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Alterations of Circadian Clockworks During Differentiation and Apoptosis of Rat Ovarian Cells

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ABSTRACT

Ovarian development is related to cell proliferation, differentiation and apoptosis of granulosa cells and luteal cells under the control of various modulators including follicle-stimulating hormone (FSH), luteinizing hormone (LH) and growth factors. In the present study, the expression of clock genes and the related regulation mechanism were analyzed in different ovarian cell types during differentiation and apoptosis. We focused on the circadian expression of *Per2*, as a core clock gene for the maintenance of circadian rhythms. By using real-time monitoring system of the *Per2* promoter activity, the circadian oscillation was analyzed in the granulosa cells and luteal cells from preantral follicles, antral follicles and corpora lutea of immature *Per2* promoter-destabilized luciferase transgenic rats that were primed with diethylstilbestrol, equine chorionic gonadotropin (eCG) and/or human CG. In addition, transcript levels of *Per2*, *Bmal1, Clock* and *Nampt* were quantified by qPCR. Immunohistochemical studies revealed a strong circadian rhythmicity of the PER2 protein in the luteal cells, but apparently little rhythmicity in the granulosa cells of both preantral and antral follicles. The *in vitro* monitoring of the promoter activity showed generation of several oscillations in the luteal cells after exposure to dexamethasone (DXM), whereas the oscillatory amplitudes in immature and mature granulosa cells were rapidly attenuating. The circadian rhythm of the *Bmal1* transcript levels, but not the *Per2* transcript, was very weak in the granulosa cells, as

compared to that in the luteal cells. Granulosa cells gained a strong circadian rhythm ability of the *Per2* promoter activity after stimulation with FSH for 3 days. In contrast, LH had little effect upon circadian rhythm before the stimulation of granulosa cells with FSH, probably owing to lack of the LH receptor. In luteal cells, the induction of apoptosis by inhibiting progesterone synthesis resulted in the deregulation of the *Per2* circadian oscillation. Transcript levels of *Bmal1* and *Clock*, but not *Per2* and *Nampt*, were significantly decreased in apoptotic luteal cells. The *Bmal1* transcript level was particularly reduced. Consequently, these results strongly suggest that the circadian clockwork alters in ovarian cells during follicular development, luteinization and apoptosis, and expression of *Bmal1* may be related to the switch-on and switch-off of the circadian oscillation.

Keywords: Ovarian cells; Clock genes; Circadian oscillation; Differentiation; Apoptosis

INTRODUCTION

In mammals, ovarian growth is involved in follicular development, luteinization and luteolysis accompanied by proliferation, differentiation and apoptosis of granulosa cells and luteal cells. FSH and LH exert influences on immature and mature granulosa cells, respectively, which promote proliferation and differentiation of the follicular cells and synthesis of sex steroid hormones. The clock system may contribute to the progress of ovarian growth through fluctuating hormones. This is because the clock genes, such as *Period1-2* (*Per1-2*), are differently expressed in various ovarian cell types (He et al., 2007; Nakao et al., 2007). The *Per2* gene may be regulated by various hormones in particular, because many regulatory elements are located in the 5' flanking region of *Per2* promoter. These include steroid hormone response element-half sites and the cAMP response element (CRE) as well as E-box and D-box elements.

Circadian rhythms are governed by the master clock located in the suprachiasmatic nucleus of the hypothalamus (Morse & Sassone-Corsi, 2002; Reppert & Weaver, 2002) and serve to synchronize multiple molecular, biochemical, physiological and behavioral processes (Delaunay & Laudet, 2002; Storch et al., 2002). The circadian system is composed of three components: input pathways, central pacemaker, and output pathways. The input pathways transmit environmental signals to the central pacemaker, which coordinates the external

signals with the central endogenous rhythm of the body by neural, hormonal and behavioral cues (Schibler & Sassone-Corsi 2002). Through expression of the clock genes at the molecular level, the circadian system generates circadian changes in cellular functions via identified transcriptional and posttranscriptional regulatory processes. The CLOCK and BMAL1, associated as heterodimers, bind to the E-box enhancer and positively drive expression of the *Period* genes (*Per1-3*) and the *Cryptochrome* genes (*Cry1-2*) (Shearman *et al*. 2000, Ueda *et al*. 2005). Further adding to the complexity, CLOCK-BMAL1 heterodimers induce expression of a nuclear orphan receptor Rev-erbα, resulting in the repression of the transcription of *Bmal1* through direct binding to the Rev-erbα response element (RRE) located in the *Bmal1* promoter (Albrecht & Eichele, 2003; Brown et al., 2005). Once translated in the cytoplasm, PER and CRY proteins heterodimerize and are phosphorylated. The PER-CRY complexes translocate to the nucleus and repress the activity of CLOCK-BMAL1 heterodimers. Over several hours, a number of PER-CRY complexes are degraded and eventually the CLOCK-BMAL1 heterodimers are released from feedback inhibition. These oscillations persist, or free run, under constant conditions, indicating the presence of a self-sustaining clock. These clock-driven events recur approximately, but not precisely, every 24 h (Allada et al., 2010). The peripheral oscillators, synchronized by the central clock, control the expression of downstream clock-controlled genes in tissue-specific relationships (Abe et al., 2002; Yamazaki et

al., 2000).

Recent studies suggest that the circadian system is not only required for proper growth control, but also involved in the circadian regulation of cell proliferation and apoptosis. It has been reported that 2-10% of all mammalian genes are clock-controlled (Le et al., 2001; Panda et al., 2002; Storch et al., 2002). Most of these genes are involved in organ functions and show tissue-specific expression (Takahashi et al., 2008). Only a small set of clock-controlled genes are expressed in multiple organs. Among them are genes that encode key regulators of cell cycle progression (Duffield et al., 2002; Kornmann et al., 2001). The deregulation of the circadian clock may disturb expression of the clock-controlled genes and can have a profound influence on organ functions. Recent studies have demonstrated that the circadian clock function is very important for cell cycle, DNA damage response, and tumor suppression *in vivo* (Fu & Lee, 2003; Lévi et al., 2010; Sun et al., 2010). Proapoptotic drugs such as docetaxel displayed the least toxicity and highest antitumor efficacy following administration during the circadian rest phase in mice, suggesting that cell cycle and apoptotic processes could be controlled under the circadian clock (Granda et al., 2005). Our recent studies report that progesterone plays an especially pivotal role in the circadian clocks of rat uterus endometrial stromal cells (Hirata et al., 2009) and *Per2* is up regulated in rat prostate mesenchymal cells during flutamide-induced apoptosis (Yoshida et al., 2010).

