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Perfusion-culture-based secreted bioluminescence reporter assay in living cells

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Abstract

Bioluminescence reporter proteins have been widely used in the development of tools for monitoring biological events in living cells. In this study, we describe the development of a reporter system with secreted *Cypridina noctiluca* luciferase (CLuc) for a pharmacological assay that is based on targeted promoter activity. A model cell line was established with Rat-1 fibroblasts expressing CLuc driven by the promoter of a circadian clock gene, *Bmal*1. To accurately assay for temporally secreted CLuc activity, a perfusion culture in which the promoter activity was sequentially monitored by the reporter activity in the perfusate was adopted. By pulsing with dexamethasone (DEX), a glucocorticoid (GC) analog, the profile of the reporter activity successfully showed diurnal fluctuation, which is a canonical expression pattern of the *Bmal*1 gene. Trial studies illustrated that the DEX-pulsed circadian oscillation was reasonably attenuated by RU486, a GC receptor antagonist. Moreover, SP600125, a c-Jun N-terminal kinase inhibitor, caused phase shifting of the rhythmicity. We conclude that the CLuc reporter assay in combination with perfusion culture is a suitable pharmacological tool for drug discovery.

Keywords: Secreted bioluminescence reporter; *Cypridina* luciferase; Perfusion culture; Pharmacological assay; Circadian clock

Bioluminescence and fluorescence reporters have emerged as powerful tools for the continuous and real-time monitoring of the intracellular dynamics of gene expression in living cells. Firefly luciferase (FLuc)[1] is the most widely used reporter protein for noninvasive and quantitative monitoring. However, FLuc is not well suited to pharmacological reporter assay in living cells owing to the difficulties in controlling the intracellular concentrations of the substrates, luciferin and ATP, and the pH, all of which affect the intensity and stability of the bioluminescence [1–3].

These difficulties can be circumvented by procuring secreted bioluminescence reporters that allow for measurement of reporter activity in the extracellular space. As an artificial system, FLuc fused to a secretory signal peptide was shown to be secreted by a transfected parasite, *Plasmodium falciparum*, and transported to the cytosol of infected erythrocytes [4]. Seapansy *Renilla* luciferase, when fused to a secretory signal peptide, was also successfully used to trace promoter expression in the culture media of mammalian cells [5–7].

Naturally occurring secreted luciferases from luminous organisms, such as the marine ostracods *Vargula hilgendorfii* and *Cypridina noctiluca*, the decapod shrimp *Oplophorus gracilirostris,* and the marine copepods *Metridia longa*

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[1] *Abbreviations used:* FLuc, firefly luciferase; VLuc, *Vargula hilgendorfii* luciferase; Cry, cryptochrome; Per, period; SCN, suprachiasmatic nuclei; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; CLuc, *Cypridina noctiluca* luciferase; CLucPer system, *Cypridina noctiluca* luciferase reporter system with perfusion culture; IRES, internal ribosome entry site; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BPB, bromophenol blue; DEX, dexamethasone; GC, glucocorticoid; GR, glucocorticoid receptor; SD, standard deviation; PEEK, poly ether ketone.

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and *Gaussia princeps*, have been identified for use as reporter enzymes in mammalian cell systems [8–12]. Among them, the ostracod luciferases are the best characterized, generating blue luminescence ($\lambda_{max} = 465$ nm) by a simple enzyme-catalyzed reaction that involves only luciferase, luciferin, and molecular oxygen [11,13–15]. *V. hilgendorfii* luciferase (VLuc) is a useful reporter enzyme in studies of gene expression monitoring and real-time imaging in both cultured mammalian cells and live mouse embryos [16–18]. Moreover, we reported that VLuc is applicable to the intramolecular bioluminescence resonance energy transfer system [19]. Thus, secreted bioluminescence reporters have considerable potential to be used in a practical manner for probing biological events. Furthermore, we cloned CLuc, the activity of which was much higher than that of VLuc in living cells [11].

