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Cellular DBP and E4BP4 proteins are critical for determining the period length of the circadian oscillator

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ABSTRACT

The phenotypes of mice carrying clock gene mutations have been critical to understanding the mammalian clock function. However, behavior does not necessarily reflect cell-autonomous clock phenotypes, because of the hierarchical dominance of the central clock. We performed cell-based siRNA knockdown and cDNA overexpression and monitored rhythm using bioluminescent reporters of clock genes. We found that knockdown of DBP, D-box positive regulator, in our model led to a short-period phenotype, whereas overexpressing of DBP produced a long-period rhythm when compared to controls. Furthermore, knockdown and overexpressing of E4BP4, D-box negative regulator, led to an opposite effect of DBP. Our experiments demonstrated that D-box regulators play a crucial role in determining the period length of *Per1* and *Per2* promoter-driven circadian rhythms in Rat-1 fibroblasts.

1. Introduction

Mammalian circadian rhythms are driven by a circadian oscillator consisting of intertwined positive and negative transcription/translation feedback loops involving a set of clock genes that interact through three major clock regulatory elements: the E-box, RRE, and D-box [1,2]. In the core feedback loop, CLOCK and BMAL1 heterodimerize and induce the transcription of *Per* and *Cry* genes through E-boxes [3–7]. The PER and CRY proteins accumulate in the cytosol and are then translocated, following phosphorylation, into the nucleus where they inhibit the activity of CLOCK and BMAL1. This core feedback loop is modulated by a second feedback loop composed of ROR and REV-ERB, which drives a circadian rhythm in *Bmal1* transcription [8–11]. DBP and E4BP4 are able to activate and suppress transcriptional activity, respectively, through the same sequence called as D-box [12]. These additional loops might contribute to stabilize and fine-tuning of core PER/CRY feedback loop.

The phenotypes of mice carrying clock gene mutations have been critical to understanding the role of each clock component in the overall functionality of the molecular clock [13]. However, behavior does not necessarily reflect cell-autonomous clock phenotypes [14]. The mammalian circadian system contains both central and peripheral oscillators. The suprachiasmatic nucleus (SCN) hierarchical dominance can compensate for severe intrinsic genetic defects in peripheral clocks [15]. Indeed, the negative limb of the core clockwork, *Per1*, *Per2* and *Cry1* are required to sustain circadian rhythm in peripheral tissues [14]. The role of PER/CRY feedback loop and interlocking RRE regulatory loop, consisting of ROR and REV-ERB that regulate *Bmal1* expression, has been well-characterized [16]. By contrast, the contribution of the D-box regulatory loop remains relatively uncharacterized with respect to the cell-autonomous circadian oscillations. Therefore, it is necessary to examine the roles of DBP and E4BP4 on the cell-autonomous transcriptional oscillation of clock gene promoters.

In the present study, we performed cell-based small-interfering RNA (siRNA) knockdown and complementary DNA (cDNA) overexpression, and monitored rhythm using bioluminescent reporters of clock genes. Here, we provide demonstration that cellular DBP and E4BP4 proteins are critical for determining the circadian period length.

2. Materials and methods

2.1. Cell culture

Rat-1 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA), and grown in the recommended medium and conditions.

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2.2. Plasmid construction

Construction of reporter (*mPer1* and *mPer2*-dLuc) and expression vectors (pCMV-*DBP* and pCMV-*E4BP4*) were described previously [2,11,17].

2.3. siRNA

We purchased the following pre-designed siRNAs and negative controls for knockdown experiments from Ambion (Austin, TX) or Qiagen (Valencia, CA): *siBmal1* (Ambion GGAUCAAA AAUGC AAG GGAtt); *siClock* (Qiagen r(GGCAAAUCAUGUUACUAUA)dTdT); *siCry 1* (Qiagen r(CCUUUAUGACUUAGACAGA)dTdT); *siRev-erbAα* (Ambion CCUAUGCCCAUGACAAAUUtt); *siDbp* (Ambion GGUACAAGAA-CAAUGAAGCtt); *siE4bp4* (Ambion GCUCCGGAUUAAAGCCAAGtt); and negative control (Ambion catalog number AM4611; Qiagen catalog number 1027280).

