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Functional implications of the mammalian clock gene, *mPer* expression in the peripheral tissues

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ABSTRACT

It is well established that circadian clocks are present in the peripheral tissues as well as in the brain and they confer rhythmic behavior and physiology. It is also evident that interactions between clock genes and their products are necessary in the clock functioning. In this study, we investigated the expression of clock genes in the periphery by using skeletal muscles from *mPer1* or *mPer2* gene knockout mice and the control group. We found that either *mPer1* or *mPer2* knockout mice maintained the same phase relationship to the control mice. Clock gene expressions were still rhythmic in the level of mRNA expression in skeletal muscles in contrast to those in the brain. All together, our results suggest that *mPer* genes participate in the maintenance of rhythms differently in the brain and in non-neural peripheral tissues.

Key words : circadian clock, clock genes, *mPer*, suprachiasmatic nucleus, phase shift, knockout mouse

Introduction

Most living organisms on the earth show circadian oscillations in their physiology and behavior. In mammals, these rhythms are governed by circadian pacemaker neurons that reside in the brain region called suprachiasmatic nucleus (SCN)(1,2). Through intense analysis of circadian clock functioning at molecular levels, researchers have recently identified several so called clock genes from model organisms such as *Neurospora*, fruitflies, and mice(3-7). The circadian clock is regarded as a self-sustaining transcriptional/ translational feedback loop in which the expression of clock genes is suppressed periodically by their protein products(8).

The Drosophila *period* (*per*) gene is the first identified clock gene by aid of molecular genetics studies(5). In Drosophila, PER interacts with another protein, TIM from clock gene *timeless* (*tim*)(7). Once bound, PER-TIM dimers enter the nucleus and block their own transcription via inhibiting the DNA binding activity of two transcription factors called dCLOCK and CYCLE. Both dCLOCK and CYCLE are basic helix-loop-helix (bHLH) type transcription factors that bind to E-box (CANNTG) sequences present in the promoters of *per* and *tim*(3,9). PER and TIM also undergo progressive phosphorylations by the action of two specific kinases called DOUBLETIME (DBT) and SHAGGY (SGG), respectively. These posttranslational modifications on PER and TIM are thought to accelerate protein turnover, which allow dCLOCK-CYCLE to be free from inhibition and to begin new round of protein synthesis(8,10).

It is not surprising that circadian clocks are conserved throughout biological systems during evolution. Indeed, based on the structural and functional homology, several clock genes are also identified in mammals. For example, three mammalian *per* genes, named *mPer1*, *mPer2*, and *mPer3*, respectively, that show similar sequences to Drosophila *per* were identified(6,11-13). Among these, *mPer1*

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and *mPer2* were also implicated to light-induced clock shifts(11,14). For the mammalian counterpart of dCLOCK-CYCLE, CLOCK and BMAL1 were also cloned and both were implicated in the production of other clock genes including three *mPer* genes (4,15,16). In addition, two strong negative regulators of CLOCK-BMAL1, and possibly circadian clock-specific photoreceptors, *mCryptochrome* (*mCry1* and *mCry2*) are found(17,18; Fig. 1).

