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Construction of a Rhythm Transfer System That Mimics the Cellular Clock

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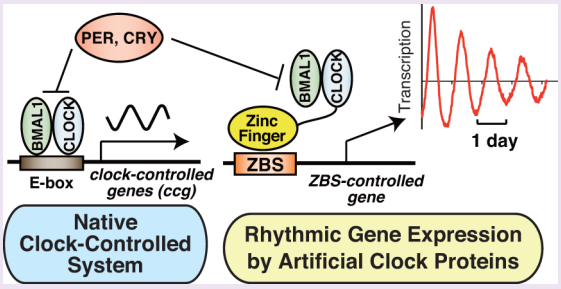
Supporting Information

ABSTRACT: Creation of an artificial oscillating gene expression system is one of the most challenging issues in synthetic biology. Here, we constructed a simple system to manipulate gene expression patterns to be circadian, reflecting the intrinsic cellular clock, by fusing a core clock protein, BMAL1 or CLOCK, with a zinc finger-type DNA binding domain. Circadian rhythmic gene expression was induced only when the target gene contained zinc finger-binding sequences. To our knowledge, this simple approach is the first to manipulate gene expression patterns into circadian rhythms and would be applicable to various endogenous genes.



The creation of an oscillating gene expression system for genes of interest is challenging. Synthetic gene oscillators have been constructed in which artificial genes constitute interlocked feedback loops.[1−3] These systems, however, contain multiple parameters and limited adaptations, resulting in difficulties in manipulating the expression patterns of desired genes. To manipulate gene expression, artificial zinc-finger transcription factors have been used widely because it is possible to design artificial zinc-fingers that bind desired genomic sequences.[4−13] To date, efforts to design such transcription factors have focused solely on changing the expression levels of target genes. In this study, we created a new type of transcription factor that changes gene expression patterns so that they are rhythmic based on the cellular clock system.

The self-sustained oscillations of the cellular clock system are driven by a circadian core oscillator, represented by the BMAL1/CLOCK and PER/CRY feedback loops through E-box (CACGTG) elements (Figure 1, see also Supplementary Figure S1 for details).[14,15] Both BMAL1 and CLOCK have a basic helix−loop−helix (bHLH) DNA binding domain and form a heterodimer with each other.[16] In the E-box-mediated system, the BMAL1/CLOCK heterodimer activates *Per* and *Cry* by binding to E-box elements in their promoters.[14,15,17−19] The expressed PER and CRY proteins in turn work as negative regulators of BMAL1/CLOCK, resulting in the rhythmic expression of the core clock genes (Supplementary Figure S1).[20,21] Other than core clock genes, various genes, called clock-controlled genes (CCGs), also exhibit circadian patterns of expression through the E-box-mediated transcriptional regulation by core clock oscillators (Figure 1b).[22,23] Mimicking the naturally occurring CCGs, a simple rhythm-inducing system was constructed using a zinc finger DNA binding domain and core clock proteins.

Rather than binding the E-box through the bHLH domain of BMAL1 and CLOCK, we devised a strategy whereby BMAL1 or CLOCK are fused with an external DNA binding domain (DBD) that binds to a specific DNA sequence on a desired promoter. We created artificial clock proteins, Zif-BMAL and Zif-CLOCK. BMAL1 or CLOCK was fused with a C_2H_2−zinc finger-type DNA binding domain (Zif) derived from a transcription factor, Zif268, binding to a specific DNA sequence (Figure 1a). We anticipated that exogenously introduced Zif-BMAL or Zif-CLOCK proteins would form heterodimers with endogenous CLOCK or BMAL1, respectively, at the zinc finger-binding sequence (ZBS). We further expected that endogenous PER/CRY would repress transcriptional activity of the heterodimer at the ZBS, resulting in the rhythmic expression of ZBS-consisting genes (Figure 1b).