2.4. Real-time monitoring of bioluminescence and analysis

Rat-1 cells were plated onto a 35-mm dish at a density of 3×10^5 cells/dish 24 h before transfection. The cells were transfected with the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. For the cDNA expression experiments, the cells in each dish were transfected with 1.0 μg *mPer1* and *mPer2*-dLuc plus 0, 0.1, 0.3, or 1.0 μg of the expression vectors. We used the pCI-neo or pCMV-Sport6 plasmids to adjust the total amount of DNA in each transfection to 2.0 μg. For the siRNA knockdown experiments, the cells in each dish were transfected with 1.0 μg of the *mPer2*-dLuc plus 50 nM target siRNAs or negative control siRNA. At 24 h post-transfection, dibutyryl cyclic AMP (dbcAMP; Wako Pure Chemical Industries Ltd.) was added at the final concentration of 1 mM. In the presence of 0.1 mM luciferin (Promega), light emission was measured with photomultiplier tube detector assemblies (Hamamatsu Photonics, Hamamatsu, Japan), and luminescence was observed continuously for ~5 days at 30 °C. The period length was analyzed by calculating the value from the third to the eighth peak of oscillation as described in previous studies [17,18].

2.5. Quantitation of mRNAs by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative PCR (Q-PCR) was performed as described previously [2]. The glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression levels were quantified and used as an internal control. The oligonucleotide DNA primers were described at Supplementary Table 1.

2.6. Statistical analysis

Group differences were determined using Student's *t*-test. Multiple comparisons among group mean differences were checked using Dunnett's test.

3. Results

We first confirmed that the phase of *Bmal1* oscillation in cultured Rat-1 cells after stimulation with dbcAMP was almost the opposite phase of *Per2*, whereas *Clock* mRNA levels did not display a clear rhythm (Fig. 1). These observations were consistent with data of mRNA expression in mouse peripheral tissue [2]. In order to estimate subtle rhythm change, we adopted a real time luciferase reporter system.

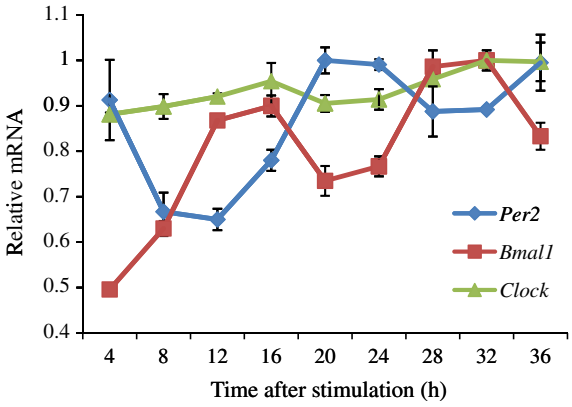

Fig. 1. Temporal expression profiles of clock genes in Rat-1 fibroblasts after dbcAMP treatment. Quantification of temporal changes in *Per2*, *Bmal1*, and *Clock* mRNA. Top levels of each mRNA were arbitrarily set to 1. The time shown is after dbcAMP stimulation.

