

The disruption of circadian clockwork in differentiating cells from rat reproductive tissues as identified by *in vitro* real-time monitoring system

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Abstract

The circadian clock, regulating hormonal secretion and metabolisms in accordance with the environmental light–dark cycle, resides in almost all peripheral tissues as well as in the superchiasmatic nucleus. Clock gene expression has been found to be noncyclic during spermatogenesis and the differentiation of thymocytes. However, currently little is known about how cell differentiation could affect circadian clockwork. We performed this study using the *in vitro* real-time oscillation monitoring system to examine the clockwork in several types of differentiating cells originated from reproductive tissues of transgenic rats (constructed with *Period* gene 2 (*Per2*) promoter–destabilized luciferase reporter gene). After treatment with dexamethasone (DXM), persistent oscillation of *Per2* expression was observed in both gonadotropin-induced and pregnant ovarian luteal cells,

proliferative uterine stromal cells (USCs), and nondifferentiating testicular interstitial cells, with a cyclic period of ~24 h. In contrast to these cell types, only one cycle of oscillation was sustained in granulosa cells undergoing differentiation. Additionally, *Per2* oscillation was irregular in USCs undergoing decidualization induced by medroxyprogesterone acetate plus N6, 2-*O*-dibutyl adenosine 3':5'-cyclic monophosphate. Furthermore, no oscillation of *Per2* expression was evoked by DXM in Leydig cells and thymocytes. In conclusion, the present study characterized the oscillation of *Per2* gene expression in several types of ovarian, uterine, and testicular cells, and it is strongly suggested that circadian clockwork is affected during cellular differentiation.

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Introduction

The circadian clock is primarily synchronized with environmental time by the 12 h light:12 h darkness cycle (Reppert & Weaver 2001). In mammals, the time-keeping system is composed of a central clock in the superchiasmatic nucleus that coordinates the subsidiary oscillators in extensive peripheral tissues (Reppert & Weaver 2001, Schibler & Sassone-Corsi 2002, Yamamoto *et al.* 2004). At molecular level, the clock system is composed of interlocked transcriptional and translational feedback loops. The CLOCK and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1), 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, Ueda *et al.* 2005). Furthermore, post-translational processes involving the phosphorylation and degradation of proteins, and regulated nuclear import

and export, are crucial for sustaining the ~24 h duration (Lee *et al.* 2001, Harms *et al.* 2004).

The peripheral clock plays an essential role in synchronizing local physiology to operate in a circadian manner via regulation of the expression of functional genes (Storch *et al.* 2002, Zvonic *et al.* 2006). These physiological processes mainly include hormonal secretion and metabolisms like gluconeogenesis and lipogenesis (Lemos *et al.* 2006, Wijnen & Young 2006). Interestingly, cultured cell lines could also exhibit several cycles of oscillations of the clock genes when treated with dexamethasone (DXM), a serum shock or other potential stimuli (Balsalobre *et al.* 1998, 2000). However, it has been suggested that the circadian clock does not work in the thymic tissue (Alvarez & Sehgal 2005) and during murine spermatogenesis (Alvarez *et al.* 2003, Morse *et al.* 2003). Alvarez *et al.* proposed that cellular differentiation might cause the suspension of the cyclic expression of clock genes. In addition, it has been reported that the DNA-binding activity of CLOCK/BMAL1 heterodimers is regulated by the redox state of NADH cofactors (Rutter *et al.* 2001). However, currently little is known about whether and how circadian

clockwork could be affected by changes of cellular physiological state.

The mammalian reproductive tissues are characterized with ever-changing physiology. Ovarian granulosa cells (GCs) undergo proliferation and differentiation during folliculogenesis under the regulation of follicle-stimulating hormone and locally produced factors (Richards 1994). The uterus is another highly dynamic tissue. In rodents, the endometrial stromal cells undergo proliferation and decidualization in response to steroid hormones and blastocyst implantation at the early stage of pregnancy (Dey *et al.* 2004). In the mammalian testicular and thymus tissues, the interstitial Leydig cells and the thymocytes (T lymphocytes) both undergo sequential stage-wise cellular differentiation (Touraine *et al.* 1977, Penit & Vasseur 1988, Mendis-Handagama & Ariyaratne 2001). The *in vitro* real-time oscillation monitoring system is a valuable tool for the investigation of circadian clockwork in cultured cell lines (Fujioka *et al.* 2006, Nakahata *et al.* 2006). In the present study, the real-time monitoring system was employed to evaluate the clockwork in several types of differentiating cells originated from the reproductive tissues of transgenic rats that have been constructed with *Per2* promoter-destabilized luciferase reporter gene.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), and 100× penicillin–streptomycin solution (PS) were purchased from GIBCO; diethylstilbestrol (DES), trypsin, trypan blue, EGTA, medroxyprogesterone acetate (MPA), N₆, 2-*O*-dibutyl adenosine 3':5'-cyclic monophosphate (db-cAMP), and luciferin were from Sigma. Collagenase was purchased from Invitrogen. DNase I was from Roche Diagnostics. Equine and human chorionic gonadotropin (eCG and hCG) were obtained from Teikoku Hormone Mfg. Co. (Tokyo, Japan).

