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Dual-Color Luciferase Mouse Directly Demonstrates Coupled Expression of Two Clock Genes†

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ABSTRACT: We have established a dual-color transgenic mouse that simultaneously reports the expression of two clock genes, *Bmal1* and *Per2*, in a single tissue. The expression of the two genes is monitored with green- and red-emitting beetle luciferases with a single luminescent substrate. Antiphasic oscillations of *Bmal1* and *Per2*, consistent with their endogenous mRNA profiles, were clearly monitored in the suprachiasmatic nucleus (SCN), the master circadian pacemaker, and in the peripheral tissues, demonstrating that the system allows the long-term, quantitative, and simultaneous monitoring of the expression of the two genes. We also showed that although the expression patterns of *Bmal1* and *Per2* in each organ are strictly antiphasic, the recorded circadian phases and periods of both genes varied between organs. The phase shifts in the expression of both genes in the SCN, induced by a change of medium, also occurred in a similar manner. Therefore, this dual-color luciferase mouse allows noninvasive and continuous monitoring of the coupled expression of two clock genes. This system provides a simple technique with which to unravel the complex interactions of two genes in the body.

Bioluminescent reporters have become an essential tool for studying various aspects of biological functions, including gene expression, posttranscriptional modification, and protein−protein interactions, because the sensitivity and range of the linear response are superior to those of other reporters, including β-galactosidase, chloramphenicol acetyltransferase, and fluorescent proteins (*1*−*3*). In particular, luciferases are used as sensitive probes to monitor gene expression noninvasively, quantitatively, and longitudinally in living cells, explant tissues, and *in vivo* (*4*−*6*).

Fluorescent proteins have contributed immensely to the advancement of cell biology and are used as powerful probes to monitor an extensive array of entities, ranging from single molecules to whole organisms. However, fluorescent reporters require exogenous illumination to emit light, making them unsuitable for the long-term quantitative monitoring of gene expression because the reporter is bleached and the subject can suffer phototoxic damage caused by repetitive exogenous illumination.

The luciferase enzyme emits light by oxidizing its substrate luciferin in a specific manner (*7*, *8*). Among the possible luciferase/luciferin reactions, the beetle luciferase and D-luciferin (benzothiazole) pair is the best probe for the long-term and noninvasive detection of cellular events, because the luminescence generated by the reaction is highly quantitative and has an extremely low background, and D-luciferin is highly stable and easily permeates cells and tissues (*9*−*11*). Moreover, no external illumination is required for bioluminescent reactions. Therefore, the characteristic properties of the beetle luciferase/luciferin reaction allow cellular events to be monitored longitudinally and quantitatively.

Of the luciferases identified to date, the firefly luciferase from *Photinus pyralis* is most commonly used as a bioluminescent reporter. Firefly luciferase produces light by oxidizing D-luciferin, with a high quantum yield of approximately 40% (*12*). Although this reporter has been extensively used to monitor multiple cellular events in cell extraction assays, cell-based assays, and *in vivo* imaging (*1*−*6*), only one target object (or the expression of one gene) can be monitored at a time. Recent advances in luciferase technology, involving improvements in both the luciferase and the detection system and a newly cloned luciferase gene, allow us to monitor the expression of multiple genes simultaneously when luciferases are used that induce differently colored emission spectra in the catalysis of a single D-luciferin substrate. These mixed emission spectra are measured simultaneously and can be quantified by splitting them with optical filter(s). This system has been used in a mammalian cell extract system (*13*−*17*), cultured plant tissues (*18*), a bacterial system monitored in real time (*19*), and cultured mammalian cells (*20*). However, the generation of a transgenic animal by introducing multicolored bioluminescent reporters and/or monitoring the simultaneous expression of multiple genes at the tissue or whole-organism level has not been reported.

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Circadian rhythm research is a field in which luciferase is frequently used to monitor gene expression in real time, because extremely long-term, quantitative monitoring of gene expression is more often required than in other types of biological research (*5*). Circadian rhythms are periodicities with a length of approximately 24 h and are essential physiological functions in almost all organisms. They include the sleep−wake cycle, body temperature fluctuations, and variations in hormonal secretion and are generated by endogenous "biological clocks" (*22*−*24*). The suprachiasmatic nucleus (SCN)[1] of the hypothalamus is the principal circadian pacemaker in mammals. It not only drives daily physiological rhythms but is also entrained by external time cues, such as light−dark cycles. Such daily rhythms are controlled by genetically determined and self-sustaining circadian clocks, which are composed of networks of interlocking autoregulatory feedback loops in the expression of the clock genes. In mammals, the molecular clock occurs not only in the SCN but also in the peripheral tissues. Briefly, *Per2* transcription is activated during the day by the CLOCK/BMAL1 heterodimer through an E-box enhancer that occurs in its promoter region. The gene product, together with the cryptochrome (*Cry*) protein, suppresses its own gene transcription in the night. Conversely, *Bmal1* transcription is regulated by an additional interlocking loop. Its transcription is activated in the night by retinoic acid receptor-related orphan receptors (RORs) through the ROR response elements in the *Bmal1* promoter, whereas it is suppressed during the day by REV−ERBs. Consequently, the expression patterns of *Per2* and *Bmal1* oscillate in antiphase (*25*−*29*).

To analyze the molecular mechanism underlying these autofeedback loops, several transgenic animals have been established, in which firefly luciferase is expressed under the control of the promoters of clock genes or clock-controlled genes, and have been extensively studied in explant tissue cultures (*21*). In particular, *Per2* and *Bmal1* have been studied independently to some extent. Although detailed expression profiles of both genes can be demonstrated with a real time monitoring system using a single reporter, any comparison of the promoter characteristics of individual genes is very difficult. Therefore, monitoring the simultaneous expression of multiple genes is important in analyzing the molecular mechanism underlying the feedback loops involving *Per2* and *Bmal1*. However, no tools have been available to monitor the oscillations of these loops or the expression of these genes simultaneously in tissues or whole organisms.

To overcome the technical limitations inherent in studying the interactions of these two genes, we generated a dual-color luciferase mouse that carries transgenes encoding green-emitting luciferase (ELuc, λ_{max} = 538 nm) (*30*−*32*) expressed under the control of the *Bmal1* promoter and red-emitting luciferase (SLR2, λ_{max} = 630 nm) (*13*, *15*, *33*) expressed under the control of the *Per2* promoter. We were able to monitor the antiphasic oscillations of *Bmal1* and *Per2* expression simultaneously in a single tissue of the dual-color luciferase mouse by splitting their emissions. We also confirmed that the expression of these two genes is strictly antiphasic and that the recorded phases and periods of both varied between organs. We also demonstrated the parallel phase responses of the two genes to a change of medium, used to perturb their circadian phases.

