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Mammalian Cultured Cells as a Model System of Peripheral Circadian Clocks

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The mammalian circadian system consists of multiple oscillators with basically hierarchical relationship, in which the hypothalamic suprachiasmatic nucleus (SCN) is the master pacemaker and the other oscillators in the periphery are subordinate. Although peripheral oscillators have been preceded by the SCN in circadian studies, accumulating data have revealed the importance and characteristics of peripheral oscillators. Cultured cell lines have also provided valuable information about intracellular mechanisms of circadian rhythms. This review outlines the properties of peripheral clocks in several perspectives such as the mechanisms of autonomous oscillations, the clock resetting, and the clock outputs, and describes the usefulness of immortalized cultured cells as a model system of mammalian circadian clocks by introducing some fruits of related works.

Key words: circadian rhythm, clock, cultured cell, periphery, SCN.

Overview

Circadian rhythm is a universal feature of most organisms living on the earth (*1*, *2*). In mammals also, a variety of physiological and behavioral activities show circadian rhythms. These rhythms are primarily controlled by a small portion of the brain called the suprachiasmatic nucleus (SCN). There are several lines of data identifying the SCN as the master circadian pacemaker (*3*). In SCN cells, a set of clock genes such as *per*, *cry*, *bmal1*, *clock*, *CKI*ε forms transcriptional feedback loops, resulting in oscillations of expression levels of clock and clock-controlled genes with ~24-h cycles (*4*). This intracellular feedback loop is believed to provide time information to the entire body with some kind of humoral signals, which are yet to be totally identified. Interestingly, clock genes forming feedback loops are also expressed in peripheral tissues throughout the body and their expression levels oscillate as in the SCN (*5*). Furthermore, circadian rhythms are also observed in immortalized cultured fibroblasts after a variety of stimuli such as a serum shock. If the SCN pacemaker controls overall rhythms throughout the body, what is the raison d'etre of peripheral oscillators? How useful are cultured cell lines for investigating the unknown mechanisms of circadian rhythms? We will introduce some researches on circadian rhythms of cultured fibroblasts and evaluate the usefulness of cultured cells as a model system of peripheral oscillators.

Keeping the rhythm at a single-cell level

In mammals, significant advances have been achieved in understanding of the molecular machinery of circadian rhythms since the identification of mouse *clock* gene in 1997 (*6*, *7*). These advances include the discovery and the functional analysis of three period genes (*per1*, *2*, and *3*), two cryptochrome genes (*cry1* and *2*), *bmal1*, *clock*, and *CKI*ε. These genes form transcriptional feedback loops, in which CLOCK:BMAL1 heterodimers transactivate *per* genes through the E-box sites in their promoter regions and then PER proteins, in turn, have an inhibitory effect on CLOCK:BMAL1 activity (*8*–*17*). It seems that this interlocked negative feedback loop is the core mechanism generating circadian rhythms. CRY proteins are also the essential components for maintaining the rhythms. They interact with PER proteins and inhibit the transactivation by CLOCK:BMAL1 (*16*). CKIε is able to phosphorylate PERs and might regulate their stabilities and their subcellular localizations (*17*–*21*). Some other genes such as *Rev-erb*α, *Npas2*/*Mop4*, and *Dec* have been identified as candidate components of the core clock machinery. *Rev-erb*α is a nuclear orphan receptor and it is indicated to be a component of core clock feedback loops (*22*). NPAS2/MOP4, a CLOCK analog, is a partner of BMAL1 and positively regulates E-box-mediated transcriptions (*23*, *24*). *Dec1* and *2* are transcription factors that were recently shown to have important role in keeping the rhythm in the SCN (*25*). There are various kinds of post-transcriptional regulations that determine the stability and the period length of circadian rhythms (*26*). However, it is not totally understood what makes circadian rhythms run with a nearly 24-hour period, because there are many factors that affect the period length such as accumulation speed of PER and CRY proteins and the timing of their nuclear entry, phosphorylation and degradation. Up to now, many researchers have tried to figure out the molecular clockworks by using genetical and biochemical methods with cultured cells. Recently established systems might help us to investigate the intracellular mechanisms relevant to the period length of circadian rhythms. The transgenic mice carrying a transgene in which destabilized GFP is driven by the *mper1* promoter were produced (*27*). Other transgenic mice and

