学位論文

Cellular stress responses regulate the mammalian circadian clock

（哺乳類の概日時計を制御する細胞ストレス応答）

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Abstract

In mammals, daily rhythms are generated by the circadian clock composed of clock genes and the encoded proteins, such as CLOCK and BMAL1, forming transcriptional/translational feedback loops (TTFLs). Particularly, posttranslational regulation of clock proteins plays crucial roles in determining the circadian period and phase, and thereby circadian clocks respond flexibly to environmental changes. A wide variety of stimuli are known to evoke cellular stress responses through phosphorylation signaling. However, it is elusive whether the circadian clock responds to cellular stress through protein kinase signaling, and if so, what is the key molecule that transmits the cellular stress to the circadian clockwork. In the first part of this study, I paid attention to the mechanism of BMAL1 phosphorylation, and identified c-Jun- N-terminal kinase (JNK) as a novel circadian clock-related kinase, which regulates phosphorylation level of BMAL1 and the period of circadian rhythm. Based on the results, in the second part of this study, I focused on Apoptosis Signal-regulating Kinase (ASK), a member of MAP kinase kinase kinase (MAPKKK), and identified it as a key mediator for determining the circadian period and phase of cultured fibroblasts in response to osmotic changes of the media. The physiological significances of ASK signal were shown in the photic response of the behavior rhythms of mice. Moreover, I found that *Ask* is rhythmically expressed in mice liver and is essential for the clock to respond to changes of the intracellular redox state. These results demonstrated that ASK mediates cellular stress such as osmotic or oxidative stress and photic signaling to circadian clock.

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Chapter 1.

Introduction

Like sleep–wake cycles, many aspects of physiologies and behaviors exhibit daily rhythms even under constant conditions. The circadian rhythms are governed by the circadian clock, a biological oscillator that is conserved among a wide range of organisms (Asher and Schibler, 2011; Hastings et al., 2003; Reppert and Weaver, 2002). In mammals, it had been believed that the circadian oscillator resides only in the neurons of hypothalamic suprachiasmatic nucleus (SCN) for the long time because ablations of the SCN eliminate the circadian behavior rhythm of rodents. However, recently it was revealed that cell-autonomous circadian oscillators, termed peripheral clocks, are distributed across the peripheral tissues and even in cultured fibroblasts (Balsalobre et al., 1998, 2000; Hirota and Fukada, 2004; Yagita et al., 2001). At the organismal level, the circadian rhythms are generated by hierarchical network of the central SCN-clock and peripheral clocks (Fig. 1). In individual cells, clock genes and their encoding products, clock proteins, form transcriptional/translational feedback loops (TTFLs), which generate a wide variety of oscillating transcripts (Bass and Takahashi, 2010; Dunlap, 1999; Fig. 2). In the molecular clock, BMAL1 and CLOCK form the heterodimer complex as basic helix-loop-helix-PAS transcriptional factors and bind to specific DNA *cis*-elements, E-box (5'-CACGTG-3') to activate transcription of a series of clock-controlled genes. This set of genes includes members of the negative limbs of the feedback loop such as *Per* and *Cry* genes. Translated PER and CRY proteins negatively regulate the transcriptional activity of BMAL1-CLOCK complex and thereby inhibit their own transcription. Freed from repression by PER/CRY, BMAL1-CLOCK then rebinds to the E-boxes and start a new day (Bass and Takahashi, 2010; Dunlap, 1999; Fig. 2). In order to maintain the feedback loops with the 24-h periodicity, clock proteins are finely regulated by post-translational modifications (Gallego and Virshup, 2007; Hirano et al., 2016; Kon et al., 2014; Lee et al., 2001; Yoshitane et al., 2009; Fig. 2).

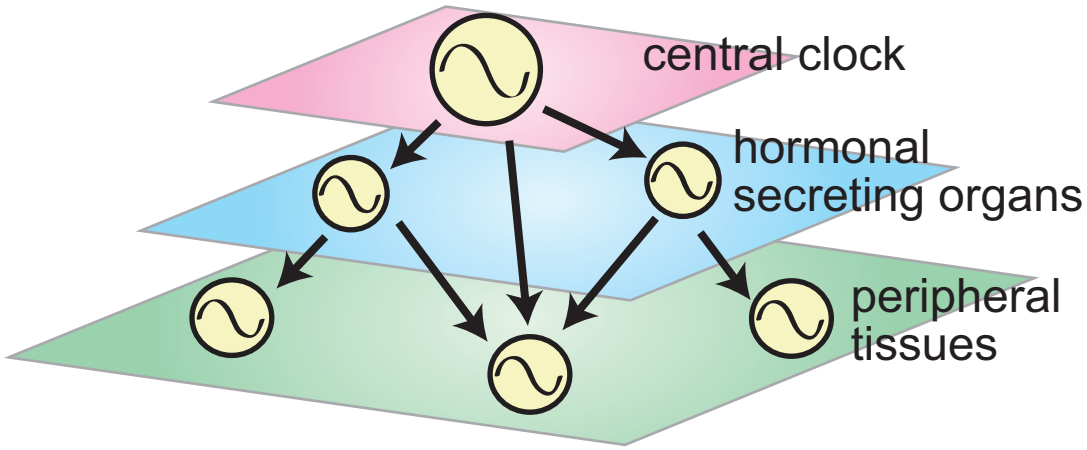

Figure 1. Hierarchical network of mammalian clock system.
Circadian clocks are resided in almost all cells of various organs (tissues). The master clock is located in the SCN (top tier) and synchronizes the downstream peripheral clocks. The hormonal secreting organs such as pineal gland and adrenal gland (middle tier), which secrets melatonin or glucocorticoid respectively, regulate the other peripheral clocks. As with a symphony orchestra, the circadian orchestra is hierarchically organized, with the SCN of hypothalamus as its conductor.

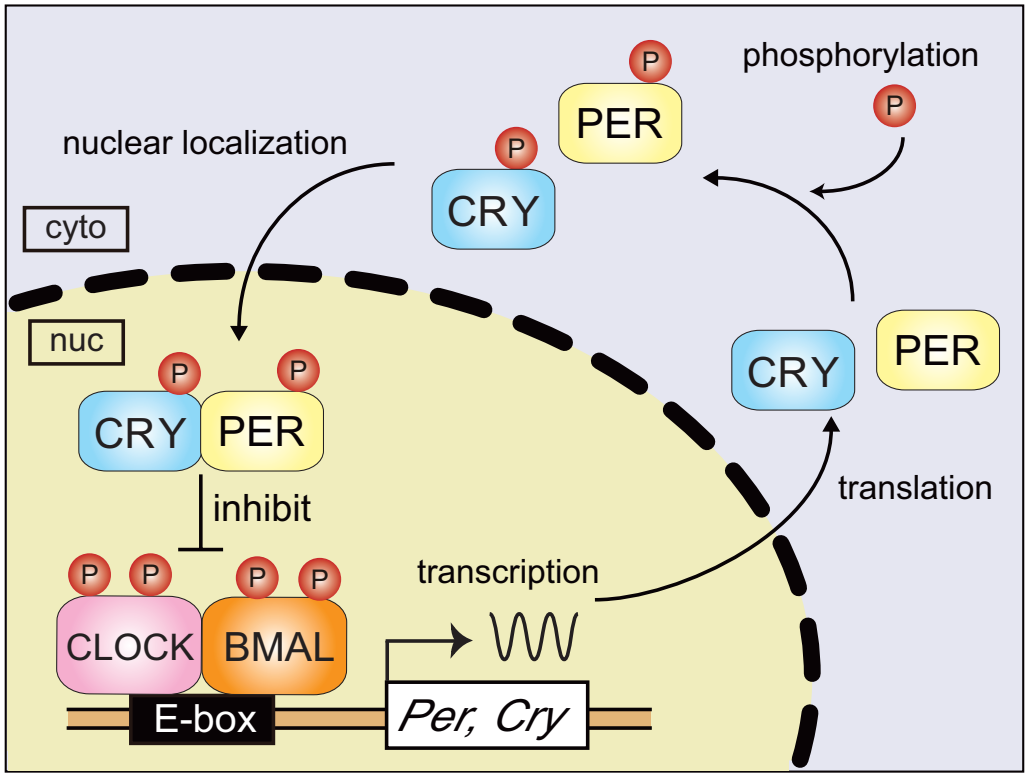

Figure 2. A model for mammalian molecular clock.
Mammalian molecular clock consists of multiple autoregulatory transcriptional/translational feedback loops (TTFLs). In particular, *Clock*, *Bmal1*, *Per* and *Cry* genes are known as "core clock gene" because deletion of these genes results in a loss of circadian rhythm of behavior and gene expression. In the molecular oscillation of the clock, posttranslational modifications such as phosphorylation of clock proteins play crucial roles in stability and robustness of clock proteins. In addition to the core loop, several feedback loops are involved in the stabilization of the oscillation and tuning the period length.

The molecular oscillation based TTFLs and post-translational modifications of clock proteins, which confer robustness on the circadian clock even under constant conditions. On the other hand, the circadian period and phase respond flexibly to a wide variety of environmental time cues in order to synchronize, or entrainment, with the external 24-h cycles. The most consistent environmental time cue is the 24-h cycles of light and darkness (LD) and most information about entrainment derives from studies using LD cycles in the central SCN clock (Hirota and Fukada, 2004; Johnson et al., 2003; Reppert and Weaver, 2002). The photic signal captured by retina is transmitted to the SCN neurons and transduced therein by mitogen-activated protein kinase (MAPK) and other kinase signaling (Fig. 3). The kinase signaling eventually upregulates cAMP response element (CRE) promoter activity and induces several immediate early gene expressions including *Per1* and *Per2* (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). Previous studies showed that the peripheral clocks are entrained by non-photic signals such as humors (Schibler and Sassone-Corsi, 2002), temperature (Buhr et al., 2010; Isojima et al., 2009) and change of extracellular pH (Kon et al., 2008) through various kinase signaling. Intriguingly, sustained activation of the MAPK cascade is sufficient to trigger the induction of circadian gene expression in mammalian cultured cells, suggesting that the MAPK cascade is involved in the resetting of circadian gene expression in peripheral clocks (Akashi and Nishida, 2000). Collectively, protein kinase signals play a crucial role in the two defining properties of the circadian clocks, *i.e.* robustness and flexibility.

In the present study, I found that c-Jun N-terminal kinase (JNK) and Apoptosis signal-regulating kinase (ASK), both of which are members of MAPK family, are involved in the circadian clockwork. The phase and period of the circadian clocks is determined by these key kinases. From the perspective of cell signaling, the present findings expand the previously reported roles of MAPK signaling toward regulation of the circadian clock.

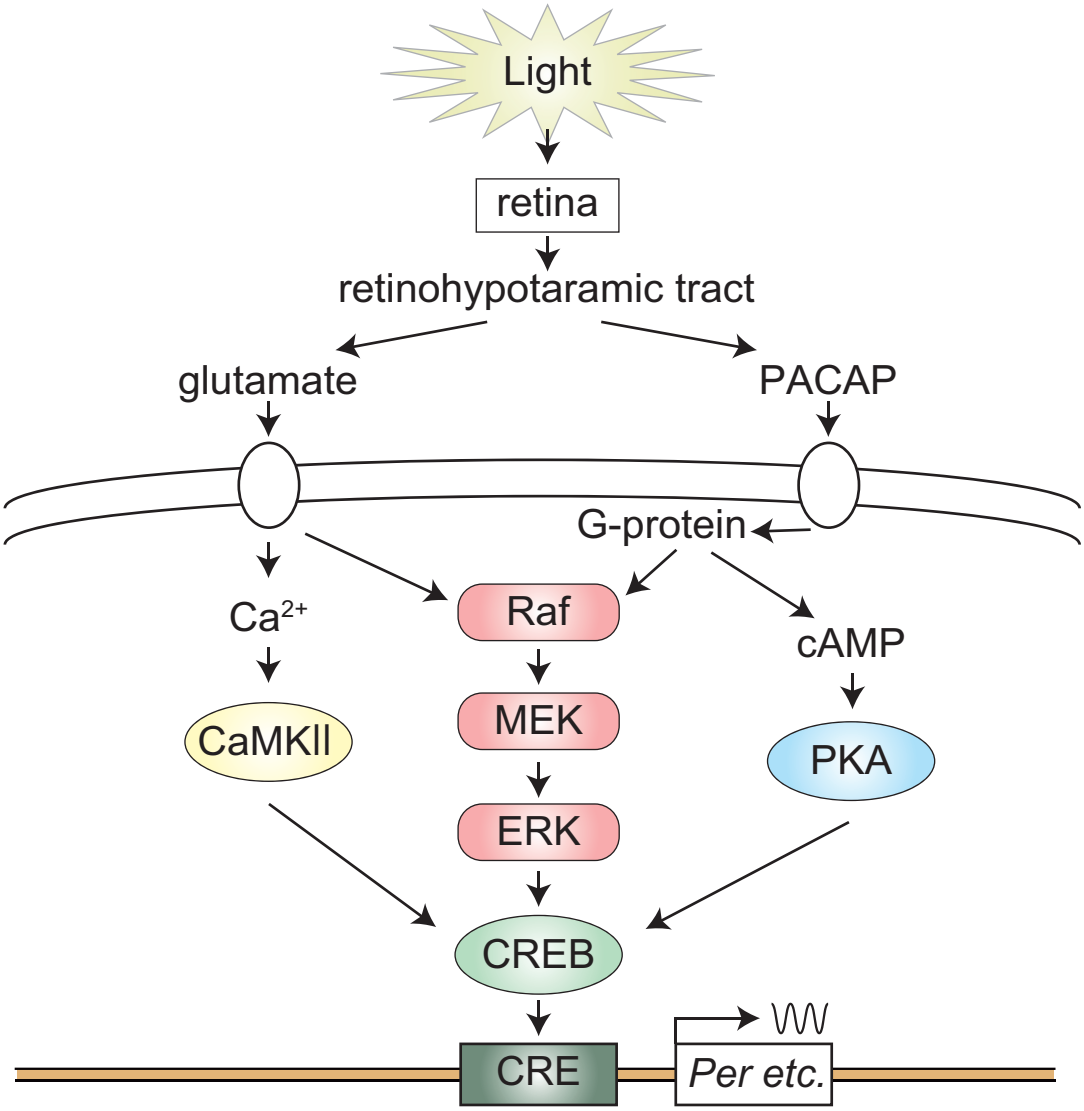

Figure 3. Photic input signal transduction pathways in the SCN neuron.
In light-mediated clock resetting, the neurotransmitters glutamate and pituitary adenylyl cyclase-activating polypeptide (PACAP) are released from the retinohypotharamic tract (RHT) terminal onto SCN neurons. In the SCN neuron, several kinase signaling such as the CaMKII, PKA and MAPK appear to play an important role in transducing the photic signal to the core oscillator. Finally, these kinase signals induce cAMP response element (CRE)-mediated gene expressions through activation of CRE-binding protein (CREB), which is responsible for the induction of the immediate early genes, including *Per1* and *Per2* clock genes.

Chapter 2.

JNK regulates phosphorylation of BMAL1 and circadian oscillation speed

2.1 Introduction

In the molecular oscillation of the circadian clock, the positive limbs of the feedback loop, BMAL1 and CLOCK, show no clear daily variations at the protein levels (Lee et al., 2001; Ueda et al., 2005; Yoshitane et al., 2009). On the other hand, the abundances and the phosphorylation states of the negative limbs of the feedback loop, PER and CRY proteins, show striking temporal changes (Kurabayashi et al., 2010; Lee et al., 2001; Ueda et al., 2005). Since the daily cycles of PER and CRY had been thought to guarantee the autonomous molecular oscillation with a stable period of ~24 h, the mechanisms of phosphorylation dependent degradation and localization in PER and CRY proteins had been well studied (Lee et al., 2001; Reppert and Weaver, 2002; Sato et al., 2006; Ueda et al., 2005). Thereafter, however, it was found that even if the protein level of CRY was artificially clamped, the molecular oscillation of the circadian clock did not arrest (Fan et al., 2007; Yamanaka et al., 2007). Moreover, it was reported that the molecular activity of the BMAL1-CLOCK heterodimer to bind to E-box-containing DNA probes exhibits circadian changes (Ripperger and Schibler, 2006). The previous studies in our laboratory showed that BMAL1and CLOCK are phosphorylated in a circadian manner and the phosphorylation regulates transactivation of the CLOCK-BMAL1 complex (Huang et al., 2012; Yoshitane et al., 2009). Collectively, even if the protein levels of CLOCK and BMAL1 are constant throughout the day, the circadian phosphorylation of CLOCK-BMAL1 regulates its transcriptional activity.

Molecularly, BMAL1 is phosphorylated by various kinases at the multiple residues. For example, it was reported that CKI directly interacts with and phosphorylates BMAL1 *in vitro* (Eide et al., 2002). CKII also phosphorylates BMAL1 and mutation of the conserved CKII-phosphorylation site in BMAL1, Ser90, results in impaired nuclear BMAL1 accumulation and disruption of the circadian clock function (Tamaru et al., 2009). The

phosphorylation of BMAL1 at Ser17 and Thr21 by GSK3 leads to ubiquitination of BMAL1 and its accompanying proteasome degradation (Sahar et al., 2010). In our laboratory, *in vitro* experiments demonstrated that extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinase (MAPK) family, phosphorylates chicken BMAL1 at Ser527, Thr534 and Ser599 (Sanada et al., 2002). Thereafter, an inhibitor screening revealed that treatment of NIH3T3 cells with SP600125, an inhibitor of c-Jun N-terminal kinase (JNK; Bennett et al., 2001), significantly decreased the phosphorylation levels of BMAL1, whereas no significant change in the phosphorylation was observed by treatment with U0126, an inhibitor of the ERK pathway (Yoshitane et al., 2012).

The three members of MAPK family members, *i.e.* ERK, p38 kinase and JNK, are all shown to be phosphorylated for activation not only in a circadian fashion but also in response to photic stimuli in the SCN of rodents (Nakaya et al., 2003; Obrietan et al., 1998; Pizzio et al., 2003). The inhibition of ERK phosphorylation (or activity) by using ERK kinase inhibitor attenuates the light-induced phase-shift in the central SCN clock (Butcher et al., 2002; Coogan and Piggins, 2003). ERK also has a role in circadian input pathways of peripheral clocks in mammals (Akashi and Nishida, 2000). Moreover, ERK phosphorylates several clock proteins to modulate their stability and function (Sanada et al., 2002, 2004). In contrast to the accumulating evidences for important roles of ERK in the mammalian clockwork, far less is known about the roles of JNK and p38, known as the stress-activated protein kinase (SAPK; Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999).

In the present study, I hypothesized that JNK kinases regulate the circadian clockwork through phosphorylation of BMAL1. RNAi-mediated depletion of JNK attenuated the phosphorylation levels of endogenous BMAL1 in NIH3T3 cells. Moreover, simultaneous knockdown of JNK isoforms by specific shRNAs lengthened the circadian period in NIH3T3 cells. Thus, my presented data strongly suggested that JNK has a regulatory role for the oscillation speed of the cellular clock by phosphorylating BMAL1.

