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Zhang, Jing

(Degree)
博士（医学）

(Date of Degree)
2005-03-25

(Date of Publication)
2013-02-14

(Resource Type)
doctoral thesis

(Report Number)
甲3371

(URL)
https://hdl.handle.net/20.500.14094/D1003371

Kobe J. Med. Sci., Vol. 50, No. 4, pp. 101-109 , 2004

Molecular Signals of Mammalian Circadian Clock

JING ZHANG, XIN DONG, YOSHITO FUJIMOTO,
and HITOSHI OKAMURA

Division of Molecular Brain Science, Department of Brain Sciences,
Kobe University Graduate School of Medicine

Received 5 January 2005/ Accepted 19 January 2005

Key Words: clock genes; transcription regulation; clock proteins; ubiquitination

The circadian rhythm is originally generated by a transcription-translation based oscillatory loop composed of a set of clock genes in most organisms. The clock gene oscillation is generated by the core loop in each neuron of the hypothalamic suprachiasmatic nucleus. Phosphorylation and ubiquitination of clock proteins play the crucial role for the rhythmic transcription of clock genes. The core clock oscillation is conducted at the cellular levels by E-box or by D-box of clock controlled genes.

The period lengths of biological cycles are very similar to environmental cycles: menstrual period follows the lunar cycle; sleeping, awakening, and secretion of cortical hormones follows the 24 h rhythm of day and night, and the secretion of growth hormone and gonadotropin releasing hormone is hourly. Among these periodical cycles, the 24 h rhythm is best investigated. Although this rhythm seems to occur passively by the day/night changes of the solar energy, but actually, this behavior is elicited actively by the organisms which contain an internal pacemaker (biological clock) that generates an endogenous rhythm without any cues from the environment.

Most organisms living on earth have an internal clock and thus circadian rhythm represents a basic feature of life. In mammals as in many organisms, the circadian oscillation is driven by a transcription/translation-based core feedback loop of a set of clock genes which is dynamically regulated by clock proteins. The feature of the circadian system is the prevalence of the oscillation at the levels of genes reflects at cells, tissues, and system levels. Oscillation of cell clocks is performed by the intracellular molecular core feedback loop of clock genes. The oscillation starts first at the transcription of two main oscillators, *mPer1* and *mPer2* (3, 29, 45). The promoter regions of *mPer1* and *mPer2* genes exhibit circadian rhythms of histone acetylation (10), and to the E-box of these promoters, heterodimers formed by the bHLH-PAS proteins (CLOCK and BMAL1) bind (20), and initiate the transcription of these *mPer* genes (12). Activated transcription results in the formation of *mPer1* and *mPer2* mRNAs, which are translated in the cytoplasm to mPER1 and mPER2 proteins. These proteins translocate into the nucleus, and form negative complex that comprises mCRY1, mCRY2, mPER1, mPER2, mPER3 and mTIM, which suppresses the transcription of the *mPer1* and *mPer2* genes by binding to the positive factors (CLOCK/BMAL1). Since *mCry1/mCry2* double knock-out mice and *Bmal1 (Mop3)* knock-out mice (6) show the immediate loss of behavioral rhythm in constant darkness, *mCry1/mCry2* and *Bmal1* play a key role for making up the core loop.

If the concentration of negative factors determines the time for the shut off of the transcription, the question remains what mechanisms determine the concentration of clock proteins. The mPERs are made in the cytoplasm, translocate into the nucleus, and form a

Phone: 81-78-382-5340 Fax: 81-78-382-5341 E-mail: okamurah@kobe-u.ac.jp

negative complex comprised of mCRY1, mCRY2, mPER1, mPER2, mPER3 and mTIM that suppresses the transcription of the *mPer1* and *mPer2* genes by binding to the positive factors. Phosphorylation of PER1 and PER2 by casein kinase Iε (CKIε) is crucial for determining the circadian period length (21, 22, 33). Experiments leading to this conclusion and others point strongly to the importance of posttranscriptional and posttranslational regulatory mechanisms in the cell clock. Furthermore, there are growing evidences suggesting that clock proteins are regulated dynamically in both spatial (nuclear and cytoplasm) and temporal (production and degradation) dimensions. The main clock oscillatory protein mPER2 usually shuttles between the cytoplasm and the nucleus and is easily degraded by ubiquitination and the proteasome pathway (38). Ubiquitination of mPER proteins is inhibited by the presence of mCRY proteins. Since mCRY protein can also be ubiquitinated when mPER proteins are absent (38), the mPER/mCRY dimer is stabilized, suppresses *mPer1* and *mPer2* transcription, and shuts off mPER translocation **(**Fig. 1**)**. Since it is speculated that the transcription level of