After the *mPer2*-dLuc reporter and a siRNA specific for one of the clock-related genes were co-transfected in Rat-1 cells, bioluminescence was measured. At 24 h post-transfection, dbcAMP was added for stimulation. The circadian oscillation of *mPer2* was abolished by *siBmal1* and *siClock* (Fig. 2A–C). *siCry1* had little effect on the period length of the *mPer2* oscillation, although bioluminescence was significantly reduced (Fig. 2A–C). *siRev-erbAα* had little effect on the amplitude and period length of the *mPer2* oscillation (Fig. 2A–C). *siDbp* caused a shorter period as compared to the negative control (0.96 ± 0.24 h), whereas *siE4bp4* caused a longer period (2.03 ± 0.77 h) (Fig. 2A–C). To better visualize the phase differences, data sets were de-trended by subtracting the 24 h running average from the raw data (Fig. 2B). The depletion of the D-box regulators led to a modulation of the period length of the *mPer2* oscillation (Fig. 2C). In all treatments, we observed 40–90% decrease in gene expression of the targeted gene (Fig. 2D). DBP and E4BP4 knockdown was confirmed by western blotting (Supplementary Fig. 1). These results suggest that E-box regulators generate the rhythm oscillation, and D-box regulators modulate the period length.

In order to examine the roles of DBP and E4BP4 on the cell-autonomous transcriptional oscillation of *mPer2* gene, we performed our in vitro-rhythm assay using cDNA overexpression of these genes in Rat-1 cells. We confirmed that exogenously transfected transgenes (DBP and E4BP4 protein) are functional in conventional reporter assay (Supplementary Fig. 2). The overexpression of DBP resulted in a longer period (0.90 ± 0.14 h), whereas E4BP4 overexpression resulted in a shorter period (0.63 ± 0.09 h) (Fig. 3A–C). The overexpression of the D-box regulators led to a modulation of the period length of the *mPer2* oscillation in a dose-dependent manner (Fig. 3C). In terms of the period length, DBP and E4BP4 showed opposite effects in the siRNA-knockdown and cDNA-overexpression studies, indicating that these genes control the period length of the circadian rhythm.

To avoid the possibility that the effects of the period length were *mPer2* specific phenomena, we performed cDNA overexpression studies of DBP and E4BP4 with a real-time luciferase-monitoring assay using the *mPer1* promoter-driven luciferase reporter. DBP and E4BP4 overexpression caused a longer and a shorter period as compared to the negative control, respectively (Fig. 4A–C), which is consistent with the response observed in the cDNA overexpression studies using the *mPer2* promoter reporter (Fig. 3).

The D-box cis element was included in *mPer1* and *mPer2* promoter construct. If the intrinsic circadian clock oscillators might be affected in response to the manipulation of the D-box