2.2 Materials and Methods

2.2.1 Plasmid

Full-length mouse *Jnk1α1*, *Jnk2α1* and *Jnk3α1* cDNA were cloned from the total cDNA of the mouse SCN (C57BL/6J) by RT-PCR analysis with gene-specific primers. The primers used were: *Jnk1α1* Fw; 5' -GCGGC CGCAT GAGCA GAAGC AAACG TG- 3', *Jnk1α1* Rv; 5' -GCGGC CGCTC ATTGC TGCAC CTGTG C- 3', *Jnk2α1* Fw; 5' -GCGGC CGCAT GAGTG ACAGT AAAAG CGATG- 3', *Jnk2α1* Rv; 5' -GCGGC CGCTT ACTGC TGCAT CTGTG CTG- 3', *Jnk3α1* Fw; 5' -GCGGC CGCAT GAGCC TCCAT TTCTT ATAC- 3', *Jnk3α1* Rv; 5' -GCGGC CGCTC ACTGC TGCAC CTGTG C- 3'. Mammalian expression vectors for expression of Flag epitope-tagged JNK isoforms were generated by inserting the *Jnk* cDNAs into the pSG5 vector, with a slight modification to create NotI sites, a Kozak sequence, and a Flag epitope. For knockdown of JNK1 and JNK2, shRNA was designed using siDirect (http://sidirect2.rnai.jp/), a web-based software, and the following sequences were used: shJNK1; 5' -GAGAA CUAGU UCUUA UGAAG U- 3', shJNK2; 5' -GUAUA UUACU GUUUG GUAUG A- 3', and shJNK1/2; 5' –GGAAU AAAGU UAUUG AACAG C- 3'. The oligonucleotides to express the shRNA were inserted into the pBS-mU6 vector (modified by Dr. Kimiko Shimizu from pBS-hU6 vector).

2.2.2 Antibodies for immunoprecipitation and immunoblot analysis

Anti-CLOCK monoclonal antibody (mAb), CLNT1, was used for immunoprecipitation as described previously (Yoshitane et al., 2009). In immunoblot analysis, antibodies used were, BIBH2 anti-BMAL1 mAb (Yoshitane et al., 2009), anti-JNK2 (Millipore, #05-986) and anti-JNK1/3 (Santa Cruz Biotechnology, C17). The bound primary antibodies were detected by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Kirkegaard & Perry Laboratories).

2.2.3 Cell culture and transient transfection

NIH3T3 cells (RIKEN cell bank) were maintained at 37°C under 5% CO_2, 95% air in Dulbecco's modified Eagle's medium (Nissui) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. For detection of endogenous BMAL1 in NIH3T3 cells, the cells treated with 0.1 μM dexamethasone for 2 h were washed with PBS, and were solubilized in ice-cold IP buffer (20 mM HEPES-NaOH, 137 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM DTT, 2 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4; pH 7.8). The cell extracts were then centrifuged for 10 min, 21,600 xg, and the supernatant was subjected to immunoprecipitation with CLNT1 anti-CLOCK mAb (Yoshitane 2009) and Protein G-Sepharose (Amersham Biosciences). For transient transfection, the cells were plated in the wells of 12-well plates 24 h before the experiments, and were transiently transfected by using Lipofect AMINE PLUS Reagent (Invitrogen) according to manufacturer's directions. Cells were washed with PBS 36 h after the transfections, and solubilized in RIPA (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4; pH 8.0). The cell extracts were then centrifuged for 10 min, 21,600 xg, and the supernatant was subjected to immunobolotting.

2.2.4 Real time monitoring of circadian rhythms of cultured cells

Real-time monitoring of the cellular bioluminescence rhythms was performed as previously described (Kon et al., 2008) with minor modifications. Briefly, NIH3T3 cells were plated on 35-mm dishes (1.0×10^6 cells/dish) and were transiently transfected by *Bmal1*-luc/pGL4.12, a firefly luciferase reporter under regulation of the mouse *Bmal1* promoter region (nucleotides from -95 to +168; +1 is the transcription start site). After 24 h, the cells were treated with 0.1 μM (final) dexamethasone for 2 h, and then the media were replaced by a recording media

[phenol-red free Dulbecco's modified Eagle's medium (SIGMA) supplemented with 10% fetal bovine serum, 3.5 g/l glucose, 25 U/ml penicillin, 25 mg/ml streptomycin, 0.1 mM luciferin (Promega), and 10 mM HEPES-NaOH; pH 7.0]. The bioluminescence signals of the cultured cells were continuously recorded for 5-10 days at 37ºC in air with Dish Type Luminescencer, Kronos (ATTO, AB-2500) or LumiCycle (Actimetrics). The raw data of bioluminescence rhythms were detrended by subtracting their 24-h moving averages and were smoothed by their 2-h moving averages. The circadian period was calculated from 5 or more time points of peaks and troughs of the bioluminescence rhythms.

2.3. Results

2.3.1 Cloning of JNK isoforms

In mammals, JNK protein kinases are encoded by three genes; *Jnk1* and *Jnk2* are ubiquitously expressed in adult tissues, whereas *Jnk3* is predominantly expressed in the nervous system (Martin *et al*., 1996). Each gene produces four kinds of splice variants, α1, α2, β1 and β2, so that a total of 12 isoforms exist (Fig. 4A). Among the 12 isoforms, *Jnk1α1, Jnk2α1* and *Jnk3α1* were cloned from mouse SCN by RT-PCR because α1 isoforms are expressed in dominant, and the differences between the four splice variants in their function were considered relatively small (Gupta et al., 1996). Each cloned gene was constructed to the expression vector in which Flag-tag was inserted.

2.3.2 Design and characterize of shRNAs for knockdown of JNK isoforms

While SP 600125 is widely used as a JNK inhibitor (Bennett et al., 2001), this compound has a very wide range of targets and inhibits a number of protein kinases simultaneously (Bain et al., 2003). In other words, the phenotype observed by treatments with SP600125 in the circadian clockwork seems to be the effect of overlapping effects of inhibition of multiple kinases. I investigated a role of JNKs for the circadian clockwork by performing knockdown experiments in NIH3T3 cells, which express *Jnk1* and *Jnk2* but not *Jnk3* (Isojima et al., 2009; Yoshitane et al., 2012). *Jnk1* and *Jnk2* each produce four splice variants, for which I designed short hairpin RNA (shRNA) constructs targeting all the variants (termed shJNK1, shJNK2 and shJNK1/2; Fig. 4B).

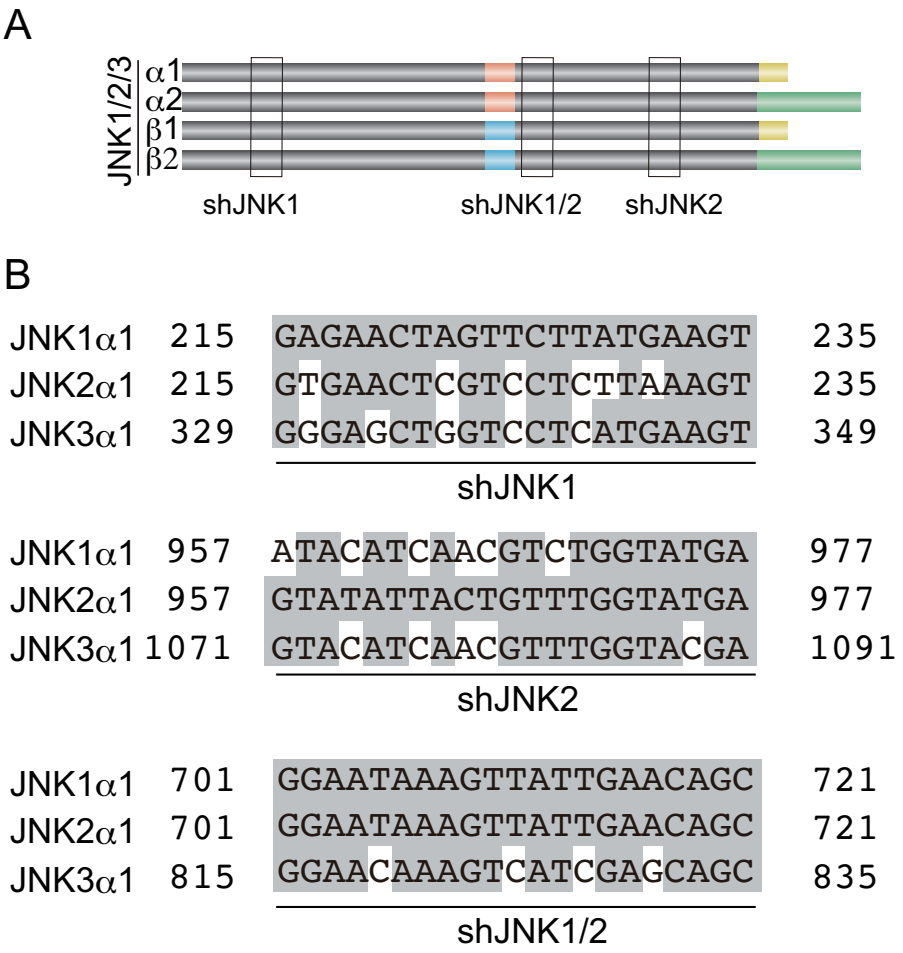

Figure 4. Design of shRNAs for knockdown of JNK isoforms.

(A) Schematic drawings of the JNK family isoforms. The regions used for the design of shRNAs are surrounded. The alternative splice sites are indicated by color bars. Each colors indicates differences in mRNA sequences. (B) Two shRNA constructs, shJNK1 and shJNK2, respectively, target 4 splice variants of JNK1 and JNK2, while shJNK1/2 targets all the 8 variants of JNK1 and JNK2. Nucleotide numbers starting from initiation codon are shown.

Expression level of transfected JNK1 in NIH3T3 cells was markedly reduced by coexpression of shJNK1 or shJNK1/2 (Fig. 5A, B). Similarly, JNK2 level was largely decreased by shJNK2 or shJNK1/2 (Fig. 5A, B). Moreover, each shRNAs also decreased the endogenous expression levels of JNK1 and JNK2 in accordance with their specificity in NIH3T3 cells (Fig. 5C).

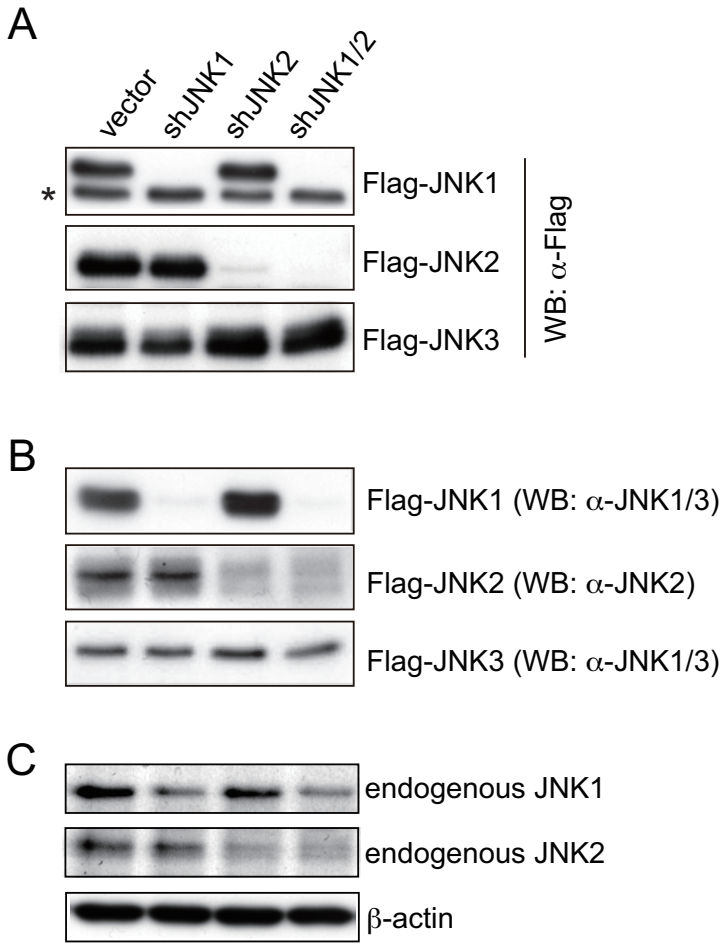

Figure 5. Evaluation of JNK knockdown by RNAi.
(A, B) NIH3T3 cells were transfected by Flag-JNK1α1/pSG5, Flag-JNK2α1, or Flag-JNK3α1 in combination with shJNK1/pBS-mU6, shJNK2, or shJNK1/2. For detection of JNKs, anti-Flag antibody (A) or anti-JNK antibodies (B) were used. Asterisk indicates non-specific band. A major band in panel B indicates JNK2α1 and the other minor bands indicate endogenous splice variants. (C) NIH3T3 cells were transfected with shJNK1/pBS-mU6, shJNK2, shJNK1/2 or the empty vector. The endogenous JNK1 and JNK2 were detected.

2.3.3 Knockdown of JNKs decrease the phosphorylation level of BMAL1

It is previously showed that phosphorylation level of endogenous BMAL1 is maximized 24 h after synchronizing the cellular rhythm by treatment with dexamethasone (Dex) in NIH 3T3 cells (Yoshitane et al., 2009). The cell extracts were subjected to co-immunoprecipitation 24 h after the Dex treatment to purify the BMAL1 protein as a CLOCK-BMAL1 complex, and then Western blot analysis were performed to investigate the phosphorylation level of BMAL1 (Fig. 6). Among the two visible bands of BMAL1 (black and white arrowheads), the slower-migrating species was identified as phosphorylated form of BMAL1, previously (Yoshitane et al., 2009). Importantly, transient transfection of shJNK1/2 significantly reduced not only endogenous protein levels of JNK1 and JNK2, but also phosphorylation levels of endogenous BMAL1 in NIH3T3 cells (Fig. 6). These data indicated that JNK regulates phosphorylation levels of endogenous BMAL1.

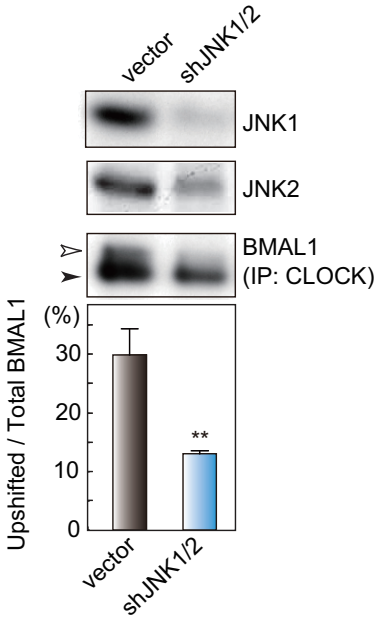

Figure 6. Effects of knockdown of JNK1 and JNK2 on BMAL1 phosphorylation. NIH3T3 cells were transfected with shJNK1/2 or the empty vector. After 48-h incubation, the cells were synchronized by 2-h dexamethasone (Dex) pulse. The cells were collected 24 h after the synchronization, and were subjected to immunoprecipitation (IP) and immunoblot analysis. The ration of upshifted band intensity (phosphorylated BMAL1) to total band intensity was quantified. Data are means with s.e.m. (n=4; **P<0.01, Student's *t*-test, versus empty vector)**.**

2.3.4 Knockdown of JNKs lengthens the circadian period

To investigate the role of JNK on the circadian oscillation, all 8 kinds of JNK isoforms expressed in NIH3T3 cells were knocked down by the shRNAs and the cellular rhythms were recorded. In order to monitor the circadian gene expression in living cells, NIH3T3 cells were transiently transfected by a firefly luciferase reporter under the regulation of *Bmal1* promoter region (-95 to +168; +1 is the transcription start site; Hirota et al., 2010; Kon et al., 2008). After synchronization with 0.1 μM dexamethasone (Dex) pulse, the bioluminescence rhythms were recorded continuously by a dish-type luminescencer (Fig 7A). I found significant lengthening of the circadian period when both JNK1 and JNK2 were concomitantly knocked down by shJNK1/2 (Fig. 7B). The circadian periods with and without shJNK1/2 were 22.15 ± 0.14 and 21.31 ± 0.16 h, respectively. The significant period lengthening was also observed by co-transfection of shJNK1 and shJNK2 (with and without shJNK1 and shJNK2 were 22.99 ± 0.28 and 20.68 ± 0.05 h, respectively; Fig. 7C). Notably, knockdown of JNK1 lengthened the period of cellular rhythm but knockdown of JNK2 did not (Fig. 7D), suggesting that the contribution of JNK1 in the circadian clockwork is larger than that of JNK2, at least in NIH3T3 cells. In fact, the relative expression level of *Jnk1* mRNA is ~10 fold higher than that of *Jnk2* in NIH3T3 cells (Yoshitane et al., 2012). These results demonstrate that JNK regulates the oscillation speeds of cellular clock.

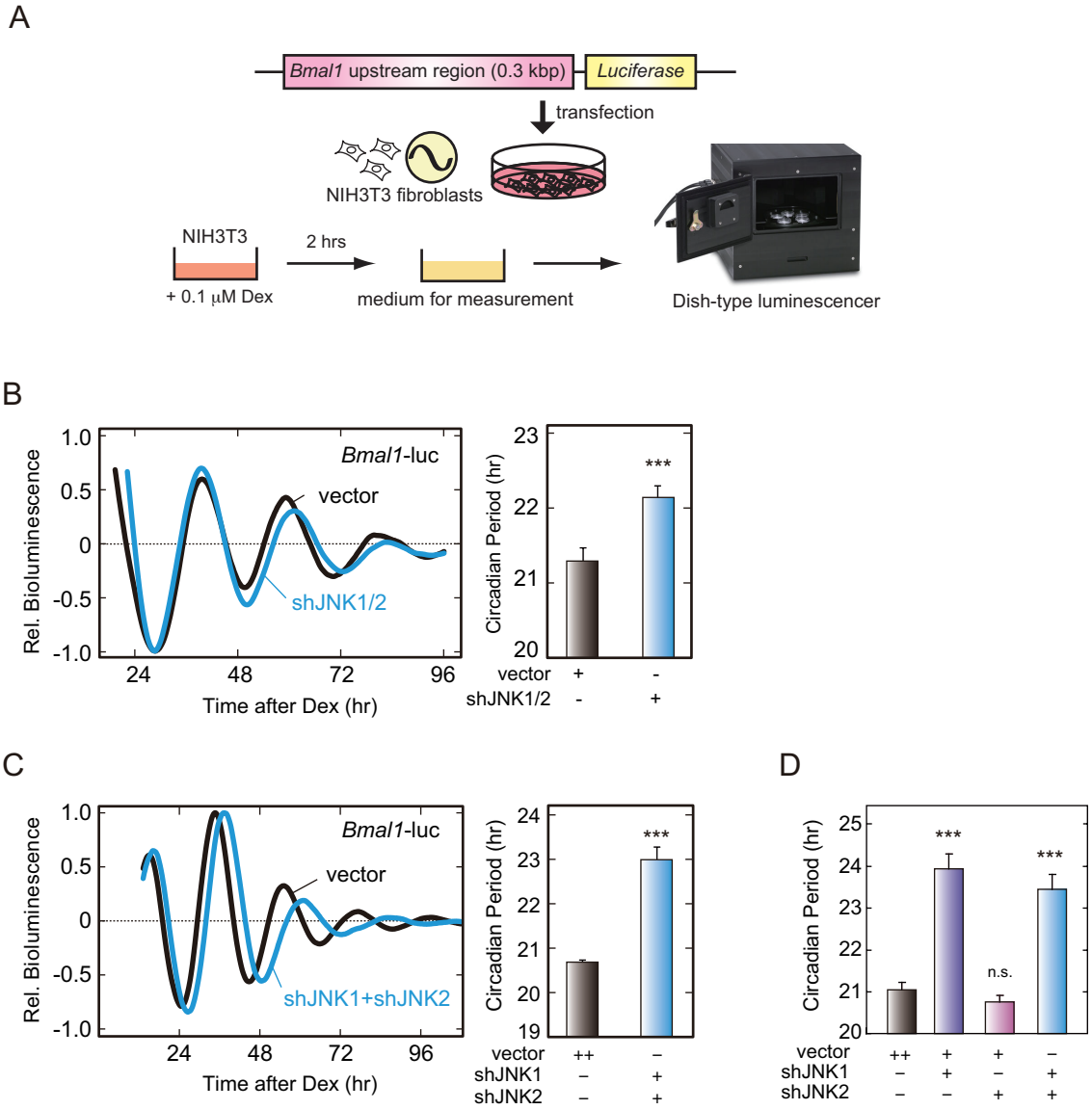

Figure 7. Effects of knockdown of JNK1 and JNK2 on cellular rhythms.

