Positive Autoregulation Delays the Expression Phase of Mammalian Clock Gene Per2

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Abstract

In mammals, cellular circadian rhythms are generated by a transcriptional-translational autoregulatory network that consists of clock genes that encode transcriptional regulators. Of these clock genes, Period1 (Per1) and Period2 (Per2) are essential for sustainable circadian rhythmicity and photic entrainment. Intriguingly, Per1 and Per2 mRNAs exhibit circadian oscillations with a 4-hour phase difference, but they are similarly transactivated by CLOCK-BMAL1. In this study, we investigated the mechanism underlying the phase difference between Per1 and Per2 through a combination of mathematical simulations and molecular experiments. Mathematical analyses of a model for the mammalian circadian oscillator demonstrated that the slow synthesis and fast degradation of mRNA tend to advance the oscillation phase of mRNA expression. However, the phase difference between Per1 and Per2 was not reproduced by the model, which implemented a 1.1-fold difference in degradation rates and a 3-fold difference in CLOCK-BMAL1 mediated inductions of Per1 and Per2 as estimated in cultured mammalian cells. Thus, we hypothesized the existence of a novel transcriptional activation of Per2 by PER1/2 such that the Per2 oscillation phase was delayed. Indeed, only the Per2 promoter, but not Per1, was strongly induced by both PER1 and PER2 in the presence of CLOCK-BMAL1 in a luciferase reporter assay. Moreover, a 3-hour advance was observed in the transcriptional oscillation of the delta-Per2 reporter gene lacking cis-elements required for the induction by PER1/2. These results indicate that the Per2 positive feedback regulation is a significant factor responsible for generating the phase difference between Per1 and Per2 gene expression.

Introduction

The circadian clock controls daily rhythms of biological activities such as the sleep/wake pattern in many organisms. The cellular mechanism of the mammalian clock has been characterized as a transcriptional-translational autoregulatory network that consists of clock genes encoding transcriptional regulators [1]. In this network, the circadian expressions of clock genes peak one after another, and their expression phases may determine the timing of internal events such as metabolism [2]. Both the Per1 and Per2 genes are rhythmically transactivated by the CLOCK-BMAL1 heterodimer, which binds to the E/E’-box motifs in their promoter regions [3–6], as well as Cryptochrome(Cry1) [7,8] and Rev-erbα [9,10], which are components of negative feedback loops in the mammalian circadian clock. The transcriptional activation of Per1 and Per2 by CLOCK-BMAL1 is repressed byCRY1 andCRY2 [5–8], whereas REV-ERBαrepressesBmal1 transcription via the transcription factor binding site RORE [9,10]. These negative feedback regulations guarantee sustainable circadian oscillations.

The Per1 and Per2 genes are essential to sustain the circadian rhythm, and the behaviors of Per1−/−/Per2−/− double mutant mice are arrhythmic [11,12]. Intriguingly, the oscillation phase of Per2 mRNA lags behind that of Per1 by approximately 4 hours in the suprachiasmatic nucleus (SCN), which is the master circadian regulator in the brain, and other peripheral tissues [13–17], though the oscillatory expressions of both Per1 and Per2 are assumed to be evoked by CLOCK-BMAL1 transactivity. The functions of PER1 and PER2 proteins (PER1/2) are partially redundant because both Per1 and Per2 single mutant mice are rhythmic under both light-dark (LD) and constant dark conditions [11,12,18–20]. However, the differing roles of PER1 and PER2 have also been documented in the different behaviors of Per1Brdm1 and Per2Brdm1 single mutant mice, which show abnormal responses to photic stimuli under light-dark conditions [20]. In this study, we investigated the mechanisms underlying the phase difference between Per1 and Per2 expression by a combination of mathematical simulations and molecular experiments. The elucidation of the regulatory mechanism of Per1 and Per2 expression should provide important clues about the robust self-sustainable oscillation and photic entrainment of the circadian clock.