Animals

Mouse *Per2* promoter region (chr1 (–): 93289505–93293019 on the Build 36 'essentially complete' assembly by National Center for Biotechnology Information and the Mouse Genome Sequencing Consortium), which is sufficient for circadian oscillation, was fused to a destabilized luciferase (*dLuc*) reporter gene (*dLuc*; luciferase gene fused with modified PEST sequence; 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 rats were maintained under 12 h light:12 h darkness cycle (light between 0800 and 2000) with water and food *ad libitum*. Adult females were mated with fertile males to produce pregnant rats

for isolation of endometrial stromal cells. The day of finding a copulatory plug or sperm in the vaginal smear was designated as day 1 of pregnancy. All the experiments were performed under the control of the Guideline for Animal Experiment in Faculty of Medicine, Kyushu University and The Law (no. 105) and Notification (no. 6) of the Government.

Preparation and culture of cells

GCs were prepared as previously described (Hattori *et al.* 1996) with minor modifications. Briefly, immature female rats at 21–23 days of age were injected subcutaneously with DES (1 mg/day) for 3 days. Ovaries were collected, incubated in DMEM/F-12 containing 6 mM EGTA for 15–30 min, and then in DMEM/F-12 supplemented with 0.5 M sucrose for 10–20 min at 37 °C. GCs were harvested by penetration of the follicles with a 27 gauge needle. The released cells were washed thrice with fresh medium. Approximately, 1×10^6 GCs were plated on a 35 mm collagen-coated dish with 2 ml culture medium (DMEM/F12 supplemented with $1 \times$ PS and 10% FBS).

Luteal cells (LCs) were prepared according to a previous report (Peluso *et al.* 2005) with slight modifications. Briefly, immature rats were treated with 50 IU eCG and 25 IU hCG 60 h later. Ovaries were collected on day 4 after hCG injection, followed by incubation in PBS containing 0.25% trypsin and 1 mM EGTA for 15 min at 37 °C. For comparison, ovaries were also isolated from the rats at day 5 of pregnancy. The corpora lutea were teased away from the connective tissues and incubated with PBS supplemented with 0.2% collagenase, 30 µg/ml DNase I and 0.5% BSA for 1 h at 37 °C. Cells were washed, and then plated on 35 mm collagen-coated dishes at the density of 1×10^6 cells/dish in 2 ml culture medium. More than 90% cells were positive to 3β-hydroxysteroid dehydrogenase (3β-HSD) staining.

Uterine stromal cells (USCs) were isolated from uterine horns of the rats at the stage of day 5 gestation. The uterine lumens were filled with PBS containing 0.1% collagenase and incubated at 37 °C for 1 h in a shaking water bath. The dissociated cells were washed and seeded at the density of 2×10^5 cells/dish. The culture medium was replaced at 15 min after cell seeding to remove epithelial cells. *In vitro* decidualization of USCs was performed as previously described (Matsui *et al.* 2004). Confluent USCs were further cultured for 6 days in DMEM/F12 supplemented with 2% FBS, 0.1 mM MPA, and 0.5 mM db-cAMP, with each medium changed every 3 days.

Testicular interstitial cells (TICs) were separated from decapsulated testes of the rats aged 2 months by collagenase digestion (Niedziela & Lerchl 1999). The dispersed cells were plated on collagen-coated dishes. After 1 h, the suspended cells were removed from adhesive TICs and subjected to the isolation of pure Leydig cells by Percoll density gradient centrifugation (Niedziela & Lerchl 1999). The Leydig cell fraction was collected, completely washed, and seeded on collagen-coated dishes at the density of 1×10^6 cells/dish.