Several studies have reported that the deregulation of *Per2* gene expression accelerates breast cancer growth by altering its circadian oscillations (Chen et al., 2005), and *Per2* overexpression induces apoptosis in murine lung and breast cancer cell lines (Hua et al., 2006).

Although numerous studies have reported the expression of circadian clock genes in reproductive tissues, including the ovary (Johnson et al., 2002; Fahrenkrug et al., 2006; Karman & Tischkau, 2006; Sellix et al., 2010), the physiological function of clock genes in the reproductive system remains largely unknown. The previous studies demonstrated reproductive dysfunctions in *Bmal1*$^{-/-}$ mice (Ratajczak et al., 2009) and *Clock* mutant mice (Dolatshad et al., 2006). For example, female *Clock*-mutant mice, which carry a 51-amino acid deletion in the transcriptional activation domain of the *Clock* gene, have an irregular estrous cycle and do not have a normal LH surge on the day of proestrus (Miller et al., 2004). However, there is little literature concerning the hormonal regulation of circadian clocks during differentiation and apoptosis of ovarian cells.

We raised the possibility of alteration in the circadian oscillation during the differentiation and apoptosis of ovarian cells. Therefore, the present study aims to analyze expression of the core clock genes and the regulation mechanism, when different ovarian cells are induced to differentiation and apoptosis by using gonadotropins and an inhibitor of steroid hormone synthesis, respectively. The

real-time monitoring system of gene expression is employed to evaluate the circadian clocks using transgenic rats constructed with mouse *Per2* promoter-destabilized luciferase (*Per2-dLuc*) reporter gene (Ueda et al., 2002) and the mRNA levels of clock genes are measured by quantitative real-time PCR. In the present study, we provide evidence that the ovarian cells display peculiar oscillations of the *Per2* gene expression during differentiation and apoptosis, and the *Bmal1* and *Clock* genes play important roles in the circadian clock of ovarian cells.

MATERIALS AND METHODS

Animals

All the experiments were performed under the control of the Guideline for Animal Experiment in Faculty of Medicine, Kyushu University and in compliance with Law No. 105 and Notification No. 6 of the Government of Japan and complied with the ethical standards of Chronobiology International (Portaluppi et al., 2010). Mouse *Per2* promoter region (chr1 (-): 93289505-93293019 on the Build 36 assembly by NCBI and the Mouse Genome Sequencing Consortium) 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 normal rats were maintained under 12-h L and 12-h D (zeitgeber time, ZT0: 08:00 h light on; ZT12:

20:00 h light off) with water and food *ad libitum*.

Cell Preparation and Culture

Immature and mature granulosa cells were prepared as in a previous report (Hattori et al., 1996) with minor modifications. Immature female rats (21–23 days) were injected subcutaneously with 1 mg diethylstilbestrol (DES: Sigma Chemical, St. Louis, MO) for 3 days for the granulosa cells from preantral follicles and ovaries were collected at ZT2 on day 4. Rats were primed with 50 IU eCG (ASKA Pharmaceutical, Tokyo, Japan) and ovaries were collected at ZT2 on day 4 for preparing the granulosa cells from the antral follicles. Ovaries were incubated in DMEM/F12 (Invitrogen, Carlsbad, CA) containing 6 mM EGTA for 15–30 min, and then in DMEM/F12 containing 0.5 M sucrose for 10–20 min at 37°C. Granulosa cells were harvested by puncturing the follicles with a 27-gauge needle. Granulosa cells ($1x10^6$) were plated on 35-mm collagen-coated dishes with 2 ml culture medium; DMEM/F12 supplemented with 1 x penicillin–streptomycin (PS: Invitrogen) and 5% (v/v) charcoal-treated FBS (Invitrogen). Luteal cells were prepared as in the previous report by Peluso et al. (2005) with slight modifications. Briefly, immature rats were primed with 50 IU eCG and followed by 25 IU hCG (ASKA Pharmaceutical) 60 h later. Ovaries were collected at ZT2 on day 5 after hCG injection, and then incubated in PBS containing 0.25% (w/v) trypsin (Invitrogen) and 1 mM EGTA for 15 min at 37°C

followed by treatment with PBS containing 0.2% (w/v) collagenase type I (Invitrogen), 30 μg/ml DNase I (Invitrogen) and 0.5% (w/v) BSA for 1 h at 37°C. The released cells were washed three times with DMEM/F12 supplemented with 5% (v/v) charcoal-treated FBS, and then luteal cells ($1x10^6$) were plated on 35-mm collagen-coated dishes (IWAKI, Tokyo, Japan) with 2 ml culture medium. Cultures were carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Real-Time Monitoring of *Per2-dLuc* Oscillations

Cultured granulosa cells and luteal cells were given the serum-free medium supplemented with 15 mM HEPES, 0.1 mM luciferin (Wako, Tokyo, Japan), 0.1% bovine serum albumin and 1 x PS with or without 100 ng/ml ovine FSH (NIH-oFSH-S16, supplied from Dr. AF Parlow, Harbor-UCLA Medical Center, Torrance, CA), 100 ng/ml ovine LH (NIH-oLH-S21, supplied from Dr. AF Parlow), 100 nM dexamethasone (DXM: Sigma Chemical) or 1 μM aminoglutethimide (AMG: Sigma Chemical) and 100 nM progesterone (Sigma Chemical). In some experiments, the monitoring was performed in the presence of 5% (v/v) charcoal-treated FBS. Luciferase activity was chronologically monitored at 37°C with Kronos AB-2500 (ATTO, Tokyo, Japan) interfaced to computer for continuous data acquisition as previously described (He et al., 2006, Hirata et al., 2009).