Because the circadian regulatory network is a self-sustainable oscillatory circuit, it is of interest not only for cellular biology but also for computational biology. Thus, many mathematical models...
have been developed through the accumulation of biological knowledge [21]. Mathematical approaches enable us to test whether our current knowledge about the regulation of Per1 and Per2 expression is sufficient for explaining the phase difference of Per1 and Per2. If the current knowledge is not sufficient, studies that incorporate mathematical models can yield predicted mechanisms that regulate gene expression to generate the phase difference, and these predictions can then be tested experimentally. By combining mathematical and experimental approaches, we report here that a new transcriptional regulation mechanism is needed to explain the phase difference in the expression of Per1 and Per2 mRNAs.

Results
In silico analysis of an mRNA expression phase in a current circadian oscillatory network model

To analyze the mechanism that generates the oscillation phase difference between Per1 and Per2, we employed a mathematical model of the circadian clock that included Per, Cry, Bmal1, and Rev-erb, as proposed by Leloup and Goldbeter with following modifications [22]. We introduced the Per1 and Per2 genes instead of Per to compare their oscillation phases because Per1 and Per2 were not distinguished and the Per gene represented both of Per1 and Per2 in the original model (Figure 1A). The kinetics equations and parameters of Per1 and Per2 were the same as those of original Per except for the translation rate coefficient, which was divided in half because the PER protein represented the sum of the translational products of both genes. All kinetic parameters and reaction rate equations for the 20 variables are indicated in Table S1 (Model1) and Text S1.

As long as the transcriptional regulation of Per1 and Per2 is the same as hypothesized in the model, the observed phase difference between Per1 and Per2 mRNA oscillations is not likely to occur. However, possible difference in synthesis and/or degradation rates may cause the phase difference between Per1 and Per2. We computationally estimated the dependency of oscillation phases on the transcription rate by varying the proportion of the Per1 transcription rate coefficient ($v_{\text{p1}}$) to the Per2 transcription rate coefficient ($v_{\text{p2}}$; Figure 1B). Similarly, the proportion of the Per1 degradation rate coefficient ($v_{\text{m1}}$) to the Per2 degradation rate coefficient ($v_{\text{m2}}$) was varied (Figure 1C). As shown in Figure 1B and C, slow synthesis or fast degradation of mRNA advanced the phase of oscillation. Indeed, the 4-hour phase lag of Per2 mRNA behind Per1 mRNA could be reproduced when the Per1 transcription was 0.8-fold lower than that of Per2 or when the Per1 mRNA degradation was 2-fold more than that of Per2. If the transcription of Per1 was much faster than that of Per2 (i.e., $v_{\text{p1}}/v_{\text{p2}}$ $>$ 1.2 in Figure 1B) or the degradation of Per1 was much slower than that of Per2 (i.e., $v_{\text{m1}}/v_{\text{m2}}$ $<$ 0.7 in Figure 1C), oscillations did not occur.

**Figure 1. Effects of mRNA transcription and degradation rates on the Per mRNA expression phase.** (A) Schematic representation of the circadian oscillatory network model used to compare the expression phases of Per1 and Per2 mRNAs, which was based on the Leloup and Goldbeter model [22]. The Per2 gene transcription and translation are additionally introduced in the shaded region. A square, wave line, and circle indicate a gene, mRNA, and protein, respectively. Details are described in Text S1. (B, C) Variation in the phase difference between the Per1 and Per2 mRNA oscillations with varied (B) the proportion of the Per1 transcription rate coefficient to the Per2 transcription rate coefficient ($v_{\text{m1}}/v_{\text{m2}}$) and (C) the proportion of the Per1 degradation rate coefficient to the Per2 degradation rate coefficient ($v_{\text{m1}}/v_{\text{m2}}$). The rate coefficient of transcription and degradation of Per2 were fixed to 2.4 nM/h and 2.2 nM/h, respectively. The phase difference of Per1 from Per2 is indicated in circadian time (CT). doi:10.1371/journal.pone.0018663.g001
and the orbit converged to the steady state. From the numerical results, we conjectured that the transcriptional activity of Per2 is higher than that of Per1 or that the rate of Per1 mRNA degradation is faster than that of Per2, which causes the observed phase difference between Per1 and Per2 mRNA oscillations.