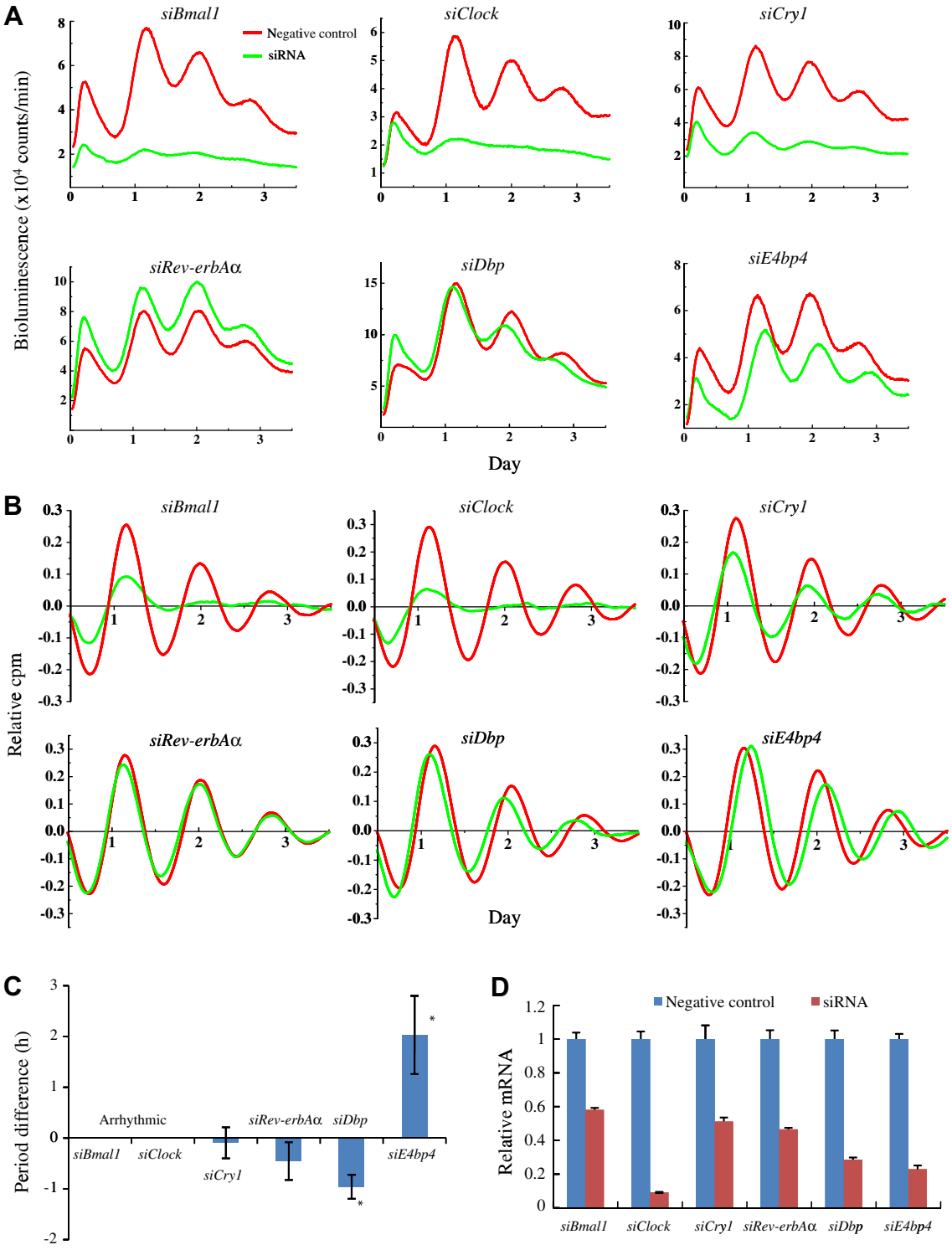

Fig. 2. Effect of the siRNA knockdown of clock and clock-controlled genes on the cell autonomous circadian transcription of *mPer2*. (A) After siRNA and *mPer2*-dLuc were co-transfected in Rat-1 cells, bioluminescence was measured with photomultiplier tube detector assemblies. The red and green lines represent 50 nM of the negative control and target siRNA, respectively. The time shown is after dbcAMP stimulation. (B) The signals obtained in (A) were de-trended. (C) Period length differences for cells transfected with siRNA as compared to cells transfected with negative control siRNA. All results are representative of three independent experiments, and the mean ± S.E. is indicated. $^{*}P < 0.05$. (D) At 24 h post-transfection, the reduction of target mRNA levels by their corresponding siRNAs in Rat-1 cells was confirmed by Q-PCR. Values are relative to that of negative control transfected cells.

regulators, clock-controlled element-driven circadian bioluminescence rhythms should be altered regardless of whether exogenously transfected reporter construct contains D-box elements. We performed cDNA overexpression studies of DBP genes with a real-time luciferase-monitoring assay using the E′-box region-SV40 driven luciferase reporter. This promoter construct did not contain the D-box elements but essential clock-controlled element to generate cell-autonomous circadian oscillation [17,18]. The