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rats carrying the promoter region of the *mper1* gene fused to the luciferase gene were also produced (*28–30*). These transgenic animals show robust circadian rhythms in the reporter activity and will be very useful in monitoring circadian rhythms in real time in intact organisms. In fact, the phase shifts of the rhythms after a light pulse and an altered feeding have been investigated by using these animals (*31*, *32*). The molecular mechanisms of circadian clockwork that determine the period length of ~24 h could be studied in more detail at an intact single-cell level by using cultured cells carrying such reporter genes.

One of the most interesting characteristics of circadian rhythms is temperature compensation. The period length of circadian rhythms is temperature-compensated over the wide range of physiological temperature. In mammals, it has been demonstrated that the melatonin secretion rhythm of cultured retinas of the golden hamster and the firing rhythm of the SCN neurons of rat are temperature-compensated in their period length (*33*, *34*). Moreover, we found that the period length of circadian gene expressions in NIH3T3 mouse fibroblasts is almost constant over the ambient temperature range of 33–42°C (*35*). Since the accumulation speed of mPER proteins is temperature-dependent, there might exist some unknown mechanisms coordinating the circadian periodicity and intracellular metabolisms. It is really interesting that the circadian periodicity is generated and temperature-compensated at the molecular level in a single cell. However, the scientific elucidation of the mechanisms determining the periodicity is quite difficult because of its intricacy and its stochastic nature. There have been some trials to understand the mechanisms regulating the circadian periodicity by using mathematical models (*36*, *37*). These approaches must be indispensable for thorough understanding of intracellular machinery of keeping time. It is hoped that the working model explaining the circadian periodicity consistent with the biological observations will come out.

Resetting of peripheral oscillators

Since the period length of circadian rhythms is nearly, but not exactly, 24 h, some entraining mechanisms are necessary to adjust the internal clock to environmental day-night cycles. The most popular time cue is light, which is perceived by eyes and translated into a neuronal signal that is able to reset the rhythms of the SCN neurons (*38*). It seems that peripheral oscillators are completely under the control of the SCN circadian pacemaker on conditions of constant darkness, constant temperature, and ad-lib feeding. Therefore, there must be some humoral signals emitted from the SCN, which are able to synchronize peripheral clocks throughout the body directly or indirectly. TGF-α and prokineticin-2 have been identified as candidates for output factors (*39*, *40*). Both genes are expressed rhythmically in the SCN and their infusions into the cerebral ventricles affect locomotor activity. However, it still remains to be examined whether or not these factors could circulate through the body and directly reset the clock in peripheral tissues. Otherwise, output factors from the SCN might act upon the only proximal regions of brain and regulate the rest/activity cycle, which, in turn, causes the rhythm of feeding behavior. Feeding is able to regulate circadian rhythms dominantly in some peripheral tissues without affecting the SCN rhythm (*41*, *42*). Thus, it is still obscure what is the real timing cue for peripheral oscillators *in vivo* under constant conditions.