After 24-h culture at 37 °C, 3 β -HSD staining was performed on both Leydig cells and adhesive TICs, with 80–90% of the cells positively stained within the former population and > 90% of the cells unstained for the latter. Approximately, 85–90% of the Leydig cells were viable as revealed by trypan blue staining.

Thymocytes were prepared from thymus tissues of 2-month old male rats as previously reported (Oka *et al.* 2000). Harvested cells were dispersed in culture medium and plated on collagen-coated dishes at the density of 1×10^6 cells/dish. After 1 h of cell seeding, the suspended cells were transferred into new dishes to remove the contamination by adhesive cells such as fibroblast cells and macrophages.

All the cultures were performed in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C until the bioluminescent monitoring with Kronos AB-2500 (ATTO, Tokyo, Japan) and the isolation of total RNA.

RT-PCR

Cultured cells were harvested at the indicated times and total RNA was extracted using Sepasol reagent according to the manufacturer's protocol. Total RNA (1 μ g) was then used for cDNA synthesis in 20 μ l mixture. The expression levels of β -actin, luteinizing hormone receptor (LHR), and decidual/trophoblast prolactin-related protein (d/t PRP) were examined by PCR using the following primers: β -actin (NM_031144), F: 5'-TTG CGC TCA GGA GGA GCA AT -3', R: 5'-ATC ATG TTT GAG ACC TTC AA-3'; LHR (NM_012978), F: 5'-AGG GAT GAATAACGA GTC TGT C-3', R: 5'-ATT GGA GTG TCT TGG GTG AAC -3'; d/t PRP (NM_022846), F: 5'-ATC CAG CGA GCT GAA GTC AT-3', R: 5'-CAT GAA GTG GTG GGT TTG TG-3'. PCR was performed in a 10 μ l mixture containing $1 \times$ PCR buffer, 0.25 U AmpiTaq-Gold enzyme, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.2 μ M of each primer, and 1 μ l of each RT product (after 5 \times dilution) as a template. Amplification was performed in a programmable thermocycler (Biometra, Germany). The following cycling conditions were applied, denaturing at 95 °C for 50 s, annealing at 50 °C for 50 s, and extension at 72 °C for 50 s (28 cycles). The PCR products were subjected to agarose (2%) electrophoresis. The bands, stained by ethidium bromide, were visualized by u.v. fluorescence. Densitometric analysis was performed using Scion Image (NIH software).

Real-time monitoring of bioluminescence

Cultured cells were treated with 0.1 μ M DXM in serum-free medium. After 2 h, the medium was replaced with 2 ml DMEM/F12 containing 5% FBS, $1 \times$ PS, 15 mM HEPES, and 0.1 mM luciferin. Real-time monitoring of bioluminescence was performed at 37 °C (34 °C for Leydig cells) using Kronos AB-2500 interfaced to computer for continuous data acquisition as previously described (He *et al.* 2006). The photon counts were integrated for 1 min at intervals of 8 min.

Data analysis

Period was calculated as previously reported (Abe *et al.* 2002). The original data were smoothed by an adjacent averaging method with 3 h running means. The peak and trough were calculated as the highest and lowest point of smoothed data respectively. The time of trough was used as the phase marker for analysis of cycle period. The data are expressed as mean \pm S.D. ($n=6$) and the differences between them were evaluated using Student's *t*-test following one-way ANOVA. $P < 0.05$ was considered significant.

Results

LCs display more stable oscillation of *Per2* expression than differentiating GCs

The 48 h cultured GCs and LCs were treated with DXM for 2 h and subjected to real-time monitoring of *Per2* oscillation. In both GCs and LCs, a peak of *Per2* expression was found at about 4 h after the initiation of monitoring. Whilst circadian oscillation of *Per2* was induced in both cell types, the oscillatory pattern was distinct between GCs and LCs. Only one cycle of oscillation was apparently sustained in GCs during 96 h monitoring (Fig. 1A). In contrast, persistent oscillation was observed in LCs, albeit with a continuous decreasing of amplitude (Fig. 1B). Furthermore, persistent *Per2* oscillation was also observed in the LCs isolated from the rat ovaries on day 5 of pregnancy (Fig. 1C).