(A) After synchronization with 0.1 μM dexamethasone (Dex) pulse, circadian rhythms were recorded from the NIH3T3 cells transfected by BMAL1 *Bmal1*us0.3/pGL4.12 with the shRNAs. (B, C) The time point of the medium change to the recording medium was defined as time 0. Shown were the detrended curves of bioluminescence rhythms. (D) The effects of simultaneous knockdown of JNK1 and/or JNK2 on the circadian period of the NIH3T3 cells. (B-D) Data are means with s.e.m. (n=4; ***P<0.001, n.s. $p \geq 0.05$, Student's *t*-test, versus empty vector).

2.4. Discussion

In mammalian clock genes, the first identified mutation affecting the normal clock oscillation was *tau*, a mutation of Casein Kinase I (CKI) regulating the degradation rate of PER proteins (Gallego and Virshup, 2007; Ralph and Menaker, 1988). Physiologically, the importance of PER phosphorylation by CKI was emphasized by the identification of 21 phosphorylated residues of PER2, including Ser659 (Vanselow et al., 2006), which is mutated in patients suffering from familial advanced sleep phase syndrome (FASPS; Toh et al., 2001). When compared with CKI-catalyzed phosphorylation of PER proteins, little is known as to how phosphorylation of BMAL1 and CLOCK controls the E-box-dependent transcription.

In the present study, I paid special attention to JNK, because JNK inhibitor SP600125 treatment significantly reduced BMAL1 phosphorylation, and chronic application of SP600125 abrogated BMAL1 phosphorylation rhythms in cultured fibroblasts (Yoshitane et al., 2012). In RNAi-based studies in cultured cells, I showed JNK is involved in regulation of the phosphorylation level of BMAL1 protein (Fig. 6). Taken together with the collaborative data in which BMAL1 was directly phosphorylated by JNK *in vitro* (Yoshitane et al., 2012), it was showed that JNK is the novel circadian related kinase responsible for phosphorylation of BMAL1. Moreover, I revealed that JNKs regulate the oscillation speeds of the cellular rhythms (Fig. 7).

In the present study, however, whether or not the JNK-mediated phosphorylation of BMAL1 regulates the circadian oscillation speed remains to be proved. The causal relationship between BMAL1 phosphorylation and regulation of the circadian oscillation speed is still obscure. The definition of the JNK target residue(s) of BMAL1 is the key to reveal the causal relationship. *In vitro* experiments previously demonstrated that ERK2 phosphorylates chicken BMAL1 at Ser527, Thr534 and Ser599 (Sanada et al., 2002). The three residues are conserved among BMAL1 proteins of other species; *e.g.*, Ser520, Thr527 and Ser592 in mouse BMAL1. It should be emphasized that JNK1-dependent

phosphorylation of BMAL1 was significantly attenuated when the three phosphorylatable residues in BMAL1 were all mutated to Ala, indicating that the three residues are candidate target of JNK (Yoshitane et al., 2012).

It was reported independently of the present study that JNK interacts with PER2 and that its activity increases PER2 phosphorylation (Uchida et al., 2012). The phosphorylation of PER2 by JNK has a significant effect on PER2 protein levels and stability. An expression of a hyperactive JNK in mouse embryonic fibroblasts (MEFs) decreases the ubiquitination level of PER2, indicating that JNK activity increases PER2 protein stability by reducing its degradation *via* the proteasome (Uchida et al., 2012). PER2 stabilization eventually resulted in an extended period of the cellular rhythm (Uchida et al., 2012), in accord with my present data.

There are almost no data demonstrating a role for p38 kinase in mammalian circadian clock, but in chick pineal, *Neurospora* and *Drosophira*, the roles of p38 were reported (Dusik et al., 2014; Hayashi et al., 2003; Vitalini et al., 2007). In the chick pineal gland, chronic treatment with the SB203580, an inhibitor of p38 kinase, lengthened the endogenous period of melatonin secretion rhythms (Hayashi et al., 2003). A lengthening of the period of the clock gene rhythms was also described in U2OS cells and C6 mouse glioblastoma cells after treatment with SB203580 (Yagita et al., 2009). Similar to the JNK, p38 may phosphorylate clock protein(s), thereby altering the oscillation speed of the molecular oscillator, although no specific interactions between p38 and clock proteins have been described (Goldsmith and Bell-Pedersen, 2013). It is possible that that the increase in period is due to simply off target effects of the SB203580. It is necessary to perform a specific knockdown experiment as in the present study to investigate the contribution of p38 kinase in the circadian clockwork.

Among the three *Jnk* genes, *Jnk1* and *Jnk2* are ubiquitously expressed throughout the body and mice lacking both *Jnk1* and *Jnk2* are embryonic lethal. On the other hand, *Jnk3* is expressed exclusively in the nervous system (Martin *et al.*, 1996), and *Jnk3*-null mice are

fertile and show no apparent abnormality in their development (Yang et al., 1997). In previous study, it was found that *Jnk3* transcript is rhythmically expressed in the SCN (Yoshitane et al., 2012). The *Jnk3*-deficient mice showed significantly longer free-running period of the locomotor rhythm in constant dark condition when compared with that of wild type mice (Yoshitane et al., 2012) , indicating that JNK3 plays an important role for normal oscillation of the circadian clock in the SCN. These results are in line with the present my data of cultured cells (Fig. 7). The peak phase of the BMAL1 phosphorylation level and activity (phosphorylation) level of JNK are match at ZT (zeitgeber time; ZT 0 is lights on) 12 in mice SCN (Pizzio et al., 2003; Yoshitane et al., 2012). Moreover, the BMAL1 phosphorylation levels were lower in the *Jnk3*-deficient SCN than WT (Yoshitane et al., 2012). Collectively, it is most probable that JNKs have a main regulatory role for the oscillation speed of the cellular clock by phosphorylating circadian component(s) including BMAL1 (Fig. 8).

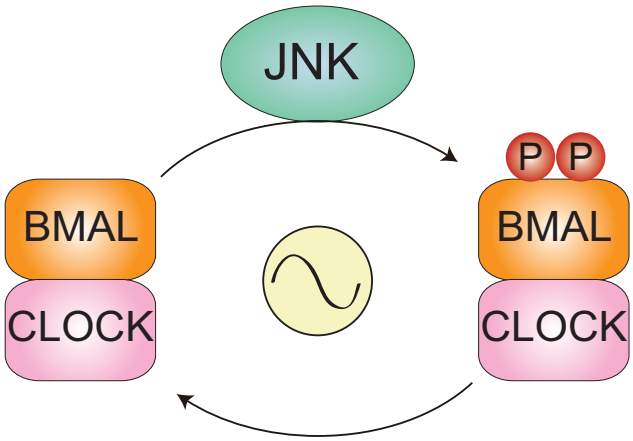

Figure 8. A model for roles of JNK in the circadian clockwork.
In the mammalian circadian clockwork, BMAL1 is phosphorylated by JNK, and its phosphorylation cycle should be important for the molecular oscillation such as *Per* transcription rhythms.

Chapter 3.

ASK is a key mediator for stress response of circadian clock

3.1. Introduction

To be adapted to the changes in environmental conditions, almost all organisms are equipped with various intracellular signal transduction systems, which sense multiple stressors from the external and internal environments and evoke a wide variety of cellular stress responses, such as DNA repair, cell cycle arrest, cytokine production and apoptosis. The mitogen-activated protein kinase (MAPK) cascades are one of the major stress-responsive signaling, which are evolutionally conserved in all eukaryotic cells (Kyriakis and Avruch, 2012; Saito and Tatebayashi, 2004; Tibbles and Woodgett, 1999). Three main MAPK cascades that converge on ERK, JNK and p38 MAPKs are highly conserved in eukaryotes and have been well characterized (Goldsmith and Bell-Pedersen, 2013; Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999). Each cascade consists of three tiers of protein kinases: MAPK, MAPK kinase (MAP2K) and MAP2K kinase (MAP3K). MAP3K phosphorylates and thereby activates MAP2K, and activated MAP2K in turn phosphorylates and activates MAPK. Among them, JNK and p38 kinase are known as the stress-responsive protein kinases (SAPK) and serve as potent regulators of cellular functions in response to a wide variety of environmental stressors, such as ultraviolet (UV) radiation, heat shock and osmotic shock (Goldsmith and Bell-Pedersen, 2013; Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999; Fig. 9)

As shown in the previous chapter, I identified JNK as a new clock-related kinase, which regulates the circadian clock. In the process of the study to investigate the physiological role(s) of JNK in the circadian clockwork, we treated cultured cells with hyperosmotic stress in order to activate endogenous JNK. The chronic hyperosmotic stimulation to cultured cells prolonged the cellular circadian period (unpublished data). On the other hand, transient hyperosmotic stimulation to the cultured cells reset the cellular circadian rhythms

(unpublished data). Moreover, it was revealed that not only osmotic stress but also various other environmental stimuli such as heat shock (Buhr et al., 2010), UV irradiation (Papp et al., 2015), influx of cationic ion (Feeney et al., 2016) and change of the extracellular pH (Kon et al., 2008), perturb the cellular clock. These accumulating data strongly suggest that cellular stress responses regulate the circadian clock *via* phosphorylation signals such as SAPK cascade. Both the cellular stress response and the circadian clock system are the most fundamental physiological functions, but it is obscure (i) whether the circadian clock responds to cellular stress through protein kinase signaling and, if so, (ii) what is the key molecule for transmitting the cellular stress to the circadian clockwork.

Extrinsic stressors disturbing cellular homeostasis are classified into the following three types; physical factors including temperature, light or UV radiation; chemical factors such as flux of ion or hypoxia, and biological factors like infection with bacteria, virus or parasite (Kourtis and Tavernarakis, 2011). The SAPK cascade and circadian clock system are both responsive to not only these extrinsic stimuli but also intrinsic stimuli. For example, intracellularly generated reactive oxygen species (ROS) or inflammatory cytokines activate SAPK cascade (Goldsmith and Bell-Pedersen, 2013; Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999). On the other hand, the levels of intracellular redox cofactor, nicotinamide adenine dinucleotide (NAD^+) regulate the circadian clockwork (Nakahata et al., 2009; Peek et al., 2013; Ramsey et al., 2009). The intrinsic NAD^+ levels cycle with a 24-h rhythm (Nakahata et al., 2009; Peek et al., 2013; Ramsey et al., 2009), and other redox-related genes or metabolites also fluctuate in a circadian manner (Edgar et al., 2012; Hirayama et al., 2007; Peek et al., 2013). Surprisingly, even in red blood cells, which have no nucleus and therefore cannot perform transcription, the Peroxiredoxin (Prx; known as antioxidant enzyme coupled with Trx) shows circadian redox rhythms (Edgar et al., 2012; O'Neill and Reddy, 2011). These accumulating data lead to an interesting idea that intracellular circadian cycle of the redox-related stressors counterbalances the molecular circadian oscillation,

transcription/translation feedback loops (TTFLs), through stress-activated signal transductions.

Here I showed that chronic hyper- and hypo-osmotic changes of the culture media decelerate and accelerate the speed of circadian oscillation, respectively, in the real-time monitoring system of cellular rhythms. I focused on the Apoptosis Signal-regulating Kinase (ASK), a member of MAP3K (Kawarazaki et al., 2014; Takeda et al., 2008), as a key mediator for determining the circadian period in response to osmotic changes of the media, because ASK is a unique bidirectional responder to osmotic stress, *i.e.* activated by hyposmotic stress and inactivated by hyperosmotic stress respectively. The bidirectional effects of osmolarity on oscillation speed were completely blocked in cells of *Ask1* (Tobiume et al., 2001), *Ask2* (Iriyama et al., 2009) and *Ask3* (Naguro et al., 2012) triple-KO. Moreover, acute osmotic changes resulted in type-0 resetting of the cellular rhythms, which was again severely diminished by the *Ask*-TKO. Physiological impact of ASK signaling was shown in wheel-running activity rhythms of *Ask*-TKO mice, which exhibited compromised phase-shifts in response to a light pulse and showed shorter periodicity of locomotor rhythms in constant light. Moreover, *Ask* is rhythmically expressed in mice and is essential for the clock to respond to change of the intracellular redox states. These findings identify cellular stress response *via* ASK as a mechanism for circadian flexibility. Together with previous reports on circadian oscillation of intracellular redox state (Edgar et al., 2012; Nakahata et al., 2009; O'Neill and Reddy, 2011; Peek et al., 2013; Ramsey et al., 2009), the present results now raise the possibility that ASK is a molecular link between TTFLs and intracellular redox rhythms.

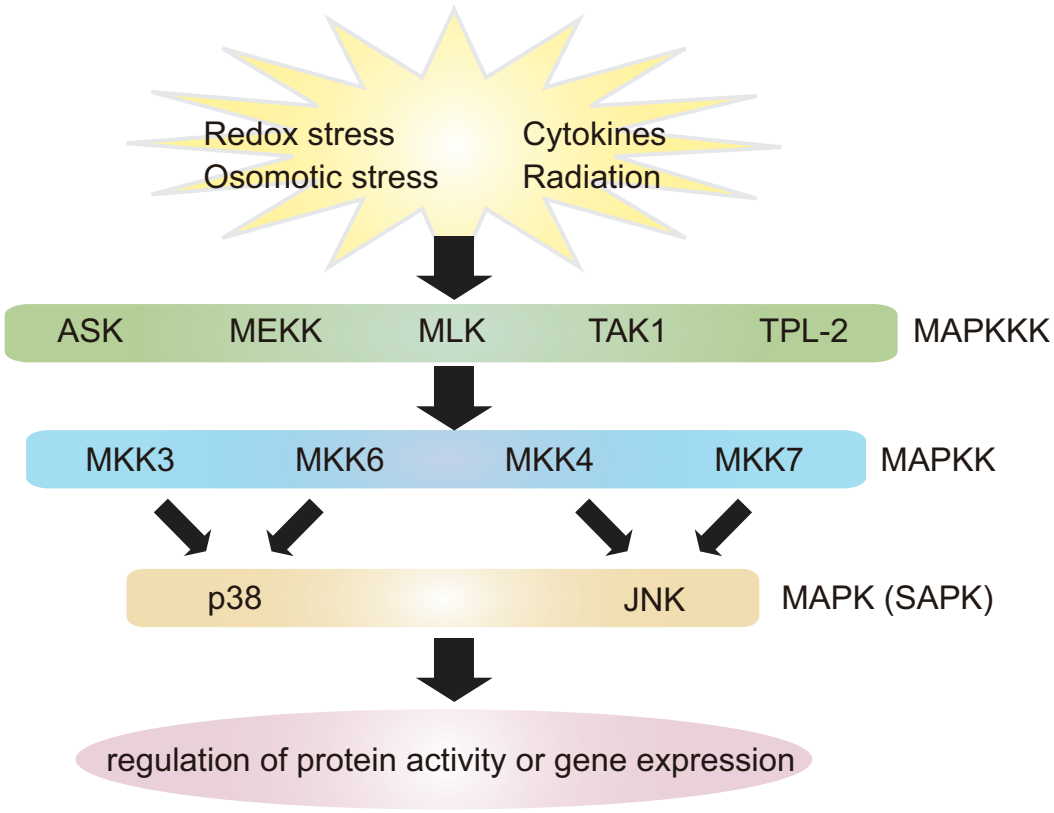

Figure 9. The hierarchy chart of the mammalian SAPK signaling pathway.
When MAP3Ks are activated in response to a particular stress, *e.g.,* redox stress, osmotic stress, they phosphorylate MAP2Ks, which in turn, activate MAPKs. ASK: Apoptosis signal-regulating kinase, MEKK: MAPK/extracellular protein kinase kinases, MLK: mixed-lineage kinase, TAK1: TGF-β-activated kinase1, TPL-2: Tumor Progression Locus 2, MKK: MAPK kinase, JNK: c-Jun N-terminal kinase.

3.2. Materials and Methods

3.2.1 Animals

Mice used in this study (C57BL/6 background) were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals at The University of Tokyo. Mice were reared in the compartments where the environmental conditions were controlled (room temperature 23 ± 1 °C). Animals had free access to commercial chow (CLEA Japan, Inc.) and tap water available *ad libitum*. *Ask*-TKO mice were generated by crossing *Ask1*-KO (Tobiume et al., 2001), *Ask2*-KO (Iriyama et al., 2009) and *Ask3*-KO (Naguro et al., 2012) mice. The *Ask*-TKO mice were bred with PER2::Luc knock-in mice (Yoo et al., 2004), and *Ask*-TKO/PER2::Luc MEF lines were prepared as previously described (Hirano et al., 2013).

3.2.2 Cell culture and real time monitoring assay

MEFs from PER2::Luc mice (Yoo et al., 2004), NIH3T3 cells (RIKEN cell bank) and U2OS cells (ATCC) were maintained at 37°C under 5% CO_2, 95% air in Dulbecco's modified Eagle's medium (SIGMA or Wako) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Real-time monitoring of the cellular bioluminescence rhythms was performed as previously described (Kon et al., 2008) with minor modifications. Briefly, PER2::Luc MEFs were plated on 35-mm dishes (1.0×10^6 cells/dish) or 24-well plates (2.5×10^5 cells/well) and cultured at 37°C under 5% CO_2. After 24 h, the cells were treated with 0.1 μM (final) dexamethasone for 2 h, and then the medium was replaced by a recording medium [phenol-red free Dulbecco's modified Eagle's medium (SIGMA) supplemented with 10% fetal bovine serum, 3.5 g/l glucose, 25 U/ml penicillin, 25 mg/ml streptomycin, 0.1 mM luciferin (Promega), and 10 mM HEPES-NaOH; pH 7.0]. The bioluminescence signals of the cultured cells were continuously recorded for 5-10 days at 37ºC in air with Dish Type Luminescencer, Kronos (ATTO, AB-2500 or AB-2550), LumiCycle (Actimetrics), or CL24A (CHURITHU). NIH3T3 cells that stably expressed the

Bmal1-luc reporter (Yoshitane et al., 2012) were used in Figure 11. U2OS cells plated on 24-well plates were transiently transfected by *Bmal1*-luc/pGL4.12, a firefly luciferase reporter under regulation of the mouse *Bmal1* promoter region (nucleotides from -95 to +168; +1 is the transcription start site; Figure 10, 11C, 13A and 13C). For transient transfection, the cells were plated in 24-well plates 24 h before the experiments, and were transiently transfected by using Lipofectamine 3000 Reagent (Invitrogen) according to manufacturer's protocol. The raw data of bioluminescence rhythms were detrended by subtracting their 24-h moving averages and were smoothed by their 2-h moving averages. The circadian period was calculated from 5 or more time points of peaks and troughs of the bioluminescence rhythms.