FLuc has been used as a convenient and promising tool in the investigation of the regulatory mechanism of circadian gene expression, which requires continuous long-term monitoring. Both bioluminescence and conventional techniques have revealed that the molecular basis for the circadian clock consists of key regulators that form autoregulatory feedback loops to maintain a 24-h rhythm in gene and protein expression. In mammals, the heterodimeric transcription activator CLOCK/BMAL1 promotes the transcription of *Cryptochrome* (*Cry*) and *Period* (*Per*) genes. The CRY and PER proteins that are imported into the nucleus function as negative regulators of CLOCK/BMAL1 activity [20]. Although the central circadian clock resides in the hypothalamic suprachiasmatic nuclei (SCN), the clock genes are expressed also in peripheral tissues to control cyclic expression [20–22]. Despite advancement in our understanding of the molecular machinery of the circadian clock, the signaling mechanisms underlying the synchronization of the clock to external cues are still poorly understood. Recent studies have shed light on the role of mitogen-activated protein kinase (MAPK), particularly on the role of extracellular regulated kinase (ERK), as a biological timekeeper. Several researchers have proposed that ERK-mediated signaling plays a role in transmitting environmental information into the intracellular clock to cause the phase resetting of clock gene expression and eventually of biological rhythms [23,24]. Pizzio et al. [25] suggested the involvement of c-Jun N-terminal kinase (JNK) and p38, which are members of the MAPK family, in the regulation of the circadian clock by showing that the activation of JNK and p38 is under circadian and photic regulation in the SCN.

In this context, we assumed that a pharmacological-assay-oriented bioluminescence reporter system would be beneficial in obtaining a deeper insight into the signaling mechanisms. Our group previously reported that a VLuc reporter assay in combination with perfusion culture was useful for monitoring the promoter activity of a growth hormone [26]. On the basis of this previous achievement, we report herein the development of a CLuc reporter system with perfusion culture (CLucPer system) for several days, which is suitable for use as a pharmacological assay and is sustained by the highly secreted bioluminescence activity of the reporter enzyme [11] and a self-fabricated cell culture chamber. These properties allowed us to monitor circadian gene expression and to evaluate the efficacy of drugs based on the circadian output as criteria.

Materials and methods

Vector construction

The DNA sequences of CLuc and the internal ribosome entry site (IRES) were amplified by polymerase chain reaction using pcDNA-CL [11] and pIRES templates (Clontech), respectively. The oligonucleotide primers, including the underlined linker sequences, were as follows: CLuc forward primer, 5′-(*Hin*dIII) ACAAGCTTGGCATTCC GGTACTGTTGGTAAAGCCACCATGAAGACCTTA ATTCTTGCCGTTG-3′; CLuc reverse primer, 5′-(*Xba*I) TTTCTAGACTATTTGCATTCATCTGGTACTTCT-3′; IRES forward primer, 5′-(*Xba*I) GGTCTAGAAATTCCG CCCCTCTCCCTCCCCCCCCCCTAACGTT-3′; and IR ES reverse primer, 5′-(*Nco*I) TGCCATGGTGTTATCAT CGTGTTTTTCAAAGGAAAACCACGTCCC-3′. The amplified products were ligated in one step into*Hin*dIII and *Nco*I sites of Bp/915-LUC [27] to give the pBmal1-CLuc vector.

Cell culture and transfection

Rat-1 fibroblast cells were grown in 24-well plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere. The medium was replaced with serum-free DMEM before transfection to minimize the background level of nonspecific luciferin chemiluminescence. Transient transfection was performed on cells at approximately 70% confluence with the use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The plasmid DNAs for transfection were as follows (per well): 350 ng of pBmal1-CLuc as the reporter, 0 to 300 ng of pCR3.1-mRORα4 [28] as the transactivator, 50 ng of phRL-TK (Promega) with the constitutive expression of *Renilla* luciferase for normalizing the transfection efficiency, and pBluescript SK(-) (Stratagene) as the additive, which was added to make a total amount of 800 ng.

For stable transfection, Rat-1 fibroblasts were cotransfected with pBmal1-CLuc and pSV-Neo as the expression vector of neomycin resistance by using Lipofectamine Plus (Invitrogen). Twenty-four hours after transfection, the cells were subcultured for selection with 500 μg/ml geneticin G418 (Nacalai Tesque). The G418-resistant cell lines were subcultured, and at more than 90% confluence, the media were replaced with serum-free DMEM for 24 h and the culture media and cell extracts were assayed for bioluminescence activities to select stable transfectants.

