings may contribute to our understanding of the coordination between the circadian oscillator and cell differentiation in the regulation of embryonic implantation and placenta formation.

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## FIGURE LEGENDS

**Fig. 1. Profiles of circadian *Per2-dLuc* oscillations in UESCs of pregnant rats at day 4.50 and day 6.50 of gestation.** UESCs were given in replaced fresh medium supplemented with DXM (each 0.1  $\mu$ M: *panels* B and D) or vehicle (as a control: *panels* A and C) and cultured for 2 h. At time 0 h, UESCs were given in replaced fresh medium supplemented with 0.1 mM luciferin. (a) Real-time bioluminescent activity was monitored for 4 days. P1-3: first to third peaks, T1-3: first to third troughs. *Panels* A and B: day 4.50 of gestation, C and D: day 6.50 of gestation (b) Differences between peak and trough were normalized to the values of the control cells treated with vehicle and data are means  $\pm$  SEM of three independent determinations. *Asterisk* indicates statistically significant different ( $p<0.05$ ).

**Fig. 2. Circadian rhythms of PER2 protein in uteri of pregnant rats at day 4.33-5.16 of gestation (implantation stage) estimated by Western blotting and fluorescent immunohistochemistry.** The pregnant rats were killed at 4-h intervals over daily cycle (ZT0, 0800h). (a) Proteins (30  $\mu$ g) prepared from a part of uterine horns were separated on SDS-PAGE, and analyzed by immunodetection using anti-mPER2 antibody. Data are means  $\pm$  SEM from three independent experiments normalized to the values given by  $\alpha$ -tubulin. Values with different letters are significantly different ( $p<0.05$ ). *Striped areas*, during the night. (b) Parts of

uterine horns at ZT4 and ZT12 were subjected to fluorescent immunohistochemistry. The square regions shown in HE (A, E) were magnified from a serial section subjected to immunofluorescent studies (B, F) and Hoechst staining (blue). PER2 and Hoechst staining merged image (C, G). The square regions shown in merged image (C, G) were magnified (D, H). *Bars*: 150  $\mu\text{m}$  (A, E), 50  $\mu\text{m}$  (B, C, F, G), 25  $\mu\text{m}$  (D, H).

**Fig. 3. Circadian rhythms of PER2 protein in uteri of pregnant rats at day 6.33-7.16 of gestation (decidualization stage) estimated by Western blotting and fluorescent immunohistochemistry.** The pregnant rats were killed at 4-h intervals over daily cycle (ZT0, 0800h). (a) Proteins (30  $\mu\text{g}$ ) prepared from a part of uterine horns were separated on SDS-PAGE, and analyzed by immunodetection using anti-mPER2 antibody. Data are means  $\pm$  SEM from three independent experiments normalized to the values given by  $\alpha$ -tubulin. Values with different letters are significantly different ( $p < 0.05$ ). *Striped areas*, during the night. (b) Parts of uterine horns at ZT4 and ZT12 were subjected to fluorescent immunohistochemistry. The square regions shown in HE (A, E) were magnified from a serial section subjected to immunofluorescent studies (B, F) and Hoechst staining (blue). PER2 and Hoechst staining merged image (C, G). The square regions shown in merged image (C, G) were magnified (D, H). *Bars*: 150  $\mu\text{m}$  (A, E), 50  $\mu\text{m}$  (B, C, F, G), 25  $\mu\text{m}$  (D, H).

**Fig. 4. Circadian rhythms of *Per2* and *Vegf* transcripts in UESCs of pregnant rats during implantation and decidualization stages.** RNA was extracted from a part of uterine horns and reverse transcribed. The resulting cDNA was used for real-time qPCR using *Per2*- and *Vegf*-specific primers. (a) *left panel*, *Per2* mRNA expression was significantly changed in UESCs of pregnant rats during implantation (day 4.33-5.16 of gestation). *Right panel*, *Per2* mRNA expression was not changed in UESCs of pregnant rats during decidualization (day 6.33-7.16 of gestation). (b) *left panel*, *Vegf* mRNA expression was significantly changed in UESCs of pregnant rats during implantation (day 4.33-5.16 of gestation). *Right panel*, *Vegf* mRNA expression was not changed in UESCs of pregnant rats during decidualization (day 6.33-7.16 of gestation). Values with different letters are significantly different ( $p<0.05$ ). *Striped areas*, during the night.