The differentiating status of cultured GCs was examined by the expression of LHR, a classical marker of GC differentiation. The 48 h cultured GCs, after exposure to DXM for 2 h, were collected at 4 h intervals within 48 h and subjected to RNA extraction and RT-PCR analysis of *LHR* expression. The result showed that *LHR* expression was present at time 0 h and reached an expression platform from 24 h onward in the DXM-treated GCs (Fig. 2).

Unstable *Per2* oscillation is not due to GCs death

Given that GCs could spontaneously undergo cellular apoptosis in culture (Tilly *et al.* 1992), we explored whether cell death is responsible for the nonpersistent *Per2* oscillation in GCs. After 48 h monitoring, the medium was replaced with fresh medium or medium supplemented with 10 μ M forskolin and GCs were subjected to continued detection. Another cycle of robust oscillation was induced by a simple medium change (Fig. 3A). Forskolin administration evoked a pattern of *Per2* oscillation mimicking that induced by DXM (Fig. 3B). Furthermore, after 48-h bioluminescent detection, GCs were detached and cell viability was directly examined by trypan blue staining. The result revealed that $\sim 90\%$ of the cells were viable (data not shown). Consequently, it is unlikely that cell death caused the cessation of *Per2* oscillation in GCs after only one cycle.

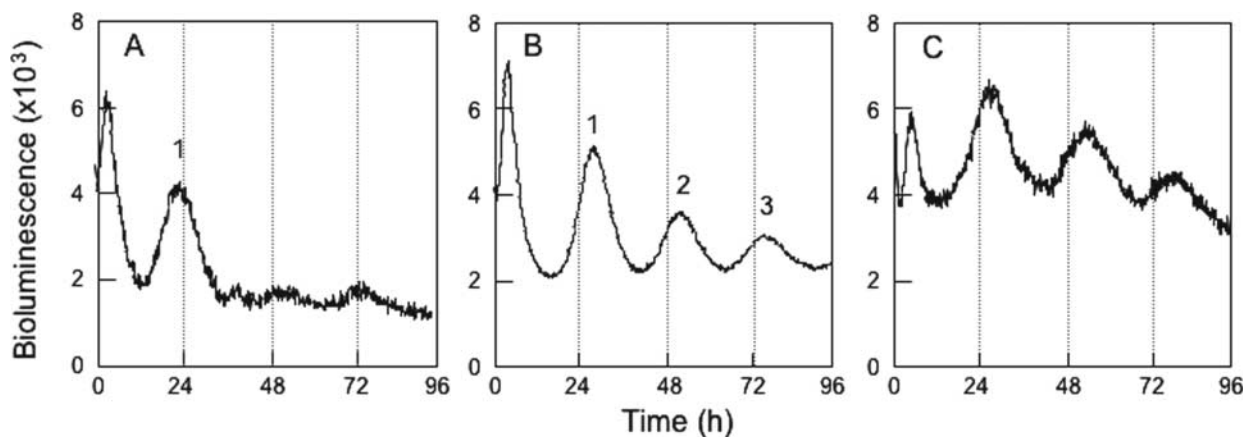


Figure 1 Circadian oscillations of *Per2* expression in GCs and LCs. GCs and LCs were cultured for 48 h, and exposed to DXM (0.1 μ M) for 2 h prior to the real-time monitoring of bioluminescence. Representative graphs show *Per2* oscillation in (A) GCs, (B) eCG/hCG-induced LCs, and (C) pregnant LCs. Each experiment was independently performed six times for (A) and (B) and thrice for (C). Numbers show the oscillatory cycles.

Per2 oscillation is irregular in USCs undergoing decidualization

USCs were cultured for 3 days to reach confluence and subjected to DXM treatment and real-time monitoring of *Per2* expression. The USC displayed similar pattern of oscillation to that obtained in LCs within 96-h detection (Fig. 4A). The decidualization of USC was induced by MPA and db-cAMP, and the differentiating status was revealed by the strong expression of *dt/PRP* gene (Fig. 4B), a useful marker of decidualization (Gu *et al.* 1994). The subsequent bioluminescent monitoring showed distinct pattern of *Per2* expression in decidualizing USC from that in nontreated USC. After DXM treatment, a visible peak of *Per2* expression was observed at about 29 h, however, followed by a prolonged falling phase

without obvious circadian or circadian-like oscillation (Fig. 4C).