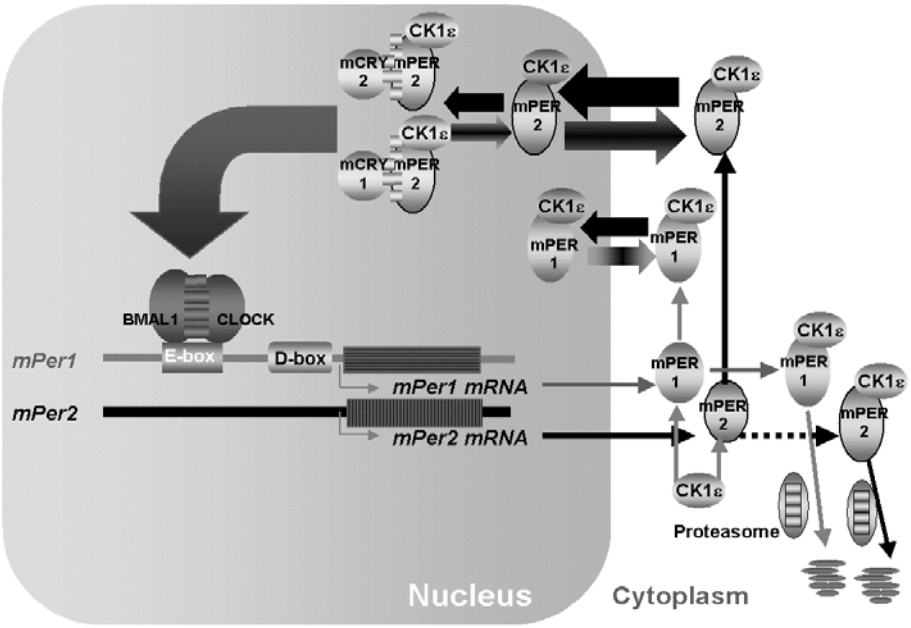

Fig. 1 A model of the core feedback loop in the mammalian circadian clock. To E-box in clock oscillating *mPer1* and *mPer2* genes, BMAL1/CLOCK heterodimer binds and initiates their transcription. The core negative autoregulatory feedback loop is regulated at protein level by negative complex consisting of mPER1, mPER2, mPER3, mCRY1 and mCRY2. mPER2 protein is produced in the cytoplasm, phosphorylated by casein kinase Iε (CKIε). mPER2 protein keeps on shuttling between nucleus and cytoplasm via the CRM1/Exportin1 nuclear export system until (i) mPER2 is ubiquitinated and subsequently degraded by the proteasome system or (ii) the stabilization of nuclear mPER2 by the binding of mCRY1 or mCRY2.

mPer genes is understandable as the concentration of mPER/mCRY dimer in the nucleus, re-starting *mPer* transcription will depend on the nuclear export of the mPER proteins. The decrease of mPER in the nucleus by the CRM1/Exportin1 nuclear export machinery causes destabilization of mCRY, and the decrease of mCRY allows *mPer1* and *mPer2* gene transcription to restart (38).

In *Drosophila per*, the main oscillatory gene in the fly, the PER protein is known to show rhythm without accompanying the rhythm at its transcription level (7, 11, 34, 43). In

mammals, we recently found that mPER2 protein accumulation in the peripheral cells showed clear circadian oscillation even in the presence of constitutive *mPer2* mRNA expression by using the fibroblast cell lines (41). This finding suggests that posttranscriptional regulation plays an important role in generating the core clock oscillation in mammals as in *Drosophila*. Since the mutation of *Drosophila slimb*, an F-box protein constituting ubiquitin ligase, shows the constant accumulation of PER protein and behavioral arrhythmicity, ubiquitin-proteasome mediated degradation will be involved in this process (13,18) **(**Fig. 2**)**. The involvement of ubiquitin ligase to circadian clock oscillatory machinery will be evolutionally conserved since FWD1, an F-box protein, regulates the ubiquitination of *Neurospora* FRQ, which is the main oscillatory component of circadian feedback loop in this species (15).