FIG. 1

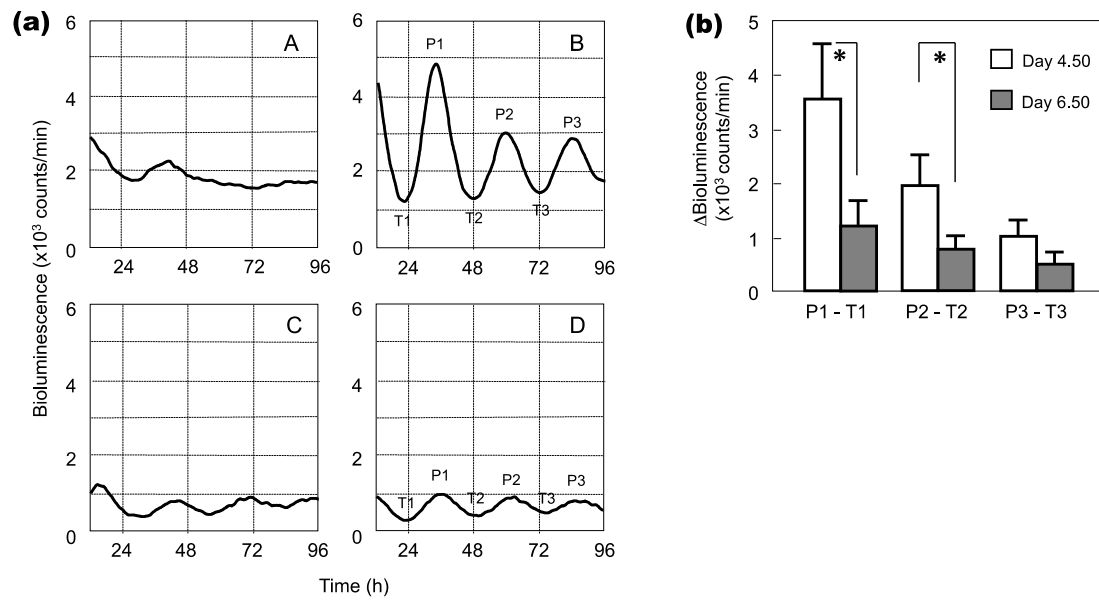


FIG. 2

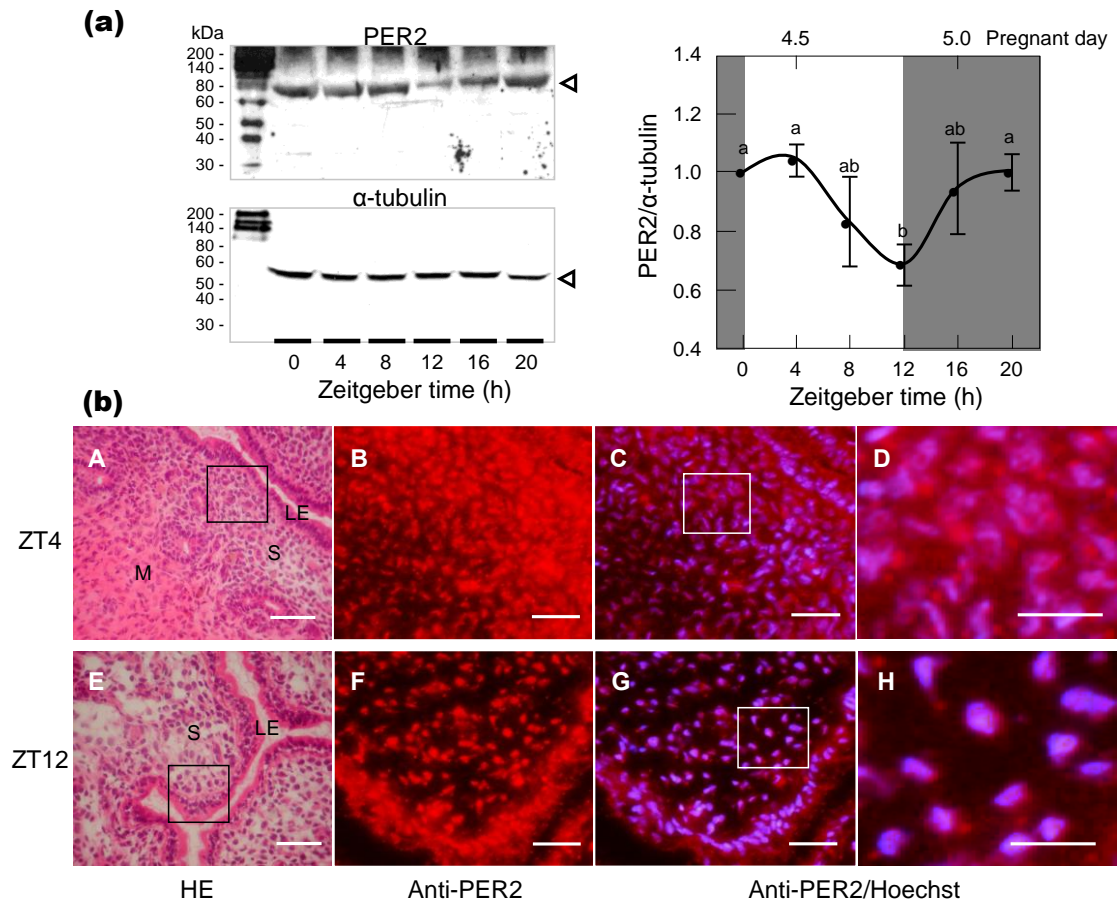