[1]Abbreviations: SCN, suprachiasmatic nucleus; *Cry*, cryptochrome; ROR, retinoic acid receptor-related orphan receptor; ATP, adenosine triphosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SLR2, red-emitting luciferase from *Phrixothrix hirtus*; ELuc, green-emitting luciferase from *Pyrearinus termitilluminans*; HBSS, Hank's balanced salt solution; cpm, counts per minute; CT, circadian time; WT, wild type; DD, constant darkness; PRC, phase response curve.

MATERIALS AND METHODS

Plasmid Constructions. To optimize the codons of the red-emitting *Phrixothrix hirtus* luciferase (*33*) (GenBank accession number AF139645) for mammalian expression, the codon usage data were obtained from the *Mus musculus* database at the Kazusa DNA Research Institute (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=10090). To delete the putative transcription factor binding sites within the cDNA sequence, the sites were identified and deleted with the MatInspector sequence analysis software (Genomatix, Munich, Germany). All nucleotide substitutions were designed so that the deduced amino acid sequence was not altered. The designed 1641-bp double-stranded cDNA was synthesized using a customized DNA synthesis service at Toyobo Gene Analysis (Osaka, Japan). The reporter plasmid mBmal1−ELuc carrying the 5′ flanking region (−816 to +99 bp, where +1 indicates the putative transcription start site) of *mBmal1* (*34*) was constructed as reported previously (*32*). The reporter plasmid mPer2−SLR2 carrying the 5′ flanking region (−2811 to +110 bp) of *mPer2* was constructed by replacing the *Nco*I/*Fse*I fragment of the *mPer2* promoter/pGL3 (*35*) with SLR2. Constructs encoding the destabilized luciferase (mBmal1−dELuc and mPer2−dSLR2), fused at their C-terminal ends with the PEST motif of mouse ornithine decarboxylase, were constructed as reported previously (*32*). To generate the transgenic mice, the reporter plasmids mBmal1−ELuc and mPer2−SLR2 were digested and linearized with *Cla*I, *Kpn*I, and *Bam*HI, respectively, and removed from the vector sequences.

Cell Culture and Transfection Procedures. Mouse NIH3T3 cells (RCB1862) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; ICN Biochemicals, Aurora, OH) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were seeded in 35 mm dishes 1 day before transfection, and the reporter plasmids were transfected using Lipofectamine PLUS (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. One day after transfection, transfected cells were treated with 100 nM dexamethasone (Nacalai Tesque, Kyoto, Japan) in the absence of FBS for 2 h, and the medium was replaced with DMEM without phenol red (Gibco-BRL, Grand Island, NY) supplemented with 10% FBS and 200 μM D-luciferin potassium salt (Toyobo) and overlaid with mineral oil (Sigma-Aldrich) to prevent evaporation.

Spectral Measurements. To measure the bioluminescent spectra in live cells, 2 μg of reporter plasmid was transfected into NIH3T3 cells, which were cultured for 1 day. After stimulation with 100 nM dexamethasone in the absence of FBS for 2 h, the medium was replaced with DMEM supplemented with 10% FBS, 25 mM HEPES/NaOH (pH 7.0; Sigma-Aldrich), and 200 μM D-luciferin potassium salt. The dish was placed on the sample stage of a spectrophotometer. Spectral measurements were made with an AB-1850 spectrophotometer (ATTO, Tokyo, Japan) for 1 min with a slit width of 1 mm.

Generation of Bmal1^{ELuc} and Per2^{SLR2} Transgenic Mice. The transgenic mice were generated at the University of Tsukuba. The procedure was strictly in accordance with protocols approved by the Animal Care and Use Committee of the University of Tsukuba. We generated transgenic mice carrying mBmal1−ELuc (*Bmal1^{ELuc}*) and transgenic mice carrying

mPer2−SLR2 ($Per2^{SLR2}$) from the oocytes of BDF1 mice (C57BL/6N Jcl × DBA/2N Jcl, F_1; CLEA Japan, Tokyo, Japan). The linearized DNA fragments were injected into the fertilized mouse eggs using standard protocols (*36*). The F_0 and F_1 generations of the $Bmal1^{ELuc}$ and $Per2^{SLR2}$ transgenic mice were transferred to the National Institute of Advanced Industrial Science and Technology (AIST). The light intensity from the cultured tissues of these transgenic mice was measured with a dish-type luminometer (AB2500 Kronos, ATTO). Among the 10 transgenic lines of $Per2^{SLR2}$ produced, we used the $Per2^{SLR2\text{-}C3}$ mouse line because it expressed the strongest SLR2 bioluminescence. Among the 10 transgenic lines of $Bmal1^{ELuc}$ produced, we selected the $Bmal1^{ELuc\text{-}A1}$ mouse line because it displayed the bioluminescence intensity closest to that of $Per2^{SLR2\text{-}C3}$. The two selected lines of transgenic mice, $Bmal1^{ELuc\text{-}A1}$ and $Per2^{SLR2\text{-}C3}$, were back-crossed to C57BL/6J Jms Slc mice (Japan SLC, Hamamatsu, Japan) to the F_4 and F_2 generations, respectively. $Bmal1^{ELuc}$:$Per2^{SLR2}$ mice were bred from an F_4 $Bmal1^{ELuc\text{-}A1}$ animal and an F_2 $Per2^{SLR2\text{-}C3}$ animal as parents. The $Bmal1^{ELuc}$:$Per2^{SLR2}$ mice thus generated were back-crossed to C57BL/6J Jms Slc mice for more than two generations. To screen the pups for the transgenes, polymerase chain reaction (PCR) was performed using the genomic DNA from the newborn mouse tails as templates and the following primer sets: for the *ELuc* gene, 5′-CCACCATGGAGAGAGAGAA-GAA-3′ and 5′-TGTAGCCACAGTTCTGGAGGCT-3′; and for the *SLR2* gene, 5′-CCATGGAAGAAGAGAACATCGT-3′ and 5′-GTTGTTGTGATCCAGGCCATAC-3′.