3.2.3 Osmotic stress treatments

The extracellular osmolality was measured by OSMOMAT 030 (Asahi Life Science). For chronic hyperosmotic stimuli, sorbitol (final 200 mM) was added to the cultured media. For pulse hyperosmotic stimuli, the cultured media (2.5 ml) were removed and 0.88 ml of the used media were rapidly returned back to the original wells after mixing with 0.12 ml of 5 M sorbitol in fresh media (final 600 mM). After 30-min incubation, the hypertonic media were replaced by the residual used media that had been kept at 37°C (see also Fig. 15). For chronic and acute hyposmotic stimuli, the cultured media were diluted by mixing with distilled water (see also Fig. 17).

3.2.4 Antibodies, Immunoprecipitation and immunoblot analysis

Anti-CLOCK monoclonal antibody (mAb), CLNT1, was used for immunoprecipitation as described previously(Yoshitane et al., 2009). In immunoblot analysis, antibodies used were CLSP3 anti-CLOCK mAb(Yoshitane et al., 2009), BIBH2 anti-BMAL1 mAb (Yoshitane et al., 2009), anti-E4BP4 (MBL), anti-PER2 (ADI), anti-REV-ERBα (MBL), anti-DBP (MBL),

anti-CRY1 (MBL), and anti-TBP (SantaCruz). The bound primary antibodies were detected by horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-guinea pig IgG antibody (Kirkegaard & Perry Laboratories).

3.2.5 Preparation of nuclear proteins

The nuclear proteins and cytoplasmic proteins were prepared as described (Yoshitane et al., 2009). Briefly, mouse tissue (1 g wet weight) was washed with ice-cold PBS and homogenized at 4°C with 9 ml of ice-cold buffer A composed of 10 mM HEPES-NaOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fruoride (PMSF), 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4. The homogenate was centrifuged twice (5 min each, 700 xg), and the resultant precipitate was resuspended in 2 ml of ice-cold buffer C composed of 20 mM HEPES-NaOH (pH 7.8), 400 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4. After being gently mixed at 4°C for 30 min, the suspension was centrifuged (30 min each, 21,600 xg) twice, and the final supernatant was used as “nuclear extract”.

3.2.6 Quantitative real time RT-PCR

Total RNA was prepared from the mouse liver or MEFs by using TRIzol reagent (Invitrogen) and RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. RT-PCR analyses were performed as previously described (Yoshitane et al., 2014). Total RNA was reverse transcribed by Go Script Reverse Transcriptase (Promega) with both anchored (dT)15 primer and random oligo primer, and the reaction mixture was treated with RNase H (TaKaRa). The cDNA was subjected to real-time PCR (Applied Biosystems, StepOnePlus™ Real-Time PCR Systems) by using GoTaq master mix (Promega) with the following gene-specific primers. For quantitative real time RT-PCR, the following sequences were used. For *Per1*

5’-CTGAC AAGCT GCTGA AGTGG-3’ and 5’-GGTAG AGCTG AGAAC ACCTG G-3’; for *Per2* 5’-GCTCA CTGCC AGAAC TATCT CC-3’ and 5’-CCTCT AGCTG AAGCA GGTTA AG-3’; for *Dec1* 5’-CTATC TCATC CCACC ATCGG-3’ and 5’-GAATC TTCTC TGTGG GTCTG C-3’; for *Dec2* 5’-AGGGA GGAAG AGTAA GAGAT GC-3’ and 5’-GGAAG GGAGT GTCAA AGGGT-3’; for *Cry1* 5’-TCCAG CGACA GAGCA GTAAC-3’ and 5’-AACAC AGACT GTCCA CGCAG-3’; for *Cry2* 5’-GCATC ATTGG CGTGG ACTAC-3’ and 5’-CCACA GGGTG ACTGA GGTCT-3’; for *Dbp* 5’-CCAAT CATGA AGAAG GCAAG G-3’ and 5’-AGGAT TGTGT TGATG GAGGC-3’; for *E4bp4* 5’-ATCGG AACAC TGGCA TCAC-3’ and 5’-TATCT GACTA CACGC CAGGC-3’; for *Rev-erb* α 5’-AGAAT GTTCT GCTGG CATGT C-3’ and 5’-TTGAG CTTCT CGCTG AAGTC-3’; for *Rev-erb* β 5’-TGAGC AAGTC TCCAT ATGTG G-3’ and 5’-AGGCC TCATT TGGAT GGTT-3’; for *Bmal1* 5’-TGGTA CCAAC ATGCA ATGC-3’ and 5’-AGTGT CCGAG GAAGA TAGCTG-3’; for *Clock* 5’-CAAGG TCAGC AACTT GTGAC C-3’ and 5’-AGGAT GAGCT GTGTC GAAGG-3’; for *Rps29* 5’-TGAAG GCAAG ATGGG TCAC-3’ and 5’-GCACA TGTTC AGCCC GTATT-3’; for *Ask1* 5’-CGTGC TGGAC CGTTT TTAC-3’ and 5’-TCTCG CACTC CAAGA TGGTA -3’; for *Ask2* 5’-CTGCT GAGCC CTGAC ATTG-3’ and 5’-ATGAT GGCCG AGTAG TCCTG-3’; for *Ask3* 5’-GAAAT CCCAG AGAGA GATAT CAGG-3’ and 5’-TGTTT GAGAT ACTTG TGCAG AGC-3’.

3.2.7 Recording of mice behavioral rhythms

Behavioral rhythms of mice were recorded as previously described (Terajima et al., 2016), with minor modifications. Briefly, 8- to 16-week-old male mice were individually housed in a polycarbonate cage equipped with a running wheel. The animals were maintained in a light-tight chamber and were entrained to the 12-h light/12-h dark (LD) cycles for at least 2 weeks. Responses of the mouse circadian clock to single light pulses were examined after

releasing it into the constant dark (DD) conditions for 14 days or longer. Single light pulses (30 min, ~400 lux) were given every 2 to 3 weeks at two different circadian times in subjective night phase (CT22 and 14) where the activity onset was designated as CT12. Responses of the circadian clock to different light intensities in LL condition were examined for 21 days or longer after monitoring the behavioral rhythm in DD for 14 days or longer. Fluorescent lamps were used in Figure 24-27 (~400 lux). Light-emitting diodes (Nichia Corp. NS2W157AR-H3) were used to coordinate light intensity exactly in Figure 29. The locomotor activity rhythms of mice were measured by wheel revolutions in 5-min bins and analyzed by using ClockLab software (Actimetrics). The circadian periods were analyzed by chi-square periodogram with the p value < 0.001, based on the locomotor activity in 14 days interval taken 7 days after the start of each condition.

3.3 Results

3.3.1 Osmotic stress regulate circadian oscillation speed bidirectionally

Mammalian cultured cells, such as mouse embryonic fibroblasts (MEFs), are useful model in studying oscillation mechanism of peripheral clocks. In order to monitor circadian oscillation of clock protein in cultured cells, MEFs had been isolated from PER2::Luc knock-in mice (Yoo et al., 2004) in our laboratory. While I performed the loss-of-function experiments and demonstrated that JNK regulates the oscillation speed of cellular rhythm in chapter 2 above, it was concurrently revealed that JNK gain-of-function *via* chronic hyperosmotic treatments lengthened the period of PER2::Luc MEFs, in our laboratory (unpublished). In fact, the lengthening of the period was also observed in U2OS cells treated with chronic 200 mM (final concentration) sorbitol (Fig. 10). The same effect on the period *via* hyperosmolality was ascertained by the other observations that the period of cellular rhythm in PER2::Luc MEFs gradually lengthened day by day during long-term cell culturing: *e.g.,* from 23.5 ± 0.3 h on day 1 to 25.5 ± 0.3 h on day 3 (Fig. 11A). The gradual lengthening effects of the period was also observed in mouse-derived NIH3T3 cells stably expressing *Bmal1*-luc, and human-derived U2OS cells transiently expressing *Bmal1*-luc (Fig. 11B, C), suggesting the underlying mechanism is common across mammalian cell strains. In the previous study, it was reported that alkalization of extracellular pH lengthened the period of cellular rhythms and specifically induced *Dec1*, a clock-related gene, through activation of transforming growth factor-β signaling (Kon et al., 2008). I validated the possibility that extracellular pH was gradually changed in the culture condition, but pH of the medium at Day 7 was almost the same as that of fresh medium under our experimental condition (Fig. 12A, B). On the other hand, the osmolality of the media increased even though the dishes were sealed with parafilm in inverse proportion to the volume of cultured media (Fig. 12B, C). When the media at day 7 were collected and reused for monitoring newly prepared cellular rhythms, the circadian period (25.1 ± 0.1 h) was significantly

lengthened as compared with that recorded in the fresh media (23.6 ± 0.1 h). The effect on the period of the reused media was abolished when the reused media were diluted with distilled water to isotonic (Fig. 12B). The results eliminate the possibility that the other factors, *e.g.* secretions from cells, glucose concentration and serum concentration, rather than osmolality lengthen the cellular period. Interestingly, the circadian period was shortened when MEFs and U2OS cells were chronically exposed to hypotonic media (Fig. 13A, B), even though the hyposmotic stress also is known to activate JNK similarly to hyperosmotic stress (Zhou et al., 2016). The cellular circadian period was unaltered between mock treatment with isotonic NaCl (140 mM) and the fresh media (data not shown). These data indicating that hyper- and hyposmotic stress regulate the oscillation speed of cellular clocks in a bidirectional manner respectively.

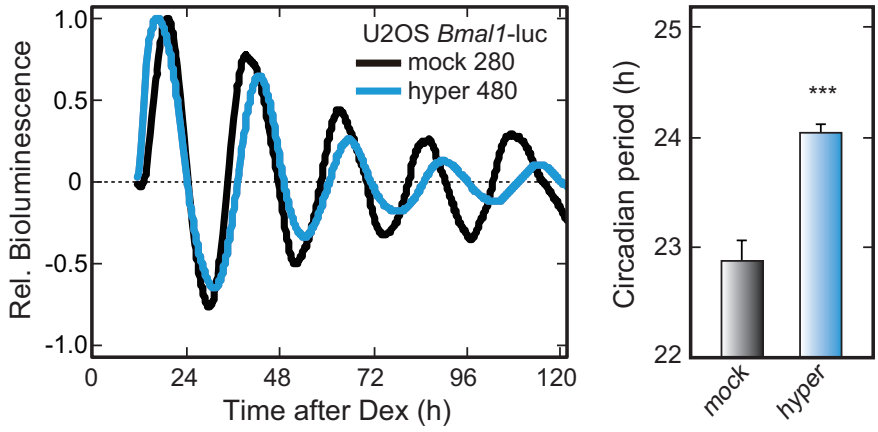

Figure 10. Effects of chronic hyperosmotic treatments on the periods of the cellular rhythms

Cellular rhythms were recorded from U2OS cells transiently expressing *Bmal1*-luc. For hyperosmotic treatment, 200 mM sorbitol was added to the recording media ~280 mOsm to give the final osmolality of ~480 mOsm (blue). For mock treatment, the same volume of recording media was added (black; n=4; Student's *t*-test, A-C)

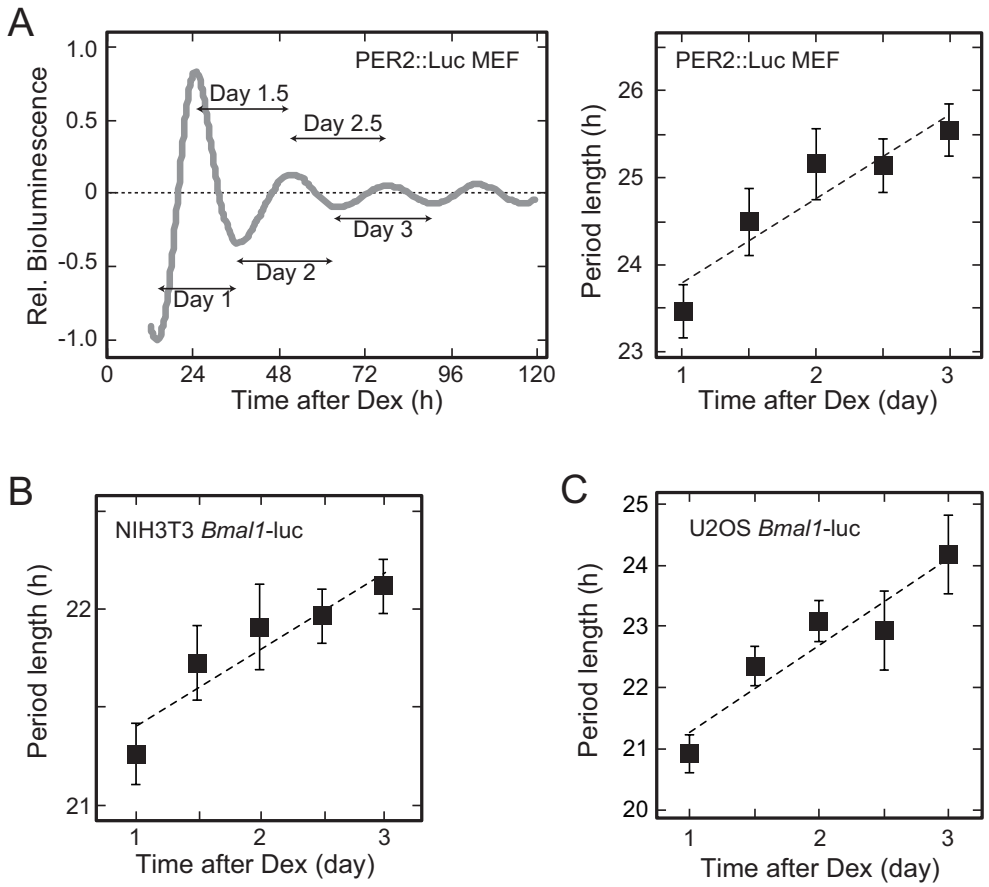

Figure 11. Effects of long-term culturing on the periods of the cellular rhythms.

(A) The change of the circadian period length in PER2::Luc MEFs during several days. The period length was calculated as time between trough (or peak) of the day and the next trough (or peak) as shown in the left panel (n=22). (B**)** The change of the circadian period length in NIH3T3 cells (stably expressing *Bmal1*-luc) during several days. The period length was calculated as in panel A (n=40). (C) The change of the circadian period length in U2OS cells (transiently expressing *Bmal1*-luc) during several days. The period length was calculated as in panel A (n=6). Data are means with s.e.m.,

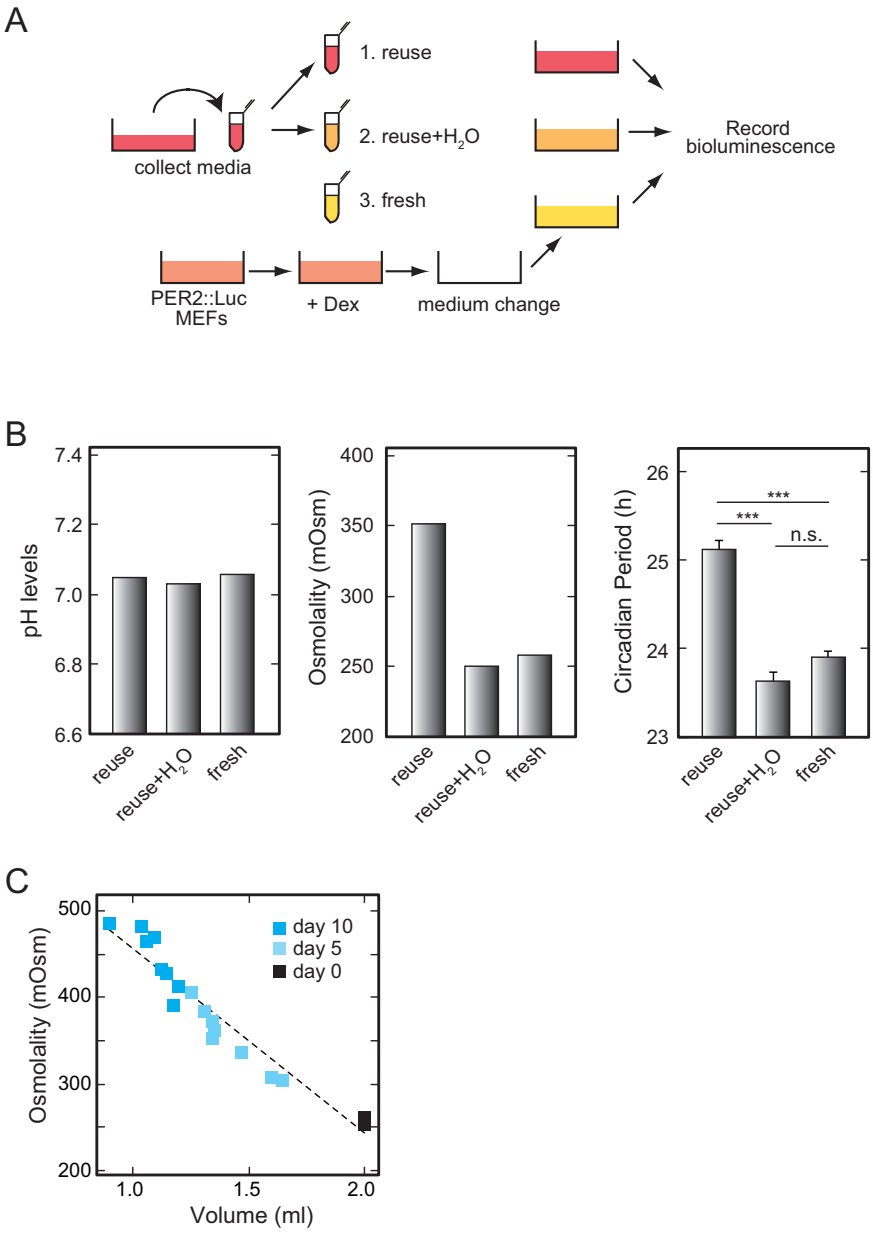

Figure 12. The periods of the cellular rhythms when the cultured media were reused. (A) The illustrated scheme of the experiment to test the period-lengthening effect of the reused media. The used media were obtained from cultures of PER2::Luc MEFs at day 7 (reuse). The reused media were diluted to isotonicity by adding distilled water (reuse+H_2O). (B) The effect of the reused media on the circadian period of the cellular rhythms. The measured pH (left panel) and measured osmolality (middle) of the indicated media were shown. (C) The measured volume and osmolality of the media recovered from the culture of PER2::Luc MEFs at day 0 (n=4), day 5 (n=8) and day 10 (n=8). Data are means with s.e.m., ***$p < 0.01$, n.s. $p \geq 0.05$ (n=8, Student's *t*-test).