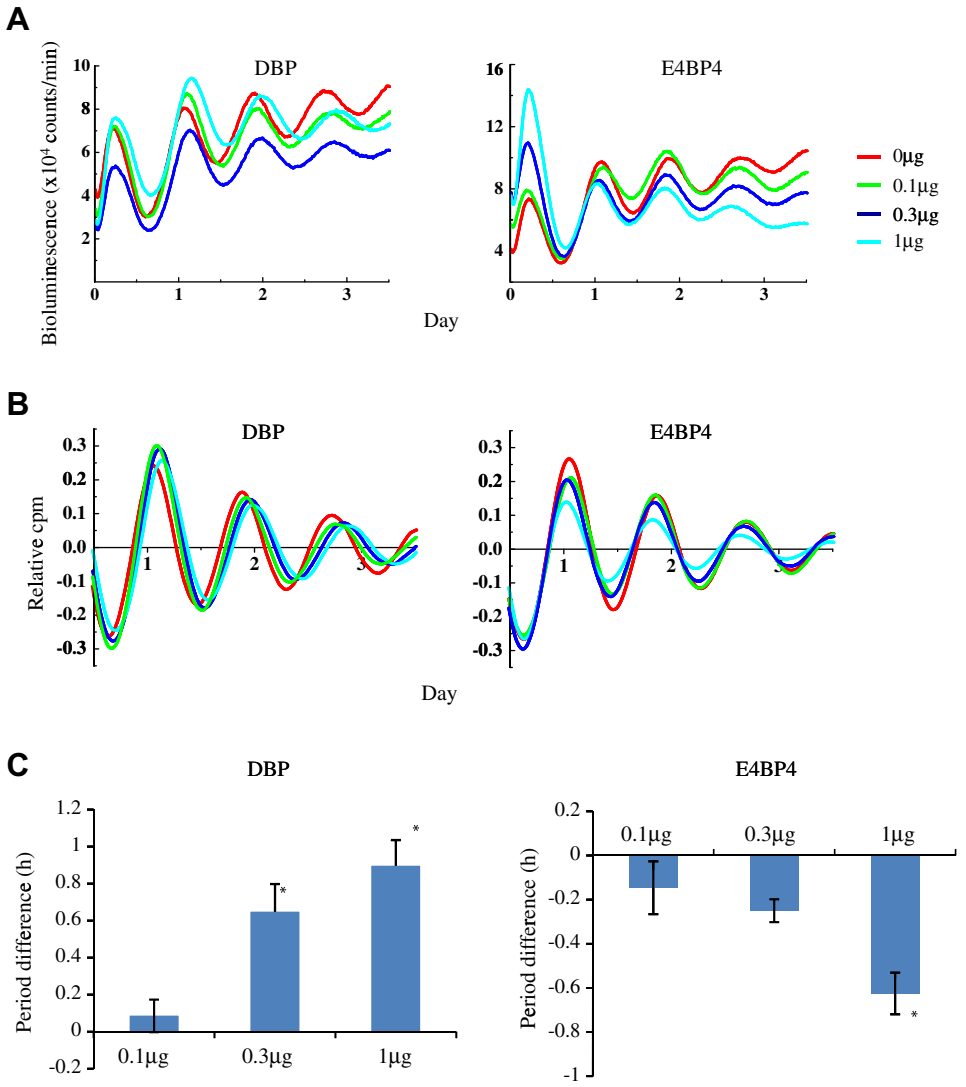

Fig. 3. Effect of overexpression of DBP and E4BP4 on the cell autonomous circadian transcription of *mPer2*. (A) After the introduction of the *mPer2*-dLuc reporter construct and the various cDNA expression vectors into the Rat-1 cells, bioluminescence was measured with photomultiplier tube detector assemblies. The red, green, blue, and sky-blue lines represent 0, 0.1, 0.3, and 1 μg of transfected cDNA, respectively. The time shown is after dbcAMP stimulation. (B) The signals obtained in (A) were de-trended. (C) Period length differences for cells transfected with varying concentrations of the expression vectors as compared to cells expressing a control vector. All results are representative of three independent experiments, and the mean ± S.E. is indicated. $^{*}P < 0.05$.