Explant Cultures. The mice were maintained under a 12 h light/12 h dark (LD12:12) regime (lights on: 07.30−19.30 h). At 14.00−15.00 h, 2−6-month-old mice were decapitated. Their brains and peripheral tissues were rapidly removed and placed in ice-cold Hank's balanced salt solution (HBSS) supplemented with 10 mM HEPES/NaOH, 1.76 mg/L $NaHCO_3$ (Invitrogen), 97 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). Coronal sections of the brain (250 μm thick) cut with a microslicer (Dosaka, Osaka, Japan) were transferred to ice-cold HBSS. The bilateral SCNs with the connected optic chiasmas were trimmed to approximately 4 mm^2. Lung, liver, adrenal gland, and ovary tissues were cut into 1−3 mm pieces with a surgical knife, and the whole eyeball, pituitary gland, and thyroid gland were removed. All of the tissues were cultured on Millicell culture membranes (PICMORG50; Millipore, Billerica, MA) with 1.2 mL of tissue culture medium (pH 7.4) composed of DMEM without phenol red (Sigma-Aldrich, D2902), 10 mM HEPES/NaOH, 3500 mg/L glucose, 352 mg/L $NaHCO_3$, 2% B27 supplement (Invitrogen), 0.1 mg/mL streptomycin, 100 units/mL penicillin, and 200 μM D-luciferin potassium salt. The individual tissue cultures were sealed in 35 mm Petri dishes with Parafilm (American National Can, Menasha, IL) to prevent evaporation. The procedure was strictly in accordance with the protocols approved by the Institutional Animal Care and Use Committee of AIST.

Real Time Monitoring of Bioluminescence. The *Bmal1*-driven ELuc and *Per2*-driven SLR2 emissions were monitored and measured simultaneously with AB2500 Kronos (ATTO) equipped with a photomultiplier tube (H7421−40; Hamamatsu Photonics, Shizuoka, Japan) with high quantum efficiency in the red wavelength range. The luminometer was placed in an incubator set at 20 °C, and the cultures were incubated at 37 °C in the luminometer. Bioluminescence was monitored for 1 min at 10−20 min intervals in the presence or absence of a >620 nm long-pass filter (R62 filter; Hoya, Tokyo, Japan). We expressed the measured bioluminescence intensities as counts per minute (cpm). The activities of the ELuc and SLR2 luciferases were calculated as described previously (*13*, *20*), using the equation:

$$\begin{pmatrix} F(-) \\ F(+) \end{pmatrix} = \begin{pmatrix} 1.0 & 1.0 \\ \kappa_{ELuc} & \kappa_{SLR2} \end{pmatrix} \begin{pmatrix} ELuc \\ SLR2 \end{pmatrix}$$

where *ELuc* and *SLR2* are the ELuc and SLR2 luciferase activities, $F(-)$ is the total counts measured in the absence of the filter, $F(+)$ is the counts that passed through the filter, and κ_{ELuc} and κ_{SLR2} are the filter's transmission coefficients for the two luciferases.

Analysis of Circadian Rhythms in Tissues. The explants were cultured in the luminometer for more than 6 days to measure their bioluminescence. The crude data (20 min bins) were smoothed with the adjacent-averaging method, with a 3 h moving average (*37*), and were detrended by subtracting the 24 h moving average from the smoothed data. The highest points in the detrended data were considered the circadian peaks. To determine the circadian periods for the eye, SCN, pituitary gland, thyroid gland, lung, and ovary, we prepared detrended data ranging from 0.5 to 5.5 days after the start of measurement, and the periods were obtained from the best-fit cosine curves using the least-squares spectrum method (*38*). To determine the circadian periods for the liver and adrenal gland, we obtained the best-fit cosine curves from the detrended data ranging from 2.5 to 5.5 days after the start of measurement, because cultivation for a few days is required to stabilize the oscillations and bioluminescence levels in these tissues.

Analysis of Phase Shifts in the SCN. The culture medium was replaced after the bioluminescence from the SCN explants had been measured for 3−6 days. After the medium had been changed, bioluminescence measurements were continued for more than 4 days. We calculated the magnitude of the phase shifts in the SCN explants using a previously described method (*38*, *39*), with slight modifications. Briefly, to estimate the phase shifts in the *Bmal1* rhythm, the crude data collected before and after the medium change were smoothed and detrended as described above. The best-fit cosine curves were obtained from (I) all of the data collected before the medium change (detrended data ranging from 0.5 days after the start of measurement to 0.5 day before the medium change) and (II) the data collected for 3 days after the medium change (detrended data ranging from 0.5 to 3.5 days after the medium change). Because the mean (±SEM) of the first peak time was 23.1 ± 0.1 h after the last lights-on, the peak time of the *Bmal1* expression rhythm was defined as circadian time (CT) 23.0, where subjective "dawn" and subjective "dusk" were referred to as CT0 and CT12, respectively. The CTs at the time of medium change were estimated from curves of both (I) and (II). We defined these as CTI and CTII. The magnitude of the phase shift was calculated as the difference between CTI and CTII. The phase shifts were plotted against the CTI. The same procedure was applied to the *Per2* rhythm. The best-fit cosine curves were constructed on the assumption that the period of the *Per2* rhythm was the same as that of the *Bmal1* rhythm in the same explants because there was no significant difference between these periods observed in the SCN explants (*Bmal1*, 23.5 ± 0.1 h; *Per2*, 23.8 ± 0.1 h; $P > 0.05$, Student's *t* test). The calculated magnitude of the phase shift was plotted against the CT of the medium change estimated from the *Bmal1* expression rhythm in the same explant. If the cpm for *Bmal1* or *Per2* expression was smaller than 200 or no cosine curve with a rejection rate (probability) of below 10^{-20}

was found, the data were excluded from the analysis. A majority of the slices (31/42) satisfied this standard in both in *Bmal1* and *Per2* expression, and these data were used for the analysis in Figure 5D. A few slices (2/42) were completely excluded from all of the analyses. In 21% of slices (9/42), only *Bmal1* expression exceeded the standard due to the low light intensity of SLR2. These *Bmal1* data were, therefore, included in the phase shift analysis of each gene (Figure 5B,C).