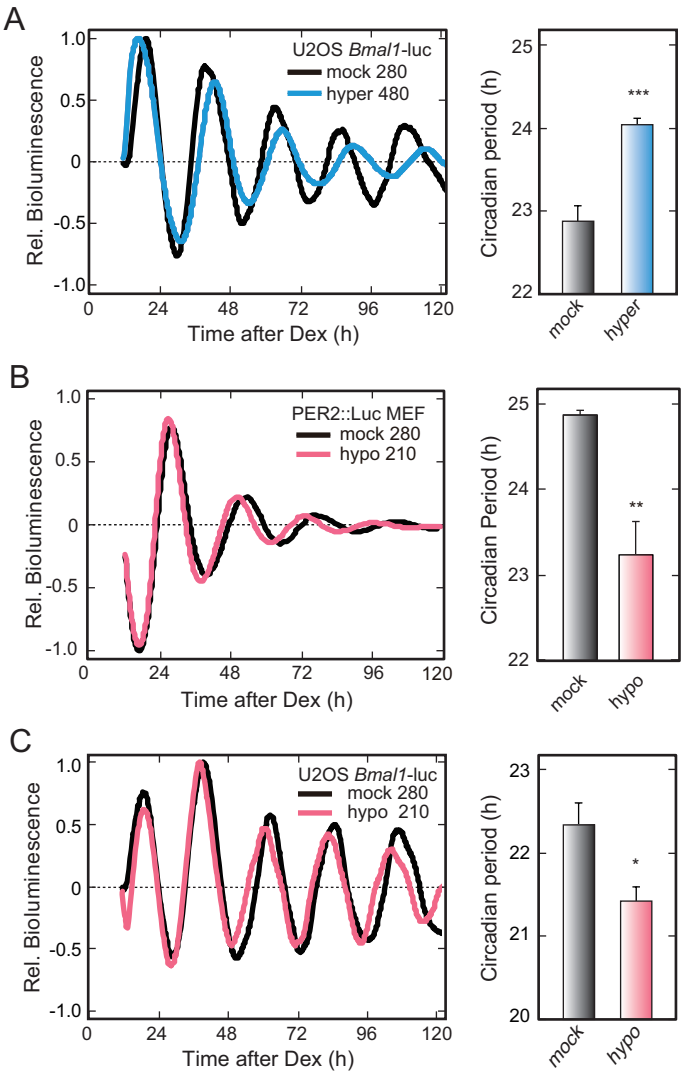

Figure 13. Effects of chronic osmotic treatments on the periods of the cellular rhythms
(A) Cellular rhythms were recorded from U2OS cells transiently expressing *Bmal1*-luc. For hyperosmotic treatment, 200 mM sorbitol was added to the recording media ~280 mOsm to give the final osmolality of ~480 mOsm (blue). For mock treatment, the same volume of recording media was added (black). (B) Cellular rhythms were recorded from PER2::Luc MEFs. For the hyposmotic treatment, the recording media were diluted by distilled water to give the final osmolality of ~210 mOsm (red). For mock treatment, the recording media were diluted to isotonicity (~280 mOsm, black) by the same volume of 140 mM NaCl solution. (C) Cellular rhythms were recorded from U2OS cells transiently expressing *Bmal1*-luc. For hypotonic treatment, the recording media were diluted to give the final osmolality of ~210 mOsm by distilled water (red). For mock treatment, the same volume of 140 mM NaCl solution was added to isotonicity (~280 mOsm, black). Data are means with s.e.m., $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s. $p \geq 0.05$ *versus* mock treatment (A, n=4; B, C, n=3; Student's *t*-test, A-C)

3.3.2 ASK is essential for osmotic stress-dependent regulation of the circadian oscillation speed

Hyper and hyposmotic stress activates (or inactivates) a wide variety of signaling pathways (Burg et al., 2007; Hoffmann et al., 2009). Among them, the mitogen activated protein kinase (MAPK) cascade play central roles in osmotic stress signaling (Kyriakis and Avruch, 2012; Saito and Tatebayashi, 2004; Tibbles and Woodgett, 1999; Zhou et al., 2016; Fig. 9). In particular, MAPK kinase kinases (MAP3K) are important upstream regulator in the osmotic stress-response. Although very few studies have indicated the functions of MAP3K in response to osmotic stress, it has been demonstrated that apoptosis signal-regulating kinases (ASK1, ASK2 and ASK3 termed ASKs in this study), members of MAP3K, have distinct and important roles in the osmotic stress response (Naguro et al., 2012). Notably, in cultured cells, hyposmotic stress activates endogenous ASK kinases while hyperosmotic stress inactivates them, when their relative activation levels were estimated from their phosphorylation levels in the activation loops (Naguro et al., 2012). This bidirectional change in its activity is rapid, sensitive and reversible (Naguro et al., 2012; Zhou et al., 2016). In fact, we evaluated activities of ASK kinases by immunoblotting and confirmed that endogenous ASKs were immediately activated or inactivated by hyposomotic treatments or hyperosmotic treatments respectively (unpublished data). Now I hypothesized that ASK kinases play crucial roles in the bidirectional clock responses to changes of tonicity. To test this, I crossed *Ask*-TKO mice, triple knockout mice deficient for *Ask1* (Tobiume et al., 2001), *Ask2* (Iriyama et al., 2009) and *Ask3* (Naguro et al., 2012), with PER2::Luc knock-in mice (Yoo et al., 2004). I obtained Ask-TKO/PER2::Luc mice, which are fertile and show no apparent abnormality in their development, and examined the effect of *Ask*s deficiency on the bioluminescence rhythms in the *Ask*-TKO/PER2::Luc MEFs. The circadian periods of the *Ask*-TKO MEFs were kept mostly constant during the long-term recording, while those monitored from control PER2::Luc MEFs were again gradually lengthened during long-term

culturing (Fig. 14A-C). Furthermore, the lengthening effect of the circadian period induced by chronic hyperosmotic treatment was abolished in the *Ask*-TKO MEFs (Fig. 15A, B), in which the shortening of the period length induced by chronic hyposmotic treatment was markedly attenuated (Fig. 15C, D). These results demonstrate that ASKs are essential for the osmolality-dependent regulation of the oscillation speed of the cellular clock.

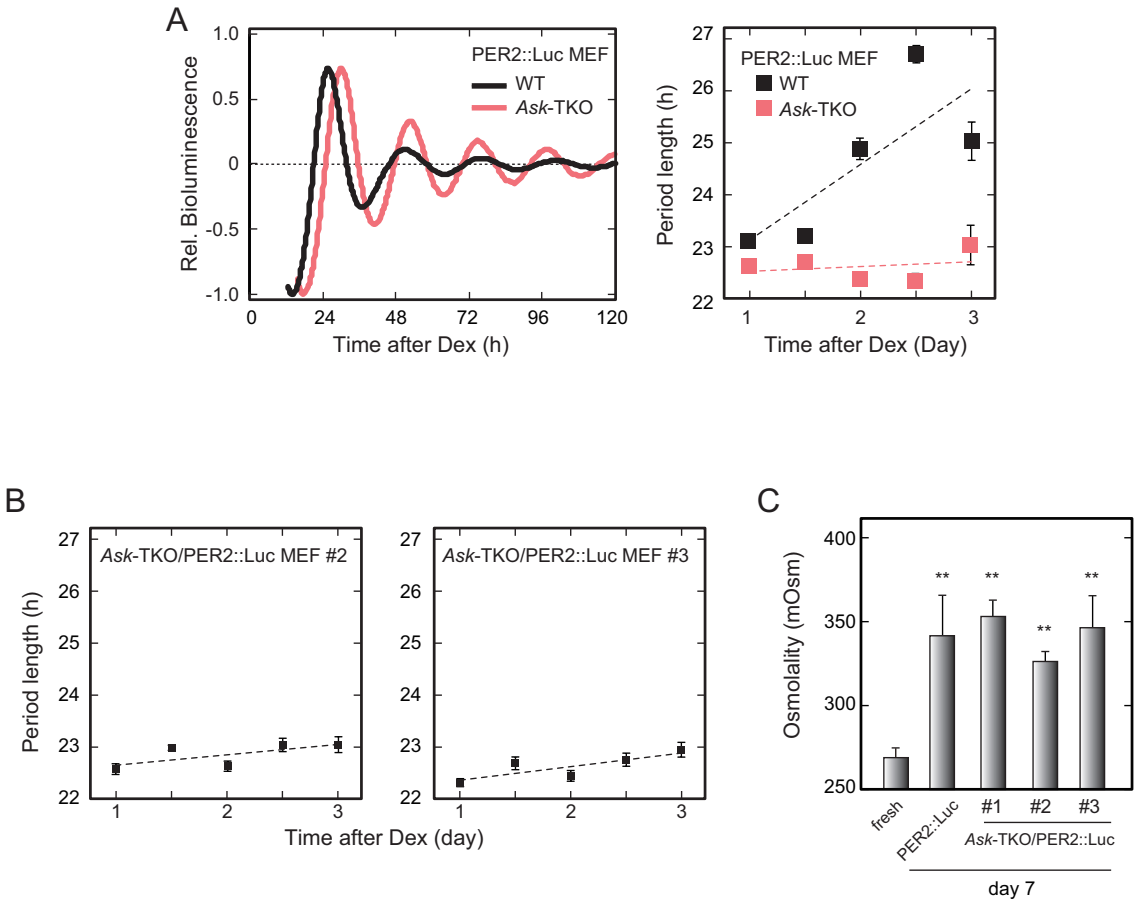

Figure 14. The osmolality changes of media during the culture affect the circadian period of the cellular rhythms through ASK signaling.

(A) The effect of *Ask*-TKO on the circadian period of the cellular rhythms. Cellular rhythms were recorded from PER2::Luc (black) and *Ask*-TKO/PER2::Luc MEFs (red). The period length was calculated as in Figure 10 panel A. (B) The cellular rhythms were recorded from *Ask*-TKO/PER2::Luc MEFs #2 and #3 isolated from independent embryos. *Ask*-TKO/PER2::Luc MEF #1 was used in this study except Figure 13 panel B and C. The period length was calculated as in Figure 10 panel A. (C) The measured osmolality of the recording media recovered from the culture of PER2::Luc MEFs or *Ask*-TKO/PER2::Luc MEFs #1-3 at day 7. Data are means with s.e.m., $^{**}p < 0.01$, *versus* fresh recording media (fresh; n=8, Student's *t*-test).

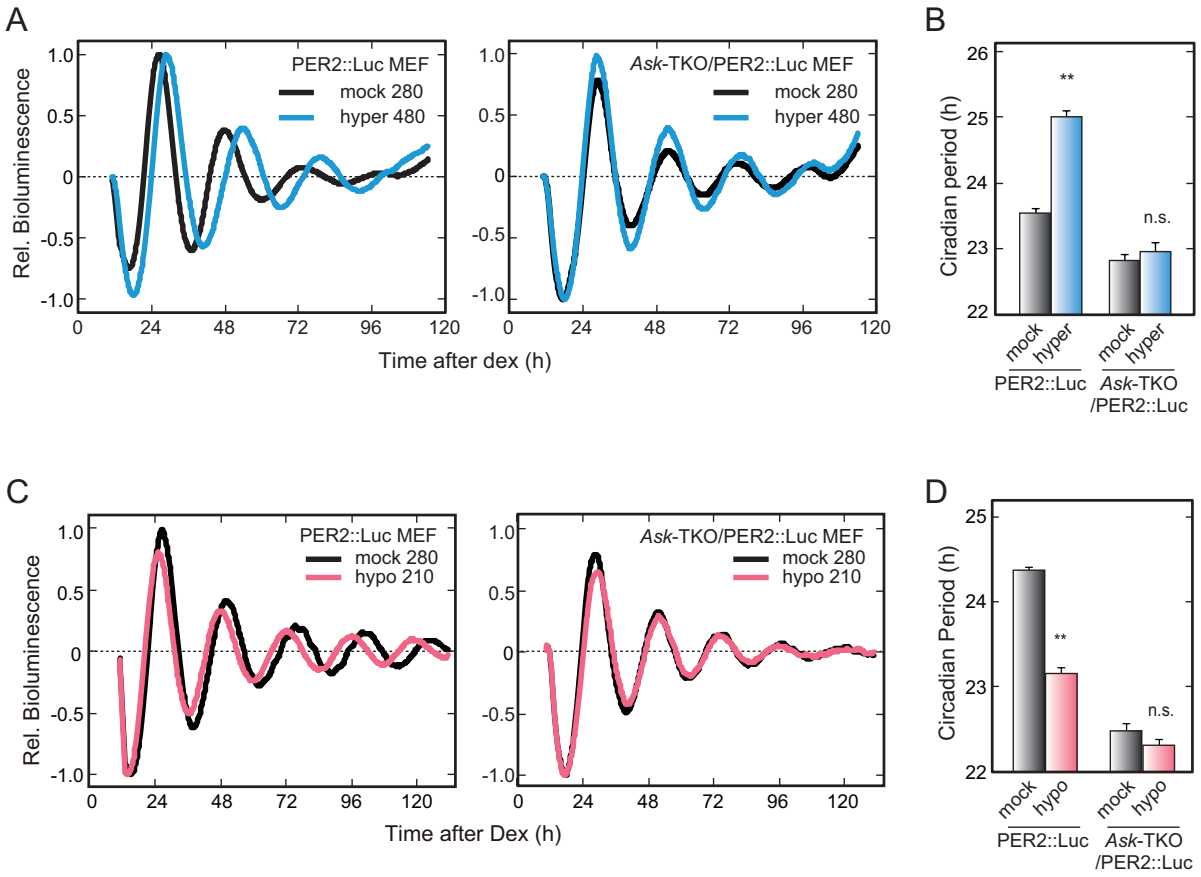

Figure 15. ASK family members are essential for the osmolality-dependent regulation of the circadian period of cellular rhythms.

The effects of chronic hyperosmotic (A, B) and chronic hyposmotic (C, D) treatment on the circadian period of the cellular rhythms in PER2::Luc and *Ask*-TKO/ PER2::Luc MEFs. For the hyperosmotic treatment, 200 mM sorbitol was added to the recording media (~280 mOsm) to give the final osmolality of ~480 mOsm (blue). For mock treatment, the same volume of the recording media was added (black). For the hyposmotic treatment, the recording media were diluted by distilled water to give the final osmolality of ~210 mOsm (red). For mock treatment, the recording media were diluted to isotonicity (~280 mOsm, black) by the same volume of 140 mM NaCl solution. Data are means with s.e.m., $^{**}p < 0.01$, n.s. $p \geq 0.05$ *versus* mock treatment (B, n=4; D, n=3; Student's *t*-test).

3.3.3 Osmotic stress regulate circadian phase through ASKs signal

Pioneering works in the chronobiological field demonstrated that the environmental time cues such as light have the following properties (Aschoff, 1960; Daan, 2000; Johnson et al., 2003; Pendergast et al., 2010), (i) chronic exposure to time cue signals affect the oscillation speed of the clock, *e.g.* constant illumination of light, and (ii) short-term exposure to time cues shift the phase of the clock in a time-of-day-dependent manner, *e.g.* light pulse during the night. The chronic exposures to hyperosmotic stress or hyposmotic stress, respectively, lengthen or shorten the period of cellular rhythm as shown in Figure 10-15. On the other hand, it was previously demonstrated that transient hyperosmotic stress strongly shifts (or reset) the phase of cellular rhythm, in our laboratory (unpublished data). Indeed, the circadian phase of cellular rhythm was drastically shifted in a time-of-day-dependent manner when the PER2::Luc MEFs were exposed to transient hyperosmotic stress by adding final 600 mM sorbitol for 30 min (Fig. 16A-C). For example, when the MEFs were stimulated at 66 h after the dexamethasone pulse for synchronization (time after Dex: TAD 66 h), the phase of the cellular rhythm was shifted to nearly anti-phase when compared with the mock treatment (Fig. 16B). Phase response curves (PRC) and phase transition curves (PTC), which are graphic descriptions of the shifts of a rhythm in response to stimuli given at various time point of the day, have been used to elucidate entrainment (or synchronization) mechanisms (Aschoff, 1960; Daan, 2000; Johnson et al., 2003; Pendergast et al., 2010). Both the PRC and PTC demonstrated type-0 phase-shift, which is characterized by resetting of the oscillator to a similar new phase after the hyperosmotic pulse delivered at any time of the day (Fig. 16C). On the other hand, no remarkable phase-shift was observed in *Ask*-TKO MEFs when they received the hyperosmotic pulse at any time (Fig.16D, E). The dexamethasone treatment induced type-0 phase-shift as normally in *Ask*-TKO MEFs, indicating their potential of circadian entrainment (Fig. 17). These results demonstrate that the hyperosmotic stress resets the cellular circadian clock through ASK signaling. Notably,

the acute hyposmotic stress by dilution with distilled water also resets the cellular rhythms (Fig.18A, B, C). Collectively, ASK kinases are essential for mediating the osmotic stress signaling as a time cue to the circadian clockwork.