In addition to the model proposed by Leloup and Goldbeter, other computational models for mammalian circadian clock, which reproduce the time-series data of clock gene mRNAs and protein expression, have been also proposed [23,24]. One of these models developed by Forger and Peskin including different kinetic parameters of Per1 and Per2 transcription did not reproduce the expression phase difference between Per1 and Per2. Another model developed by Minsky et al. reproduced the phase difference between Per1 and Per2 mRNA. Actually, the phase difference was generated by the different kinetic rates such as Hill coefficient and Michaelis constant of Per1 and Per2 transcription. However, the kinetic rates assumed in this model were not measured experimentally. Therefore tested the hypothesis that difference in kinetic rates between Per1 and Per2 dynamics can account for the phase difference by using the experimentally measured parameters.

Synthesis and degradation rates of Per1 and Per2 mRNAs in vitro

To evaluate our mathematical estimation, we next measured the promoter activities of Per1 and Per2 as well as the degradation rates of these mRNAs in vitro. The promoter activities of Per1 and Per2 were measured by using two reporter genes Per1::luc and Per2::luc, in which the Per1 [23] and Per2 [5] promoters, respectively, were fused to the luciferase gene (Figure 2A). Both reporter genes were induced by Clock and Bmal1 co-transfection; however, a 3-fold higher induction was observed in cells transfected with Per1::luc compared to Per2::luc (Figure 2B). The higher transcriptional activity of Per1 did not produce the 4-hour phase advance in Per1 expression compared to Per2 because the increase in promoter activity should have delayed the oscillation phase as estimated by the previous mathematical analysis (Figure 1B). Subsequently, we examined the degradation rates of Per1 and Per2 mRNA in a cell line derived from the rat SCN (Figure 2C) [26]. Although the faster degradation of Per1 satisfies a requirement for the advanced Per1 oscillation phase compared to Per2 in this model, neither the 1.1-fold faster rate of Per1 degradation nor the 0.9-fold slower rate of Per2 degradation estimated in vitro reproduced the 4-hour phase difference (Figure 2C).

Then, the combined effect of the transcription and degradation rate ratios on the phase difference was examined using our mathematical model. However, the oscillation phase of Per2, but not of Per1, was advanced by +5.4 hours (Figure 3). The differences observed in the promoter activities induced by CLOCK-BMAL1 and mRNA degradation rates could not reproduce the 4-hour phase difference between Per1 and Per2.

A new model including an additional feedback regulation to reproduce the phase delay of Per2

As described above, our modified model (Text S1, Eqs. S1–S20) with measured parameters could not reproduce the phase difference between Per1 and Per2 mRNA oscillations. Therefore, we hypothesized several models, including an additional transcriptional regulation that may account for the phase difference. The basic idea underlying our modeling was that a feedback regulation of Per1 or Per2 transcription by PER1/2 could be the basis for the observed phase difference between Per1 and Per2. To express this idea, we studied i) positive feedback regulation of Per2 transcription, ii) negative feedback regulation of Per1 transcription, iii) positive feedback regulation of Per1 transcription, and iv) negative feedback regulation of Per2 transcription by PER1/2. We examined whether any of these mechanisms could potentially explain the observed phase difference.

To elucidate the molecular functions of nuclear PER1/2, ten reactions were additionally assumed on the basis of the model described previously: dissociation/association of the nuclear PER-CRY complex, phosphorylation/depshorylation/degradation of nuclear PER and CRY, and association/dissociation of the nuclear CRY with CLOCK-BMAL1 (Figure 4A). All kinetic parameters and reaction rate equations, including five additional variables (nuclear PER (PN), phosphorylated nuclear PER (PmPN), nuclear CRY (CN), phosphorylated nuclear CRY (CPN), CRY-BMAL1 heterodimer (CBN)) and the modified reaction rate equation of nuclear PER-CRY complex are available in Table S1 (Model2) and Text S1.