RESULTS AND DISCUSSION

Simultaneous Monitoring of Bmal1 and Per2 Expression in Cultured Fibroblasts. The bioluminescent reporters used in this work were the red-emitting luciferase (SLR2) from a railroad worm (*P. hirtus*) (*13*, *15*, *33*) and the green-emitting luciferase (ELuc) from a Brazilian click beetle (*Pyrearinus termitilluminans*) (*30*, *31*). The cDNA sequence of the *P. termitilluminans* luciferase has been optimized for mammalian expression (*32*). To construct the dual-color luciferase mouse, we chose these luciferases as reporters for the following reasons: (i) ELuc and SLR2 display the most blue- and red-shifted spectra, respectively, among the beetle luciferases, which are the most easily separable emissions; (ii) these emit light with a single luciferin (D-luciferin); and (iii) the colors of their emissions are not affected by pH changes (*30*, *33*, *40*).

We first optimized the cDNA sequence of *P. hirtus* luciferase for mammalian expression, because the expression and light output of the wild-type (WT) form are extremely low in cultured mammalian cells (*41*). Improvements were made by deleting the putative transcription factor binding sites and optimizing the codons within the cDNA, without changing the deduced amino acid sequence (Supporting Information Figure S1). We refer to this luciferase as stable luciferase RED2 (SLR2). The sequence-optimized SLR2 luciferase exhibits dramatic increases both in its expression at the mRNA and protein levels and in the intensity of the bioluminescent signal in NIH3T3 cells (Figure 1A,B) compared with those observed for the WT luciferase.

Next, to verify whether the antiphasic oscillations of *Bmal1* and *Per2* expression can be simultaneously monitored using ELuc and SLR2, respectively, we monitored both emissions simultaneously in real time in NIH3T3 cells as the model system. The reporter plasmids mBmal1−dELuc and mPer2−dSLR2, which both encode luciferases fused to the PEST element of mouse ornithine decarboxylase, were transiently cotransfected into the NIH3T3 cells. The bioluminescence was recorded continuously for 96 h with a luminometer in the presence or absence of a >620 nm long-pass filter (R62, Hoya), and each bioluminescence intensity was calculated as described previously (*13*, *20*) (and see the Materials and Methods section). As shown in Figure 1C, both bioluminescent signals displayed clear antiphase oscillations, consistent with the intrinsic mRNA expression patterns of the two genes, as reported previously (*22*−*24*). This suggests that ELuc and SLR2 distinctly recorded the transcriptional oscillations of *Bmal1* and *Per2* associated with their intrinsic expression. Therefore, these results demonstrate that appropriateness of using these reporters and this measurement system to simultaneously monitor the circadian expression patterns of the two genes.

We next verified whether the emission spectra of ELuc and SLR2 are constant during prolonged incubation, even though we used pH-insensitive luciferases, because constancy of the emission spectra is extremely important for the simultaneous measurement of gene expression in the present study. The reporter

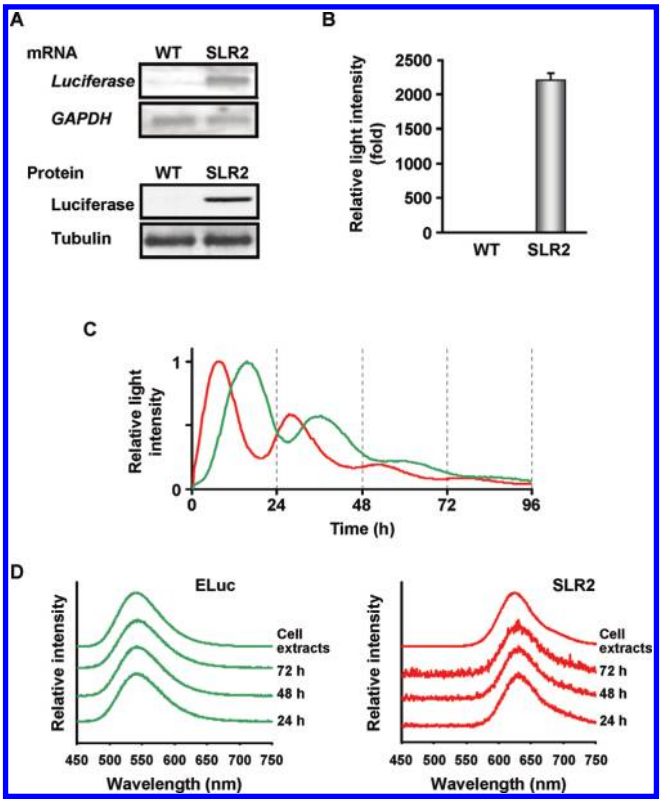

FIGURE 1: Characteristic properties of SLR2 and ELuc and the simultaneous monitoring of *Bmal1* and *Per2* expression in cultured fibroblasts. (A) Northern and Western blot analyses of the expression of WT luciferase and SLR2 in NIH3T3 cells. WT luciferase or SLR2 was transiently expressed under the control of the simian virus 40 promoter in NIH3T3 cells, and the cells were harvested and disrupted 2 days later. In the Northern blot analysis, both luciferases were detected with cDNA probes by mixing equal molar amounts of both luciferase cDNAs. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control. In the Western blot analysis, both luciferases were detected using the anti-RED luciferase antibody. Tubulin was used as the internal control. (B) Bioluminescence intensity of wild-type luciferase- and SLR2-expressing cell extracts. (C) Simultaneous monitoring of *Bmal1* (green line) and *Per2* (red line) transcriptional oscillations using ELuc and SLR2, respectively, in NIH3T3 cells. Reporter vectors mBmal1−dELuc (0.2 μg) and mPer2−dSLR2 (1 μg) were cotransfected, and the cells were stimulated with 100 nM dexamethasone. Bioluminescence was recorded for 96 h with a luminometer (AB2500; Kronos) in the presence or absence of an optical filter (R62), and the intensity of each bioluminescence was calculated as described in the Materials and Methods. The peak counts were set to 1. (D) Emission spectra of ELuc (left panel) and SLR2 (right panel) in viable cells. NIH3T3 cells were transfected with mBmal1−dELuc or mPer2−dSLR2 and incubated for 24 h. To obtain the spectra after stimulation with dexamethasone, the culture medium was replaced with DMEM supplemented with 10% FBS, 25 mM HEPES/NaOH (pH 7.0), and 200 μM D-luciferin. The spectra were then measured at 24 h intervals. To obtain the spectra of both luciferases in cell extracts, extracts of ELuc- or SLR2-expressing NIH3T3 cells were mixed with PicaGene as the substrate.

vector mBmal1−dELuc or mPer2−dSLR2 was independently transfected into NIH3T3 cells. After stimulation with dexamethasone, the bioluminescence spectra of both luciferases emitted from viable cells were noninvasively measured at 24 h intervals for 72 h using a spectrometer (Figure 1D). The emission spectra of both luciferases from viable cells were consistent with those measured in cell extracts, and these spectra remained constant during the incubation period. These results suggest the feasibility of using ELuc and SLR2 to simultaneously monitor *Bmal1* and *Per2* expression in tissues by generating a dual-color transgenic mouse.