FIG. 3

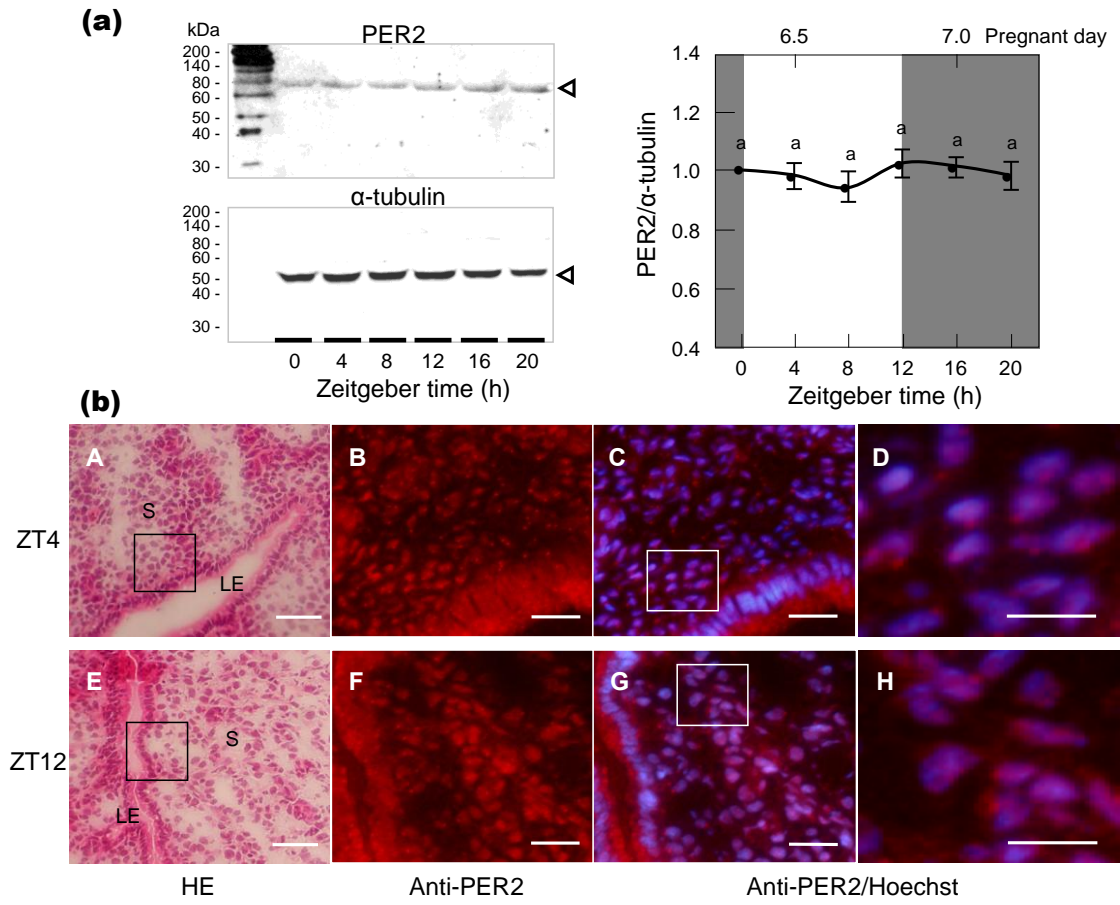
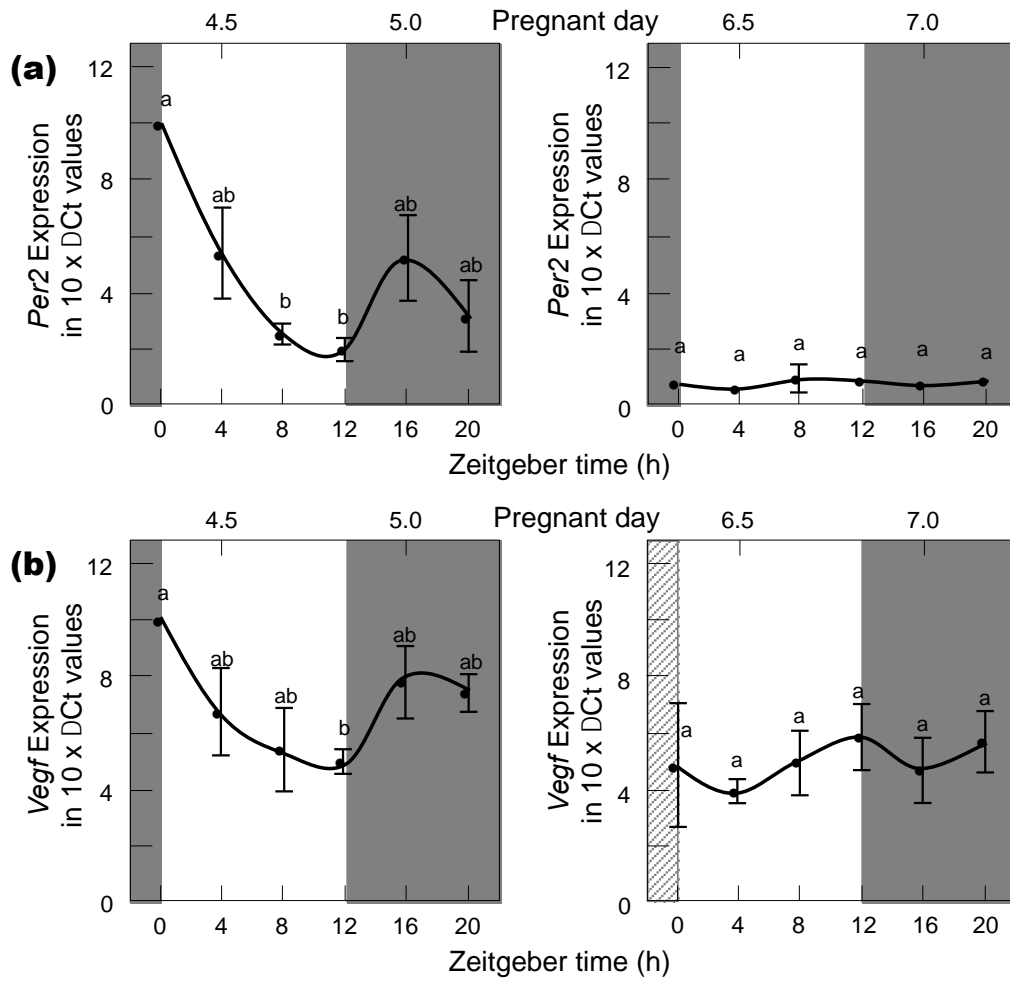