Quantitative Real-Time PCR

Cultured cells were harvested at indicated times, and total RNA was isolated using RNeasy Mini kit (Qiagen) according to the manufacture's protocol. RNA samples were treated with the RNase-free DNase I (Qiagen). The RNA concentration was determined by 260/280 UV spectrophotometry ($A_{260/280}$ ratios >1.9) and RNA integrity was assessed by agarose gel electrophoresis. An aliquot of total RNA (0.6 μg) was reverse transcribed in a 60-μl-reaction volume using the High Capacity Reverse Transcription kit (Applied Biosystems). Primer sets used for qPCR were listed in Table 1. PCR was performed in a 15-μl volume containing 30 ng cDNA sample in Master SYBR Green I mixture (Roche Diagnostics) and 0.5 μM specific primers with Mx3000P Real-time qPCR System (Stratagene) using the parameters recommended by the manufacturer (95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec, 60°C for 15 sec). All reactions were performed in triplicate and displayed amplification efficiency between 80-120%. Relative quantification of each mRNA was performed using the comparative cycle threshold (ΔCt) method. The ΔCt for each sample was normalized to *Gapdh*.

Cell Death

Cultured luteal cells were washed twice with PBS, and incubated at 37°C for 20 min

with PBS containing 5 μg/ml PI and 10 μg/ml Hoechst (Sigma Chemical). After washing twice with PBS, Hoechst-positive and PI-positive cells were counted under a fluorescence microscope (Nikon, Tokyo, Japan).

Fluorescent Immunohistochemistry

The ovaries were embedded in an OCT compound and frozen immediately in liquid nitrogen. Air-dried tissue sections of 8-μm 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 anti-PER2 antibody (1:250) (H-90: Santa Cruz Biotechnology, Santa Cruz, CA) as previously reported (Sangoram et al., 1998; Uchikawa et al., 2011). After washing with PBS, they were incubated with anti-rabbit IgG conjugated with fluorescein (Leinco Technologies, St. Louis, MO) and 0.1 μg/ml Hoechst for 1 h at room temperature (RT). Sections were subsequently washed in PBS and mounted with Mount-Quick Aqueous (Daido Sangyo, Tokyo, Japan). 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. Immunostaining was detected under a fluorescence microscope (Nikon, Japan).

Statistical Analyses

Data were expressed as means ± SEM of at least three separate experiments, each performed with triplicate samples. The one-way ANOVA was used to determine significant differences between groups followed by Student-Newman-Keuls test or Duncan's test using the SigmaPlot software (Ver. 11.2, Systat Software Inc., San Jose, CA, USA). *P* values of <0.05 was considered statistically significant.

RESULTS

Immunofluorescence Analysis of the PER2 Protein in Ovaries

The expression and distribution of the PER2 protein were investigated by fluorescent immunohistochemistry in the preantral follicles, antral follicles and corpora lutea at ZT 0 and ZT 12. A strong immunostaining signal was detected in the granulosa cells of the preantral and antral follicles at both points (Figure 1a, c, e, g). However, there was apparently little difference in the immunostaining signal (Figure 1b, d, f, h), suggesting a small cyclic expression of the PER2 protein. Conversely, in luteal cells, a strong immunostaining signal was observed in both the cytoplasm and nucleus at ZT12 (Figure 1k, l) as compared to the signal at ZT0 (Figure 1i, j). Consequently, fluorescent immunohistochemical studies might support circadian rhythmicity of the PER2 protein at least in the luteal cells.

Circadian Oscillations of *Per2* in Cultured Granulosa Cells and Luteal Cells

The circadian rhythm of *in vitro Per2* promoter activity was furthermore investigated using the granulosa cells prepared from preantral and antral follicles, and luteal cells of *Per2-dLuc* transgenic rats. The *Per2* circadian oscillation was generated by DXM. The oscillations were obviously observed in different cell types, as compared to those by fresh medium alone (control) (Figure 2A, B, C). All three-cell types displayed a rapid increase in bioluminescence within 6 h after exposure to DXM and generated a few oscillations following 24 h, albeit with a continuous decreasing of amplitude. After the second peak (P2) at ~24h, luteal cells displayed several oscillations with approximately 24 h per cycle, whereas the oscillatory amplitudes in immature and mature granulosa cells were attenuated. The oscillatory intensity, showing the difference between the peak and trough bioluminescence values (∆bioluminescence), was distinct among two granulosa cell types and luteal cells. The ∆bioluminescence values of the latter oscillations were significantly less in both granulosa cells than in luteal cells (Figure 2D). This indicated that the circadian rhythm was operative in both granulosa and luteal cells, although the granulosa cells were weak in oscillatory amplitude. Luteal cells also displayed oscillations without DXM treatment. In addition, there was a significant difference in the time of second and third peaks between both cell types (difference, ~3 h), with no difference in cycle time (Table 2).

Circadian Expression of the Clock Genes in Granulosa Cells and Luteal Cells

To further analyze the clock system in the granulosa cells from antral follicles and luteal cells, transcript levels of *Per2* and *Bmal1* were measured. The results are shown in Figure 3. Circadian rhythms of the *Per2* and *Bmal1* transcripts were obviously reverse-phase in luteal cells. The *Per2* circadian oscillation and the *Per2* transcript level agreed in both cell types. Statistical analysis showed that the *Per2* transcript level was robustly rhythmic as a function of a 24-h-cycle in granulosa cells (p=0.0001) and luteal cells (p=0.0033). In granulosa cells, however, circadian rhythm of the *Bmal1* transcript was very small but statistically significant (p=0.0406), as compared to that in luteal cells (p=0.0013).