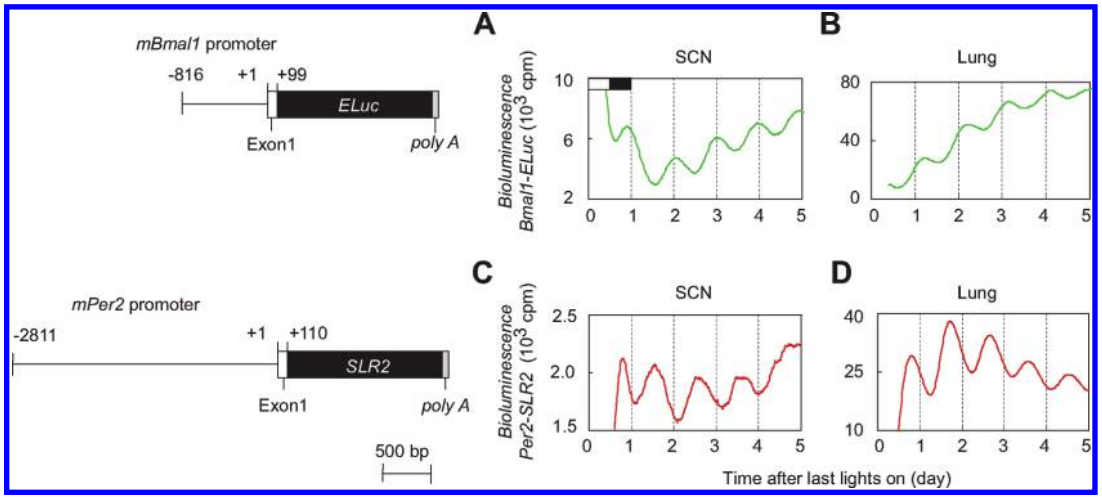

FIGURE 2: Construction of *Bmal1*^{ELuc} mice and *Per2*^{SLR2} mice. (A−D) Representative records of bioluminescence in tissues from transgenic mice containing a single transgene. The circadian rhythms in SCN (A) and lung (B) explants from *Bmal1*^{ELuc} mice and SCN (C) and lung (D) explants from *Per2*^{SLR2} mice were plotted. The *x* axis indicates the time after last lights-on. Open bar, the previous light period. Filled bar, the previous dark period. cpm, counts per minute. Schematic drawings of the reporter constructs are shown on the left, where +1 corresponds to the putative transcription start site. polyA, polyadenylation signal.

Generation of Dual-Color Luciferase Mouse. To produce a dual-color luciferase mouse, we first generated two transgenic mice, one expressing ELuc under the control of the *Bmal1* promoter, as reported previously (*32*), and the other expressing SLR2 under the control of the *Per2* promoter, and designated them *Bmal1*^{ELuc} and *Per2*^{SLR2}, respectively. To examine whether or not the bioluminescence oscillations could be monitored in these mice, we maintained the SCN and lung tissues in static cultures containing medium supplemented with D-luciferin. The bioluminescence was measured continuously in real time with a luminometer (Figure 2). Clear circadian rhythms were observed in the SCN and lung explants of both the *Bmal1*^{ELuc} mouse (Figure 2A,B) and the *Per2*^{SLR2} mouse (Figure 2C,D). The bioluminescence oscillations in the tissues from these two mice were almost antiphasic, as observed in the NIH3T3 cells (Figure 1C). These results suggest that ELuc and SLR2 reflect the expression levels in tissues of *mBmal1* and *mPer2*, respectively. These two mouse lines were then mated to establish a dual-color luciferase mouse, *Bmal1*^{ELuc}:*Per2*^{SLR2}. The *Bmal1*^{ELuc}:*Per2*^{SLR2} mice were then back-crossed to the WT mice, and both transgenes were maintained in a hemizygous state. We used the hemizygous transgenic mice for all of the following experiments.

First, we tested the wheel-running rhythms of the dual-color luciferase mice to determine whether their circadian behavior was altered (Supporting Information Figure S2A,B). The WT (n = 8) and *Bmal1*^{ELuc}:*Per2*^{SLR2} mice (n = 8) exposed to an LD12:12 cycle showed no differences in entrainment. Four different circadian pacemaker traits were examined in constant darkness (DD): the free-running period, the amplitude of the circadian rhythms, the daily activity levels, and the magnitude of the light-induced phase shifts (Supporting Information Figure S2A,B and Table S1). The *Bmal1*^{ELuc}:*Per2*^{SLR2} mice showed normal responses to light pulses (Supporting Information Figure S2C). No significant differences were detected between the WT and *Bmal1*^{ELuc}:*Per2*^{SLR2} mice in any of the parameters analyzed. These results demonstrate that the insertion of the transgenes had no obvious effect on the circadian locomotor behaviors of the *Bmal1*^{ELuc}:*Per2*^{SLR2} mice.

Expression Profiles of Bmal1 and Per2 in Tissues. To accurately monitor and profile the phase relationships between *Bmal1* expression and *Per2* expression in various tissues, we simultaneously monitored the expression of both genes in explant cultures from *Bmal1*^{ELuc}:*Per2*^{SLR2} mice. The eye, SCN, pituitary gland, thyroid gland, lung, liver, adrenal gland, and ovary were dissected and maintained in static culture. As shown in Figure 3A, the circadian oscillations of the expression of both *Bmal1* and *Per2* could be clearly monitored in the explants of various organs. We noted that the light output from ELuc was over 10-fold stronger than that from SLR2 in all tissues, which may be attributable to the high stability and expression of ELuc in the cells (*32*). We previously examined the range of linearity of the dual-color reporter system using cell extracts expressing the green- and red-emitting luciferases and estimated it to be over 2 orders of magnitude (*13*) when the respective luciferase activities display over 1×10^3 cpm (data not shown). Therefore, even though the light intensities from various tissues differed by over 10-fold, this was within the linear response range, allowing us to accurately monitor *Bmal1* and *Per2* expression simultaneously.