Another matter of general interest is the intracellular mechanisms of signal transduction from a resetting cue down to entrainment of circadian gene expressions. In 1998, it was reported that the serum shock could induce circadian oscillations of clock and clock-controlled genes in rat-1 fibroblasts (*43*). This entrainment by the serum shock in rat-1 cells probably mimics that by light in the SCN because of an observation common to both of them, an acute and immediate induction of *per1* mRNA expression. Since this remarkable finding, a variety of treatments have been reported to be able to entrain the circadian rhythms in cultured cells. Forskolin, which is able to induce the immediate expression of *per1* mRNA and trigger the circadian gene expressions in rat-1 fibroblasts, activates adenylate cyclase and increases intracellular cAMP (cyclic AMP) level, resulting in activation of protein kinase A (PKA) (*44*). This forskolin-induced transient expression of *per1* mRNA is inhibited by an inhibitor of cAMP-dependent kinases, and besides, a PKA-specific cAMP analog can induce rapid expression of *per1* (*45*, *46*). These data indicate that cAMP signal-transdustion pathway is involved in clock-resetting mechanism by forskolin. The increase of intracellular calcium levels also elicits immediate expression of *per1* mRNA, suggesting the involvement of Ca^{2+} signal-transduction pathway in resetting clock (*45*, *47*, *48*). Furthermore, we found that treatment of NIH3T3 with TPA, an activator of protein kinase C (PKC), could trigger the circadian oscillations of clock and clock-related gene expressions (*49*). This clock-resetting effect of TPA was inhibited by a MEK inhibitor and a prolonged activation of the MAPK cascade was sufficient to trigger circadian gene expression, strongly suggesting that MAPK cascade is involved in resetting of the circadian clock. Accumulating data suggest that cAMP response element-binding protein (CREB) acts upstream of the core clock components. Phosphorylated CREB might bind CRE sites in the *per1* promoter and directly enhance its transcription. As CREB can be phosphorylated by multiple kinases including PKA, calmodulin dependent kinase, and MAPK, these pathways might regulate the resetting of the clock through the activation of CREB (*50*, *51*).

Although acute and immediate induction of *per1* mRNA expression associated with the rhythm entrainment is observed in all cases described above in the SCN and in cultured cell lines, its importance and its exact role in resetting the clock are not clear. Is this acute expression of *per1* necessary and/or sufficient to reset the clocks? This question defies a simple solution. Ligands for some receptor tyrosine kinases (RTKs) such as insulin and FGF are able to trigger both induction of *per1* mRNA and following oscillation of circadian gene expressions in cultured fibroblasts (*45*, *49*). Although epidermal growth factor (EGF) is also able to induce immediate expression of *per1* mRNA, following circadian gene expressions are not observed (*45*, *49*). Unlike EGF, FGF signaling induces prolonged activation of MAPK, which might play an important role to generate the rhythms after induction of

per1 mRNA. These results suggest that acute induction of *per1* mRNA expression is not sufficient to reset the rhythm. Recently it was demonstrated that antisense oligodeoxynucleotide (ODN) against *per1* could block glutamate-induced phase shift in rat cultured SCN slices (*52*). Moreover, CRE-decoy ODN that sequesters phospho-CREB and prevents its binding to CRE sites could block light-induced phase shift *in vivo*. Taken together, light-induced phase shift in the SCN requires rapid expression of *per1* mRNA regulated by CREB but *per1* expression alone is not sufficient to induce rhythm entrainment. However, it has been suggested that there is some clock-resetting mechanisms that do not accompany immediate expression of *per1* mRNA. It has been demonstrated that a medium change is able to trigger circadian rhythms in rat-1 fibroblasts without the acute induction of *per1* mRNA expression but, rather, the down-regulation of *per1* mRNA expression, probably by glucose dependent manner (*53*). Furthermore, heat treatment is also able to trigger circadian oscillations of clock and clock-related gene expressions in NIH3T3 fibroblasts without inducing *per1* mRNA expression (*35*). These *per1*-independent mechanisms of entrainment might also play an important role in regulating the circadian clock *in vivo*.