We first ascertained the correct transcriptional regulation by the heterodimers, Zif-BMAL/CLOCK or BMAL1/Zif-CLOCK. We used the 4× ZBS/pGL3 reporter vector[12] with four tandemly repeated ZBSs in the basic promoter of the luciferase gene for a luciferase reporter assay. The Zif268 DNA binding domain (Zif), BMAL1, CLOCK, or BMAL1/CLOCK did not activate ZBS-driven luciferase gene expression (Supplementary Figure S2a). Zif-BMAL alone showed an approximately 8-fold increase in luciferase activity (Figure 2a). When both Zif-BMAL and CLOCK were expressed, luciferase activity was significantly increased in a CLOCK dose-dependent manner (Figure 2a). This indicates that Zif-BMAL

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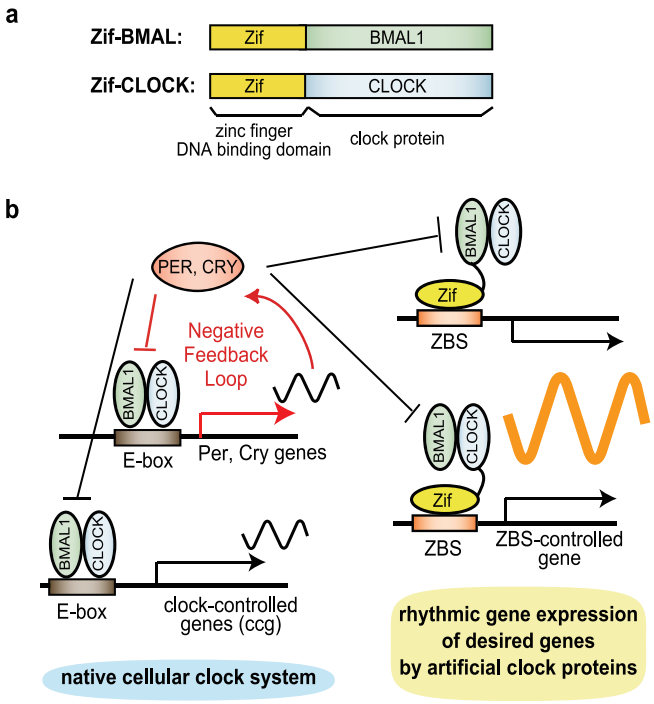

Figure 1. Schematic representation of rhythmic gene expressions by artificial clock proteins. (a) The design of artificial clock proteins, Zif-BMAL and Zif-CLOCK. (b) (left) The native cellular clock system; negative feedback regulation through E-box elements between BMAL1/CLOCK (positive factors) and PER/CRY (negative factors) (shown in red, see Supplementary Figure S1 for detail) generates rhythmic expression of E-box-driven genes and clock-controlled genes (ccg). (right) The expected regulation system of ZBS (Zif-binding site)-driven genes by Zif-BMAL or Zif-CLOCK.

and CLOCK cooperate to activate the ZBS-controlled luciferase gene. Zif-BMAL and CLOCK coexpressed with CRY1, a negative regulator of the BMAL1/CLOCK heterodimer,[20] downregulated luciferase activity. Similarly, Zif-CLOCK showed BMAL1-dependent transcriptional activity. In addition, the activity of the BMAL1/Zif-CLOCK heterodimer was significantly suppressed by coexpression of CRY1 (Figure 2b). These results suggest that the artificial clock proteins, Zif-BMAL and Zif-CLOCK, form a heterodimer with CLOCK and BMAL1, respectively, at the ZBS-containing promoter region. Because neither Zif-BMAL/CLOCK nor BMAL1/Zif-CLOCK activated luciferase activity from noZBS (no Zif binding sites)/pGL3 reporter vector (see Supplementary Figure S2b), the observed increase in luciferase activity likely resulted from DNA binding of these proteins to the ZBS region of the promoter.

The induction of circadian rhythmicity from a ZBS-controlled reporter gene was achieved by Zif-BMAL or Zif-CLOCK. This was confirmed by real-time monitoring of bioluminescence. Flag-BMAL1/Flag-CLOCK overexpression is well tolerated by the molecular oscillator;[24] therefore, we expected that constitutive expression of Zif-BMAL or Zif-CLOCK should also be tolerated. Indeed, the expression of the luciferase gene driven by the mouse *Period2* clock gene promoter (mPer2-luc) showed circadian oscillation in the presence of Zif-BMAL or Zif-CLOCK (Supplementary Figure S3).