Development of the Clock System in Granulosa Cells During Differentiation

To investigate the development of circadian rhythm during differentiation of granulosa cells into luteal cells, FSH control of the circadian rhythm was analyzed in a serum-free culture system using the granulosa cells from antral follicles. The cells displayed several oscillations in the presence of DXM and FSH, but only one cycle of oscillation was observed with LH (Figure 4A), suggesting LH has little effect in driving the circadian rhythm. On the other hand, granulosa cells treated with FSH clearly exhibited increases in the oscillatory intensity upon stimulation with DXM or LH (Figure 4B, C). The Δbioluminescence value of the second oscillation significantly increased two-fold by DXM exposure. LH newly induced the second oscillation.

Deregulation of *Per2* Circadian Oscillation in Luteal Cells During Inhibition of Progesterone Synthesis

To investigate the *Per2* circadian oscillation during apoptosis, luteal cells were treated with AMG, an inhibitor of progesterone synthesis. Treatment with the inhibitor caused a significant increase in the promoter activity 4 days after appearance of a major peak at ~30 h ($p<0.05$) (Figure 5A). Addition of progesterone (the final concentration, 100 nM) significantly suppressed the increased bioluminescence, which was reduced to the level generated by progesterone alone. Total RNA was isolated from luteal cells treated with AMG on day 4 and subjected to qPCR for the *Per2*, *Bmal1*, *Clock* and *Nampt* transcripts. There were no significant differences in transcript levels of *Per2* and *Nampt* between the presence and absence of AMG ($p>0.05$) (Figure 5B). However, transcript levels of *Bmal1* and *Clock* were significantly decreased in the presence of AMG. The *Bmal1* transcript level was especially reduced ($p=0.002$). In luteal cells treated with AMG for 4 days, cell death was increased by approximately 15%, as compared to the control (data not shown).

DISCUSSION

Our present study is the first to examine the expression profiles of core clock genes systematically in rat granulosa cells and luteal cells during differentiation and

apoptosis, and show that the *Bmal1* and *Clock* genes play important roles in the circadian clock of ovarian cells. The expression and intracellular localization of the PER2 protein were spatiotemporally analyzed by immunohistochemistry on developing ovaries. The cytoplasmic PER2 protein was differently distributed in luteal cells, but not apparently in granulosa cells, at two time points (ZT0 and ZT12), indicating the strong circadian rhythm of the PER2 protein in luteal cells *in vivo*. The real-time monitoring assay revealed that circadian oscillations were observed after exposure to DXM in luteal cells *in vitro*. Immunohistochemical analysis supported the results obtained by the real-time monitoring system of *Per2* promoter activity in luteal cells. The circadian oscillation in luteal cells was observed which was unlike that generated in granulosa cells.

Bmal1 is an essential core component of the circadian clocks, because the lack of BMAL1 protein in mice results in immediate and complete loss of circadian rhythmicity in constant darkness (Bunger et al., 2000; Wicht et al., 2010). In the present study, rhythmicity of the *Bmal1* transcript was clearly observed in luteal cells, but not in granulosa cells. This supports the finding that the *Per2* circadian oscillation was different between the two cell types. Surprisingly, however, a typical *Per2* oscillation was detected *in vitro*. This may arise from stimuli with DXM to the GRE element in the promoter of *Per2*, or other signals as yet unidentified, but not from the regulation of *Bmal1* through E-box. However, the circadian rhythms of *Per2* and *Bmal1* transcripts

were reverse-phase in luteal cells, as previously described in liver, muscle, adipose and uteri (Panda et al., 2002; Storch et al., 2002; Dolatshad et al., 2006; Zambon et al., 2003; Yang et al., 2006), but, for the *Bmal1* transcript, the circadian rhythm was very weak in granulosa cells, despite *Per2* circadian oscillation and circadian rhythms of *Per2* transcript being in complete agreement in both cell types. Thus, the circadian oscillator in luteal cells is actively driven via identified transcriptional and posttranscriptional feedback loops.

Our previous studies showed differential patterns of synchronized *Per2* oscillation in immature and mature granulosa cells (He et al., 2007). It has also been demonstrated that hCG resets a circadian pattern of *Per2* and *Bmal1* transcripts in eCG-primed ovaries of hypophysectomised rats (Karman & Tischkau, 2006). During follicular development, cell proliferation, the expression of LH receptor, and the activation of aromatase and P450 enzymes are promoted in granulosa cells under the influence of FSH. Our present study demonstrated that FSH induces the expression of *Per2* gene during the development, growth and maturation of immature ovarian follicles. However, LH could not promote the *Per2* circadian oscillation in granulosa cells owing to the lack of an LH receptor (Wakabayashi et al., 1989). The present study clearly demonstrated that the *Per2* circadian rhythm is developed during maturation in FSH-treated granulosa cells which were prepared from antral follicles. After granulosa cells were treated for 3 days, they clearly gained the ability of

circadian oscillations upon stimulation with DXM or LH. Interestingly, LH induced an additional oscillation. These findings suggest that FSH plays a critical role in the modulation of the circadian oscillator in granulosa cells. The present results support a previous report (Fahrenkrug et al., 2006) that *Per2* and the gene products are expressed in the steroidogenic cells of rat ovaries, being endowed with one or both of receptors for FSH and LH.