When PER1/2 proteins (PN) positively regulated Per2 transcription, the dynamics of Per1 and Per2 mRNA were calculated by following equations:

\[
\frac{d\text{Per1}}{dt} = \frac{v_{\text{P1}}}{K_{\text{AP1}} + B_N} - \frac{v_{\text{mP1}}}{K_{\text{mP1}} + \text{Per1}} - k_{\text{dmp}\text{Per1}} \text{Per1} \quad (1)
\]

\[
\frac{d\text{Per2}}{dt} = \frac{v_{\text{P2}}}{K_{\text{AP2}} + B_N} + \frac{k_{\text{AP2}} \text{PN}}{K_{\text{mP2}} + \text{Per2}} - k_{\text{dmp}\text{Per2}} \text{Per2}
\]

where \(v_{\text{P1}}\) and \(v_{\text{P2}}\) are the transcription rates, \(v_{\text{mP1}}\) and \(v_{\text{mP2}}\) are the degradation rates, \(K_{\text{mP1}}\) is a Michaelis-Menten coefficient, \(k_{\text{dmp}}\) is a natural degradation rate, and \(k_{\text{AP2}}\) is a rate coefficient of positive feedback regulation by PER1/2. The second term of Eq. 2 is the elementary form, which expresses an additional transcriptional induction of Per2 depending on the concentration of nuclear PER1/2 proteins. The full model is governed by Eq. 1, Eq. 2 and Eqs. S3–S25 in Text S1. The model, which includes no positive feedback regulation of Per2 by PER1/2 (i.e., \(k_{\text{AP2}} = 0 \text{ h}^{-1}\)), reproduced 23.5-hour period oscillations corresponding to the observed period length, but it did not reproduce the phase difference between Per1 and Per2 with the parameters obtained experimentally (Figure 4B). Once a feedback induction of Per2 transcription was introduced, the model reproduced the 4-hour phase difference (i.e., \(k_{\text{AP2}} = 2.4 \text{ h}^{-1}\); Figure 4C). This result suggested that the positive feedback regulation of Per2 transcription by nuclear PER1/2 contributed the phase delay of Per2 in vivo.

We also simulated the Per1 transcriptional repression by PER1/2, which was one of the alternative ways to differentiate the promoter activity pattern of Per1 from that of Per2. The oscillation phase of Per1 expression was advanced with the increase of repression intensity; however, it did not occur ahead of Per2 expression (Figure S1 and Text S1; see discussion). Moreover, when the positive feedback regulation of Per1 or the negative feedback regulation of Per2 was assumed, the phase of Per1 mRNA always lagged behind that of Per2 mRNA within a range of feedback strength that can yield sustainable oscillations (see details in Text S1). In short, these three alternative models were unable to reproduce the observed phase difference between Per1 and Per2.

Positive feedback regulation by PER1/2 contributes the expression phase delay of Per2

The positive feedback regulation by PER1/2 suggested by the simulations was examined experimentally by co-expressing the
Per1::luc and Per2::luc reporters with CLOCK, BMAL1, PER1 and PER2 (Figure 5A). In fact, Per1::luc reporter activity was not affected by the presence of either PER1 or PER2, except that PER2 had a small effect on CLOCK-BMAL1 transactivation (Figure 5B, left panel). However, the co-expression of CLOCK and BMAL1 with either PER1 or PER2 resulted in an extensive induction of Per2::luc, while subtle inductions by PER1 and PER2 were observed (Figure 5B, middle panel). A further 3-fold increase of the CLOCK-BMAL1 transactivation of Per2::luc was induced by the presence of PER1 or PER2, indicating that Per2 transcription was positively regulated by PER1/2.

To determine the significance of the positive feedback in the Per2 oscillatory phase, we constructed a Per2::luc reporter that lacked the sequences required for the positive feedback regulation (delta-Per2::luc; Figure 5A). The region was located between two E-box-like elements in the Per2 promoter and determined by Koike et al. (in preparation). As expected, delta-Per2::luc reporter activity was induced by CLOCK-BMAL1, and the induction was not intensified by either PER1 or PER2 (Figure 5B, right panel). Then, we estimated the periods and phases of bioluminescence oscillations of these reporter genes (Per1::luc, Per2::luc, delta-Per2::luc) when they were transfected into Rat-1 cells using a cosine fitting method (Figure 5C, Table 1). The 4-hour delay observed in Per2::luc compared to Per1::luc almost disappeared in the case of the delta-Per2::luc reporter in the absence of the positive feedback regulation of Per2 transcription by PER1 and PER2 proteins. Taken together, the positive feedback regulation by PER1 and...
PER2 is indispensable for the phase delay of Per2 mRNA oscillation.