E′-box SV40-dLuc displayed clear circadian oscillation, and DBP overexpression caused a longer period (Supplementary Fig. 3). In addition, we performed cDNA overexpression studies of DBP genes with a real-time luciferase-monitoring assay using the D-box mutant of *mPer2* promoter driven luciferase reporter. A similar period change was observed with this promoter driven luciferase reporter (Supplementary Fig. 4), suggesting that the intrinsic circadian clock oscillators might be affected by DBP protein levels. These results indicate that D-box regulators contribute to maintaining the stable period length of the mammalian cellular circadian clock.

4. Discussion

Molecular phenotypes determined from peripheral tissues are strongly influenced by the state of the SCN [15]. We confirmed whether our results in cultured cells reflect the phenotypes of genetically modified animals lacking these clock-related genes. Mice homozygous for a null allele of *Bmal1* have severely disrupted behavioral rhythms [6]. Mice homozygous for a *DBP*-null allele display a shorter free-running period [19]. Consistently, *Bmal1* knockdown let to circadian disruption and *DBP* to shorter period in our model.

However, not all the phenotypes we observed are identical to those of corresponding knockout mouse. Recent genetic evidence in mice indicates that peripheral oscillators are arrhythmic without CLOCK [20], whereas CLOCK and NPAS2 have partially redundant functions within the SCN [21]. The circadian oscillation of *mPer2* was dramatically diminished by *siCry1*, whereas the behavioral rhythm is maintained in the corresponding knockout mouse [22]. There was a trend in our cell-culture system that showed more severe rhythm generation phenotypes than those seen in the mice carrying clock gene mutations. This may be due to the hierarchical dominance of the central clock, or fundamental differences in the clock mechanism between tissues.

Here, we provide demonstration that DBP and E4BP4 inversely regulate the period length of the *mPer1* and *mPer2* oscillation in cultured fibroblasts. Recent experiments revealed that PAR-domain basic leucine zipper (PAR bZip) proteins, DBP, TEF, and HLF control the expression of many enzymes and regulators involved in detoxification and drug metabolism in liver and kidney [23]. In-