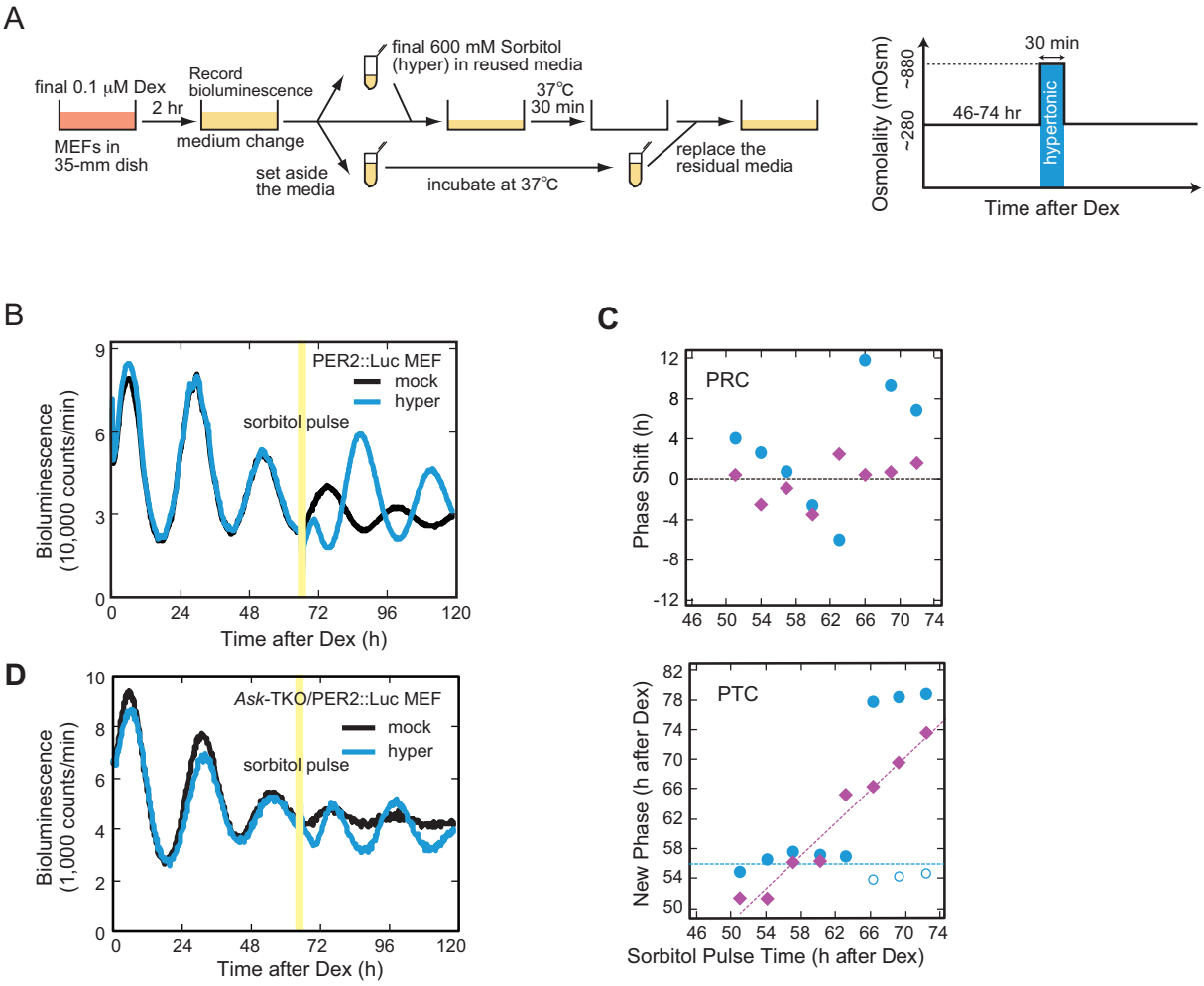

Figure 16. Transient hyperosmotic stress reset the circadian phase of the cellular rhythm through ASK signaling

(A) The experimental scheme for acute hyperosmotic in order to investigate the phase-shift of the cellular rhythms (left). For hyperosmotic pulse, the media were removed at various circadian phases, and 0.88 ml of the used media were rapidly returned back to the original well after mixing with 0.12 ml of 5 M sorbitol in fresh media (final 600 mM). For mock treatment, 0.12 ml of fresh media were added. After 30-min incubation, the hypertonic media were replaced by the residual used media that had been kept at 37°C. A time course of the osmolality changes is shown (right). (B-D) The effect of acute hyperosmotic treatment on the circadian phase of the cellular rhythms in PER2::Luc (B and C; blue circle) and *Ask*-TKO/ PER2::Luc MEFs (C and D; purple diamond). For 30-min hyperosmotic pulse, 600 mM sorbitol was added to the recording media (~280 mOsm) to give the final osmolality of ~880 mOsm (blue). For mock treatment, the same volume of recording media was added (black). (C) Phase response curve (PRC) and phase transition curve (PTC) for the cellular rhythms in response to the osmotic pulses. The open circles in PTC indicate the extrapolated new phases by subtracting intrinsic cellular periods from the actual new phases.

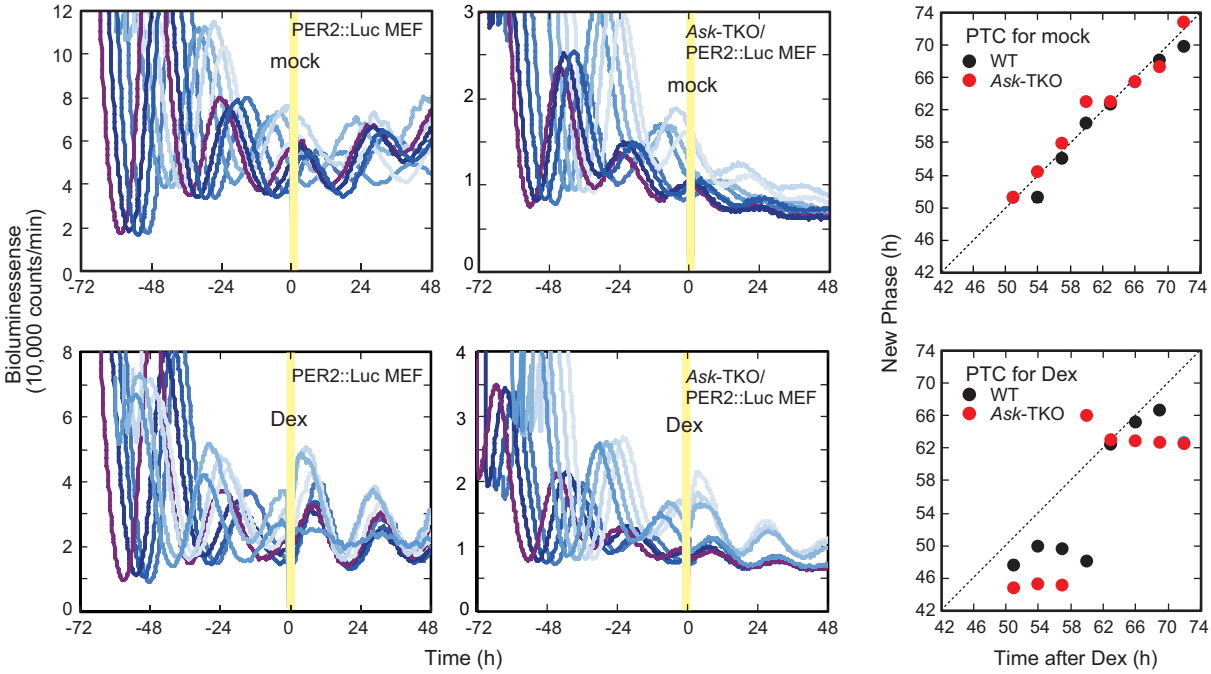

Figure 17. The Dex-induced phase-shift in *Ask*-TKO MEFs.

The phase response of PER2::Luc (left) and *Ask*-TKO/ PER2::Luc MEFs (middle) in response to dexamethasone (Dex) treatment. Dex (final 0.1 μM) or the same volume of fresh media (mock) was added to the recording media at the indicated time point, and this timing was set to Time 0 in this figure. Phase transition curves (PTC) for the cellular rhythms in response to the Dex treatment are shown (right).

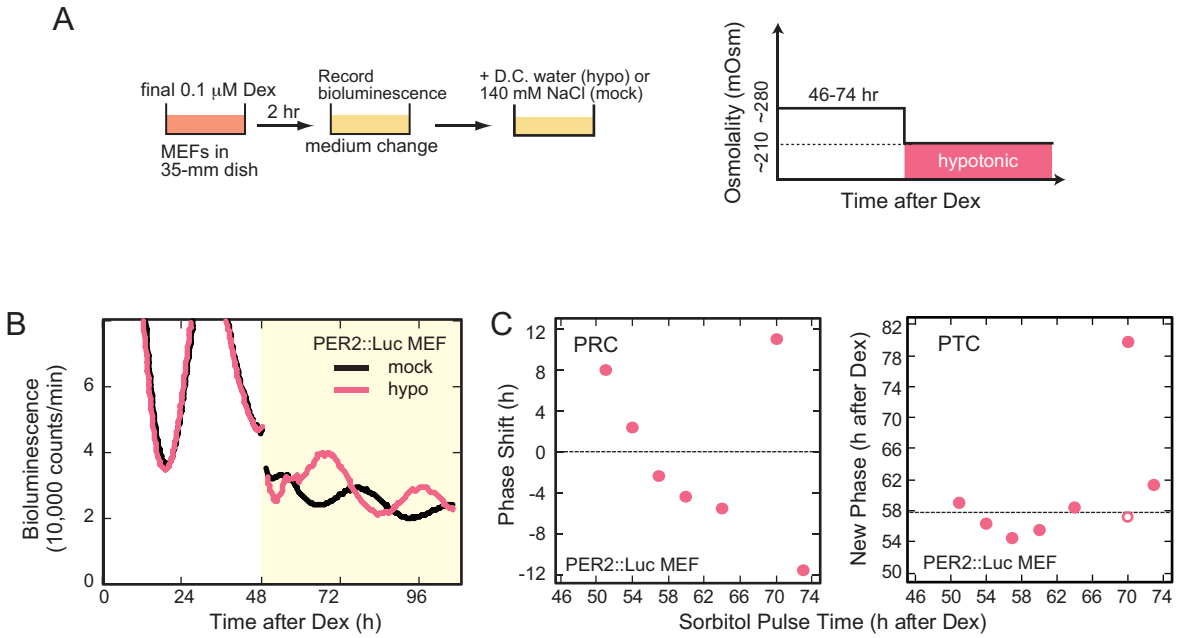

Figure 18. Acute hyposmotic stress reset the cellular clock.

(A) The experimental scheme for hyposmotic treatments in order to investigate the phase-shift of the cellular rhythms (left). For acute hyposmotic treatment, the media were removed at various circadian times, and were rapidly returned back to the original well after mixing with distilled water to give the final osmolality of ~210 mOsm. For mock treatment, the recording media were diluted by the same volume of 140 mM NaCl solution to isotonicity. A time course of the osmolality changes is shown (right). (B, C) The effect of acute hyposmotic treatment on the circadian phases of the cellular rhythms in PER2::Luc MEFs. For acute hyposmotic treatment, the recording media were diluted by distilled water to give the final osmolality of ~210 mOsm (red). For mock treatment, the same volume of 140mM NaCl solutions was added to isotonicity (~280 mOsm, black). (C) Phase response curve (PRC) and phase transition curve (PTC) for the cellular rhythms in response to the acute hypoosmotic stress. The open circles in PTC indicate the extrapolated new phases by subtracting intrinsic cellular periods from the actual new phases.

3.3.4 Non-canonical ASK signaling for regulating circadian rhythms

In the MAPK hierarchy, ASK is widely studied as major upstream of JNK and p38 (Kawarazaki et al., 2014; Takeda et al., 2008). However, in response to osmotic treatments, previous studies using various types of cells demonstrated that hyperosmotic treatment resulted in dephosphorylation (inactivation) of endogenous ASKs, whilst it caused phosphorylation (activation) of endogenous JNK and p38 (Maruyama et al., 2016; Naguro et al., 2012; Zhou et al., 2016; Fig. 19). In other words, the activation pattern of ASK and SAPK, that is JNK and p38 kinase, are not always match. Indeed, we validated that endogenous ASK was dephosphorelated in response to hyperosmotic treatment, whereas JNK and p38 were phosphorylated in HEK293A cells (unpublished data).

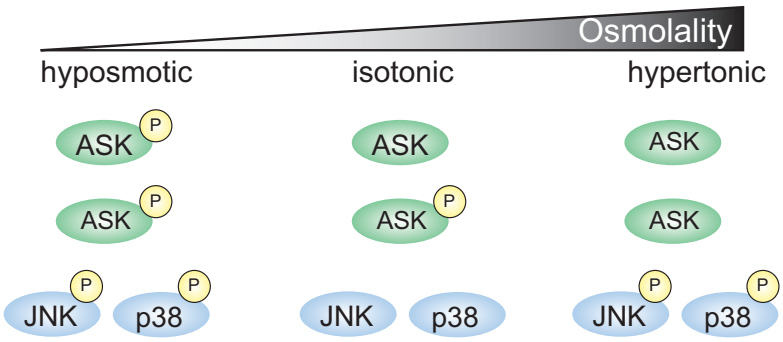

Figure 19. Regulation of MAPK signaling pathways under hyperosmotic and hyposmotic stress.
In most conditions and cell types, JNK and p38 are activated (phosphorylated) in response to hyperosmotic stress and hyposmotic stress. On the other hand, ASKs are inactivated (dephosphorylated) during hyperosmotic stress and activated (phosphorylated) during hyposmotic stress.

To examine whether an acute hyperosmotic stress resets the cellular circadian rhythms through JNK and/or p38 (termed canonical SAPK cascade in this study), I examined the phase responses to hyperosmotic pulse treatment in PER2::Luc MEFs in the presence of inhibitors, SP600125 for JNK and/or SB203580 for p38. Not only the single treatment of these inhibitors but also their simultaneous treatment caused no obvious abnormalities in the hyperosmotic stress-induced type-0 resetting of the cellular rhythms (Fig. 20A, B). It is known that the inhibitory potency of these compounds cannot be evaluated by the phosphorylation levels of JNK or p38 kinase. Instead, I calculated the cellular circadian period in the presence of the inhibitors. The obvious period lengthening *via* treatment of the compounds indicated inhibitory effects of the compounds (Fig. 20C). These results indicate that ASKs mediate non-canonical MAPK signal, which was recently proposed (Naguro et al., 2012), to the circadian input ,although this does not entirely exclude simultaneous contribution of canonical SAPK such as JNK and p38 (Fig. 21).

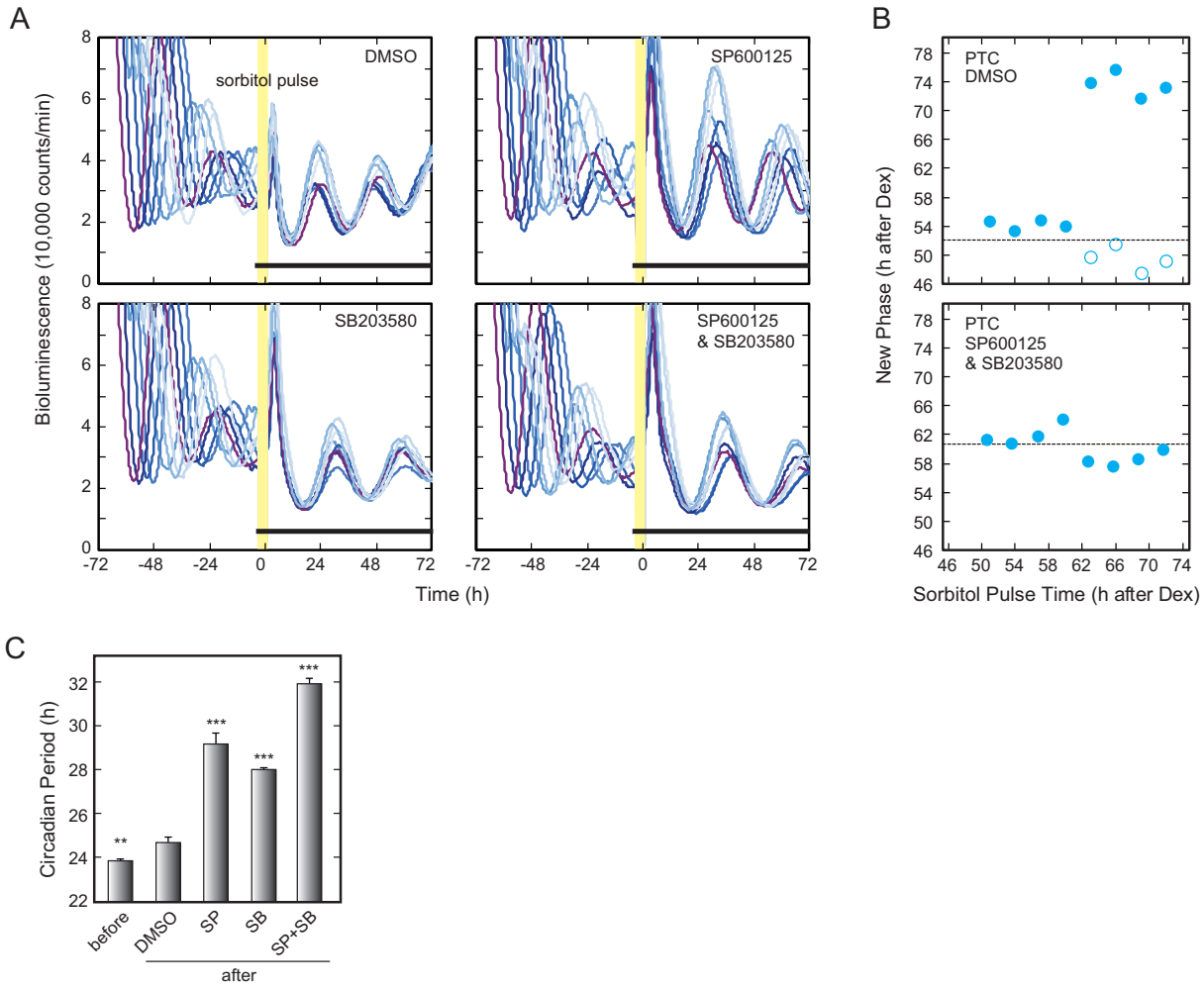

Figure 20. The Effects of SAPK inhibitors on the osmotic stress-dependent phase shift of cellular rhythm.(A) The phase response of PER2::Luc MEFs to the acute hyperosmotic treatment in the presence of inhibitors for canonical SAPK signaling. SP600125 (20 μM), SB20350 (20 μM), and/or dimethyl sulfoxide (DMSO, as control) were added to the media 1 h before hyperosmotic pulse (indicated by black lines). The cells were stimulated by 30-min pulse of 600 mM sorbitol (final) at various circadian phases, and this timing was set to Time 0 in this figure. (B) Phase transition curve (PTC) for the cellular rhythms in response to the hyperosmotic pulse in the presence of SP600125 and SB20350 (bottom). The open circles in PTC indicate the extrapolated new phases by subtracting intrinsic cellular periods from actual new phases. (C) The effect of MAPK inhibitors on the circadian period of the PER2::Luc MEFs. The circadian period was calculated from time points of peaks and troughs of the bioluminescence before and after treatment of indicated inhibitors. The significant difference between "before" and "DMSO" is consistent with Figure 10A. Data are means with s.e.m., $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* DMSO treatment (before; n=32, DMSO, SP, SB and SP+SB; n=8; Student's *t*-test).

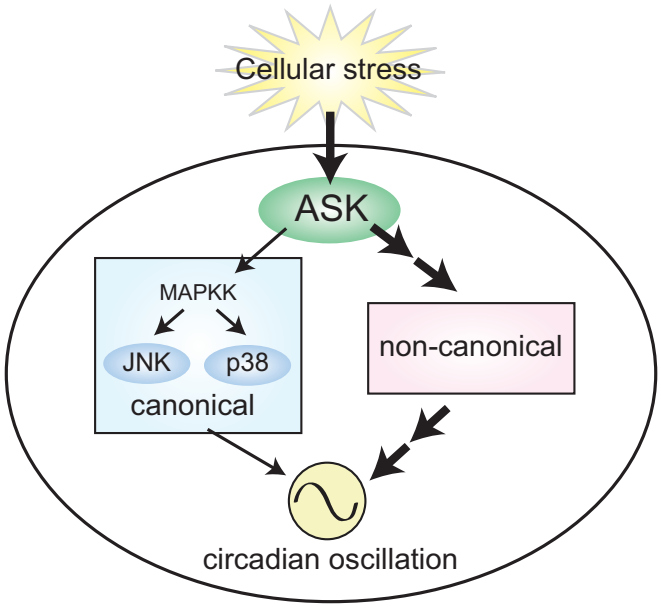

Figure 21. Non-canonical ASK signaling for regulating circadian rhythms.
A model for ASK signaling pathway as an input to the circadian oscillation. Cellular stress including change of osmolality alters ASKs activity and regulates circadian phase and period probably through non-canonical MAPK cascade.

3.3.5 *Dec1, Dec2* and *E4bp4* were immediately induced by the hyperosmotic treatment though ASK signaling.