**Discussion**

Several transcriptome analyses have revealed the circadian transcriptions of many genes with various phases [27–30]. The transcriptions of the mammalian clock genes Per1 and Per2 exhibit circadian oscillations with a phase difference of 4 hours. Jacobshagen et al. pointed out that extremely slow degradation of mRNA could reproduce a transcriptional phase delay [31]. In addition to the degradation rate of mRNA, our simulation analyses found that the transcription rate was also an important factor in determining the oscillatory phase. The significance of the difference in transcription was supported by the fact that the 4-hour phase difference was observed experimentally through bioluminescence oscillations of Per1::luc and Per2::luc ([Figure 5C](#)) and the different promoters could produce the same transcriptional and translational products of the luciferase gene. Using the synthesis and degradation rates of their mRNAs, which were measured *in vitro*, we showed that the current mathematical model is not sufficient to reproduce the phase difference between Per1 and Per2. Therefore, we predicted that an additional feedback regulation contributed to the phase difference.

In the model that included positive feedback regulation of Per2, newly synthesized PER1/2 enhanced Per2 mRNA transcription following transactivation by CLOCK-BMAL1 and caused the delay of the transcriptional peak. More importantly, this model produced the phase lag with a slight alteration in the oscillation period, and the extent of the phase delay of Per2 was dependent on factors that affected the intensity of positive feedback regulation, such as the abundance of PER1/2 ([Figure S2](#)). In addition, the circadian expressions of all genes involved in our model could be entrained to 12 h:12 h LD cycles in which Per1 and Per2 transcription rate coefficients were varied in a 24-hour period square-wave manner. Significantly, the phase of Per2 transcription also lagged behind that of Per1 in this condition. In contrast, one of three alternative models, which included Per1 transcriptional repression by PER1/2, could simulate the phase advance of Per1 ([Figure S1](#) and Text S1), but this advance was not ahead of the Per2 oscillation phase. The Per2 oscillation was almost in phase with nuclear BMAL1 oscillation in the model, which implemented the synthesis and degradation rates as estimated *in vitro*, so Per1 oscillation needed to be ahead of BMAL1 oscillation to be ahead of Per2 oscillation.
Figure 5. Per2 positive feedback regulation and its contribution to oscillatory phase delay in vitro. (A) Schematics of Per1::luc, Per2::luc and delta-Per2 promoter driving luciferase reporter (delta-Per2::luc). Delta-Per2::luc does not contain the region between two E-box like elements (115-35 bp upstream from transcription start site [5]) that contributes to positive feedback regulation. (B) PER1 and PER2 co-transfection with CLOCK and BMAL1 induced only Per2 promoter activity. Left: Per1::luc, middle: Per2::luc, and right: delta-Per2::luc. Induction intensities of Per1::luc by CLOCK-BMAL1 were 6.86 ± 0.38 without PER1/2, 6.78 ± 0.44 with PER1, and 4.64 ± 0.31 with PER2 in reference to the basal promoter activity. Induction intensities of Per2::luc by CLOCK-BMAL1 were 2.52 ± 0.08 without PER1/2, 6.57 ± 0.47 with PER1, and 7.53 ± 0.39 with PER2 in reference to the basal promoter activity. Both PER1 and PER2 proteins significantly induced the Per2 promoter in the presence of CLOCK-BMAL1, but not Per1 promoter (Student’s t-test, P < 0.01). Induction intensities of delta-Per2::luc by CLOCK-BMAL1 were 6.32 ± 0.19 without PER1/2, 5.27 ± 0.14 with PER1, and 5.76 ± 0.03 with PER2 in reference to the basal promoter activity, and there were no significant differences. Normalization was conducted with a pCNeo vector co-expression. Error bars indicate SEM determined from independent experiments in triplicate. (C) Representative bioluminescence oscillations of Per1::luc (square), Per2::luc (filled circle), and deleted-Per2::luc (open circle). The time difference from the Per1::luc to the Per2::luc expression peaks was 3.88 ± 0.14 hours (Student’s t-test, P < 0.005). The phase of delta-Per2::luc was advanced by 2.86 ± 0.39 hours (Student’s t-test, P < 0.01) compared with wild-type Per2::luc. Statistical data for the period and phase are described in the text and Table 1.