For the perfusion experiment, a stable line designated Bmal1-CL-11 was precultured in 12-well plates containing DMEM supplemented with 10% FBS, 0.1 mg/ml streptomycin, and 50 U/ml penicillin at 37 °C in 5% CO_2 for 1 day. Immediately before the perfusion, the culture medium was exchanged with perfusion medium consisting of DMEM without phenol red (Invitrogen) supplemented with 10% FBS, 10 mM Hepes (pH 7), 4 mM glutamine, 0.1 mg/ml streptomycin, and 50 U/ml penicillin.

Assembly and assessment of perfusion chamber

A silicon plug containing two ports inserted with PEEK tubing of 1-mm inside diameter (Flon Industry) was attached to each well of the 12-well plate. The resultant chamber was set at a height of approximately 9 mm by using stoppers to position the plug. The ends of the two PEEK tubes were adjusted to heights of 1–2 mm from the bottom of the well to obtain 0.5 ml of medium in the chamber during perfusion (Fig. 1).

The silicon plugs kept the chamber aseptic, and the combination of the high ceiling of the chamber with the low layer of medium provided ample room for air bubbles coming in accidentally with the flow to float on the medium, essentially preventing the cultured cells from contacting the bubbles. In a pilot study, we assessed the exchange rate of the solution in the chamber by flowing dummy perfusates of either water or 0.1% bromophenol blue (BPB) solution in turns at 1 ml/h. The measurement of optical density at 450 nm with regard to the absorption of BPB demonstrated that it takes approximately 1 h to replace the whole solution (0.5 ml) in the chamber.

Setup of perfusion apparatus

The two ports of the perfusion chamber were connected to a fraction collector (ATTO) and a three-way stopcock (Bio-Rad) that branched into a medium bottle with a screw cap (Minucells and Minutissue Vertriebs GmbH) and a vial for the drug, respectively, by using Pharmed tubes and Luer fittings (Fig. 1). The perfusion medium was supplied by a peristaltic pump (IPC-N8, ISMATEC) at 1 ml/h, and perfusate was collected every 30 min by the fraction collector. The medium containing the drugs was injected into the chamber by switching the flow path of the three-way stopcock. The whole system was placed in a 37 °C incubator without CO_2 injection, except for the fraction collector, which was refrigerated at 4 °C.

Luciferase assay

The measurements of CLuc activity in all of the fractionated perfusates were performed together because the CLuc activity could be stably maintained for at least 5 days at 4 °C. Fifty microliters of 140 nM luciferin that was purified from *V. hilgendorfii* and diluted with 100 mM Tris–HCl (pH 7.4) was mechanically injected into 50 µl of the perfusates using a Luminescencer-JNR (ATTO), and the luminescence was measured for 10 s.

The assays for CLuc and FLuc activities in the cell extracts and media after plate culture were performed as previously described [11,28].

Data analysis

Quantitative measurement of phase shifts was conducted using a modification of published methods [29,30]. Briefly, the circadian profile of the CLuc activity was smoothed by a five-point moving average, and the long-term trend was removed by subtracting the running average of values between 12 h before and after each point. To confirm the results, the data were also tested for other than 24-h fluctuations: values between 6, 10, 15, 20, or 24 h before and after each point (data not shown). The times of half-fall of the first circadian peak to half-rise of the third peak (a total of four points) were used as phase reference points. To calculate the magnitude of the phase shift, the time of each phase reference point was subtracted from the corresponding time in the controls.

Chemicals

DEX was purchased from Nacalai Tesque, and RU486 and SP600125 were obtained from Sigma.

Results

Establishment of model cell line

To establish a model cell line to evaluate the CLucPer system, we chose fibroblasts transfected with a CLuc