To precisely profile the circadian expression of *Bmal1* and *Per2* in each tissue, all raw data were detrended with a 24 h moving average method. Representative raw data of each tissue (Figure 3A) and the detrended data (Figure 3B) were shown. As shown in Figure 3B, the antiphase expression of *Bmal1* and *Per2* was stably maintained in all tissues. Among the tissues examined, the SCN displayed the most robust antiphase oscillations, with a higher amplitude than those in the other tissues, consistent with a previous reports (*42*−*44*), although the light intensities of both *Bmal1*-driven ELuc and *Per2*-driven SLR2 luminescence were moderate. We constructed phase maps for all of the tissues, using the peak times of the detrended data during the 5 days in culture (Figure 4A). During the 20−40 h interval after the last lights-on for the animals, the peak *Bmal1* and *Per2* expression appeared at 23.1 ± 0.4 and 35.4 ± 1.8 h, respectively (n = 12 slices from seven animals), in the SCN, demonstrating that *Bmal1* and *Per2* expression was almost exactly 180 degrees out of phase. We also observed the tissue-specific mean peak times for both *Bmal1* and *Per2* expression in the various tissues examined. For example, there was a 4 h phase delay in *Per2* expression between the SCN and the liver.

Next, we estimated the circadian periods of *Bmal1* and *Per2* expression from the best-fit cosine curves using the least-squares

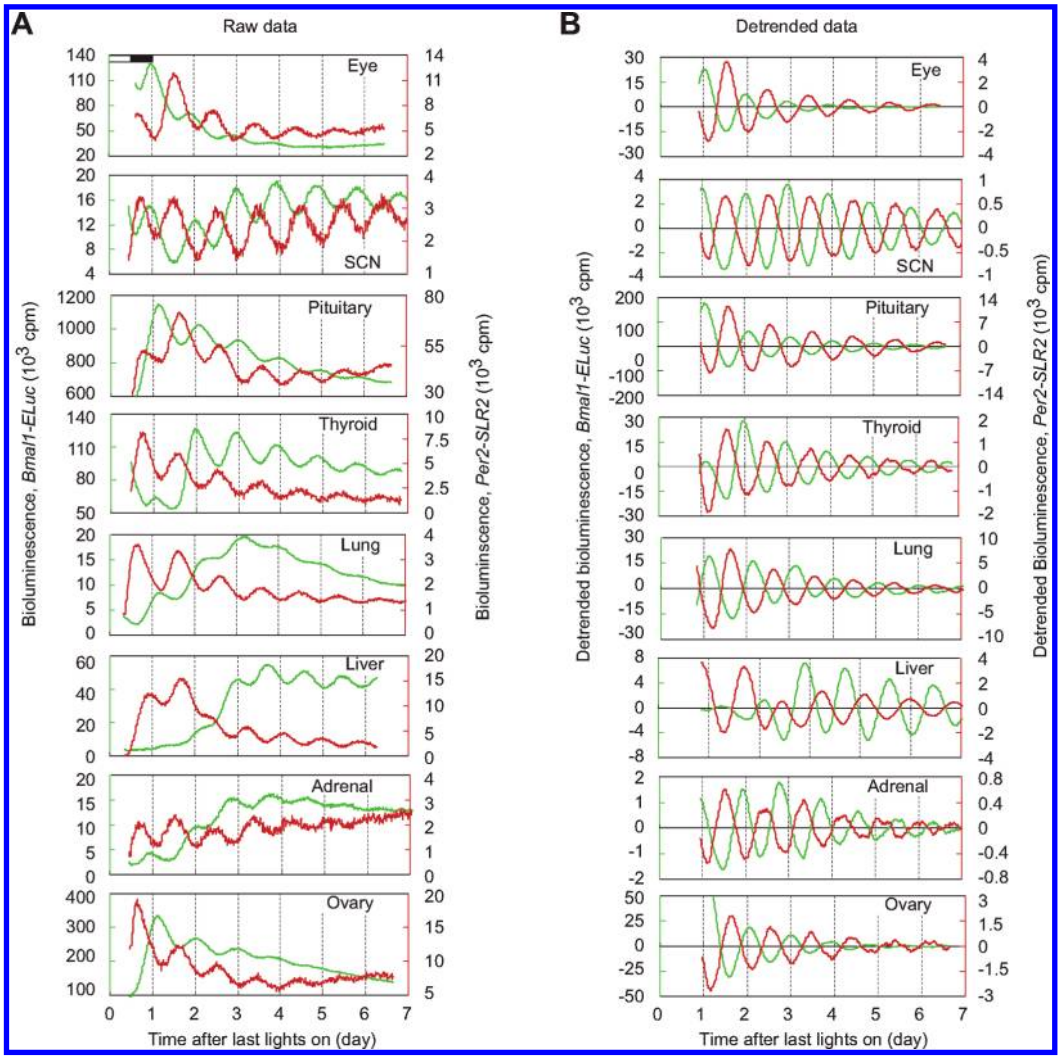

FIGURE 3: Simultaneous recording of the circadian expression of *Bmal1*-driven ELuc and *Per2*-driven SLR2 bioluminescence from dual-color luciferase mice. (A) Representative records of bioluminescence showing the circadian profiles of *Bmal1* and *Per2* expression in various tissues of the $Bmal1^{ELuc}$:$Per2^{SLR2}$ transgenic animals. Data for eye, SCN, pituitary gland, thyroid gland, lung, liver, adrenal gland, and ovary are shown. The bioluminescence of *Bmal1*-driven ELuc (cpm) is plotted with green lines and shown on the left *y* axis. The bioluminescence of *Per2*-driven SLR2 (cpm) is plotted with red lines and shown on the right *y* axis. The *x* axis shows the time after last lights-on for the animals. Open bar, previous light period. Filled bar, previous dark period. (B) Data shown in (A) were smoothed and detrended (see Materials and Methods section).