There are some factors known as the timing cue for peripheral oscillators *in vivo*. One of them is glucocorticoid signaling. The glucocorticoid analog dexamethasone is able to entrain circadian rhythms in rat-1 fibroblasts and in the periphery but not in the SCN, consistent with absent of receptor expression in the SCN (*45*, *54*). As described above, feeding is the dominant timing cue for peripheral oscillators, and thus glucocorticoids might participate in the resetting of peripheral clocks induced by feeding. However, situation is more complicated. Glucocorticoids level in serum shows diurnal change in mice fed *ad libitum* and these endogenous glucocorticoids have inhibitory effect on the uncoupling of peripheral and central oscillators induced by altered feeding time (*55*). Therefore, it takes several days to adapt peripheral clocks to the altered feeding schedule. This buffering effect indicates the existence of complex metabolic mechanisms regulating peripheral clocks. It is more likely that feeding will reset peripheral clocks by inducing the changes of redox state in the cells, which can affect the binding intensity between DNA and CLOCK:BMAL1 heterodimer and/or NPAS2:BMAL1 heterodimer (*24*, *56*). Another *in vivo* timing cue is ambient temperature. It has been demonstrated that ambient temperature cycles can entrain circadian gene expressions in rat-1 cells and in the liver without affecting the SCN (*57*). Further, ambient temperature entrains the core body temperature of mice. Thus, ambient temperature cycles might entrain peripheral oscillators both directly and indirectly by affecting the core body temperature cycles. Retinoic acid is also *in vivo* resetting factor that can phase-shift the rhythm in vascular cells, and the nuclear hormone receptors, RARα and RXRα, interact with CLOCK:BMAL1 heterodimer in hormone-dependent manner resulting in negative regulation of CLOCK:BMAL1-mediated transactivation (*58*). As described above, peripheral oscillators are regulated by multiple and synergistic effects of various factors and there should be much more regulations in controlling peripheral oscillators against the SCN pacemaker. Figure 1 shows possible regulations that can reset or phase-shifts circadian gene expressions *in vitro*.

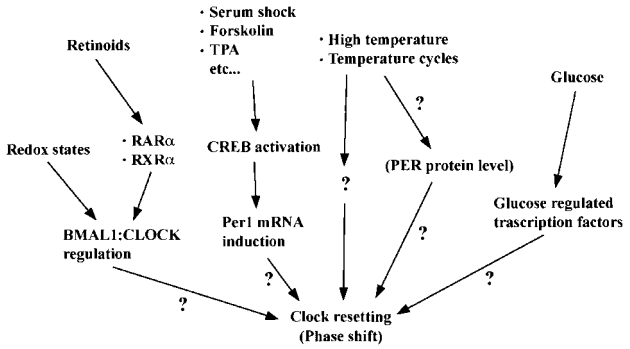

Fig. 1. **Possible intracellular regulation in clock resetting.** A variety of intracellular signaling pathways leading to clock resetting have been reported previously. Various stimuli have been shown to reset or phase shift the clock, but it is still obscure how the rhythmic expression of clock genes is generated.

Taken together, it seems that peripheral oscillators are more susceptible to external timing cues than the SCN and it might indicate the flexible characteristics of peripheral clocks to adapt to transient environmental changes. Cultured cell systems have been useful tools in understanding the characteristics of peripheral oscillators in clock-resetting mechanisms and so are expected to be in future studies.

Circadian outputs from peripheral oscillators

As for the SCN, several factors are known as candidate outputs described in previous section. They might entrain the clocks outside the SCN and control hormonal secretion from the SCN and the other regions of brain. Then what is the output from peripheral oscillators? A number of genes have been found to be regulated downstream of the core clock components, which include some transcription factors such as *Dbp* and *E4bp4*. They are expressed in circadian fashion in the peripheries as well as in the SCN and regulate various gene expressions. It is also indicated that they are involved in the core feedback loops (*59*, *60*). DBP has been rather well investigated and shown to control circadian expression of *Cyp2a4* and *Cyp2a5* in mouse liver (*61*). CYP2A4 and CYP2A5 catalyze one of the hydroxylation reactions leading to further metabolism of the sex hormone testosterone in liver. A series of large-scale profiling of diverse gene expressions by microarray analysis have revealed that a substantial number of genes are expressed in circadian manner and the sets of oscillating genes considerably differ among the SCN, liver, heart, and cultured fibroblasts (*62–67*). These oscillating genes include, for example, the components of cellular vesicle trafficking and cytoskeletal structure and detoxification, energy metabolism and cholesterol metabolism in liver. It would make sense in terms of adaptation for daily changes of environment that the daily vital functions in peripheral tissues are not controlled directly by the SCN but by peripheral oscillators that are the more flexibly entrained clocks than the SCN. In fact, hepatic clock, unlike the SCN, can be phase-shifted throughout the 24-h day (*54*).