A few reports have suggested that apoptotic processes are physiologically organized along the 24 h timescale at the molecular level (Tampellini et al., 1998; Fu et al., 2002). It is well known that progesterone is a suppressor of apoptosis in luteal cells (Okuda et al., 2004), so the survival of luteal cells absolutely depends on the action of progesterone. AMG inhibits the synthesis of progesterone by blocking the conversion of cholesterol to pregnenolone through inhibition of the enzyme P450scc and consequently reduces the production of steroid hormones (Yacobi et al., 2007). In a previous study (Hua et al., 2006), the overexpression of PER2 protein reduces cellular proliferation and rapidly induces apoptosis in several cell lines such as a lung carcinoma cell line and a mammary carcinoma cell line. Our present results showed the deregulation of *Per2* circadian oscillation in luteal cells during treatment with AMG. Interestingly, when luteal cells were treated with AMG plus progesterone, the deregulated oscillation was recovered to a regular oscillation which was observed in control and progesterone-treated cells. Transcript levels of several core clock genes

were measured for analysis of the deregulated oscillation in apoptotic luteal cells. Interestingly, *Bmal1* and *Clock* transcript levels were significantly decreased. In contrast, however, transcript levels of *nicotinamide phosphoribosyltransferase* gene (*Nampt*) and *Per2* genes were not significantly altered. A previous study implicated a role of cellular NAD^+ in the regulation of CLOCK activity (Jared et al., 2001). NAMPT-mediated NAD^+ biosynthesis may promote a robust oscillation of clock target gene expression (Ramsey et al., 2009), indicating the NAMPT/NAD^+-driven pathway modulates circadian transcription patterns in mammals. On the other hand, although *Bmal1* and *Clock* were also inhibited during the process of apoptosis, the *Per2* transcript level was not decreased until at least 4 days after AMG treatment. This finding led us raise the intriguing possibility of a mechanism on maintenance of *Per2* transcript level under the deregulation state of a negative feedback loop. However, this aspect remains to be investigated. In addition, our previous report described up-regulation of *Per2* oscillation in prostate mesenchymal cells during flutamide-induced cell death (Yoshida et al., 2010). Additionally, *Per1* and *Per2* are deregulated in several human cancers (Fu & Lee, 2003). Taking together with current results, *Per2* up-regulation may become an important prognostic marker in apoptotic cells (Hua et al., 2006).

In conclusion, the present study demonstrated several characteristic circadian oscillations of a core clock gene *Per2* in granulosa cells and luteal cells during cell

differentiation and apoptosis as follows. Firstly, the granulosa cells from preantral follicles displayed a weak circadian oscillation of *Per2* gene owing to a low expression of the *Bmal1* gene. Secondly, FSH influenced the development of circadian oscillator in granulosa cells, followed by an obvious circadian oscillation induced by LH. This provides a fascinating topic of study regarding the circadian oscillation during the differentiation of ovarian cells in vitro because the oscillation is likely to be intrigued by LH surge through the LH receptor. Thirdly, circadian oscillation was deregulated in apoptotic luteal cells, induced by inhibiting progesterone synthesis, in which the expression of *Bmal1* and *Clock* genes was greatly reduced. Consequently, the circadian clockwork alters in ovarian cells during follicular development, luteinization and apoptosis. The expression of *Bmal1* may be related to the switch-on and switch-off of circadian oscillation.

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FIGURE LEGENDS

FIGURE 1. Observation of the PER2 protein in preantral follicles, antral, and copora lutea of ovaries by fluorescent immunohistochemistry. DES-primed, eCG-primed and eCG/hCG-primed immature rat ovaries were collected at ZT0 and 12 and were subjected to fluorescent immunohistochemistry using anti-PER2 antibody and Hoechst. PER2 staining (a, c, e, g, i, k) (*red*); Hoechst staining (*blue*); magnification of PER2 and Hoechst staining merged image (b, d, f, h, j, l). GC, granulosa cell; O, oocyte; LC, luteal cell. Scale bar, 50 μm.

FIGURE 2. Bioluminescence recording of granulosa cells from preantral and antral follicles and luteal cells. Ovarian cells were prepared from DES (preantral)-primed (A), eCG (antral)-primed (B), and eCG/hCG (luteal cells)-primed (C) *mPer2-dLuc* transgenic rats. Cells were cultured for 48 h and then exposed to 100 nM DXM for 2 h prior to the real-time monitoring of bioluminescence. Cells cultured in medium only were taken as control. Real-time bioluminescent activity was monitored for 4 days. P2-4: second to fourth peaks, T2-4: second to fourth troughs. (D) Differences between the respective peaks and troughs were normalized to the values of each second oscillation (P2-T2) and data are means ± SEM of three independent determinations. Third oscillation, P3-T3; fourth oscillation, P4-T4. Values with different letters are significantly different ($p<0.05$). N.D., not detectable.

FIGURE 3. Circadian rhythms of *Per2* and *Bmal1* transcripts in granulosa cells from antral follicles and luteal cells *in vitro*. Ovarian cells were prepared from eCG (antral)-primed (A) and eCG/hCG (luteal cells)-primed (B) *mPer2-dLuc* transgenic rats. Cells were cultured for 48 h and then exposed to 100 nM DXM for 2 h prior to the real-time monitoring of bioluminescence. Total RNA was isolated from cultured cells at the indicated times during the real-time monitoring of bioluminescence (*blue line*) and reverse transcribed. The resulting cDNA was used for real-time qPCR analysis of transcript levels of *Per2* and *Bmal1* using their specific primers. *Gapdh* was used as an internal control. Each value represents the mean ± SEM of three separate experiments. Values with different letters are significantly different ($p<.05$).

FIGURE 4. Development of circadian *Per2-dLuc* oscillation in granulosa cells during treatment with FSH. Ovarian cells were prepared from eCG (antral)-primed *mPer2-dLuc* transgenic rats. (A) Cells were cultured for 48 h in the presence of 5% FBS and granulosa cells were subjected to analyze the amplitude of circadian *Per2-dLuc* oscillation during treatment with 100 nM DXM, 100 ng/ml FSH, and 100 ng/ml LH in the serum-free medium (before FSH). (B, C) Cells were also cultured with FSH for 3 days and then exposed to DXM and LH (after FSH). P1-2: first to second peaks, T1-2: first to second troughs. Differences between the respective peaks and

troughs were normalized to the values of each first oscillation (P1-T1) and data are means ± SEM of three independent determinations. Second oscillation, P2-T2. Values with different letters are significantly different (p<0.05). N.D., not detectable.