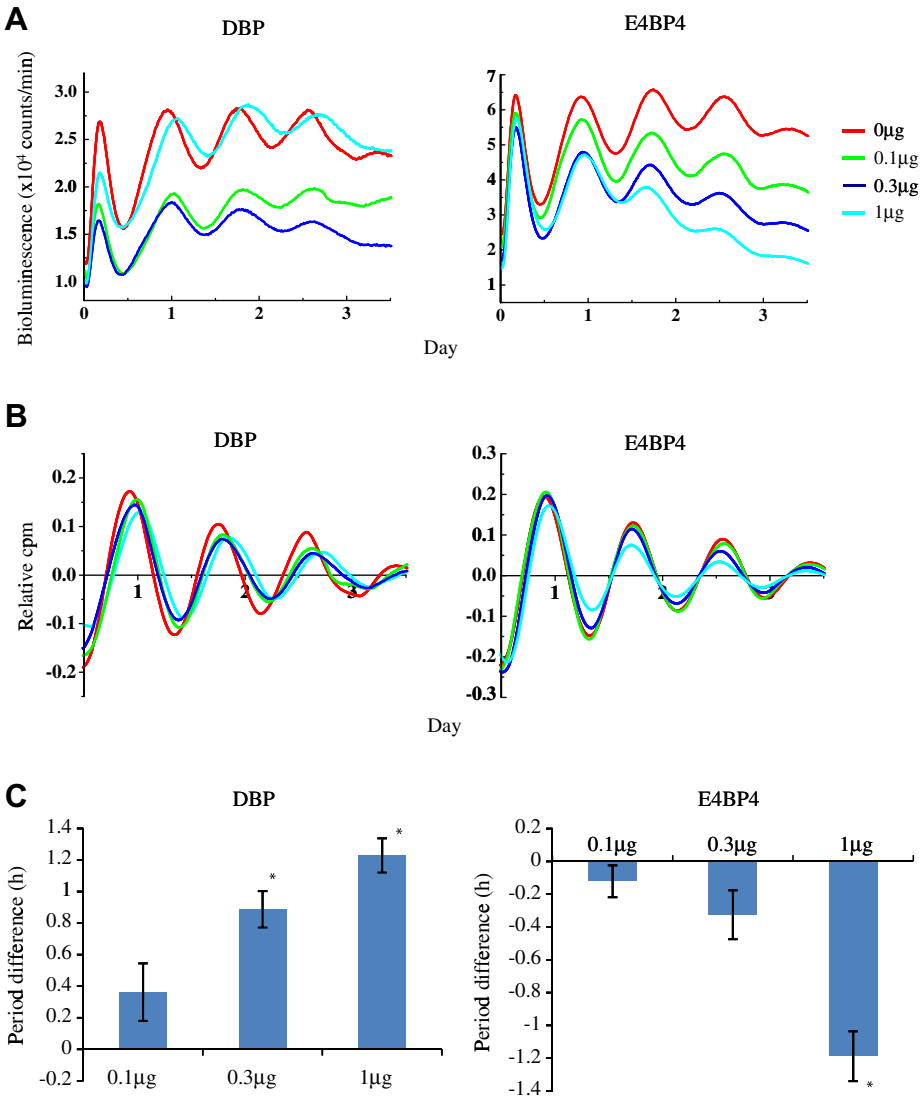

Fig. 4. Effect of overexpression of DBP and E4BP4 on the cell autonomous circadian transcription of *mPer1*. (A) After the introduction of the *mPer1*-dLuc reporter construct and the various cDNA expression vectors into the Rat-1 cells, bioluminescence was measured with photomultiplier tube detector assemblies. The red, green, blue, and sky-blue lines represent 0, 0.1, 0.3, and 1 μg of transfected cDNA, respectively. The time shown is after dbcAMP stimulation. (B) The signals obtained in (A) were de-trended. (C) Period length differences for cells transfected with varying concentrations of the expression vectors as compared to cells expressing a control vector. All results are representative of three independent experiments, and the mean ± S.E. is indicated. $^{*}P < 0.05$.

deed, PAR bZip triple knockout mice are hypersensitive to xenobiotic compounds, and display a deficiency in detoxification [23]. We have reported that abnormal rhythmicity of DBP and its target metabolic enzyme such as CYP7A1 deteriorates hypercholesterolemia in rats with irregular feeding [24]. Therefore, it has been recognized that the D-box regulators seem to be a component of the circadian output pathway rather than a master gene of the clock. However, our results suggest that the period length of *mPer2* oscillation is more sensitive to modulation of the amounts of the D-box regulators rather than the E-box and RRE regulators, raising the possibility that, among the three clock elements, D-box regulators play a prominent role in determining the period length in the cellular circadian clock.

D-box cis element-dependent transcription might play a pivotal role in determining the cellular period length. Indeed, after introducing *siDbp* and *siE4bp4* into Rat-1 cells, endogenous *Per2* mRNA levels decreased to 70% and increased to 130% of the negative control, respectively (Supplementary Fig. 5). On the other hand, we suggest that protein–protein interaction might play an important role in this system. Ohno et al. reported that E4BP4 protein interacts with PER2 and CRY2 [25]. These associations might affect its repression activity and regulate the core circadian clock network. While the mechanism by which DBP and E4BP4 proteins regulate the circadian period length has not been fully clarified, these multiple pathways might contribute to the cellular period length.

We are ultimately interested in the impact and role of altered rhythms in human disease. Some studies have shown that clock genes polymorphisms are associated with several sleep disorders such as familial advanced sleep phase syndrome [26,27] and delayed sleep phase syndrome [28]. To our knowledge, polymorphism analysis of the D-box regulators had never before been reported. These findings should initiate further studies into the clock-related perturbations to human disease, including not only drug reactivities and metabolic disorders but also circadian rhythm sleep disorders.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.038.

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