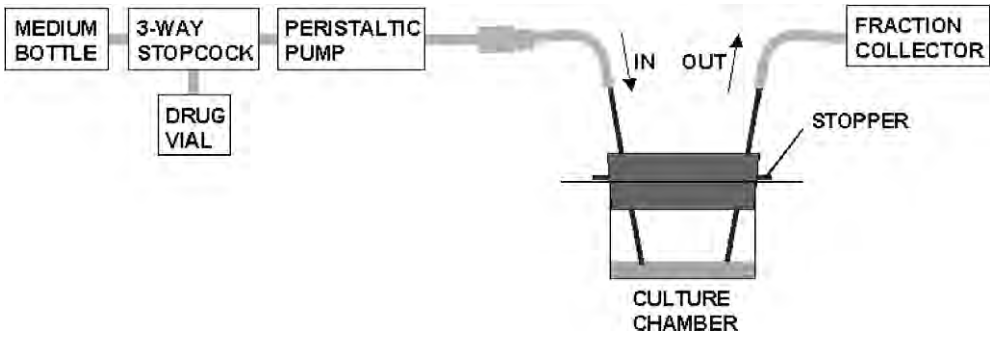

Fig. 1. The CLucPer system. Note that the drug vial contains a mixture of drug and culture medium.

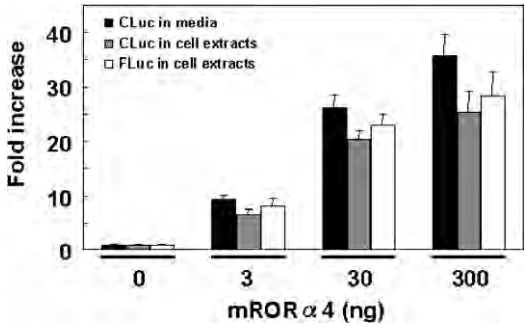

Fig. 2. Dose-dependent induction of *Bmal*1-promoter-driven transcription by RORα4. Rat-1 fibroblasts were cotransfected with reporter plasmid pBmal1-CLuc and different doses of pCR3.1-mRORα4. The CLuc activities in the media (black bars) and in the cell extracts (gray bars) and the FLuc activity in the cell extracts (white bars) were measured as described under Materials and methods. All data are normalized by the values of *Renilla* luciferase expressed from phRL-TK and are indicated as fold increases (means ± SD, $n = 6$).

reporter gene that is driven by the *Bmal*1 promoter because the reporter expression of transfectants should show a circadian fluctuation whose periodicity can be used as an authentic expression profile [31,32].

We checked the ability of the transfection vector pBmal1-CLuc to confer the appropriate expression of the reporter enzymes to the cells. The *in vitro* assays showed that Rat-1 fibroblasts transiently transfected with this vector can express CLuc and that 86% of the bioluminescence activity is detected in the culture medium. As shown in Fig. 2, the CLuc activity was increased in response to RORα4, which was identified as a transactivator of the *Bmal*1 gene [28,33,34]. The dose dependencies of CLuc in media and cell extracts were similar to each other and to that of the FLuc expression from the IRES-mediated dicistronic fusion, which was incorporated into the vector to evaluate whether the CLuc reporter expression was reasonable. These results indicate that the CLuc reporter activities indeed reflect the transcriptional activity of the *Bmal*1 promoter.

Monitoring of Bmal1 gene expression with the CLucPer system

We previously showed that the secretory property of CLuc is applicable to tracking the promoter activity continuously in a plate culture [11]. However, realizing an accurate assay with the enzyme is difficult owing to the enzyme's stability, which causes it to accumulate in the culture medium. Therefore, we decided to perfuse the culture medium and measure the CLuc activity in the perfusate using the CLucPer system.

We first tested whether the CLucPer system can trace *Bmal*1 gene expression in the stable transfectants treated with the GC analog DEX, a drug that is known to induce the oscillation [31,35]. After the exposure of the transfectants to 0.1 μM DEX for 2 h using the medium change procedure prior to perfusion, the bioluminescence activity of secreted CLuc was found to fluctuate in an approximately 24-h cycle. The rhythmicity could be monitored throughout the 4-day test, but the amplitude tended to dampen and the activity became irregular with time. Thus, the CLucPer system was applicable to the continuous monitoring of circadian promoter activity in living cells.

DEX-pulsed oscillation of Bmal1 gene is antagonized by RU486

One potential benefit of the CLucPer system is the feasibility of using transient injections of drugs for the examination of the transcriptional responses of cells. To this end, medium containing 0.1 μM DEX was supplied to the culture chamber by switching the flow for 2 h, and the resultant transcriptional response was monitored with regard to the CLuc activity. Based on the results of the exchange rate using the dummy perfusates (see Materials and methods), the DEX concentration in the chamber should gradually increase, reach a plateau (0.1 μM), and then gradually decrease over a period of approximately 1 h for each process. As shown in Fig. 3, DEX treatment after culture for 8 h increased the diurnal fluctuation of the CLuc activity. This timing for the treatment was chosen because it could generate a phase opposite that of the weak fluctuation in vehicle-treated cells, which is probably induced by the medium change before the perfusion [32]. The results suggest that the CLucPer system is suitable for monitoring the promoter activity in response to drug treatment.