spectrum method (Figure 4B). Interestingly, although the periods of both expression patterns were almost identical in a specific tissue, the periods were tissue specific. The endogenous mRNA expression patterns of both *Bmal1* and *Per2* have been profiled extensively with *in situ* hybridization, real time PCR, and DNA microarray analysis (*22*−*24*, *45*). Moreover, transgenic mice that express firefly luciferase under the control of the *Bmal1* or *Per2* promoter have been established (*42*−*44*). The peaks and periods of the *Bmal1* and *Per2* expression patterns determined in this work showed almost the same tendencies as those estimated from endogenous mRNA profiles or when explant tissues from the transgenic mice were monitored in real time. For example, the 4 h phase delay in *Per2* expression between the SCN and the liver is faithfully reported in $mPer2^{Luc}$ knockin mice (*43*) and in transgenic mice expressing firefly luciferase under the control of *Bmal1* promoter fragments (*44*). The results show that the *Bmal1*- and *Per2*-driven bioluminescence oscillations associated with endogenous transcription could be simultaneously monitored in the dual-color luciferase mice established in this study. Recently, Nishide et al. generated a dual-luciferase transgenic mouse in which firefly luciferase and a secreted luciferase from *Vargular hilgendorfii* are expressed under the control of the *Bmal1* and *Per1* promoters, respectively. This was the first transgenic mouse carrying two luciferase genes, and the antiphase oscillations in the expression of both genes were successfully monitored in a single SCN slice (*44*). However, in this system, the culture medium must be collected periodically to measure the secreted luciferase. Therefore, our monitoring system, in which the two luciferase activities are measured simultaneously in real time, seems to be relatively simpler. The combination of the dual-color mouse and a system in which different color-emitting luciferases are measured simultaneously allows us to profile two gene expression patterns conveniently and accurately in various organs using explant tissues.

Monitoring the Phase Shifts in Bmal1 and Per2 Simultaneously in SCN Slices. *Bmal1* and *Per2* are known to play important roles not only in the generation of circadian rhythms but also in resetting circadian phases to synchronize them with environmental cycles (*26*, *27*, *46*). The acute expressions of the *Per1* and *Per2* genes in the SCN, culminating in a phase shift in the expression of clock genes and clock-controlled gene, are considered to correlate closely with resetting and entrainment at

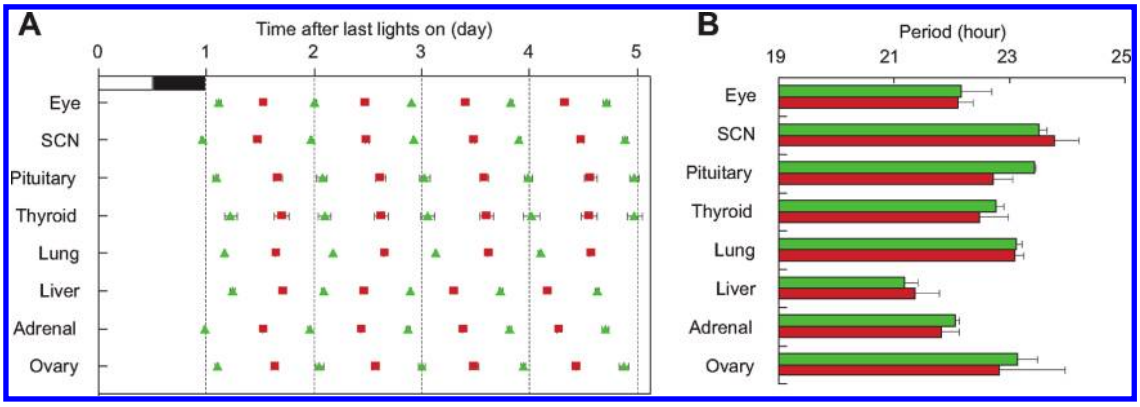

FIGURE 4: Circadian phases and periods of *Bmal1* and *Per2* expressions in tissues from *Bmal1*^{ELuc}:*Per2*^{SLR2} mice. (A) Phase map of the circadian rhythms in the cultured tissues of *Bmal1*^{ELuc}:*Per2*^{SLR2} mice. The peak times of the circadian oscillations were determined during the interval between the first day and fifth day in culture. Average peak times (±SEM) of *Bmal1*-driven ELuc rhythm (green filled triangles) and *Per2*-driven SLR2 rhythm (red filled squares) were plotted against the time after last lights-on. (B) Mean periods (±SEM) of the circadian rhythms of *Bmal1*-driven ELuc expression and *Per2*-driven SLR2 expression in various tissues from *Bmal1*^{ELuc}:*Per2*^{SLR2} transgenic mice are plotted. The mean peak times and periods were obtained for eye ($n = 6$), SCN ($n = 12$), pituitary gland ($n = 4$), thyroid gland ($n = 5$), lung ($n = 6$), liver ($n = 6$), adrenal gland ($n = 8$), and ovary ($n = 6$). One or two explants were removed from each tissue of an animal.