In mammals, cell-autonomous circadian clocks are generated by a transcriptional/translational-based negative feedback loops (TTFLs) composed of several clock genes (Dunlap, 1999; Ueda et al., 2005). In many cases known to date, the circadian entrainment has been shown to dependent on immediate upregulation of clock genes. In particular, photic stimuli is accompanied by immediate induction of *Per* genes and input to the central SCN clock (Hirota and Fukada, 2004; Reppert and Weaver, 2002). I then focused on transcriptional circuitry of the oscillation system and investigated upregulation (or downregulation) of clock (-related) genes to clarify the gene(s) that functions as an input point for the acute osmotic stress. Among the various major clock-related genes, *Dec1*, *Dec2* and *E4bp4* were remarkably induced within 30 min after the hyperosmotic pulse treatment (Fig. 22). The hyperosmolality-dependent induction of these three genes was blocked in the presence of a transcription inhibitor, actinomycin D, but not by treatment with a protein synthesis inhibitor, cycloheximide, suggesting immediate-early transcriptions of *Dec1*, *Dec2* and *E4bp4* toward the osmotic stress (Fig. 23A). Although the responses were comparatively low, the three genes were also immediately induced by acute hyposmotic treatments (Fig. 23A). Importantly, the osmolality-induced transcriptions of the three genes were not observed in the *Ask*-TKO MEFs (Fig. 23B). These data indicate essential roles of ASK signaling for the immediate-early responses of the three genes.

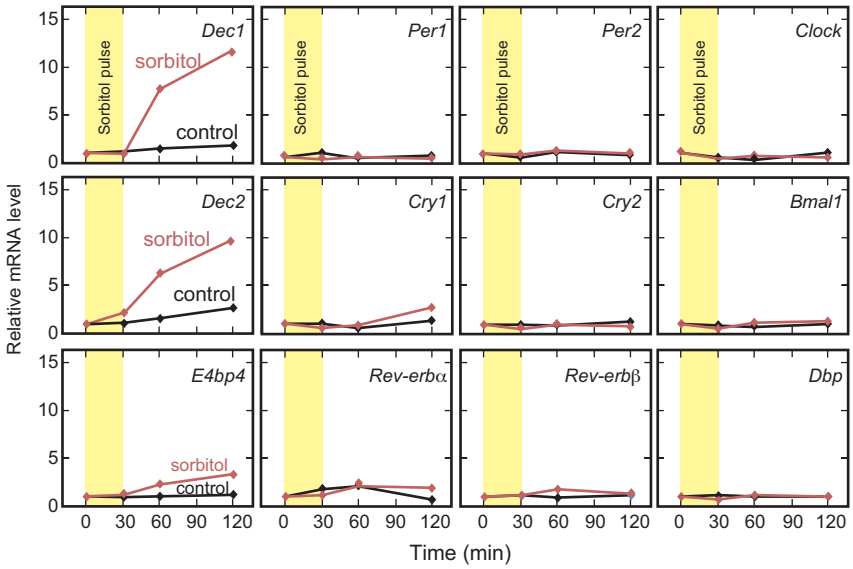

Figure 22. Temporal changes in mRNA levels of clock related genes after the hyperosmotic treatment.

PER2::Luc MEFs were cultured in the recording media and the hyperosmotic treatment was given for 30 min (illustrated by the yellow zone in each panel) at TAD 66 h. For mock treatment, the same volume of the recording media was added. The cells were collected at 0 min (start point of the treatment), 30 min (end point of the treatment), 60 min and 120 min (after the treatment), and the relative mRNA levels at time 0 were set to 1.

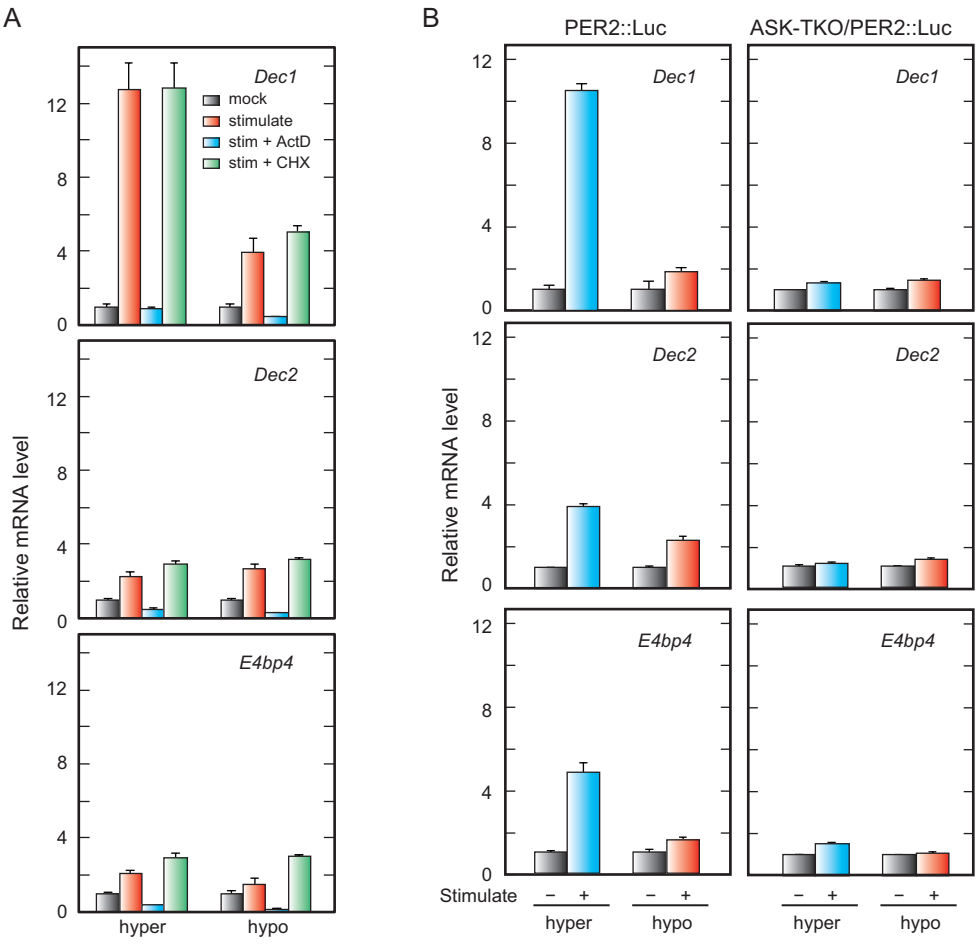

Figure 23. *Dec1*, *Dec2* and *E4bp4* were immediately induced by the hyperosmotic treatment though ASK signaling.

(A) The effect of cycloheximide (CHX) and actinomycin D (ActD) on the osmolality-dependent inductions of *Dec1*, *Dec2* and *E4bp4* transcripts. CHX (0.8 μM) or ActD (50 μM) was added 1 h before the hypertonic treatment. (B) Changes of *Dec1*, *Dec2* and *E4bp4* mRNA levels after the hyperosmotic or hyposmotic treatment in *Ask*-TKO/PER2::Luc MEFs. (A, B) The cells were cultured in the recording media and the hypertonic or hypotonic treatment was given for 30 min at TAD 66 h. For mock treatment, the same volume of the recording media was added. The cells were collected at 60 min (30 min after the end point of the treatment). The mean value of each mock was set to 1.

3.3.5 Physiological roles of ASK in regulating circadian phase and period *in vivo*.

The osmolality-dependent effects of ASKs on the circadian period and phase in the cellular clock are reminiscent of photic effects on the central SCN clock in mammals. Physiological aspect of osmotic stress to the central clock is suggested by pioneering works that hyperosmotic (Zatz and Wang, 1991a) or hyposmotic (Zatz and Wang, 1991b) treatments induced phase-shifts of the melatonin secretion rhythms in cultured chick pineal cells, as was induced by a light pulse or a dark pulse, respectively. Moreover, previous study in our laboratory showed that lengthening of the circadian period of SCN clocks in the brain section prepared from PER2::Luc mice when final 200 mM sorbitol was added to the culture media (unpublished data). In the SCN section from *Ask*-TKO mice, however, the period lengthening dependent on hyperosmotic treatment was considerably attenuated (Fig. 24).

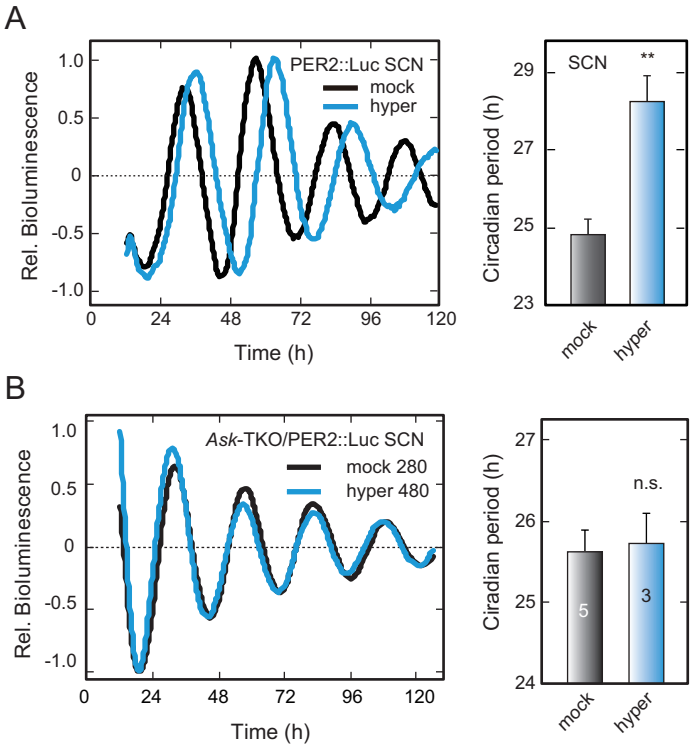

Figure 24. The effect of chronic hyperosmotic treatment on the circadian period in SCN sections from PER2::Luc mice.

For hyperosmotic treatment, 200 mM sorbitol was added to the recording media (blue). For mock treatment, the same volume of recording media was added (black). Data are means with s.e.m., $^{**}p < 0.01$ *versus* mock treatment.

To evaluate physiological roles of ASKs in the circadian clockwork *in vivo*, I monitored wheel-running rhythms of *Ask*-TKO mice. The mutant mice exhibited robust daily behavioral rhythms under 12-h light/12-h dark (LD) conditions and constant dark (DD) conditions normally (Fig. 25, 26). No remarkable changes in temporal profiles of clock proteins and gene expressions was observed between wild type (WT) and *Ask*-TKO mice liver (Fig. 27, 28), supporting the possibility that ASKs are not required for circadian normal oscillation at steady state. However, significant abnormalities of *Ask*-deficient mice were observed in light-induced phase-responses of the behavioral rhythms in DD conditions. It is well known that light pulses presented in the subjective day (circadian time; CT 0-12) have little or no effect on the phase of activity rhythm on subsequent days. By contrast, light presented during the subjective night phase-shift the activity rhythm. During the first half of the subjective night (~CT12-18), light pulses phase-delay the activity rhythm, *i.e.,* subsequent activity starts later. During second half of the subjective night (~CT18-24), light pulses phase-advance the activity rhythm, *i.e.,* subsequent activity starts earlier (Aschoff, 1960; Daan, 2000; Johnson et al., 2003; Pendergast et al., 2010). Indeed, I observed phase delay and advance in response to a 30-min light pulase of 400 lux given at early (CT14) or late (CT22) subjective night, respectively, in WT mice (Fig. 25A, B). In *Ask*-TKO mice, a remarkable reduction of the phase-shift was observed when the light pulse was given at early or late subjective night (Fig. 25A, B). On the other hand, such a light pulse particularly at subjective late night caused a noticeable phase-dependent period-shortening in WT mice (Fig. 25C), known as 'aftereffect' (Pittendrigh 1976). The aftereffect has an adaptive significance, because the light pulse modulates the circadian period towards a direction that reduces the phase-shift (Pittendrigh 1976). I found that the free-running period of *Ask*-TKO mice was unaltered even after receiving the light-pulse at the late subjective late night (Fig. 25C). In constant light (LL) condition of 400 lux, WT mice exhibited remarkably longer free-running periods (Fig. 25A, B; 23.8 ± 0.2 in DD and 25.5 ± 0.4 in LL), as was previously observed

(Aschoff, 1960; Pendergast et al., 2010; Yoshitane et al., 2012). This property is widely recognized as Aschoff's rule; the higher the light intensity in LL is, the longer the circadian period is in WT mice (Aschoff, 1960; Yoshitane et al., 2012; Fig. 29). In spite of the evolutionary and physiologically importance, Aschoff's rule has not been explained at the molecular level. Intriguingly, the period-lengthening effect of light was decreased in the *Ask*-TKO mice, which showed a shorter period than WT mice in 400-lux LL condition (Fig. 26A, B; 24.8 ± 0.3 in LL). *Ask1* single-KO mice showed mild phenotype relative to *Ask*-TKO mice but significantly shorter behavior rhythms than wild type mice in LL (data not shown), indicating functional redundancy between ASK family members in the regulation of circadian clock. Although *Ask*-TKO mice showed the free-running period nearly equal to that of WT in dim white light (10 lux), the period of the mutant was dissociated from that of WT in 300 lux or 1,000 lux (Fig. 29). This data suggests that ASKs function under comparatively strong intensity of light. Collectively, these results demonstrate that ASKs play an important role in the photic regulation of the circadian period *in vivo*.

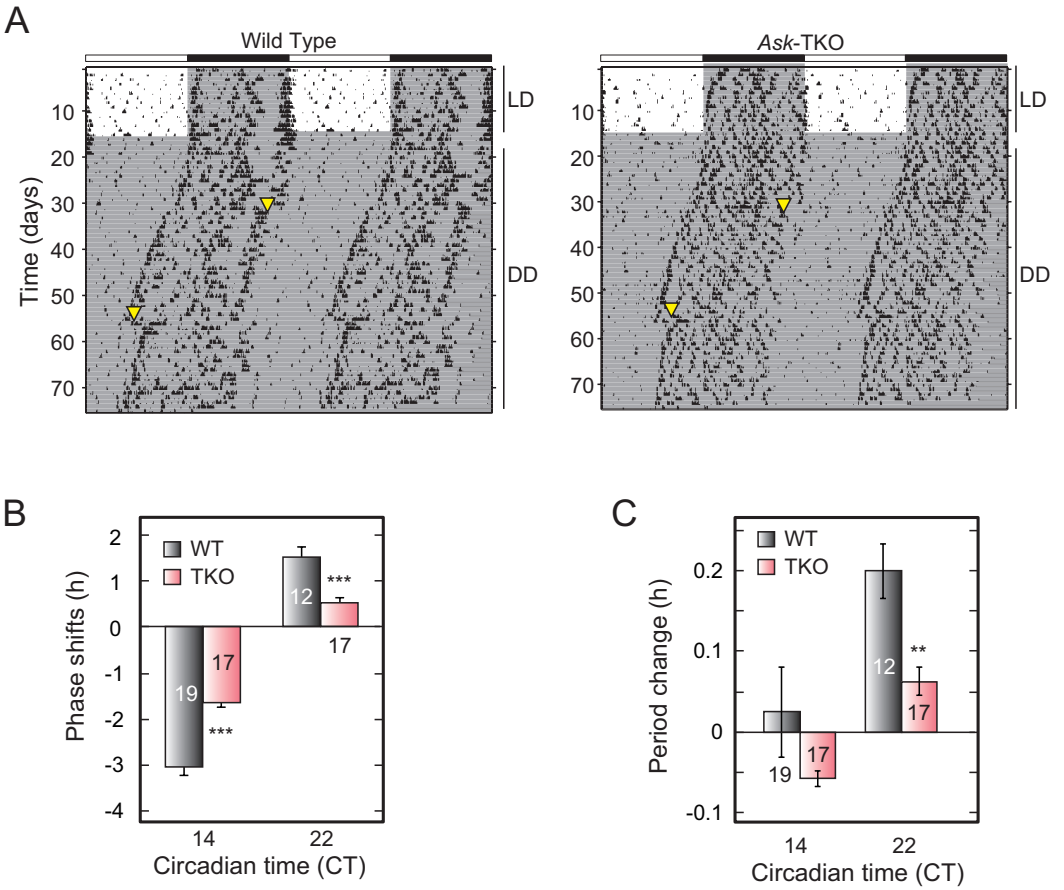

Figure 25. The effect of light pulse on the circadian behavior rhythm of *Ask*-TKO mice.

(A**)** Double-plotted wheel-running activity rhythms of representative wild-type (left) and *Ask*-TKO mice (right) in 12-h light/12-h dark (LD) and constant dark (DD) conditions. Horizontal black and white zones above each actogram indicate the dark and light phase in LD cycles, respectively. Shaded and bright areas in the actograms indicate the dark and light conditions, respectively. Yellow triangles in panel A indicate time points when the light pulse was given (~400 lux, 30 min). (B) Phase response to brief light pulses (~400 lux, 30 min) given at circadian time (CT) 14 (subjective early night) and 22 (subjective late night). Phase-advance and phase-delay shifts are expressed in positive and negative values, respectively. (C) Changes of circadian periods after the brief light pulses. Lengthening and shortening of the circadian period are expressed in negative and positive values, respectively. (B, C) The numbers in the bars indicate each sample size. Data are means with s.d., $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* wild type (Student's *t*-test).

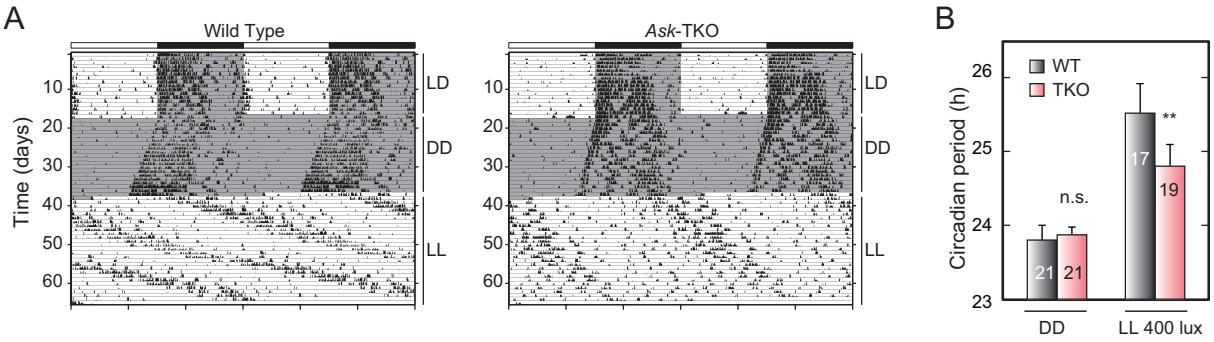

Figure 26. The effect of constant light stimulation on the circadian behavior rhythm of *Ask*-TKO mice.

(A**)** Double-plotted wheel-running activity rhythms of representative wild-type (left) and *Ask*-TKO mice (right) in 12-h light/12-h dark (LD) constant dark (DD) and constant light (LL) conditions. Horizontal black and white zones above each actogram indicate the dark and light phase in LD cycles, respectively. Shaded and bright areas in the actograms indicate the dark and light conditions, respectively. (B) Circadian free-running periods of the behavior rhythms under DD and LL conditions in wild-type and *Ask*-TKO mice. The numbers in the bars indicate each sample size. Data are means with s.d., $^{**}p < 0.01$, n.s. $p \geq 0.05$ *versus* wild type (Student's *t*-test).