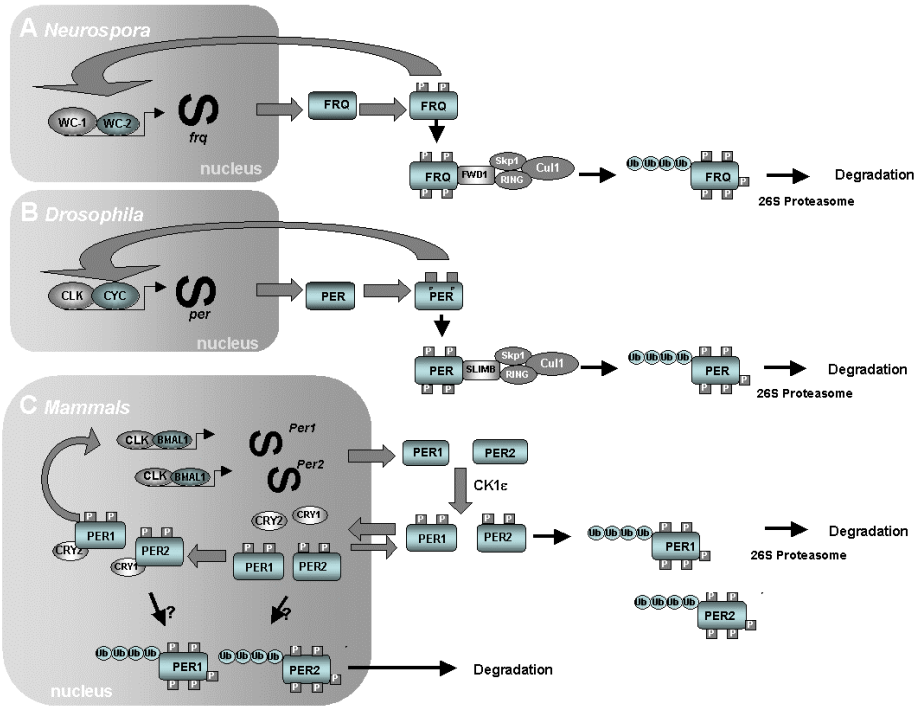

Fig. 2 Involvement of ubiquitin-proteasome system on the circadian systems in Neurospora, Drosophila and mammals. In Drosophila, phosphorylated PER is recognized by SCF type E3 ubiquitin ligase such as SLIMB, ubiquitinated and finally degraded by proteasome. In Neurospora, transcription of *frq* was accelerated by WC-1/WC-2 heterodimers. Produced FRQ protein was phosphorylated and suppresses transcription activation of WC-1/WC-2. FRQ is recognized by SCF type E3 ubiquitin ligase FWD-1, ubiquitinated and finally degraded by proteasome. In mammals, PER1 and PER2, which are unconjugated with CRY1 and CRY2, are ubiquitinated and finally degraded.

To reveal the role of ubiquitin-proteasome system in mammals, we examined the expression of ubiquitin-related substances in the suprachiasmatic nucleus (SCN), the mammalian circadian center by in situ hybridization. The animals used for the in situ hybridization were male Balb/c mice at 6 weeks age. They were housed under standard 12h:12h light-dark (LD) cycles, and the expression of ubiquitin-related enzymes at ZT4 (ZT stands for Zeitgeber time in a LD cycle; ZT0 is lights-on and ZT12 is lights-off and thus ZT4 means 4 hours after the light onset). ^{33}P-UTP (New England Nuclear, Boston, MA) labeled

antisense probe to UchL1, valosin-containing protein (VCP) and β-TRCP were made with a standard protocol for cRNA synthesis. Mice were deeply anesthetized with ether, and intracardially perfused with 10 ml of autoclaved ice cold saline, followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH7.4). For the animals housed in darkness, anesthesia was performed under safe dark red light. The brains were removed, postfixed in the same fixative for 24 h at 4°C and placed in 0.1 M PB containing 20% sucrose for 48 h. These brains were frozen using dry ice and stored at -80°C until use. Mouse brain sections were made 40 μm in thickness by a cryostat. In situ hybridization histochemistry was performed as described previously (29). Briefly, tissue sections were processed with 1 μg/ml proteinase K and 0.25% acetic anhydride. The sections were then incubated in the hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris–HCl, pH 8.0, 1 μM EDTA, pH 8.0, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 mg/ml transfer RNA, 1×Denhardt's and 0.25% sodium dodecyl sulphate) containing the ^{33}P-UTP-labeled antisense cRNA probes for 16 h at 60°C. After hybridization, these sections were rinsed in 2×SSC/50% formamide for 45 min at 60°C, and rinsed in 2×SSC/50% formamide for 15 min at 60°C and the sections were treated with a solution containing 10 μg/ml Rnase A for 30 min at 37°C. After rinsing, sections for free floating in situ hybridization were mounted onto gelatin-coated microscope slides, air-dried, and dehydrated.