FIGURE 5. Effect of progesterone synthetic inhibition on circadian *Per2-dLuc* oscillation in luteal cells. A, Luteal cells were prepared from eCG/hCG (luteal cells)-primed *mPer2-dLuc* transgenic rats and subjected to analyze circadian *Per2-dLuc* oscillation during cultivating with fresh medium (as a control), 100 nM progesterone (P4), 1 μM AMG, and progesterone (P4) plus AMG. The bioluminescence value was calculated and normalized to a value given by each peak (*triangles*). Results represent the mean ± SEM of three separate experiments. Values with different letters are significantly different (p<.05). B, Total RNA was isolated from control and AMG-treated cells for 4 days and subjected to real-time qPCR analysis of transcript levels of *Per2*, *Nampt*, *Bmal1*, and *Clock* using their specific primers. The mRNA levels of clock genes were normalized to *Gapdh* and data are means ± SEM of five separate experiments.

TABLE 1. Oligonucleotide primers

Gene	Accession No.	Sequence 5′ - 3′	Amplicon (bp)
Bmal1	NC_005100	F: CCGATGACGAACTGAAACACCT	215
		R: TGCAGTGTCCGAGGAAGATAGC	
Clock	NM_021856	F: GACAGCCCCACTGTACAATACG	81
		R: TGCGGCATACTGGATGGAAT	
Nampt	NM_177928	F: AGTTGCTGCCACCTTACCTTAGA	94
		R: CACTTTTTCTGTTTCATTCCCTCG	
Per2	NM_031678	F: GACGGGTCGAGCAAAGGA	90
		R: CCCTTTTCAGGTGTATAGGTAAGT	
Gapdh	NM_017008	F: AACCTGCCAAGTATGATGACATCA	111
		R: ACAACTTCGGCGTCCTCTGTTGGA	

TABLE 2. Profiles of circadian oscillations in rat granulosa cells and luteal cells after treatment with DXM

Cells	2nd Peak time h	3rd Peak time h	Cycle time h
Granulosa cells	26.57 ± 0.29	52.29 ± 0.36	25.72 ± 0.46
Luteal cells	29.67 ± 0.64 *	55.08 ± 0.41 *	25.41 ± 0.55

Data are expressed as mean ± SEM (n=5). * Statistical significance (p<0.01).

FIG. 1

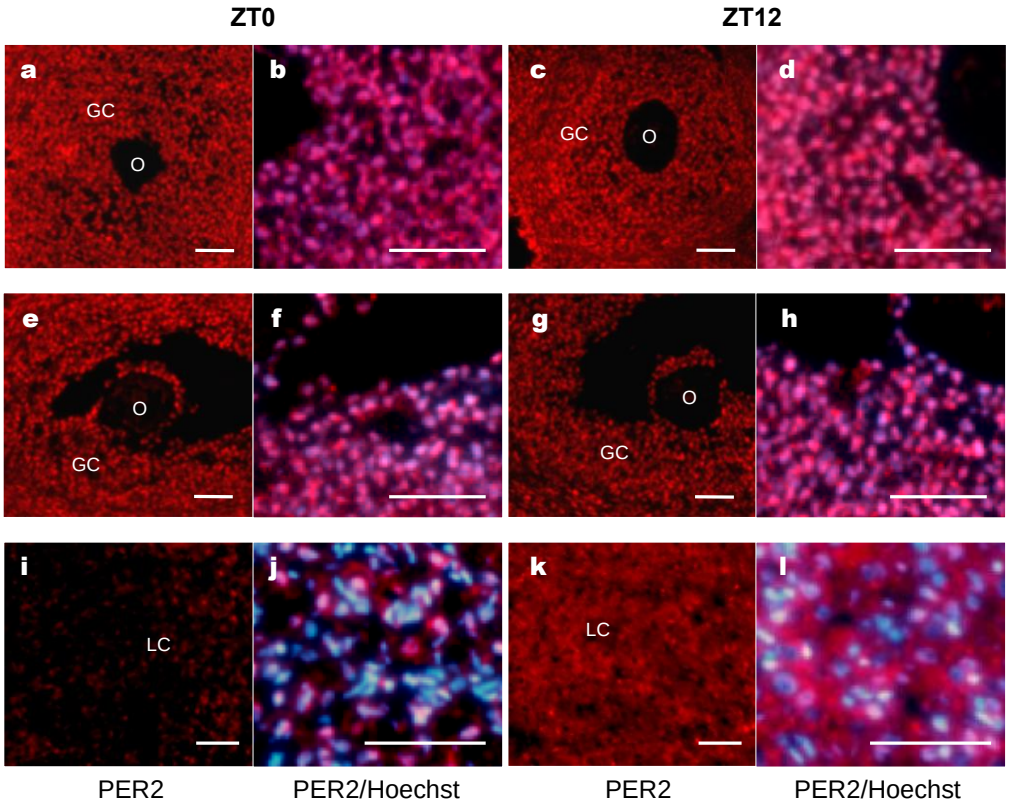

ZT0
ZT12
a
GC
O
b
c
GC
O
d
e
O
GC
f
g
O
GC
h
i
LC
j
k
LC
l
PER2
PER2/Hoechst
PER2
PER2/Hoechst