No oscillation of *Per2* expression is evoked in Leydig cells and thymocytes

Adhesive TICs were cultured for 3 days and Leydig cells were cultured for 24 h prior to DXM treatment and subsequent bioluminescent monitoring. *LHR*, a useful marker of Leydig cell differentiation (Siril Ariyaratne *et al.* 2000), was abundantly expressed in the Leydig cells but not in the adhesive TICs (Fig. 5A), which suggested the differentiating status of Leydig cells. Strikingly high amplitude of *Per2* oscillation was observed for adhesive TICs (Fig. 5B). In contrast, no obvious oscillation of *Per2* expression was evoked in Leydig cells by DXM (Fig. 5C). Further no significant oscillation was also observed in Leydig cells treated with forskolin (data not shown). The potential of clockwork in

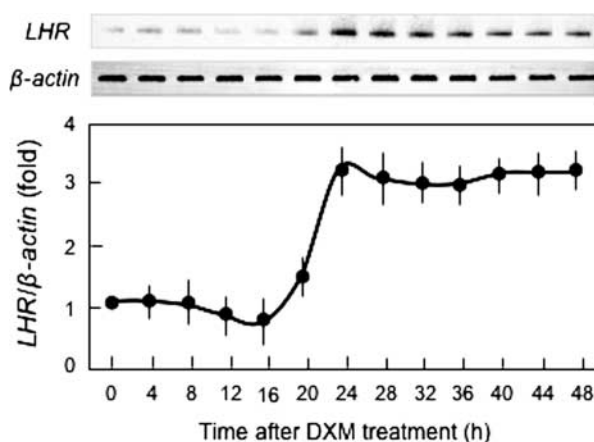


Figure 2 The expression of *LHR* in GCs after DXM treatment. The 48-h cultured GCs were treated with 0.1 μ M DXM for 2 h and harvested at the indicated time points. Total RNA was isolated and subjected to RT-PCR analysis of the transcript level of *LHR*. β -actin was used as an internal control. Data represent the mean \pm s.d. of three independent experiments (basal set as 1).

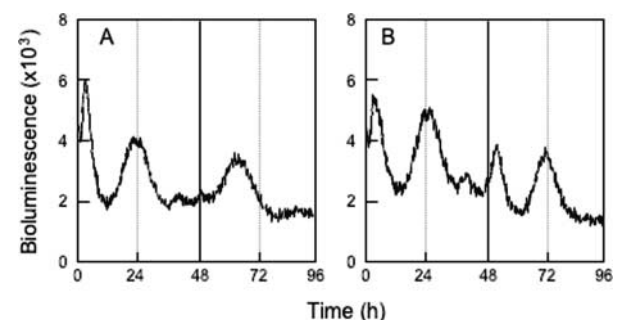


Figure 3 The oscillations of *Per2* expression in GCs after medium changes. Medium changes were performed at 48 h (solid vertical line) after the initiation of bioluminescent monitoring. Graphs represent the oscillatory patterns of *Per2* in GCs with a medium change of fresh DMEM/F12 (A) or DMEM/F12 containing 10 μ M forskolin (B). Each experiment was independently performed at least three times.