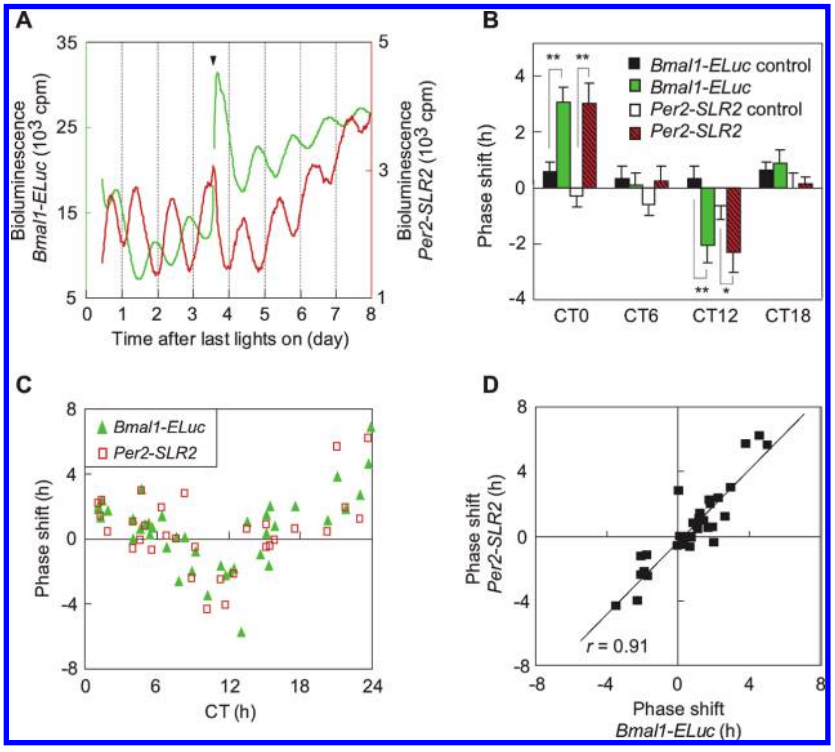

FIGURE 5: Circadian rhythms of *Bmal1*-driven ELuc bioluminescence and *Per2*-driven SLR2 bioluminescence in SCN slices from *Bmal1*^{ELuc}:*Per2*^{SLR2} mice in response to medium change. (A) Representative bioluminescence recorded in an SCN explant from a *Bmal1*^{ELuc}:*Per2*^{SLR2} mouse. In this record, the medium was changed at CT16.7, and this resulted in a phase shift of less than 1 h. The arrowhead indicates the time when the medium was changed. The bioluminescence of *Bmal1*-driven ELuc and *Per2*-driven SLR2 is shown in green and red lines, respectively, and the values are shown on the left and right *y* axes, respectively. (B) Mean phase shifts in *Bmal1*-driven ELuc expression (green bars) and in *Per2*-driven SLR2 expression (shaded bars) were compared with that of the untreated control. The magnitudes of the phase shifts (mean ± SEM) when the medium was changed at CT0 ± 3.0, CT6 ± 3.0, CT12 ± 3.0, and CT18 ± 3.0 ($n = 6$–13) were averaged. For the untreated control cultures ($n = 6$), the medium was assumed to have been changed at CT0 ± 3.0, CT6 ± 3.0, CT12 ± 3.0, and CT18 ± 3.0, and the phase shifts in *Bmal1*-driven ELuc expression (black bars) and *Per2*-driven SLR2 expression (white bars) were estimated with the same method. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). (C) PRC after medium changes in SCN explants from *Bmal1*^{ELuc}:*Per2*^{SLR2} mice. The phase shifts in the bioluminescence rhythms of *Bmal1*-driven ELuc (green filled triangles, $n = 40$) and *Per2*-driven SLR2 (red squares, $n = 31$) are shown. The *x* axis shows CT when the medium was changed. Phase advances are expressed as positive values and phase delays as negative values. (D) The magnitudes and directions of the phase shifts in *Bmal1*-driven ELuc expression and *Per2*-driven SLR2 expression were compared. The magnitude of the phase shift in *Per2*-driven SLR2 was plotted against that of *Bmal1*-driven ELuc ($n = 31$). The regression line ($y = 1.1x - 0.4$, $P < 0.01$; simple regression analysis) is shown. The magnitudes of the phase shifts show a positive correlation ($r = 0.91$, Pearson's correlation coefficient).

the behavioral level (*47*–*49*). Using the firefly luciferase reporter, the phase shifts in *Bmal1* (*50*) and *Per2* (*51*) expressions in SCN explants induced by medium change, used as a stimulant of their phase shift, were studied independently in mice. In those studies, the phase shifts in the expression of both genes showed phase dependence and displayed a type 1 phase response curve (PRC). Nevertheless, it is very difficult to merge their independent data. To precisely examine the relationship between the phase

responses in the expression of both genes, we therefore cultured SCN explants of the *Bmal1*^{ELuc}:*Per2*^{SLR2} mouse and observed *Bmal1* and *Per2* expression simultaneously after culture medium replacement. The SCN explants were cultured for 10−14 days, and the bioluminescence was measured with a luminometer. The medium was changed after 3−6 days in culture. A representative response of the SCN slice to medium change is shown in Figure 5A. The medium change induced phase shifts in the circadian rhythms of both *Bmal1* and *Per2* expression. The magnitude and direction of the phase shifts depended on the time of the medium change (Figure 5B). The medium change induced phase advances in subjective morning (CT0 ± 3.0) and phase delays in subjective night (CT12 ± 3.0) in both *Bmal1*-driven ELuc and *Per2*-driven SLR2 luminescence rhythms, whereas little effect was noted in the subjective day (CT6 ± 3.0) or at midnight (CT18 ± 3.0). The effects of the medium change are summarized in a PRC (Figure 5C), where phase advances are shown as positive values and phase delays as negative values. For both *Bmal1* and *Per2* expression, the mean phase shifts at CT0 ± 3.0 and CT12 ± 3.0 were significantly different from those in the untreated control, which is almost identical to the results of previous studies (*50*, *51*).

To analyze whether the rhythms of *Bmal1* and *Per2* expression respond to the medium change in a parallel manner, we examined the correlation between the magnitudes of the phase shifts in the expression of these two genes. The magnitudes of the phase shifts of *Per2* were plotted against those of *Bmal1* in the same explants (Figure 5D). The regression line obtained from the data clearly demonstrates that there is an almost direct correlation between the phase shifts of *Bmal1* and *Per2* ($r = 0.91$); i.e., the magnitudes and directions of the phase shifts in the two expression patterns are parallel. It has been reported that when SCN explants from mice carrying *Bmal1* or *Per2* firefly luciferase constructs were subjected to medium change, they displayed a type 1 PRC, consistent with the results of the present study. However, we monitored *Bmal1* and *Per2* expression simultaneously and showed closely correlated phase responses to this stimulus, which was not possible in previous systems.

CONCLUSIONS

In this study, we generated dual-color luciferase mice in which a *Bmal1*-driven green-emitting luciferase (ELuc) and a *Per2*-driven red-emitting luciferase (SLR2) were expressed. By combining these mice with a simultaneous monitoring system, we demonstrated that each of the tissues examined has unique circadian phases of *Bmal1* and *Per2* expression, even though the expression of the two genes is strictly antiphasic. The phase shifts of both genes in the cultured SCN slice triggered by medium exchange occurred in a similar manner. Therefore, we conclude that the dual-color mice established in the present study allow for the precise analysis of genetic networks in feedback loops that regulate the time-keeping system. In addition, the simultaneous monitoring system is readily applicable to trace the relationships between "input and oscillator" or "oscillator and output" by simultaneously monitoring gene expressions within respective systems. The system will also help us to understand the role of the circadian clock system in other biological systems, such as the cell cycle, tumor suppression, and the immune system.

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SUPPORTING INFORMATION AVAILABLE

Nucleotide sequences of *P. hirtus* WT and sequence-optimized luciferase, SLR2 (Figure S1), representative wheel-running activity records of WT and *Bmal1*^{ELuc}:*Per2*^{SLR2} transgenic mice (Figure S2), circadian phenotypic characteristics of *Bmal1*^{ELuc}:*Per2*^{SLR2} mice (Table S1), and additional experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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