To evaluate the expression rhythm of a ZBS-containing gene at the promoter region by Zif-BMAL or Zif-CLOCK, we used ZBSluc as the reporter vector with the 4× ZBS located upstream of the luciferase gene SV40 promoter. mtZBSluc and noZBSluc were employed as negative controls (Figure 3a). mtZBS contains a 4× mutated ZBS, to which Zif is bound with more than 10-fold lower affinity,[25] and noZBS lacked the Zif binding sites. The reporter and expression vectors were cotransfected into NIH3T3 cells, and luciferase activity was monitored in real time after synchronization by forskolin stimulation, which resets the clock of each of the cultured cells at irregular phases and to elicit circadian gene expression.[26] Zif-BMAL and Zif-CLOCK induced persistent circadian oscillations of luciferase gene expression downstream of the ZBS sequences (Figure 3b,c; see also Supplementary Figure S4). The detrended bioluminescence data (Figure 3b,c), with a normalized baseline, indicate that Zif-BMAL and Zif-CLOCK induced oscillating gene expression from the ZBSluc reporter vector with approximate 24 h periodicity. Conversely, the fusion proteins did not induce such oscillatory expression patterns from the mtZBSluc or noZBSluc reporter vectors (Figure 3b,c and Supplementary Figure S4), indicating that DNA binding affinity is important for induction of circadian rhythmicity. Expression from the ZBSluc reporter vector itself did not show an obvious rhythm (Figure 3b,c and Supplementary Figure S4). In addition, none of Zif, CLOCK, or BMAL1 could induce rhythmicity from the ZBSluc reporter vector (Supplementary Figure S5a). These data indicate that circadian rhythmic gene expression is only generated when Zif-BMAL or Zif-CLOCK bind the target ZBS sequences in the promoter region. Further, neither Zif-BMAL nor Zif-CLOCK induced rhythmicity from the ZBSluc reporter without forskolin-stimulated synchronization (Supplementary Figure S5b), suggesting that Zif-BMAL and Zif-CLOCK utilize an intracellular clock system. Zif-BMAL- and Zif-CLOCK-induced rhythmicity of the ZBS-driven luciferase gene was also demonstrated in mouse embryonic fibroblasts (MEF) after stimulation by dexamethasone, one of the well-known circadian synchronizers[27] (Supplementary Figure S6). This finding strongly demonstrates the versatility of the artificial clock proteins. To our knowledge, this is the first demonstration of a

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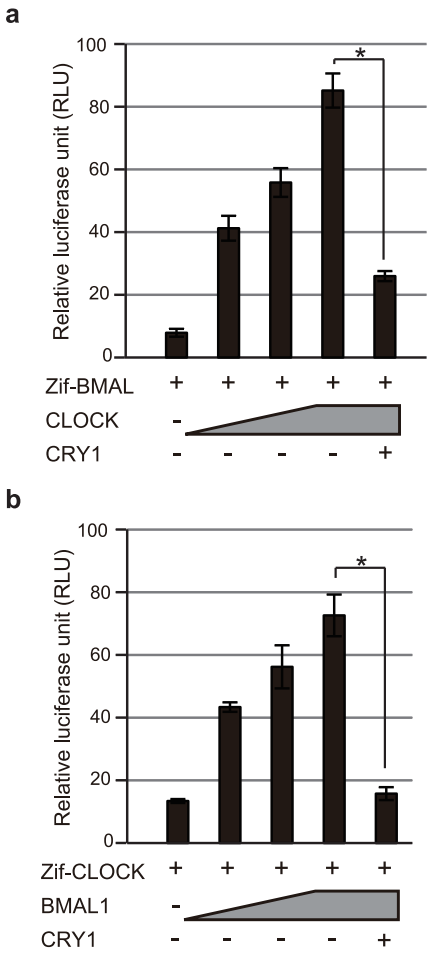