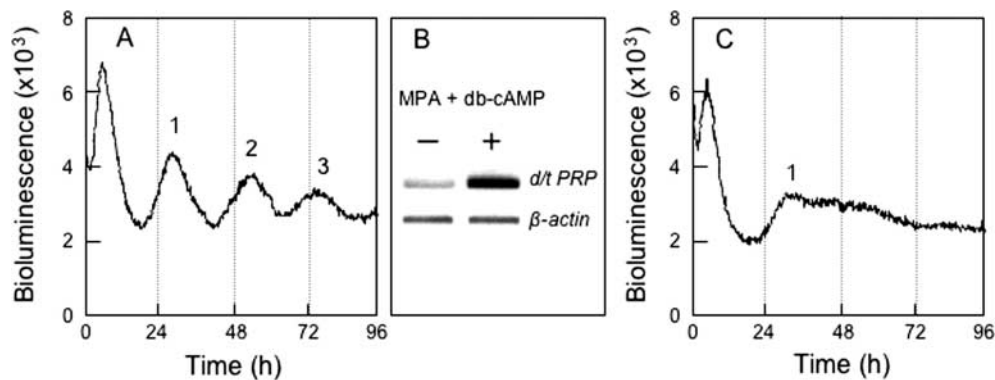


Figure 4 Oscillations of *Per2* expression in proliferative and decidualizing USC. Proliferative USC were derived from the uterine horns on day 5 of pregnancy and cultured for 3 days prior to any treatment. Decidualizing cells were obtained by treating confluent USC for 6 days with 0.1 mM MPA plus 0.5 mM db-cAMP. The mRNA level of *dtPRP*, a marker for USC decidualization, was analyzed by RT-PCR in treated and nontreated control (B). Both types of cells were likewise treated with DXM for 2 h prior to bioluminescent monitoring. Representative graphs show the oscillatory patterns of *Per2* in proliferative (A) and decidualizing (C) USC. Each experiment was independently performed six times and numbers show the oscillatory cycles.

differentiating thymocytes was also examined for comparison with that exhibited in Leydig cells. Thymocytes cultured for 24 h were likewise treated with DXM and subjected to the monitoring of *Per2* expression. The thymocytes also did not exhibit any evidence of *Per2* oscillation (Fig. 5D).

Statistical analysis of the oscillations of *Per2* in various cells

The mean time of the first peak of *Per2* oscillation in GCs appeared at ~24 h, which was significantly ($P < 0.05$) earlier than that observed in other cell types (Table 1). The first peak time could not be calculated in Leydig cells and thymocytes due to the lack of any visible oscillation. The mean period length of each oscillatory cycle was ~24 h in GCs, LCs, proliferative USC, and adhesive TICs (Table 1). Determination of the cyclic period could not be made in differentiating USC, Leydig cells, and thymocytes.

Discussion

In the present study, several types of cells, originated from the reproductive tissues of *Per2-dLuc* transgenic rats, were employed to demonstrate whether and how circadian clockwork could be affected during cellular differentiation. Our results for the first time showed distinct pattern of *Per2* oscillation in differentiating cells from that in proliferative or terminally differentiated cells.

In GCs, only one cycle of oscillation of *Per2* expression was induced after exposure to DXM, with the peak appearing at ~24 h. In contrast, persistent *Per2* oscillation was observed in both eCG/hCG-induced LCs and pregnant LCs. This distinctiveness is thought to be related with each cellular physiology. It has been suggested that GCs spontaneously undergo cellular apoptosis in culture (Tilly *et al.* 1992).

However, it is unlikely that cell death caused the cessation of *Per2* oscillation in GCs after only one cycle, as 90% of the cells were still viable, and another cycle of oscillation could be again evoked by replacement with fresh medium or stimulation with forskolin. This is consistent with a previous report (Yamazaki *et al.* 2000). Alternatively, GCs could potentially undergo cellular differentiation during *in vitro* culture, which is unlike LCs and is terminally differentiated. Indeed, *LHR*, a classical marker of GCs differentiation, was found to be abundantly expressed in cultured GCs from 24 h onward after DXM treatment. It is thus more likely that cell differentiation is responsible for the lack of persistent *Per2* oscillation in GCs. We speculate that circadian clockwork in differentiating GCs might be highly susceptible to the changes of environmental conditions, e.g., pH, depletion of nutrients, and accumulation of metabolic substances.

Several laboratories have focused on the expression of clock genes in the ovarian follicles and/or corpora lutea *in vivo*, but their findings were mostly conflicting. Studies on the circadian clock in the *Drosophila* ovary suggested that period and timelessness were constantly expressed in the follicular cells (Beaver *et al.* 2003). Fahrenkrug *et al.* (2006) reported the diurnal rhythm of *Per1* and *Per2* expression in the corpora lutea of rat ovaries. Conversely, Karman & Tischkau (2006) reported that *Bmal1* and *Per2* were rhythmically expressed in the rat ovarian follicles but not in the corpora lutea. Our present findings to some extent suggest that, *in vivo*, circadian clockwork might be somewhat influenced in the follicular GCs during the process of cellular differentiation or maturation. By contrast, it is likely that a circadian clock operates more stably in the corpora lutea.

We further extended the studies on uterine cells to prove the possibility that cellular differentiation interferes with clockwork in differentiating cells. The USC isolated from the uterus on day 5 of pregnancy are proliferative in culture.