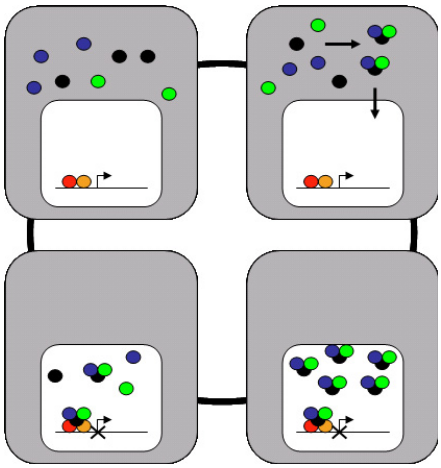


Fig 1. A schematic diagram of showing how a mammalian clock cell generates the rhythmic clock gene expression. CLOCK-BMAL1 dimers in the nucleus bind to the promoter regions of clock genes and begin to transcribe them (upper left). Once reached to the threshold level, clock gene products such as PER, CRY, and TIM interact with each other to form multimeric complexes and be translocated to the nucleus (upper right). In the nucleus, these clock proteins block the action of CLOCK-BMAL1 by physically interacting and somehow repressing their transactivation (bottom right). Meanwhile, the amounts of clock protein decline due to the lack of transcription mediated by their own negative feedback and also to protein turnover (bottom left). Then, repression by them is relieved and CLOCK-BMAL1 can initiate new round of transcription. Two cellular compartments are shown as a rectangle either in gray (cytoplasm) or in white (nucleus). Different clock proteins are marked as filled circles in colors (CLOCK: red, BMAL1: orange, PER: blue, TIM: green, CRY: black). The X indicates the transcriptional inhibition mediated by PER-TIM-CRY protein complexes.

It is now well known that every cell in organisms, even in cultured cells, expresses these clock genes rhythmically(6,8,19). Therefore, the circadian clock is ubiquitous and has unicellular moiety. Previously, we generated mice with targeted disruption of either *mPer1* or *mPer2* and showed that these two clock genes functioned distinct roles in the SCN circadian clock(12). In this report, we extended our study to better understand the importance of mPER expression in circadian clockworks, and compared the expression profiles of clock genes from mice with the loss of either *mPer1* or *mPer2* in non-neural peripheral tissues.

Materials and Methods

Animals

The animals used in the study were treated in accordance with the NIH guidelines for the care and use of experimental animals. Animals were raised within a temperature and humidity controlled room and were exposed 12-h light: 12-h dark (LD) cycles. All mice studied were of the same isogenic 129/sv genetic background. Genotype was determined by polymerase chain reaction (PCR) amplification of genomic DNA extracted from tail biopsies as previously described(12).

In situ hybridization

Eight to ten week-old male 129/sv mice were housed in LD cycles at least 10 days prior to analysis. Mice were sacrificed by carbon dioxide inhalation and decapitation. At each indicated time, brains were dissected and frozen at -80℃. Gene expression in the SCN was assessed by *in situ* hybridization using methods described previously(12). Briefly, coronal brain sections (15μm thickness) were hybridized with ^{35}S-labeled riboprobes generated by *in vitro* transcription (Promega). The template DNAs for both sense and antisense probe generation were PCR-amplified cDNA fragments subcloned into TA vector (Invitrogen). Probe for *mPer2* were nucleotides (nt) 9-489 of GenBank accession number AF035830. Image analysis was performed using NIH Image and Photoshop software as previously described(20).

Northern blot analysis

Mice were kept in LD cycles before analysis, and the light

was turned off at the day of tissue collection. Skeletal muscles from mice were collected and stored at -80℃ in every 4 hour intervals over one day long. Tissues were homogenized and total RNA was extracted using the Ultraspec RNA isolation reagent (Biotex Labs). Polyadenylated RNA was prepared using oligotex dT spin columns (Qiagen). 1μg of RNA of each time point were electrophoresed through a 1% agarose-formaldehyde gel, transferred onto GeneScreen (NEN) and cross-linked by a UV-crosslinker (Stratagene). The blots were hybridized with ExpressHyb solution (Clontech) and processed following the manufacturer's protocol. Probes used were *mPer1* (nt 468-821 of accession number AF022992), *mPer2* (nt 9-489 of accession number AF035830), *mCry1* (nt 1081-1793 of accession number AB000777), and *Bmal1* (nt 864-1362 of accession number AF015203). As a loading control, a probe for human β-actin, purchased from Clontech, was used. Blots were exposed at -80℃ to Kodak BioMax film with two intensifying screens.

Results/ Discussion

Our previous studies with either mPER1 or mPER2 knockout mice indicate that both of these proteins are essential for the maintenance of circadian rhythmicity in behavior when measured by wheel running activity. While both mice were similar on the behavioral phenotypes, the molecular characteristics were distinct from each other. Mice with the loss of mPER1 showed the normal level of SCN clock gene expression but reduced peak number of mPER2 and mCRY1 immunoreactive nuclei. In contrast, disruption of mPER2 resulted in reduced levels of clock gene expression in the SCN(12). In the present study, we measured the clock gene expressions in the peripheral tissues from these mPER1 or mPER2-deficient mice and compared them to those in the brain and we found that the temporal rhythms of gene expression in the periphery were maintained in both of these mPER-deficient mice.