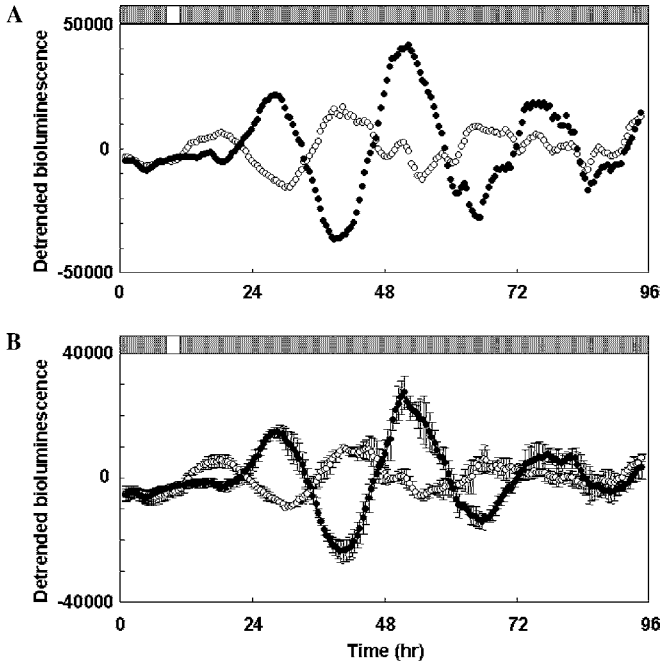

Fig. 3. Monitoring of circadian oscillation induced with a transient DEX pulse. Bmal1-CL-11 cells were preperfused for 8 h and pulsed with flowing medium containing vehicle (open circles) or 0.1 μM DEX (closed circles) for 2 h at the time indicated by the open bar at the top of each graph. Shown are results from single (A) and quadruplicate experiments (B) where each point represents the mean ± SD ($n = 4$). The long-term trends of CLuc bioluminescence were removed as described under Materials and methods.

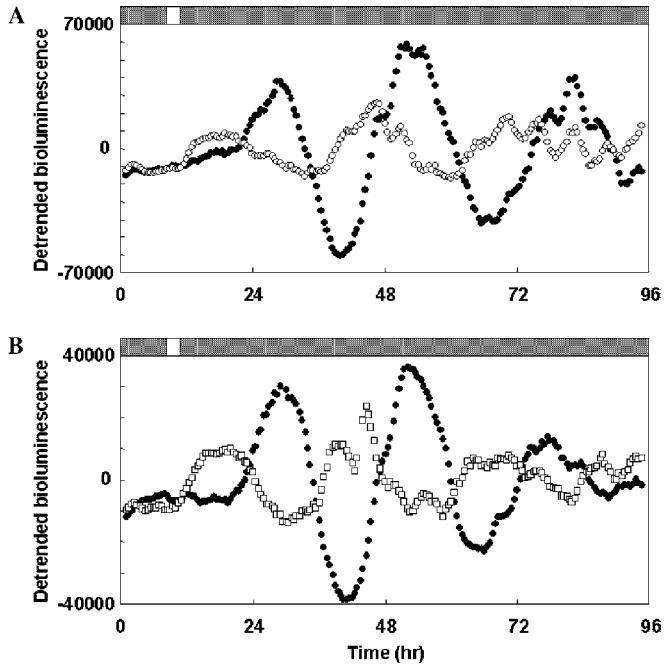

Fig. 4. Circadian oscillation induced with a transient DEX pulse is attenuated by RU486. Bmal1-CL-11 cells were preperfused for 8 h and pulsed with flowing medium containing 0.1 μM DEX (closed circles in B), 10 μM RU486 (open squares), or 0.1 μM DEX with (open circles) or without (closed circles in A) 10 μM RU486 for 2 h at the time indicated by the open bar at the top of each graph. Triplicate experiments were performed, and representative data are shown.