Furthermore, expression levels of some cell cycle regulators such as *Cyclin D1*, *Cyclin D3*, *Cdk4*, and *Ring3* were found to oscillate in a circadian manner (*62*, *65*). These observations suggest that circadian rhythms play some role in cell cycle regulation. In addition, it was reported that *mPer2* mutant mice show increased tumor development after γ radiation and have altered expression profile of *c-Myc* that play a key role in cell proliferation and apoptosis (*68*). On the contrary, there have been several data indicating the independency between the circadian rhythm and the cell cycle. For example, mutant mice of clock genes develop normally and fibroblasts show circadian gene expressions even when the cell cycle is arrested (*43*). Thus, circadian rhythms might regulate cell proliferation and DNA damage response following unpredicted hazard, while having little effect on the normal cell cycle. Even if circadian rhythms have some effect on the normal cell cycle *in vivo*, it should be ignorable or compensated by some other system(s).

Cultured cell line from the SCN

Peripheral oscillators are different from the SCN in lacking self-sustaining ability. Under constant conditions, circadian rhythms of peripheral cells are attenuated in a few days (*28*). In contrast, the SCN neurons have self-sustained rhythms. This difference might come from the existence of autocrine and/or paracrine output signals. In 1999, it was reported that an immortalized cell line derived from fetal rat SCN is established (*69*, *70*). This cell line, SCN 2.2, shows robust circadian rhythms in clock gene expressions and uptake of 2-deoxyglucose, and transplantation of SCN 2.2 cells to SCN-lesioned rats restores their activity rhythm (*69*, *71*). These observations indicate that SCN 2.2 cells have distinct properties as circadian clock pacemaker. Furthermore, when cocultured with SCN 2.2 cells, NIH3T3 mouse fibroblasts show robust circadian rhythms with delayed phase to SCN 2.2 cells by 4–12 h (*71*). In contrast, serum-shocked NIH3T3 cells are not able to entrain cocultured NIH3T3 cells. These results indicate that some diffusible output signal that can sustain the rhythm is secreted from the SCN. It is interesting that although SCN 2.2 cells and NIH3T3 cells are cocultured in same medium, there is a time lag between their phases. Possibly there might exist several output factors from the SCN neurons; one is more effective to the SCN cells themselves and another to NIH3T3 cells. Anyway, this SCN-derived cell line must be a powerful tool to understand the property of the SCN, to explore output signals from the SCN, and to investigate the direct relationship between the SCN and peripheral oscillators *in vitro*.

Conclusion

It seems that mammalian circadian clock is realized in well-balanced system between the central and peripheral oscillators (*72*). Although peripheral clocks are basically under the control of the SCN that sustains the robust rhythms according to the day-night cycles, they can be flexibly entrained to changes of fending behavior and ambient temperature (Fig. 2). Cultured cells, as a model system of *in vivo* circadian oscillators, would contribute to the further studies on molecular clockworks of mammalian circadian rhythms.

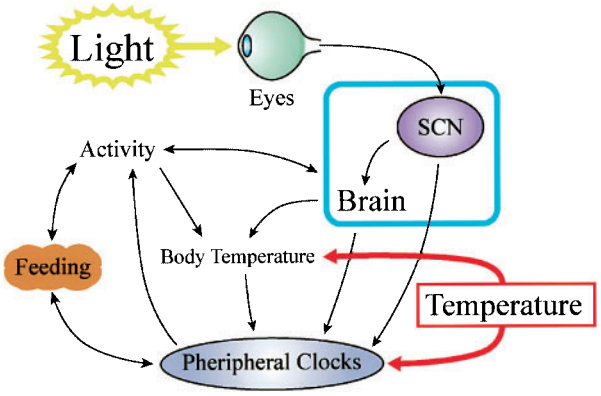

Fig. 2. **Complex interactions of mammalian circadian system.** Mammalian circadian regulation is very complicated because various factors correlate with each other. Light and ambient temperature are external timing cues ever known. Feeding also affects peripheral clocks and activity rhythms and in turn, activity and metabolic rhythms might bounce back on feeding behavior. Overall regulation of circadian clockworks in the whole organism is yet to be revealed.

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