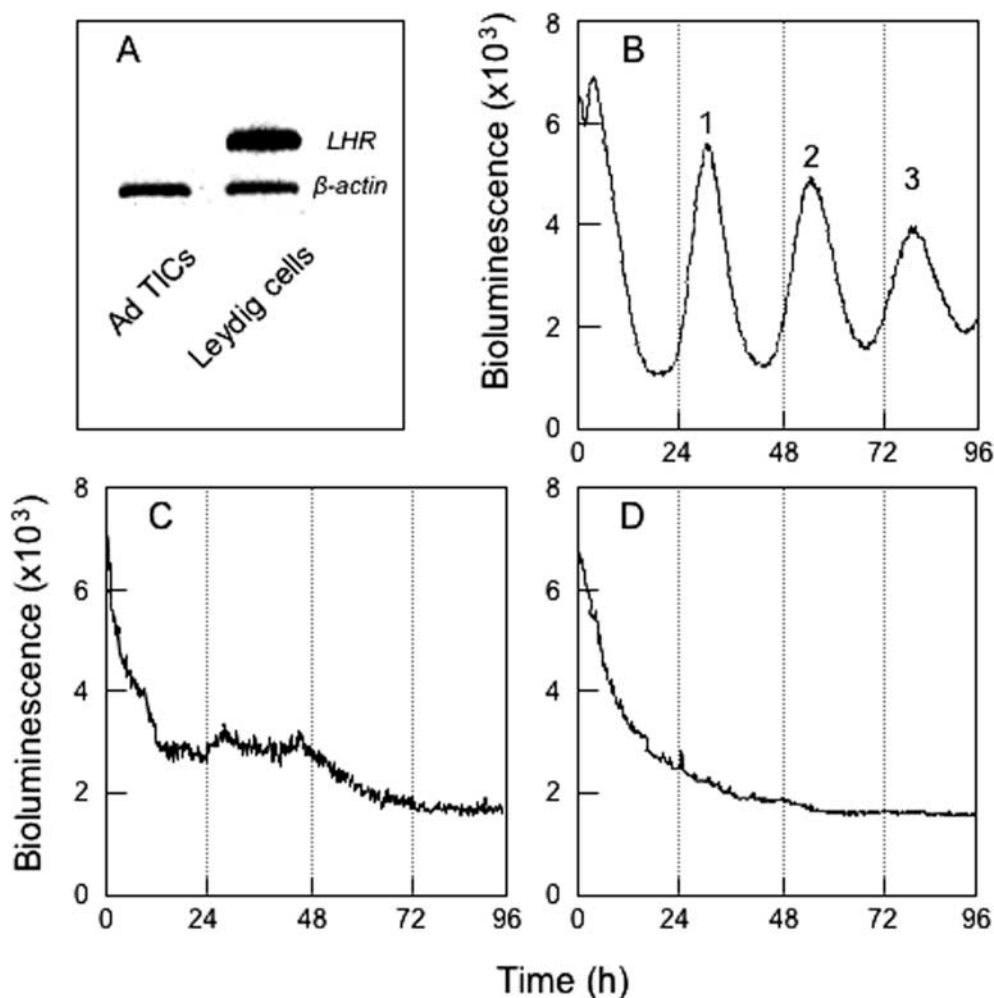


Figure 5 Representative expression patterns of *Per2* in testicular interstitial cells and thymocytes. The expression of *LHR* was examined in adhesive TICs and Leydig cells (A). Adhesive TICs (B), Leydig cells (C), and thymocytes (D) were treated with DXM for 2 h, and *Per2* expression was real-time monitored. Each experiment was independently performed six times. The numbers in B show oscillatory cycles.

Decidualization of USCs was induced by MPA and db-cAMP (Matsui *et al.* 2004), and the strong expression of *d/t PRP* indicated the differentiating status of USCs. Indeed, there was marked difference in the pattern of *Per2* oscillation between proliferative and decidualizing USCs. Persistent *Per2* oscillation observed in nontreated USCs disappeared in the latter, suggesting that normal clockwork might be disturbed during cellular differentiation. Previously, it has been reported that clock genes were rhythmically expressed in the rat uterus (Nakamura *et al.* 2005, Dolatshad *et al.* 2006). To the best of our knowledge, however, 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 predicts that, *in vivo*, the circadian rhythms in the endometrial stroma may be impaired during decidualization.