FIG. 4



**Table 1. Oligonucleotide primers**

Gene	Accession No.	Sequence 5' - 3' (position)	Amplicon (bp)
<i>Per2</i>	NM_031678	F: GACGGGTCGAGCAAAGGA (40-57) R: GGGAAAAGTCCACATATCCATTCA (129-106)	90
<i>Vegf</i>	NM_001110333	F: ACGACAGAAGGGGAGCAG (1099-1116) R: AGATGTCCACCAGGGTCTCA (1195-1176)	78
<i>Gapdh</i>	NM_017008	F: AACCTGCCAAGTATGATGACATCA (821-844) R: TGTTGAAGCCGCAGGAGACAACCT (932-909)	112

**Table 2. Characterization of the circadian oscillations of *Per2* gene in the UESCs of pregnant rats during the stages of implantation and decidualization**

The cultured UESCs from pregnant rats at day 4.5 and day 6.5 of gestation were given in replaced fresh medium supplemented with 5% charcoal-treated FBS, 1% PS, 15 mM HEPES, 0.1 mM luciferin, and 100 nM DXM dissolved in absolute ethanol (time 0). Monitoring of bioluminescence was performed for 4 days at 37°C using Kronos AB-2500 interfaced to computer for continuous data acquisition. The experiments were independently repeated 3 times, and data are expressed as means  $\pm$  SEM of three independent experiments.

Pregnant	P1 time, h	P2 time, h	P3 time, h	Cycle time (P2-P1), h	Cycle time (P3-P2), h
Day 4.5	32.75 $\pm$ 2.06	58.10 $\pm$ 1.96	82.15 $\pm$ 1.60	25.36 $\pm$ 0.45	24.07 $\pm$ 0.44
				$p > 0.05$	$p > 0.05$
Day 6.5	32.90 $\pm$ 2.61	58.70 $\pm$ 3.40	82.90 $\pm$ 2.10	25.79 $\pm$ 1.08	24.31 $\pm$ 2.11