β-TRCP, a mammalian homologue of *Drosophila slimb*, was only slightly expressed in the SCN. This suggests that this F-box protein seems not to be involved in the oscillation of the master clock in the SCN. Thus, the involvement of F-box protein on mammalian circadian system waits for further future analysis. Contrary to β-TRCP, in the SCN, we found the high levels of expression of UchL1, a main member of deubiquitinating enzyme in the brain **(**Fig. 3A**)**. UchL1 recycles ubiquitin from ubiquitin-protein complexes or

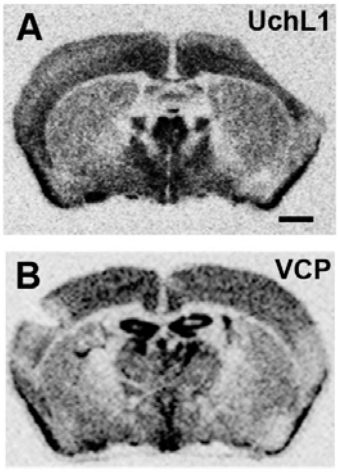

Fig. 3 Abundant expression of UchL1 mRNA (A) and VCP mRNA (B) in the suprachiasmatic nucleus. In situ hybridization histochemistry using radiolabeled specific antisense cRNA probes. VCP is strongly expressed in the SCN, the piriform cortex and the hippocampus. UchL1 shows the strong signals in the SCN, bed nucleus of stria terminalis, and the thalamic paraventricular nucleus. Bar = 1 mm.

polyubiquitin chains by cleaving the amide linkage neighboring the C-terminal glycine of ubiquitin (19). We also found a dense accumulation of molecular chaperone VCP in the SCN

(Fig. 3B**)**. More broader and deeper analyses are needed for elucidating the involvement of ubiquitin-proteasome on mammalian circadian system.

It should be in mind that the time signals produced by this oscillatory loop are differently processed unique to cell types. Gene array studies have demonstrated that hundreds of genes are controlled by circadian clock (26, 31) with its tissue specificity. From the core oscillatory loop to these clock controlled genes (ccgs), two routes are commonly used. The first is E-box (CACGTG, CACGTT) mediated mechanisms directly controlled by CLOCK:BMAL1 heterodimers. This is observed in many genes and its representative is the vasopressin gene regulation (17). The second route is an indirect pathway of D-box (RTTAYGTAAY: R, purine; Y, pyrimidine), consisting of antagonistic regulation of PAR proteins and E4BP4 (23, 39), which is also used as the accessory feedback loop of *mPer* genes. Albumin, cholesterol 7α hydroxylase, cytochrome P450 (*Cyp2A5*) and possibly aromatic L-amino acid decarboxylase are regulated by the D-box mechanism (16), in which the positive PAR proteins and the negative E4BP4 switch back and forth between the on-off conditions of the target genes.

In the SCN, clock genes keep timing with a clear circadian rhythmicity *in vitro* as *in vivo* (2, 42). Fibroblast cell line does not show rhythms without any stimulation, but external stimuli such as high concentration of serum, TPA and endothelin can induce the circadian expression of the clock genes for several cycles (1, 4, 5, 37). Are there any differences of the composites of core oscillatory loop involving a set of clock genes in the central and peripheral cells? Yagita et al. (37) tried to address this by using wild type and *cryptochrome* (*mCry*)-deficient cell lines derived from *mCry1/2* double deficient mice. Since, (i) temporal expression profiles of all known clock genes, (ii) the phase of the various mRNA rhythms (i.e. antiphase oscillation of *Bmal1* and *mPer genes*), (iii) the delay between maximum mRNA levels and appearance of nuclear mPER1 and mPER2 protein, (iv) the inability to produce oscillations in the absence of functional *mCry* genes, and (v) the control of period length by mCRY proteins, they concluded that molecular oscillatory components and their oscillatory mechanism of central clocks (in the SCN) and peripheral clocks (represented by fibroblasts) are mostly identical.

Thus at least in some adequate conditions, peripheral tissues *in vivo* and *in vitro* can show rhythmic expression of clock genes. The concept that most peripheral cells are oscillating was already reported in *Drosophila* (9) and zebrafish (35), in which the peripheral clocks can be entrained directly by light (30, 36). *In vivo*, clock genes are oscillating in various organs including liver, lung, and blood vessels (24, 25). Among them, liver hosts a powerful peripheral oscillator (28), where circadian clock is entrained by a restriction of feeding (8). This feeding-related entrainment is also observed in SCN-lesioned animals, which indicates that the process in liver is independent from the central clock (14).