FIG. 2

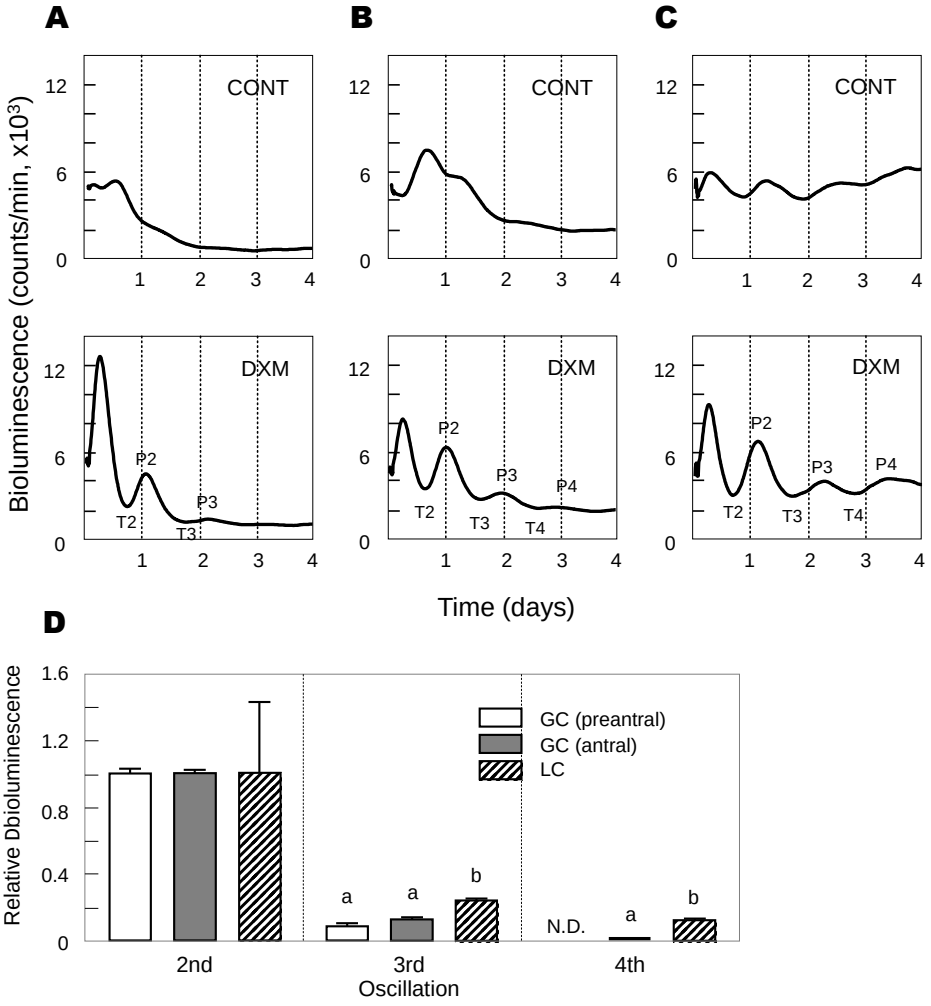

A
B
C
CONT
DXM
Bioluminescence (counts/min, x10³)
P2
P3
P4
T2
T3
T4
Time (days)
D
Relative Dbioluminescence
GC (preantral)
GC (antral)
LC
a
b
N.D.
2nd
3rd
4th
Oscillation

FIG. 3

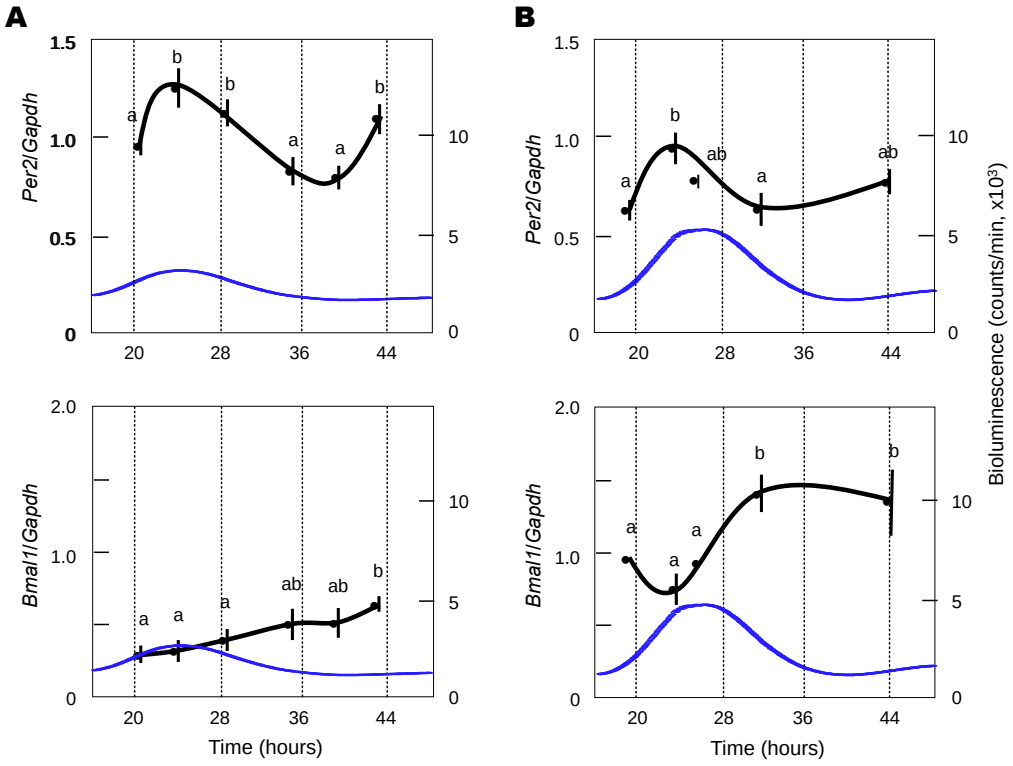

A
B
Per2/Gapdh
Bmal1/Gapdh
Bioluminescence (counts/min, x10³)
Time (hours)
20
28
36
44
0
0.5
1.0
1.5
2.0
5
10
a
ab
b