As mentioned before, three *mPer* genes are identified in mammals. In the SCN, all of these three genes show their peak expression in the morning and trough in the night. Consistently with the previous report, we could observe that the level of *mPer2* transcripts oscillate in a circadian manner in wild-type mice when measured at either ZT6 or ZT18 (where zeitgeber time 6 [ZT6] is defined as 6-h after lights-on time and ZT18 as 6-h after lights-off time during LD cycles) (Fig. 2A). In addition, we found that the loss of *mPer2* alone could reduce the level of clock gene expression in LD cycles(12). By performing 3′-RACE, we previously noticed that our *mPer2* mutant mice also expressed a little amount of small-sized transcript that ended in exon4 of the gene. Because *mPer2* probes we used were spanning 5′ region (nt. 9-489, covering from exon2 to exon4) of the gene, as expected, we could observe a minor but specific signal to those transcripts in both *in situ* and Northern hybridization experiments (12; Fig. 2B and 3B).

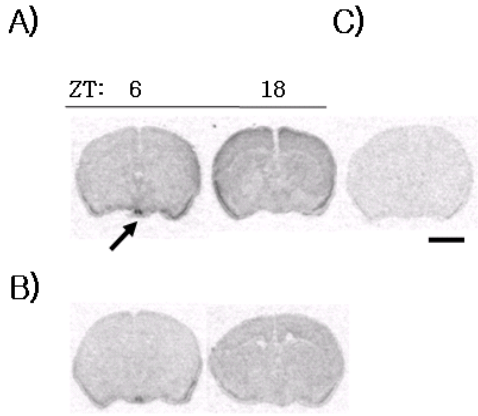
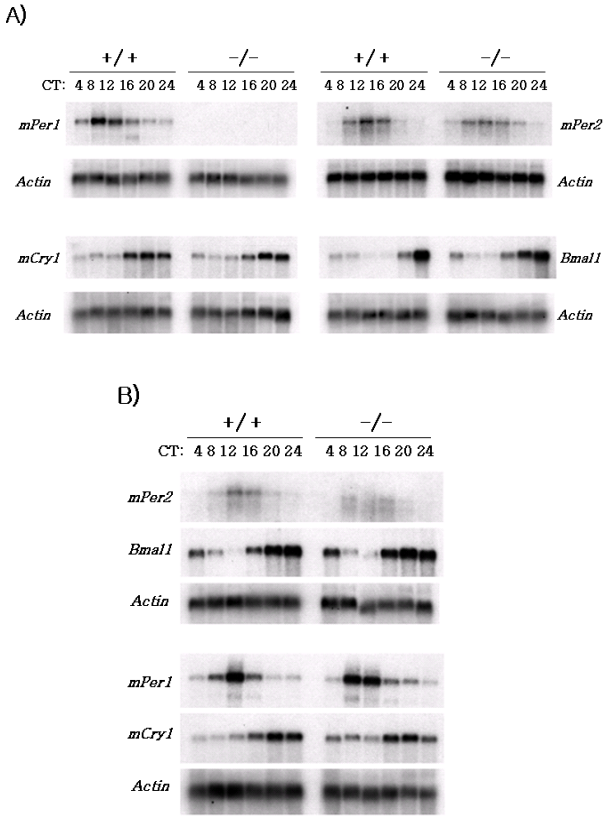


Fig 2. Representative brain images showing changes in abundance of *mPer2* transcript in the SCN from wildtype (A, C) and *mPer2* knockout mice (B). Brains were collected at either ZT6 or ZT18 under 12-h light: 12-h dark (LD) cycles and coronal brain sections were prepared. In situ hybridization on the mouse brain sections was performed by using antisense (A, B) or sense (C) probes to *mPer2*. The SCN is indicated by an arrow. A scale bar, 2.5mm, is also shown.