Figure 2. Cooperative transcriptional activation by Zif-BMAL/CLOCK or BMAL/Zif-CLOCK. (a) Zif-BMAL and (b) Zif-CLOCK activated a ZBS-driven reporter gene together with CLOCK and BMAL1, respectively. The expression vectors shown below the graph, a ZBS-driven reporter vector (pGL3-TA/4xZBS-I) [12] and a control vector (pRL−SV40), were transfected into NIH3T3 cells. Luciferase activity was measured using a dual luciferase assay system. The luciferase activity when the empty vector was used instead of the expression vector was set to 1. Data are presented as mean ± SD; *, $p < 0.01$.

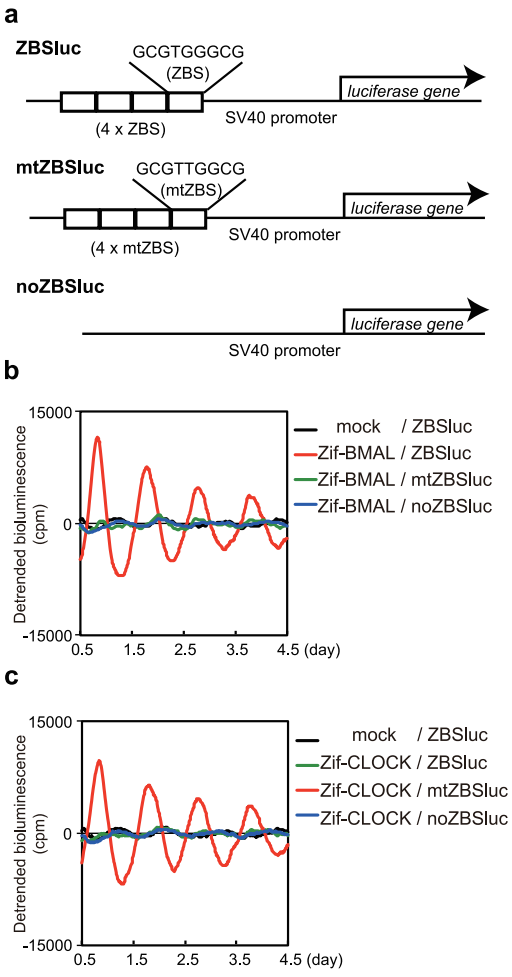

Figure 3. Zif-BMAL and Zif-CLOCK induce circadian expression patterns of the ZBS-driven reporter gene. (a) Schematic representation of the reporter vectors used in this assay. (b,c) Real-time monitoring of luciferase activity of ZBSluc, mtZBSluc, and noZBSluc reporter vectors, expressing Zif-BMAL (b) and Zif-CLOCK (c) in NIH3T3 cells after synchronization by forskolin. The detrended bioluminescence (cpm) was obtained by subtracting the 24 h moving average from the raw data (see Supplementary Figure S4). Representative examples are shown.

circadian pattern of gene expression without the presence of an E-box element to serve as a binding site for BMAL1/CLOCK heterodimers. Thus, the importance of feedback repression for mammalian circadian clock functioning rather than BMAL1/CLOCK DNA binding was emphasized, using our system without destroying the native core clock machinery, different from previous reports in which BMAL1/CLOCK themselves were mutated.[24,28]

