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論文題目	Mechanism of circadian oscillation of the mammalian core clock gene *Per2* （時計遺伝子*Per2*の発現制御機構）		

（論文内容の要旨）

Daily rhythms in behavior and physiology are controlled by an internal self-sustained molecular oscillator, referred to as the circadian clock. Circadian rhythms are generated in a cell-autonomous manner through transcription/translation-based autoregulatory feedback loops, wherein protein products of three *Period* genes (*Per1*, *Per2* and *Per3*) periodically suppress their own transcription. Genetic studies suggest that, of the three *Period* genes, *Per2* has a dominant role in both humans and mice. However, little is known about the regulatory mechanism of this gene. In this thesis, I investigate the molecular mechanism driving the circadian transcription of the mouse *Per2* gene. I find that *Per2* promoter contains a circadian *cis*-element, termed Rhythm-box (R-box) (**Chapter 1**). Furthermore, I generated novel mutant mice carrying a point mutation on the sequence of the *Per2* promoter R-box (**Chapter 2**). Analysis of the mutant mice reveals a previously unknown *in vivo* function of this element in regulation of circadian transcription of the *Per2* gene (**Chapter 3**).

Chapter 1: Autoregulatory feedback model of *Per2* transcription through R-box

Firstly, I determined the circadian oscillation profiles of *Per2* mRNA and protein in the mouse livers. Mice were sacrificed at 4 h intervals over 24-h cycle. Quantitative RT-PCR and Western blot analyses revealed that the levels of *Per2* mRNA and protein exhibit an overt circadian oscillation with a typical phase-relationship. PER2 protein began to increase 8 hours behind mRNA expression and reached its peak when the mRNA levels were decreased. These observations corroborate the current negative feedback model in which PER2 represses its own transcription. In an attempt to further test this model, I next explored the regulatory *cis*-element on the *Per2* promoter. The mouse embryonic fibroblasts were transfected with a luciferase reporter vector containing the 1.7 kb fragment of the 5'-flanking region of *Per2*, and its activities were monitored using a real-time luminometer system. I observed a robust circadian oscillation of the *Per2* promoter activity over three cycles. Furthermore, I demonstrated that a single point mutation that was introduced into the R-box sequence located in the vicinity of the transcription start site of *Per2* resulted in a complete abrogation of this rhythmicity. These *in vitro* data strongly suggest that this single R-box element is indispensable for the circadian activation and repression of *Per2* gene.

Chapter 2: Generation of mice carrying a point mutation of the *Per2* R-box

To investigate the role of R-box in regulation of *Per2* oscillation *in vivo*, I generated *Per2* R-box mutant (P2Rmut) mice. To introduce a point mutation into this element, I employed *piggyBac* (*PB*) transposon system. *PB* is a moth-derived DNA transposon whose functionality retains in mammals. Unlike the well-established methods relying on the Cre/*loxP* and Flp/*FRT*-based recombination systems, PB transposase (PBase)/*PB*-based system achieves a 'seamless' excision of a *PB*-flanked target sequence without any residual 'footprint'. This allows, for example, 'complete' removal of a neomycin resistant gene-coding cassette from a mutant mouse genome without leaving any unwanted residual sequence. I therefore constructed the targeting vector, carrying a mutant R-box sequence and a neomycin resistant cassette flanked by *PB* terminal repeats. Targeted mouse embryonic stem cell clones were injected into blastocysts to generate chimeric mice. To remove the *PB*-flanked neomycin cassette from the genome, F1 heterozygotes were intercrossed with ROSA26-PBase mice that carry a ubiquitously expressed PBase gene at the ROSA26 locus. I performed Southern blot analysis and confirmed that the neomycin cassette was deleted without reintegration into the host genome. Finally, a seamless excision of the cassette was confirmed by DNA sequencing. The sequence analysis also verified that a point mutation was indeed introduced into the R-box. Moreover, the established mutant mice were backcrossed to the C57BL/6J background over ten generations, and at the end of backcrossing, I confirmed that 64 microsatellite markers covering all individual mouse chromosomes were replaced with those of the C57BL/6J mouse strain.

Chapter 3: Characterization of *in vivo* role of the *Per2* R-box

To evaluate whether the R-box mutation *in vivo* abrogates rhythmicity of *Per2* expression, I measured circadian profiles of *Per2* mRNA in the mouse liver samples collected every 4 hours in constant darkness. Quantitative RT-PCR analysis revealed that the point mutation of the *Per2* R-box caused alteration of the circadian rhythm of *Per2*. This mutation impairs circadian repression of *Per2* expression and thereby elevates the levels of the baseline. Similar results were also observed for the suprachiasmatic nucleus (SCN) in the brain, which serves as the mammalian circadian center. Thus, this R-box appears to be important for the repression of *Per2* transcription *in vivo*. I therefore hypothesized that this R-box might play a role in recruitment of the negative circadian transcriptional regulators. To test this hypothesis, I performed chromatin immunoprecipitation (ChIP) assays. In the wildtype mouse liver, the negative regulators, PER1, CRY1 and CRY2 were all recruited to the R-box at the time of *Per2* repression. However, in the mutant mice, no recruitment was observed throughout the day, demonstrating that this R-box is required for binding of the negative regulators. Based on these results, I conclude that the R-box is responsible for circadian repression, rather than activation, of *Per2* transcription *in vivo*.

（続紙 2 ）

（論文審査の結果の要旨）

生体リズムを司る生体時計の振動は、時計遺伝子の転写調節を介した自己制御型フィードバック機構によって生み出される。すなわち、転写促進因子であるCLOCK:BMAL1 複合体が *Period*（*Per*）遺伝子の転写を促進し、翻訳された PER 蛋白質が転写抑制因子 CRY と複合体を形成して核内へ移り、CLOCK:BMAL1 複合体の転写活性を抑制する。これにより PER 蛋白質が減少すれば、転写抑制の効果が弱まり、再び CLOCK:BMAL1 複合体による転写が開始されることになる。申請者は、本論文の第一章において、哺乳類の時計機能において中心的な役割を担うと考えられている *Per2* 遺伝子に着目し、本遺伝子の mRNA 並びに蛋白質レベルの発現変動をマウス肝において追跡し、上述のフィードバック理論が成立することを明らかにした。さらに、申請者は、PiggyBac トランスポゾン系による遺伝子改変によって目的の点変異マウスを作製することに成功した。さらに、第三章においてクロマチン免疫沈降法による解析から、点変異によって転写抑制因子群が *Per2* プロモーターへリクルートされなくなっていることを明らかにした。これは、遺伝子発現制御部位の変異で初めてリズム変動が起こることを明らかにした研究で有り、リズム形成機構に新たな発見がもたらされたといえる。よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、平成 27 年 2 月 23 日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（当分の間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日： 平成 27 年 5 月 23 日以降