FIG. 4

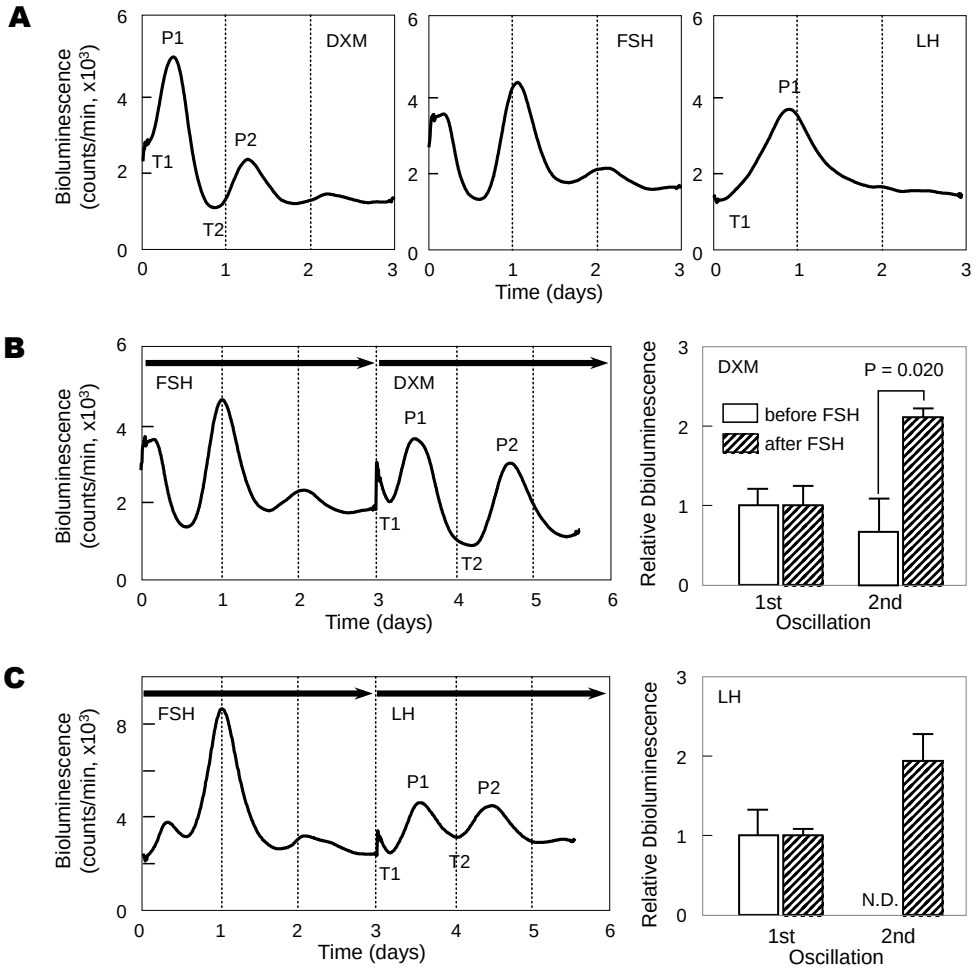
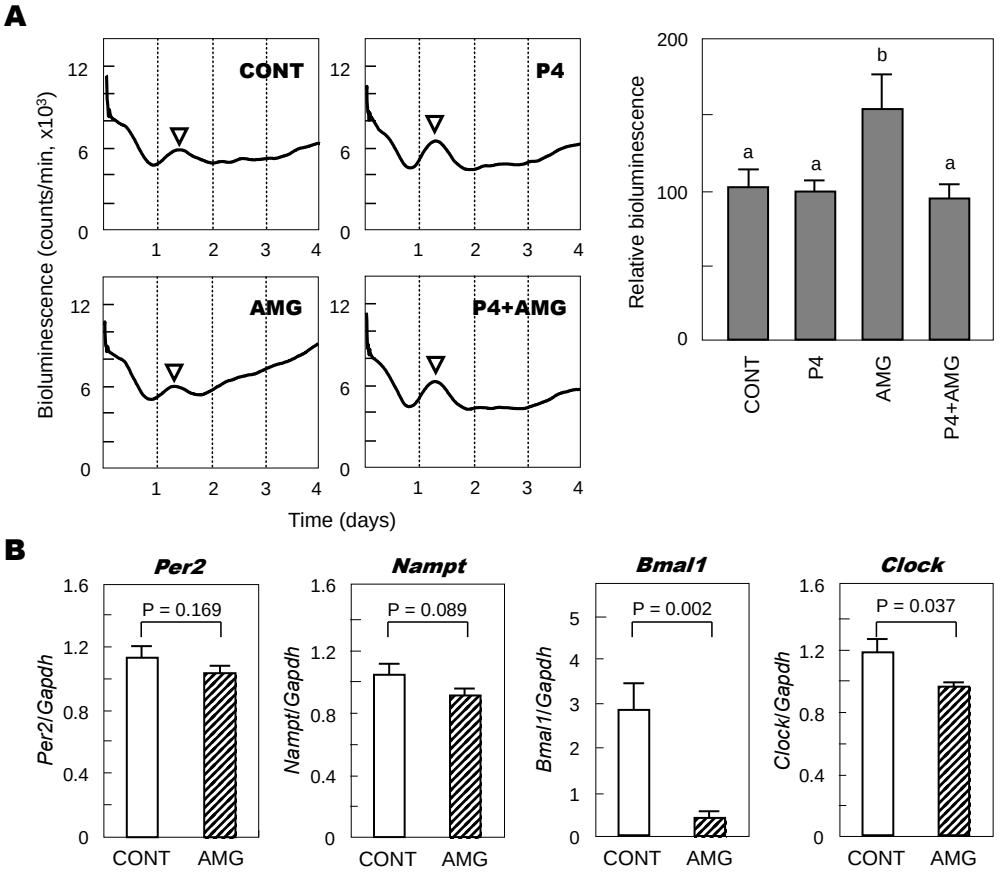

A
Bioluminescence (counts/min, x10³)
P1
DXM
T1
P2
T2
FSH
LH
Time (days)
B
FSH
DXM
Relative Dbioluminescence
P = 0.020
before FSH
after FSH
1st
2nd
Oscillation
C
LH
N.D.

FIG. 5


A
CONT
P4
AMG
P4+AMG
Bioluminescence (counts/min, x10³)
Time (days)
Relative bioluminescence
a
a
b
a
CONT
P4
AMG
P4+AMG
B
Per2
Nampt
Bmal1
Clock
P = 0.169
P = 0.089
P = 0.002
P = 0.037
Per2/Gapdh
Nampt/Gapdh
Bmal1/Gapdh
Clock/Gapdh
CONT
AMG