We successfully induced a circadian expression pattern from a promoter of a naturally occurring gene. We chose to model our system in the promoter of mouse *vascular endothelial growth factor-A* (*Vegfa*) because engineered zinc finger domains targeting the *Vegfa* promoter have been successfully created.[29] To elicit a rhythmic expression from the *Vegfa* promoter, VZ426-CLOCK was designed by fusing the clock proteins with the engineered zinc finger domain, mVZ+426.[29] A sequence of nine base pairs in the *Vegfa* promoter was targeted (Figure 4a). VZ426-CLOCK elicited circadian rhythmic expression of VEGFluc, a 1.7-kbp *Vegfa* promoter-driven reporter, though the amplitude was quite weak (Figure 4b, red; see also Supplementary Figure S7). Considering the importance of the DNA binding affinity of an artificial clock protein to the zinc finger-target site for the rhythm induction, we designed VZ426-CLOCK/Ala containing alanine substitutions of the DNA recognition residues in the bHLH DNA binding domain of CLOCK, which should reduce possible DNA binding to the CLOCK-target site and improve the selectivity to the zinc finger VZ426-target site. As a result of expression of VZ426-CLOCK/Ala, a circadian rhythmic gene expression from VEGFluc was clearly detected (Figure 4b, blue; see also Supplementary Figure S7). By contrast, Zif-CLOCK, targeting a Zif268 binding site that does not exist in the *VEGF* promoter did not elicit circadian rhythmic expression (Figure 4b, green; see also Supplementary Figure S7). Thus, our design strategy could be applicable not only to an artificial promoter, but to a naturally occurring one as well.

We demonstrated that circadian rhythmic gene expression can be generated from a ZBS-controlled gene, which is not a native clock-controlled gene. This was accomplished by fusing BMAL1 or CLOCK with a zinc finger DNA binding domain

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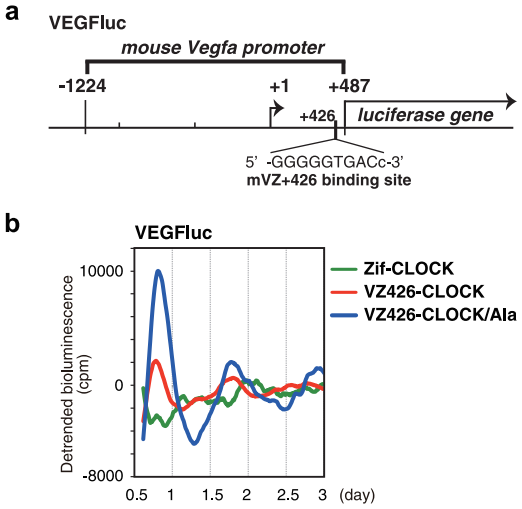

Figure 4. Induction of a circadian expression pattern of the VEGF promoter-driven reporter gene. (a) Schematic representation of the reporter vector, VEGFluc. (b) Real-time monitoring of luciferase activity of VEGFluc, expressing VZ426-CLOCK (red), VZ426-CLOCK/Ala (blue), or Zif-CLOCK (green) in NIH3T3 cells after synchronization by forskolin.

that binds to the ZBS on the promoter region of a gene of interest, thus making use of intrinsic clock networks. This is the first demonstration of a circadian pattern of gene expression without the presence of an E-box element to serve as a binding site for BMAL1/CLOCK heterodimers. Moreover, the applicability of this system to a naturally occurring promoter was exemplified by the successful rhythmic expression from the *Vegfa* promoter. To our knowledge, this simple approach is the first to manipulate gene expression patterns into circadian rhythms. Though greater optimization is required, our results suggest that any endogenous gene expression can be oscillated by artificial clock proteins using designed zinc finger domains that target desired genes.[4−6,9−11,13] This study may offer a new direction not only for chronobiological research to examine the function of BMAL1/CLOCK or the effects of rhythm induction of nonclock controlled genes but also for gene therapy targeting disordered gene expression rhythms.

METHODS

Plasmid Construction. The Zif-BMAL and Zif-CLOCK expression plasmids, pCI/myc-Zif-BMAL and pCI/myc-Zif-CLOCK, were constructed as follows: DNA fragments encoding mBMAL1 or mCLOCK were amplified by PCR and inserted into pCI (Promega), yielding pCI/BMAL and pCI/CLOCK, respectively. The DNA fragment encoding the Zif268 zinc finger domain, Zif, was amplified by PCR using pEV-ZF3 [12] as a template and inserted into pCI/BMAL and pCI/CLOCK, yielding pCI/Zif-BMAL and pCI/Zif-CLOCK, respectively. In addition, the six-repeated myc epitope tag sequence was inserted into pCI/Zif-BMAL and pCI/Zif-CLOCK. The mVZ+426 zinc finger gene was constructed by PCR using pEV3b-Sp1ZF3 [30] as a template to encode mVZ+426.[29] The DNA fragment was inserted into pCI/myc-Zif-CLOCK, yielding pCI/myc-VZ426-CLOCK. Four residues (mouse Clock(43−46); EKKR) in the bHLH domain of CLOCK were substituted with alanine residues by PCR, resulting in pCI/myc-VZ426-CLOCK/Ala. The entire sequences of these proteins are described in the Supporting Information.