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transcriptional repression by PER1/2 surpasses CLOCK-BMAL1 transactivity at the midpoint or later within its phase, the oscillation phase of Per1 is advanced over the peak phase of CLOCK-BMAL1. However, an increase of negative feedback strength of Per1 transcription led to a decrease of PER1 protein expression, and our model did not simulate the expression pattern of nuclear PER1/2 that meets the requirement. Besides, the observed Per1 mRNA oscillation is not ahead of BMAL1 protein expression peak [32]. Thus, Per2 should be delayed to reproduce the phase difference between Per1 and Per2. From these simulation results, we predicted that the positive feedback regulation of Per2 transcription by PER1/2 could be the basis for the observed phase difference between Per1 and Per2.

The hypothesis was validated by reporter analyses using Per1::luc and Per2::luc; only the Per2 promoter, but not Per1, was activated by PER1/2. The significance of the positive regulation was verified further by the fact that the Per2::luc reporter gene that could not be transactivated by PER1/2 (delta-Per2::luc) lost the phase delay observed in wild-type Per2::luc. A recent report indicated that E-box in Per2 promoter contributes to 1.5-hour phase delay of Per2 expression [33], and this might cause a residual 1-hour delay detected in delta-Per2::luc. However, the residual delay was not statistically significant (Student’s t-test, n = 3, P > 0.05). Our results strongly demonstrated that the positive feedback regulation is a major reason for the phase delay of the Per2 mRNA oscillation.

Feedback regulation has been found in many biological systems, such as gene expression regulation and signal cascades. A recent study revealed that the positive feedback regulation slows down the kinetics of gene expression in a synthetic gene circuit and contributes to the response delay [34], indicating that the positive feedback regulation of Per2 slows down the accumulation of PER2 protein and may affect the phase of the circadian clock. Additionally, a theoretical analysis previously demonstrated that positive feedback buffers a propagated noise without a loss of sensitivity to input signal [35]; thus, the positive feedback regulation of Per2 could contribute to the improvement of the sensitivity to the photic signal that induces the expressions of Per1 and Per2 [15,36,37]. Although the functions of PER1 and PER2 proteins are still unclear, the positive feedback regulation of Per2 might be involved in photoreception and the entrainment of the circadian clock.

Materials and Methods

Simulation experiment

The Original Leloup and Goldbeter model [22], written in Systems biology markup Language (SBML), was retrieved from BioModels Database (http://www.ebi.ac.uk/biomodels-main/BIOMD0000000074) [38]. All simulation experiments and mathematical analyses were performed in the E-Cell Simulation Environment version 3.1.106 [39]. The mathematical model consisted of simultaneous differential equations and was solved by Euler’s method.

Table 1. Oscillatory period, phase, and phase difference of promoter driving luciferase reporter.

|          | Period (hour) | First peak (hour) | Relative phase (CT) | Phase difference (CT) |
|----------|---------------|-------------------|---------------------|-----------------------|
| Per1::luc| 22.36±0.08    | 31.72±0.39        | 8.28±0.39           | -                     |
| Per2::luc| 22.99±0.04    | 35.65±0.16        | 12.16±0.14          | 3.88±0.14             |
| Delta-Per2::luc| 22.38±0.11 | 32.67±0.42 | 9.30±0.40           | 1.02±0.40             |