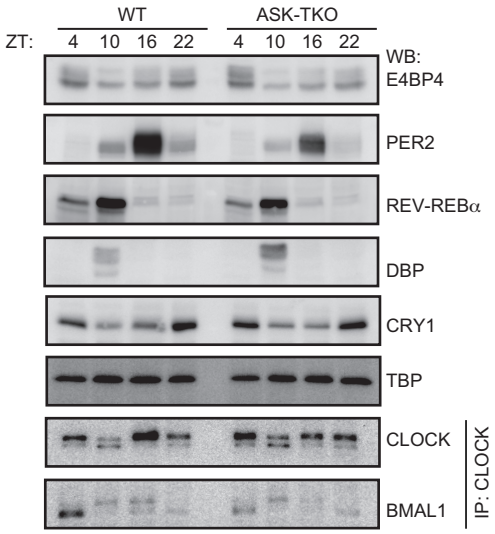

Figure 27. Temporal expression profiles of the clock proteins in the liver nucleus.
Nuclear extracts prepared at the indicated time points were subjected to immunoblot analysis. TATA binding protein (TBP) was shown as a loading control. For immunoblot of CLOCK and BMAL1, liver nuclear extracts were immunoprecipitated with anti-CLOCK mAb (IP: CLOCK; Yoshitane et al., 2009)

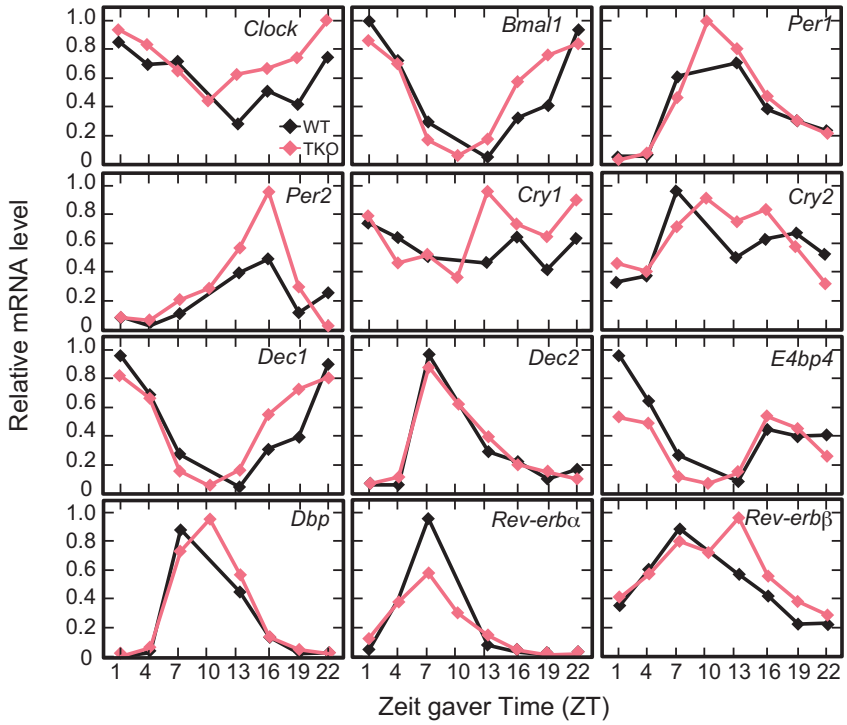

Figure 28. Temporal expression profiles of clock related genes in the liver.
The relative levels of mRNA were examined by qRT–PCR, and normalized by *Rps29*.

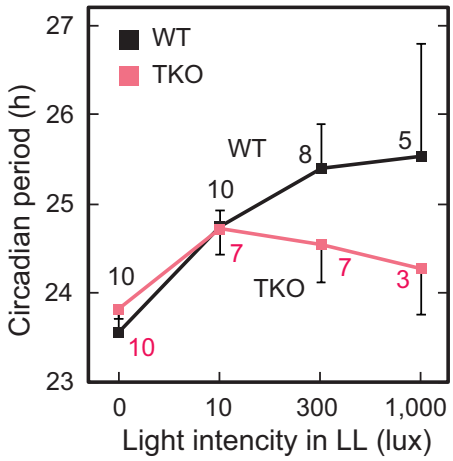

Figure 29. The change of the circadian period of behavior rhythms under the various intensities of constant light (LL) conditions.

Data are means with s.d.. The numbers near the plots indicate each sample size.

3.3.6 Interlocking interactions between the intracellular redox state and transcriptional/translational feedback loops (TTFLs) through ASK signal.

The ASKs activities are known to be regulated by a wide variety of cellular stress (Kawarazaki et al., 2014; Takeda et al., 2008), and it had previously shown in detail molecular mechanisms for regulation of the ASKs activity in response to oxidative stress (Matsuzawa et al., 2005; Nishitoh et al., 1998; Noguchi et al., 2005). In mammalian clockwork, clock genes and the encoded proteins form transcriptional/translational feedback loops (TTFLs), while it has been proposed that intracellular redox cycling underlies the oscillatory mechanism even in the absence of the transcription rhythm (Cho et al., 2014; Edgar et al., 2012; O'Neill and Reddy, 2011). However, it is not known whether these two oscillators are interlocked with each other at the intracellular level, and if so, what is the key molecule that links between TTFLs and intracellular redox rhythms (Stangherlin and Reddy, 2013). In order to approach another physiological aspect of ASKs, I focused on the effect of changes in intracellular redox states on the circadian clockwork. I treated PER2::Luc MEFs with 3-amino-1, 2, 4-aminotriazole (ATZ), an inhibitor of catalase that is a key enzyme catalyzing the decomposition of hydrogen peroxide to water and oxygen, and change the intracellular redox state to oxidative. I found that the circadian period of the cellular rhythms was significantly lengthened by adding final 20 mM or 80 mM ATZ (Fig. 30). Importantly, the period-lengthening effect by ATZ was abolished in the *Ask*-TKO MEFs (Fig. 30). From the viewpoint of circadian phase, pulse treatment with hydrogen peroxide (oxidative stress) caused type-0 reset in PER2::Luc MEFs, indicating that oxidative stress is a circadian time cue (unpublished data). These results demonstrate that intracellular redox changes affect circadian transcriptional rhythms through ASK signaling. My finding that ASK signaling controls the period and phase of the circadian oscillation of TTFLs raises the possibility that ASKs are in tern controlled by TTFLs. Indeed, I noticed rhythmic CLOCK binding to the first intron region of *Ask1* (*Map3k5*) loci in our previous CLOCK ChIP-Seq

data (Yoshitane et al., 2014), and I identified canonical-type (CACGTG) and non canonical-type (CACATG) E-box sequences just near the CLOCK-binding region (Fig. 31). Furthermore, previous RNA-Seq analysis (Terajima et al., 2016) detected circadian expression of *Ask1* and *Ask2* mRNAs in the mouse liver. Based on the results, I performed qRT-PCR analysis and showed the circadian cycles of *Ask1* and *Ask2* mRNA expressions with a peak at the timing of transition from day to night, in the mouse liver under DD conditions (Fig. 32). The *Ask1* and *Ask2* transcript levels remained low throughout the day in *Bmal1*-KO liver, in which expression rhythms of clock-controlled genes were abolished and circadian molecular oscillation was abolished (Fig. 32). These data indicated that fluctuate of ASK signals not only perturb TTFLs but also are controlled by TTFLs (Fig. 33). Intriguingly, the peak time of *Ask1* and *Ask2* genes expressions are consistent with that of redox-related metabolites and proteins (Edgar et al., 2012; Peek et al., 2013). Taken together, these data provide an interesting model that molecular clock generate expression (or activity) rhythms of ASKs, which in turn relay intrinsic redox signal to TTFLs (Fig. 33).

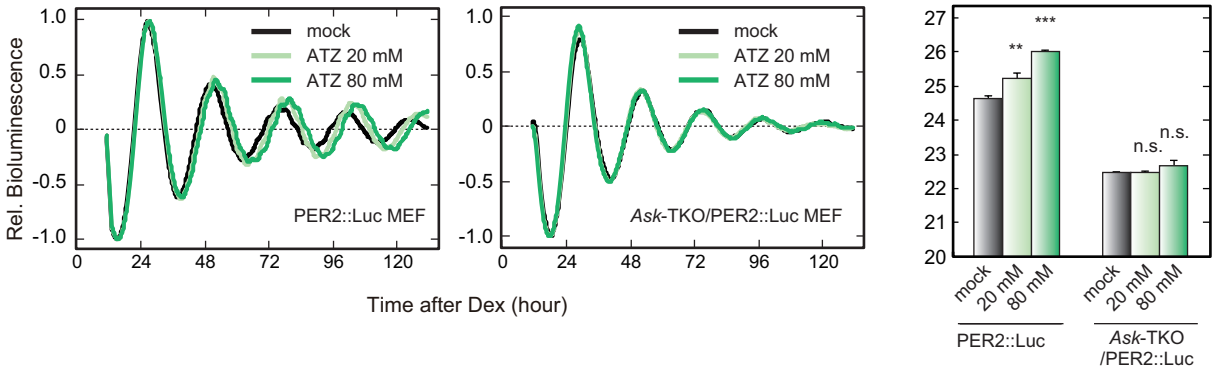

Figure 30. The effects of chronic ATZ treatment on the circadian period of the cellular rhythms.

The cellular rhythms were recorded from PER2::Luc (left) and *Ask*-TKO/ PER2::Luc MEFs (middle). Data are means with s.e.m., $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. $p \geq 0.05$ *versus* mock (fresh media) treatment (n=4, Student's *t*-test).

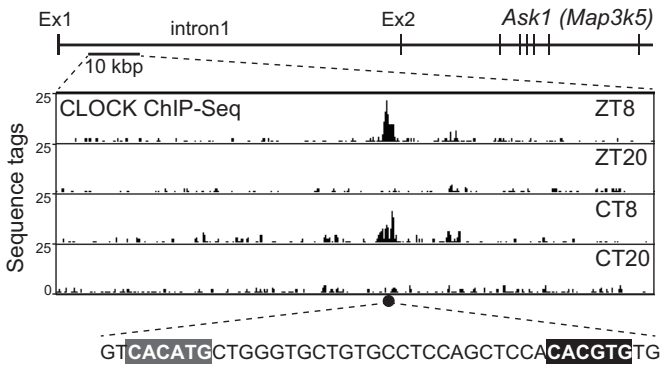

Figure 31. CLOCK ChIP-Seq data at the *Ask1* gene loci.

Canonical (black) and non-canonical E-box sequences (gray) near the CLOCK-binding regions are highlighted.

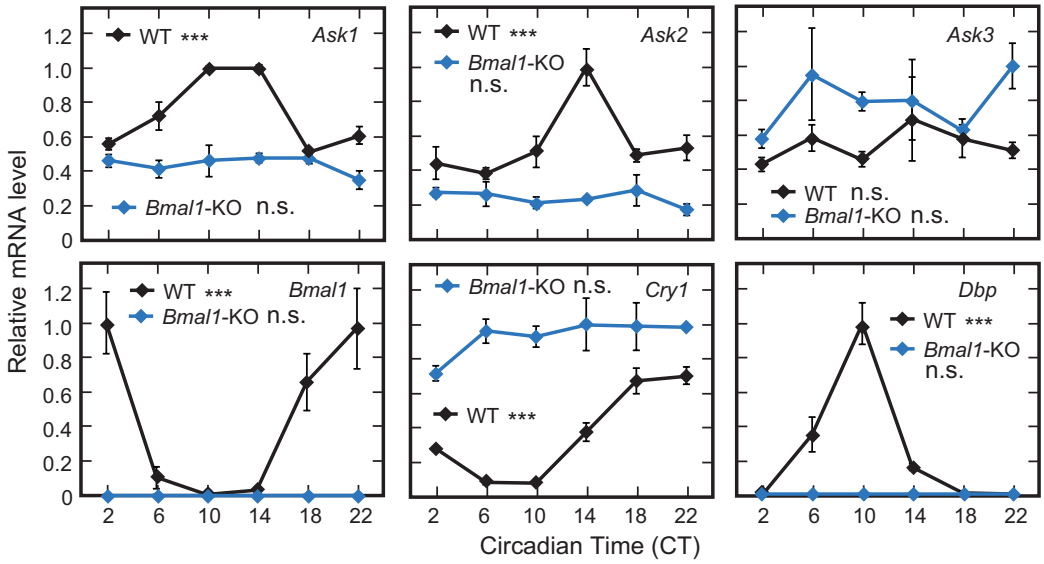

Figure 32. Temporal expression profiles of *Ask*s and typical clock genes in the mouse liver.

Gene expression levels are determined by qRT-PCR analysis in wild-type (black) and *Bmal1*-KO (blue) mice livers. Temporal changes were analyzed by one-way ANOVA; ***$p < 0.001$, n.s. $p \geq 0.05$. The signals from qRT-PCR analysis were normalized to *Rps29* (n=3).

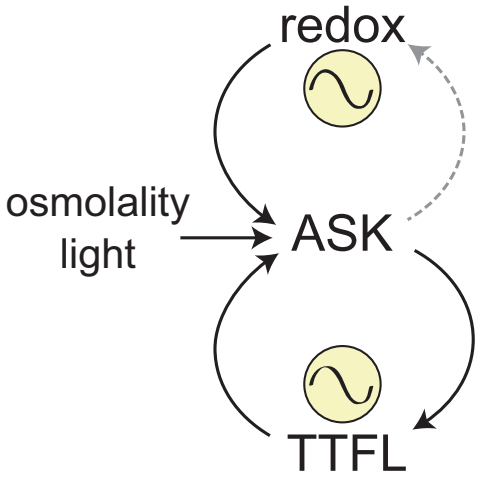

Figure 33. Interlocking interactions between the intracellular redox state and transcriptional/translational feedback loops (TTFLs) through ASK signal.
A model for the interlocking interaction between the redox state and TTFLs through ASK kinases.

3.4. Discussion

In the present study, I demonstrated that chronic hyperosmotic stress and hyposmotic stress changes the period of the mammalian circadian clock bidirectionally (Fig. 10-13), and the period changes were completely blocked by the deficiency of ASK family members (Fig. 14, 15). On the other hand, acute change of the osmolality reset the cellular clock, and loss-of-function experiments demonstrated that ASKs are key players mediating the cellular stress response and controlling the circadian phase (Fig. 16). Furthermore, the behavioral analysis revealed physiological aspects of the ASKs signaling that regulates a core characteristic of the circadian clock by controlling the oscillation speed and the phase in response to light (Fig. 25, 26, 29). Collectively, the similar response of circadian clock to osmotic stress and light exposure support my hypothesis that these time cues share a common molecular pathway at least partially for the clockwork regulation through ASKs signaling. Theoretically, the parametric and non-parametric entrainment models (Daan, 2000; Johnson et al., 2003; Pendergast et al., 2010) support my idea that cellular stress response signal through ASKs acts as a time cue. The parametric model assumes that entrainment involves changes of the clock's oscillation speed by continuous stimuli of time cues. That is, the acceleration or deceleration of the oscillation speed could allow the circadian pacemaker to continuously adjust its cycle length to that of environment. On the other hand, the non-parametric model assumes that entrainment occurs *via* acute phase-shifts with accompanying pulse stimuli of time cues. In our results, the chronic osmotic stress regulated the circadian period concordantly with the parametric model (Fig. 10-15) and acute osmotic stress reset the phase according to the non-parametric model (Fig. 16) as well as light (Fig. 25, 26, 29).

As shown in the chapter 2, I identified JNK as a new clock related kinase and which regulates the circadian clock (Yoshitane et al., 2012). However, in this study, in the phase responses to acute hyperosmotic stress, no remarkable alterations were observed by the inhibition of JNK and p38 (Fig. 20). These results strongly suggest pivotal contribution of

non-canonical SAPK cascade in the cellular-stress responses (Fig. 21). Recently, it was showed that ASK3 regulates kinase activity of WNK1, in the kinase activity-dependent manner, but not through canonical SAPK pathway in response to osmotic stress (Naguro et al., 2012). The ASK3-WNK1 pathway has a strong impact on water/salt reabsorption in renal cells that are directly exposed to massive osmotic changes (Naguro et al., 2012), therefor the ASK-mediated regulation of the circadian clockwork in response to osmotic changes could be physiologically important in the renal clock. The renal clock is known to control a large number of specific renal functions, and alteration of the clock is associated with several diseases including hypertension and type II diabetes (Bonny and Firsov, 2013).

In this study, I showed the essential function of ASKs in cellular circadian clockwork by controlling the osmolality of media and change the activities of ASKs. The ASKs activities are also known to be regulated by a wide variety of cellular stress (Kawarazaki et al., 2014; Takeda et al., 2008), and it had previously shown molecular mechanisms for regulation of the ASKs activity in response to oxidative stress (Matsuzawa et al., 2005; Nishitoh et al., 1998; Noguchi et al., 2005). On the other hand, the intracellular amount of the redox-related metabolites and activities of antioxidant enzymes are reported to be rhythmic in mouse liver (Edgar et al., 2012; Peek et al., 2013). Even in red blood cells, which have no nucleus and therefore have no transcriptional rhythms, intracellular redox states exhibit circadian rhythms (O'Neill and Reddy, 2011). Intriguingly, light induces the production of ROS that acts as the second messenger coupling photoreception to the zebrafish circadian clock (Hirayama et al., 2007). These lines of evidence support the fascinating idea that intracellular rhythm of redox state may regulate the circadian clock through activities of ASKs *in vivo* (Fig. 33).

Chapter 4.

Conclusion

In chapter 2 of the present study, I identified c-Jun N-terminal kinase (JNK) as a new circadian clock-related protein kinase. JNK is responsible for phosphorylation of BMAL1, a positive limb of circadian molecular oscillation. Adding to this, JNK regulates the circadian oscillation speeds. In the process of the study to investigate the physiological function of JNK, it was demonstrated that hypertonic treatment regulates the circadian period and phase in cultured cells. These data and previous studies concerned with circadian input signal proposed me a hypothesis that cellular stress responses regulate the circadian clock.

In chapter 3, I focused on the linkage between cellular stress response and the circadian clock. In accord with previous results, increase in osmotic pressure accompanying long-term culture lengthened the circadian period of the cellular rhythms. Intriguingly, I found that hyposmotic stress shorten the period of cellular rhythms. It was reported Apoptosis signal-regulating kinase (ASK) is a unique bidirectional responder to osmotic stress, so now I hypothesize that ASK kinases play crucial roles in the bidirectional clock responses to osmolality changes. Detailed examination of the culture conditions has eventually revealed that continuous- or acute-osmotic stresses alter the oscillation speeds or the phases of cellular clocks respectively through ASK activity. Physiologically, ASK signal is responsible for photic responses of the behavioral rhythm of mice. I showed another physiological impact of ASK as a mediator for redox signaling to the circadian clock. In summary, I demonstrated that the circadian clock responds to cellular stress, such as osmotic stress and oxidative stress, through ASK kinase signaling.

Chapter 5.

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Chapter 6.

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