Reporter plasmids for real-time monitoring of luciferase expression, ZBSluc, mtZBSluc, and noZBSluc, namely, pGL4.12-SV40/ZBS, mtZBS, and noZBS, were prepared as follows: the SV40 promoter was excised from pGL3-Control (Promega) by *Bgl*II and *Hin*dIII and inserted into pGL4.12 (Promega), yielding pGL4.12-SV40. The 4× ZBS sequence was excised from pGL3-TA/4xZBS-I [12] and inserted into pGL4.12-SV40. The 4× mtZBS, noZBS, and their complementary oligonucleotides were annealed and inserted into pGL4.12-SV40. The sequences are described in the last section of the Supporting Information. The 1.7 k mouse VEGF promoter (Gene ID: 22339) was amplified using the primer set 5′-caccaggctcgagTGTTTAGAA-GATGAACCG-3′ and 5′-gccgccaagcttgCGCGACTGGTCCGATG-3′, and genomic DNA from NIH3T3 cells as a template. The amplified DNA fragment was digested by *Xho*I and *Hin*dIII and inserted into pGL4.12, resulting in VEGFluc.

The plasmids were confirmed by sequencing using ABI prism 3130 (Applied Biosystems).

Cell Culture. The NIH3T3 cell line (RIKEN Cell BANK) was cultured in DMEM (Nissui) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries), L-glutamine (GIBCO), and $NaHCO_3$.

Luciferase Reporter Assay. Transcriptional activation was evaluated using the dual luciferase assay system (Promega). pCI/myc-Zif-BMAL, pCI/myc-Zif-CLOCK, pCI/BMAL, pCI/CLOCK, pCMV/ZF3,[12] and/or pcDNA3.1/mCry1 were used as expression vectors. Expression vectors or pCI empty vector (total 450 ng; 75 ng pCI/myc-Zif-BMAL or pCI/myc-Zif-CLOCK; 100, 150, and 250 ng pCI/CLOCK; 10, 20, and 80 ng pCI/BMAL; and 120 ng pcDNA3.1/mCry1), 50 ng of pGL3-TA/4xZBS-I,[12] and 5 ng of control plasmid (pRL-SV40; Promega) were transiently cotransfected into NIH3T3 cells using the LipofectAMINE LTX and PLUS reagent (Invitrogen) according to the manufacturer's instructions. Firefly and *Renilla* luciferase activities were measured using the dual luciferase assay system, and luminescence was determined by normalization to the transfection control.

Real-Time Monitoring of Bioluminescence. One day before transfection, NIH3T3 cells (3.0×10^5) were seeded in 35 mm culture dishes. Cells were transfected with 1400 ng expression vector (pCI/myc-Zif-BMAL and pCI/myc-Zif-CLOCK) and 200 ng reporter vector (pGL4.12-SV40/ZBS, mtZBS, and NBS) using LipofectAMINE 2000 (Invitrogen). After 5 h, the medium was replaced with 2 mL of culture medium (10% FBS/DMEM) supplemented with 0.1 mM beetle luciferin. After 48 h, it was replaced with culture medium containing 0.1 mM beetle luciferin and 10 μM forskolin. Luciferase activity was monitored with an LM-2400 (Hamamatsu Photonics). For monitoring the VEGFluc reporter activity, 800 ng expression vector and 800 ng reporter vector (VEGFluc) were used.

ASSOCIATED CONTENT

Supporting Information

Supplementary figures and sequences of the proteins and reporter vectors. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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