Cell culture and measurement of mRNA half-life using real-time PCR

Total RNA was extracted from rat SCN-derived cultured cells, named RS182 [26]. A total of 1.0×10⁶ cells per 35-mm cell culture polystyrene dish (IWAKI) were proliferated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C. After a 4-day proliferation period, the cells were differentiated in Neurobasal medium (Gibco) supplemented with 2% B27 supplement (Invitrogen) and 1% antibiotics (insulin-streptomycin, Invitrogen) at 39°C. Half of the dishes were treated with 10 μM actinomycin D (an mRNA synthetic inhibitor), whereas the remaining dishes were treated with DMSO (vehicle control). Total RNA was extracted at 0, 0.5, 1.0, and 2.0 hours after treatment using an RNeasy Mini Kit (Qiagen) and an RNase-Free DNase Set (Qiagen). Extracted total RNA (500 ng) was reverse-transcribed for stability with 500 μg oligo(dT)₁₂-₁₈ (Invitrogen) using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Quantification of Per1 and Per2 mRNAs was performed using the ABI PRISM 7900HT, SYBR Green PCR Master Mix (Applied Biosystems), and 200 nM forward/reverse primers. The primer sequences were as follows; Per1 forward 5'-cctgg ccaat aaggc agaga -3' and reverse 5'-gccg caggt ctagt gg -3', and Per2 forward 5'- gcctt ggctt cttc -3' and reverse 5'- egca cagaa actgg tgg -3'.

Dual-luciferase reporter gene assay

COS-7 cells [40] were cultured in DMEM supplemented with 10% FBS, 50 mg/ml penicillin, and 50 U/ml streptomycin at 37°C. Cells were seeded the day before transfection at 4.0×10⁴ cells per well in 24-well plates and transfected with a total of 200 ng of plasmid using 1 μl of FuGENE6 (Roche). At 48 hours after transfection, cells were lysed, and luminescence was measured using the Dual-Luciferase® Reporter Assay System (Promega) and a Luminescencer-JRN II AB-2300 (ATTO BIO-INSTRUMENT) according to the manufacturer’s instructions.

Real-time monitoring of luciferase expression in cultured cells

Rat-1 cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomyacin at 37°C. Cells were seeded 48 hours before transfection at 4.0×10³ cells per dish with 2 ml of medium in 35-mm dishes and transfected with 1.6 μg of plasmid using 5 μl of FuGENE6 (Roche). At 24 hours, the medium was replaced with culture medium containing 100 μM luciferin. At 48 hours after transfection, cells were treated with 100 nM dexamethasone for 3 hours, and then the medium was replaced with culture medium containing 100 μM luciferin. Bioluminescence was measured using photomultiplier tube detector assemblies (LM2420; Hamamatsu) and the time series bioluminescent data of triplicate samples, which were measured from 0.5 to 3.8 hours after the medium change, was fitted to a cosine curve using R version 2.9.1.
Supporting Information

Figure S1 Analysis of the effect of PER1/2 negative feedback regulation on expression period and phase. The Per1 mRNA expression phase variation that depended on the intensity of additional PER1/2 negative feedback regulation, was mathematically simulated using the negative feedback regulation model (see Text S1). (A) Schematic representation of a model hypothesized Per1 negative feedback regulation. (B) The oscillation period of Per1 was increased by 12 hours, while the phase difference between Per1 and Per2 varied by 6 hours. (C) The Per1 expression phase advanced as the negative feedback strength became larger. However, the phase advance was saturated when the expression phase of Per1 was close to that of Per2. X-axis: strength of the negative feedback regulation, namely the rate coefficient, \( k_{\text{Per1}} \), of the transcriptional equation (Text S1, Eq. S1b, the first term).

(TIF)

Figure S2 Analysis of the effect of PER positive feedback regulation on expression period and phase. The Per2 mRNA expression phase variation that depended on the intensity of additional PER1/2 positive feedback regulation was mathematically simulated using the positive feedback regulation model (see Text S1). X-axis: strength of the positive feedback regulation, namely the rate coefficient, \( k_{\text{Per2}} \), of the transcriptional equation (Text S1, Eq. S2a, the second term). (A) The oscillation period of Per2 varied within \( \pm 1 \) hour, while the phase difference between Per1 and Per2 varied \( \pm 6 \) hours. (B) The Per2 expression phase laged behind the Per1 expression phase when the strength of positive feedback regulation caused \( k_{\text{Per2}} \) to be greater than or equal to 0.8 h\(^{-1}\), and stronger positive feedback regulation increased the phase difference.

(TIF)

Text S1 (DOC)

Table S1 (DOC)

Author Contributions

Conceived and designed the experiments: YO NK GJ TS MT HT. Performed the experiments: YO. Analyzed the data: YO. Contributed reagents/materials/analysis tools: NK. Wrote the paper: YO NK GK HT.

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