It is already known that the SCN clock genes oscillate sustaining the original rhythms for more than a month in slice cultural conditions (2, 42). Recently Yoo et al. (44) demonstrates the sustained peripheral rhythms for over 20 cycles in culture, although a previous study reported that rhythms of peripheral tissues damped after a few cycles (42). Thus, both central and peripheral oscillators have the rhythmic sustained oscillatory ability with their own oscillatory machinery. In the SCN, the clock gene oscillation generated by the core loop in each SCN neuron produces the robust spike rhythms at each cell level. Thousands of multi-phased, clock oscillating cells synchronize and produce a stable and robust rhythm (40), which is transmitted to the peripheral tissues. Since peripheral tissue cultures *en bloc* show the circadian rhythm of clock genes (44), there must also be synchronizing ability among cells in peripheral tissues although they still remain unknown at present.

In contrast to these *in vitro* findings, *in vivo*, SCN is the sole powerful tissue which can induce the rhythms of clocks in peripheral tissues: the destruction of the SCN abolishes rhythms in clock gene expression in the liver (27) or disorganizes the rhythms of clock gene in cultured liver tissues (44). Thus, now it is thought that mammalian clock system displays a complex hierarchical structure of cell clocks at various levels headed by the oscillating SCN cell clocks at the top **(**Fig. 4**)**.

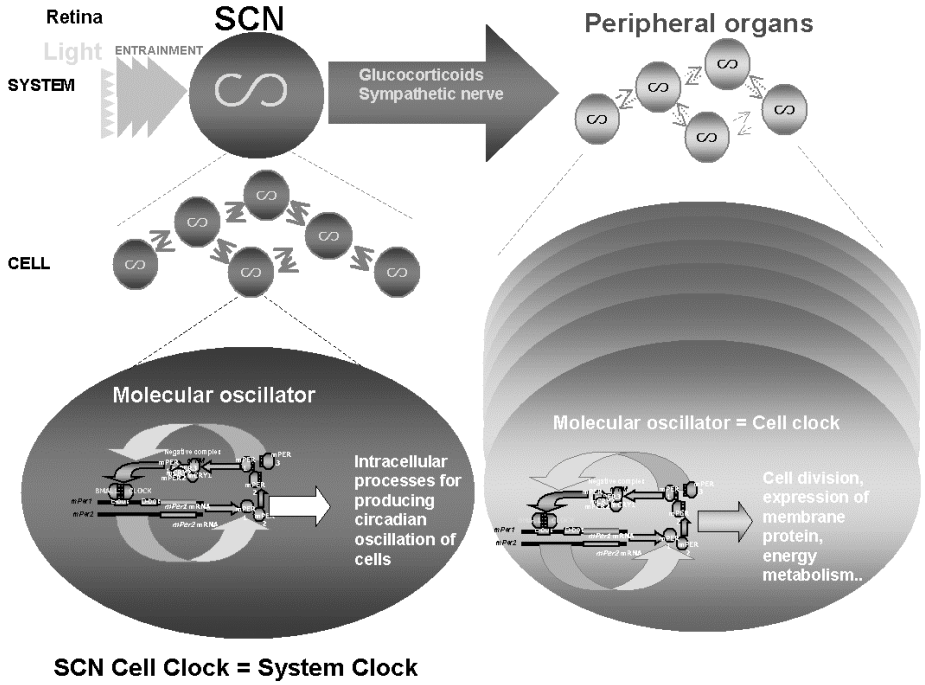

Fig.4 Molecular core oscillator and cell clock in the central and peripheral clock. Molecular oscillator is common in both central and peripheral clocks. Central clock in SCN is the only clock which can regulate another clock at system level. Outputs of SCN regulate behavioral rhythms via non-SCN brain clocks, and peripheral clocks via glucocorticoids and the sympathetic nerve.

The unique feature of circadian biology is that the gene transcription occurring in the SCN reflects the behavioral and physiological rhythms. This means that the clock gene oscillation generated by the core loop in each SCN neuron is coupled and amplified, and spread into the whole brain and to all those peripheral organs including liver through oscillation conducting systems, where glucocorticoids and sympathetic nerves play important roles (5, 32). In the peripheral organs, arriving clock signals entrain the cell clocks, and the intracellular oscillating molecular loop coordinates the timing of the expression of a variety of genes with specific cellular function.

Does TIME developed by the cellular core oscillator have cellular functions? The proteins are key players of cellular function, and for single functional proteins (5-30 nm in diameter), cell space is a huge space (10-50 μm in diameter). Thus, if one protein is hypothesized to correspond to our human size, the boundary of edge of cells is about 1000 m far away. Thus to perform effective cellular functions, things must be organized in ensemble in time dimension as well as in space dimension. Intracellular clock oscillating loop may be

worth existing for controlling cellular events into proper and adequate time organization. TIME is a key word for clock genes to perform non-clock cellular functions.

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