We next examined the effect of RU486, a selective GC receptor (GR) antagonist [36], on DEX-pulsed *Bmal*1 oscillation. The coapplication of 10 μM RU486 with 0.1 μM DEX led to an attenuated fluctuation compared with the treatment with 0.1 μM DEX alone while treatment with 10 μM RU486 had no significant effect on the *Bmal*1 oscillation (Fig. 4). This antagonistic effect was restored by adding an excess amount of DEX (100 μM), which gave a profile similar to that of the treatment with DEX (0.1 μM) alone (data not shown). These results suggest that GR-mediated signal transduction may be involved in the induction of *Bmal*1 oscillation and demonstrate that the CLucPer system is suitable for the evaluation of agonist–antagonist interactions.

JNK inhibitor SP600125 causes phase shifting of Bmal1 oscillation

To test the performance of the CLucPer system in pharmacological screening, we next focused on JNK, which was found to act antagonistically on GR functions [37–40]. As a trial study, we tested SP600125, a selective JNK inhibitor [41] that has not been evaluated in regard to its effect on circadian gene expression. Treatment with 50 μM SP600125 had no effect on the induction of *Bmal*1 oscillation (Fig. 5B). The coapplication of 50 μM SP600125 and 0.1 μM DEX for 6 h caused a phase delay of the circadian rhythm compared with the rhythm generated by DEX alone (Fig. 5A), whereas no significant effect was detected with the 2-h application (data not shown). Although the

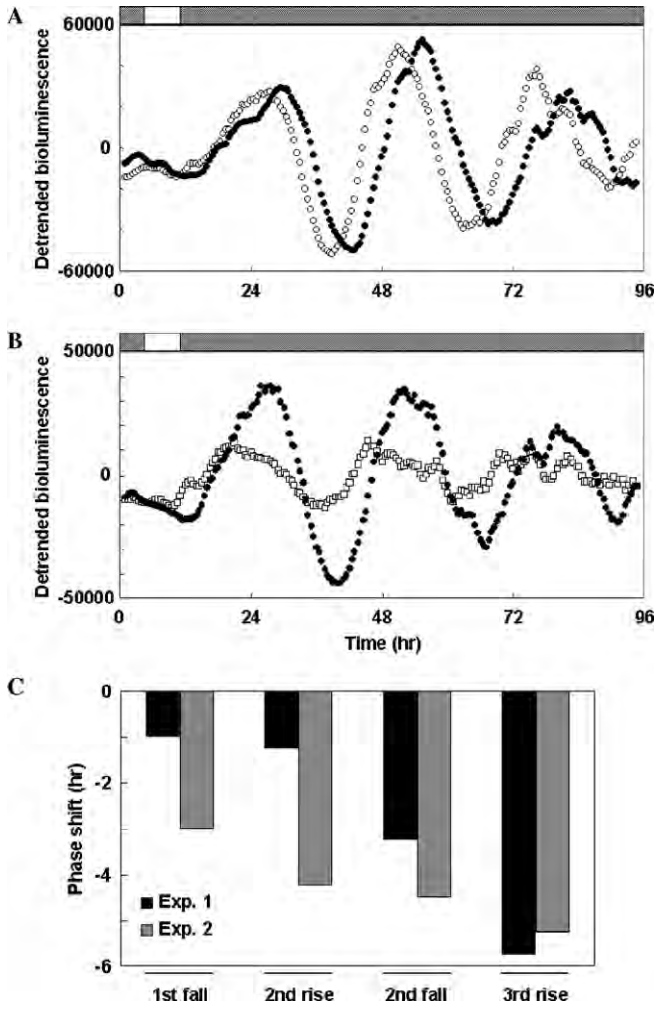

Fig. 5. Effect of SP600125 on DEX-pulsed circadian oscillation. (A) and (B) Bmal1-CL-11 cells were preperfused for 4 h and pulsed with flowing medium containing 50 μM SP600125 (open squares) or 0.1 μM DEX with (closed circles) or without (open circles) 50 μM SP60125 for 6 h at the time indicated by the open bar at the top of the graph. Triplicate experiments were performed, and representative data are shown. (C) The magnitudes of the phase shifts caused by the SP6000125 treatment were calculated at the four phase reference points from the half-fall of the first peak to the half-rise of the third peak in two experimental cases.

degree of phase delay was dependent on the experiment, the observation that the delay time increased as the circadian cycle progressed was reproducible (Fig. 5C). The findings in the trial study suggest that SP600125 potentially causes the phase resetting of *Bmal*1 oscillation. The results show that the CLucPer system can be used to identify the effects of drugs that cause changes in the temporal pattern of promoter expression and the magnitude of activity.