We performed Northern blot analysis to examine the temporal expression profiles of clock gene transcripts in skeletal muscles from mice with either *mPer1* or *mPer2* knockout mice and from the control mice. As shown in Figure 3A and B, all four clock mRNA levels that we measured oscillated in a circadian manner. For instance, the level of *mPer2*

showed its peak expression around CT 12 and its trough at CT24. While the circadian rhythmicity of either *mPer1* or *mPer2* transcript in skeletal muscles showed its peak expression around CT8-12, *Bmal1* showed its peak at CT24, which is similar phase relationship to that in the SCN. On the contrary, *mCry1* in the periphery maintained a delayed peak expression up to 8 hours when compared to that in the SCN (Fig. 3A and B). Therefore, the time course of either *mPer1* or *mPer2* transcript cycling in skeletal muscles was antiphase to that observed for *mCry1* and *Bmal1* RNAs. Surprisingly, there was no difference in the level of clock gene expression between genotypes.



Fige 3. Northern blot analysis of circadian clock gene expression in the skeletal muscle from either *mPer1* (A) or *mPer2* (B) knockout mice. Transcripts from wildtype (+/+) or knockout mice (-/-) were prepared at the indicated circadian time (CT) and the blot were probed with specific probes marked next to each autograph. Human β-actin (Actin) probes were used as an internal loading control.

What will be the roles of these mPER proteins? Both mPER1 and mPER2 are initially implicated in mediating clock phase shifts in the SCN to light pulses in a time-of-day specific manner. While *mPer1* mRNA is rapidly induced in the brain by a single light pulse both early and late in subjective night, *mPer2* induction is restricted to early subjective night(11). Treatment of antisense oligonucleotides specific to either *mPer1* or *mPer2* reduced the response to light(21). However, *mPer1* mutant mice ($mPer1^{psc}$) from other research group exhibited normal phase-shifting responses to light pulses(22). Consistently, our studies with mPER knockout mice did not support the involvement of mPER proteins in phase-shifting responses to light(14). Although they still may contribute to the molecular response to light in the SCN, it is not clear whether they influence the magnitude or shape of the phase response curve.

It is well established that, in the SCN in mammals, two bHLH-type transcription factors, CLOCK and BMAL1, function as dimers to bind to the promoter regions of clock genes and to transcribe them. Among these, clock gene products such as mPER and mCRY interact with each other to form protein complexes and be shuttled into the nucleus. Once in the nucleus, these proteins block their own transcription by physically interacting with CLOCK-BMAL1 and repressing their action. Both the repression of transcription and protein turnover contribute to the decline of clock protein levels, which can relieve CLOCK-BMAL1 from inhibition and let them initiate new round of transcription(2,8). Our previous studies suggest that *mPer1* and *mPer2* gene products in the SCN contribute to the stability of this circadian feedback loop by distinct mechanisms. mPER1 appears to be involved in post-translational stabilization of other clock proteins such as mPER2 and mCRY1, while mPER2 positively regulate BMAL1 at the transcriptional level(8,12,15). However, present results indicate that neither of these two mPERs is absolutely necessary for the rhythmic expression of circadian clock genes in the peripheral tissues, which is in stark contrast to their proposed roles in the brain SCN. It implies that the core of the circadian clock is composed of CLOCK-BMAL1 as positive factors, and mCRY as the negative factor(15-17). Nevertheless, mPER1 and mPER2 play modulatory roles in the circadian clockwork, and both are required for the main-

tenance of rhythmicity in the SCN(8,12). Therefore, even though armed with the same members of clock genes, two types of circadian clock, one in the brain and the other in the periphery, may function in a different way to manifest the rhythmic oscillation in behavior and physiology.

Conclusions

In conclusion, the present work reveals that mPER1 and mPER2 are not necessary for the rhythmic gene expression in the peripheral tissues such as skeletal muscles. In contrast to its proposed role in the brain, mPER2 seems not function as a transcriptional activator via affecting Bmal1 expression in skeletal muscles. In view of these, it awaits to identify the distinct clock mechanisms in which these two mPERs take part in the brain and in non-neural tissues.

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