In vivo, cellular differentiation occurs in GCs and USCs only at a certain developmental stage i.e. later folliculogenesis and blastocyst attachment respectively. Herein, a circadian clockwork study was also performed in testicular Leydig cells and thymocytes, which are characterized with sequential stage-wise differentiation (Touraine *et al.* 1977, Penit & Vasseur 1988, Mendis-Handagama & Ariyaratne 2001). The presently prepared Leydig cell population may comprise Leydig cell lineage at different developmental stages (Mendis-Handagama & Ariyaratne 2001). Remarkably, after exposure to potent resetting cues, no obvious synchronized *Per2* oscillation could be observed in these differentiating Leydig cells, which is in dramatic contrast to the high-amplitude of *Per2* oscillation in the adhesive TIC population mainly consisting of nondifferentiating fibroblast-like (mesenchymal) cells, macrophages, and endothelial cells

Table 1 Characterization of oscillation profiles of *Per2* expression in various cell types

	First peak time (h) (n=6)	Cycle time (h, mean \pm s.d., n=6)		
		1	2	3
Cells				
Granulosa cells	23.71 \pm 0.35*	23.45 \pm 0.49	N.D.	N.D.
Luteal cells	28.24 \pm 0.31	23.88 \pm 0.41	24.26 \pm 0.27	24.13 \pm 0.22
Uterine stromal cells	28.57 \pm 0.39	23.63 \pm 0.37	23.95 \pm 0.32	24.02 \pm 0.43
Decidualizing uterine stromal cells	28.92 \pm 0.52	N.D.	N.D.	N.D.
Adhesive testicular interstitial cells	29.50 \pm 0.36	24.45 \pm 0.24	24.25 \pm 0.46	24.13 \pm 0.35
Leydig cells	N.D.	N.D.	N.D.	N.D.
Thymocytes	N.D.	N.D.	N.D.	N.D.

Cycle numbers are as marked in Figs 1, 4 and 5. Luteal cells are those from eCG/hCG-primed ovaries. N.D., not detected; * $P < 0.05$ versus other cell types.

(Niedziela & Lerchl 1999, Mendis-Handagama & Ariyaratne 2001). The lack of *Per2* oscillation in Leydig cells is similar to what was observed in differentiating thymocytes, wherein clock gene expression has been previously demonstrated to be noncyclic by *in vivo* examinations (Alvarez & Sehgal 2005). However, the secretion of testosterone from Leydig cells has long been known to have a low-amplitude of diurnal rhythm (Goodman *et al.* 1974, Mock *et al.* 1978). The underlying mechanism is not yet clear. Our findings suggest that this diurnal rhythm is less likely to be due to control by the local clock system in Leydig cells. Rather, the previously reported diurnal LH level (Albertsson-Wikland *et al.* 1997, Mitamura *et al.* 1999) might contribute to the regulation of the diurnal rhythm of testosterone production.

Although the present study identified a distinct manner of clockwork in differentiating cells, much work is still required to clearly elucidate the underlying mechanism. Two possible explanations are proposed. One is that regular interaction of clock proteins and chromosomal DNA is disrupted or impaired at the level of gene transcription due to the requirement of differentiation-specific gene transcription. A second possibility is that, during cellular differentiation, the post-translational modifications and subsequent nucleocytoplasmic shuttling of clock proteins (Lee *et al.* 2001, Harms *et al.* 2004) are affected by the physiological state. Notably, Yoo *et al.* (2004) suggested that transgenic constructs do not always produce robust/sustained oscillations in peripheral tissues as compared with knock-in constructs. Therefore, the employment of knock-in constructs may provide further insights into the circadian clockwork in differentiating Leydig cells and thymocytes. Furthermore, future study using the method of bioluminescence imaging of individual cell will help to elucidate whether the destroyed coordination among differentiating cells during purification and nonadhesive culture is responsible for the quick desynchronization of DXM-evoked *Per2* oscillation in Leydig cells and thymocytes *in vitro*.

In conclusion, using *in vitro* bioluminescence monitoring system, we characterized the oscillation of *Per2* gene expression in several types of cells prepared from *Per2-dLuc* transgenic rats. The results demonstrated that DXM-induced

Per2 expression in differentiating cells was in dramatic contrast to that in proliferating or terminally differentiated cells. We also found obviously different *Per2* oscillations in the cells undergoing differentiation only at a certain developmental stages (GCs and USCs) compared with the cells that exhibit sequential stage-wise differentiation (Leydig cells and thymocytes). The present findings may increase our understanding of the coordination between the developmental clock and circadian clock in the regulation of development and physiology.

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