Discussion

CLucPer system has advantages for use as pharmacological assay

Using the CLucPer system, we succeeded for the first time in performing a secreted-reporter-assisted pharmacological

assay in which drugs are applied with a flow carrier. The system enabled us to monitor the response of the circadian promoter activity under conditions that mimic the environment *in vivo*, i.e., when hormones and medicines are transported and supplied by blood flow. Furthermore, the system has merits in that the reporter activity is assayed extracellularly where the reaction conditions are adjustable, valuable luciferin substrate can be saved, and the application of luciferin to cells to cause nonphysiological conditions is avoided. Thus, we propose that the CLucPer system is a useful tool for pharmacological assays.

CLucPer system reveals an aspect of drug effects on circadian gene expression

We showed that the DEX-pulsed circadian oscillation of the *Bmal*1 gene is specifically antagonized by RU486 (Fig. 4). Previous studies using mutant mice with hepatocyte-specific inactivation of the GR gene demonstrated that GC hormones play a role in the entrainment of peripheral oscillators via a tissue-autonomous mechanism [35,42]. In a recent study, DEX was shown to synchronize self-sustained but dephased oscillators in individual fibroblasts [31]. These observations support the idea that GR-mediated signal transduction is involved in the resetting mechanism of peripheral oscillators that lead a cell population to a harmonized rhythm.

The JNK inhibitor SP600125 used in the trial study was found to have a phase-shifting effect on DEX-pulsed *Bmal*1 oscillation (Fig. 5). It has been reported that JNK phosphorylates GR directly, enhancing the nuclear export of GR and inhibiting GR-mediated transcriptional activation [37–39]. On the other hand, the binding of GR to JNK is required for the inhibition of JNK activation and the induction of the inactive JNK nuclear transfer in response to GCs [40]. Moreover, the treatment of mouse hippocampal (HT22) cells with SP600125 and a JNK-specific antisense oligonucleotide was shown to enhance the GR-mediated functions, i.e., DEX-induced mouse mammary tumor virus promoter-luciferase activity and GR binding to the GC responsive element [43]. These data support the hypothesis that SP600125 influences the phase-resetting mechanism of the circadian clock system through a signal transduction pathway involving the GR-JNK interaction. However, we should take into account the fact that SP600125 is also a ligand and antagonist of the dioxin (aryl hydrocarbon) receptor [44], which might have an unknown function in the regulation of the circadian clock.

The results of the gradual increase of phase-shift time after the SP600125 pulse are still controversial (Fig. 5). In cultured *Xenopus* retinal photoreceptor layers, SP600125 was shown to have not only the phase-shifting effect on the circadian oscillation of melatonin release but also the long-lasting stimulatory effect, which makes the interpretation of the results difficult [30]. The phase-shifting effect is shared by our findings based on the expression of the clock gene, but whether the stimulatory effect is a special characteristic in the experimental system is an issue to be elucidated by a more comprehensive investigation. Nevertheless, we speculate that the long-term effect is a likely property of the SP600125 action that influences the entire period after the pulse to cause the gradual phase-shifting of the *Bmal*1 oscillation.

Perspective of the CLucPer system

As the circadian genes show an expression profile with daily cycles of rising and falling phases, the CLucPer system can be used to monitor other classes of genes with expression profiles consisting of those components. Whether the system can dissect out promoter expressions showing a more rapid change on the order of minutes remains to be studied; this requires the faster flow of medium and the fractionation of perfusate with a shorter interval, resulting in lower reporter activities in the fractions. The application of the system to suspended growth cells is another aspect to be considered because those cells are usually used as cell models to screen novel drugs and for pharmacological assay. Our results show that the CLucPer system is a potential tool for pharmacotoxicological assays that are based on the transcriptional response of targeted genes and will evolve into a microfluid-type system that requires the minimal use of cells and specimens.

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