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| 論文題目 | Mechanism of thermal entrainment of the circadian clock<br>by newly identified post-transcriptional regulation<br>（新規転写後調節機構による体内時計の温度サイクルへの位相適応メカニズム） | | |

（論文内容の要旨）

Recent genome-wide transcriptome and proteome studies have revealed that mRNA expression levels only explain approximately 40% of the variation of protein product levels in cells, suggesting a potential importance of changeable protein production efficiency. The advent of sequencing technologies for evaluating RNA modifications led to the transformation of the concept on mRNA regulation, where mRNA is not an entity mainly controlled by copy number but is recognized to be a subject regulating protein expression through post-transcriptional modifications. Post-transcriptional regulation may thus serve as a platform for the regulation of flexible protein expression. Post-transcriptional regulatory element (PTRE), located in the untranslated region (UTR) of transcript, regulates its coding protein expression without affecting mRNA expression level. The molecular mechanisms underlying PTRE-dependent post-transcriptional regulation, however, are only limitedly understood, currently. Particularly, its physiological significance is completely unknown.

The 24-hour rotation of the Earth creates temporal changes in the environment, forcing the organisms to acquire the ability to adapt to recurrent, thus anticipatable environmental changes. A variety of physiological phenomena such as thermogenesis and sleep-wake cycles have been identified to exhibit daily fluctuation. Body temperature of thermostatic animals does not stay constant but displays a regular circadian fluctuation, which has an important physiological role in maintaining homeostasis of sleep and metabolism as well as entraining the peripheral circadian clocks in the body. Indeed, a subtle circadian fluctuation in body temperature within a physiologic range in vivo (35°C to 38.5°C in mice) has the ability to adjust or entrain the phase of the circadian clock in cultured cells. Although some of the heat or cold stress-related molecular regulators, such as the heat shock factor 1 and cold-inducible RNA-binding protein, are reported to participate in this entrainment mechanism, the precise mechanism(s) by which the physiological body temperature fluctuation affects the oscillation of the molecular clock in cells have remained unclear. One of the reasons for this unclarity is that most of previous studies have mainly focused on the contribution of transcription and paid less attention to post-transcriptional regulation in the control of the circadian clock.

In this research background, I found a specific PTRE sequence in the UTR of thermosensitive clock oscillating gene (*Tsco*) (**Chapter 1**). Importantly, a mild increase in temperature in a physiological range (warming temperature shift, hereafter WTS) led to increased Tsco protein expression, which is regulated at a post-transcriptional level in a PTRE-dependent manner (**Chapter 2**). To investigate the molecular mechanism underlying the temperature response of Tsco, I performed chemical library screening and revealed that blocking TRK35 abrogated WTS-dependent Tsco protein accumulation (**Chapter 3**). Finally, I showed that TRK35 and *Tsco* PTRE have a significant contribution to the establishment of the temperature entrainment of the circadian clock (**Chapter 4**).

**Chapter 1: Discovery of *Tsco* PTRE in cells treated with a warm temperature shift**
To investigate how WTS affects *Tsco* expression, I performed customized RNA-seq (CRNA-seq) and found increased recruitment of RNA-binding proteins (RBPs) to the UTR of *Tsco* mRNA in cultured mouse embryonic fibroblast (MEF) cells when a WTS (35°C to 38.5°C) was applied. In-depth sequence analysis revealed that the accumulation signal of RBPs on the UTR of *Tsco* transcript is located within a specific PTRE sequence. Importantly, this sequence is conserved among mammalian species, which include human and mouse.

**Chapter 2: Temperature-dependent control of Tsco protein synthesis depends on PTRE.**
Physiological temperature changes might modulate Tsco expression. To test this, I performed immunoblot analysis using MEF cells and revealed that WTS induced greater expression of Tsco protein at its circadian rising phase, but not its circadian decreasing phase. Despite the change of Tsco protein expression by WTS, *Tsco* transcripts were nearly unaffected by the same treatment. WTS did not affect the Tsco protein stability. Next, I prepared mouse lung fibroblasts (MLF) from wild-type and *Tsco* PTRE mutant mice and found that WTS upregulated Tsco protein expression level in the wild-type cells but not in mutant cells. I further performed luciferase reporter assays using reporter constructs containing *Tsco* PTRE sequence and revealed that the *Tsco* PTRE sequence is sufficient for the temperature response of Tsco.

**Chapter 3: Blocking temperature responsive kinase 35 abrogates WTS response of Tsco expression.**
As a tool to identify an essential pathway for the temperature response of Tsco protein synthesis, I generated reporter knock-in cells in which a temperature insensitive luciferase gene was inserted in frame before the endogenous stop codon of *Tsco*, and using this tool, I performed chemical library screening. The screening revealed that WTS response of Tsco protein expression was attenuated in the presence of TRK35 inhibitors. Conversely, increased expression of Tsco protein was observed in the cells treated with a TRK35 pathway activator TPA. Very importantly, TPA failed to increase Tsco protein expression in *Tsco* PTRE mutant cells. These results indicate that WTS amplifies Tsco protein expression level through the TRK35-PTRE pathway.

**Chapter 4: Thermal entrainment is mediated by TRK35-*Tsco* PTRE pathway.**
I verified that temperature cycles that simulated mouse body temperature were able to enhance circadian rhythm sustainability in wild-type cells. However, the temperature cycle failed to maintain the rhythmicity in *Tsco* PTRE mutant cells. Inhibition of TRK35 also attenuated the temperature-cycle-dependent circadian sustainability of Tsco expression rhythm. Next, I examined the ability of temperature to synchronize or entrain the Tsco rhythm. The simulated mouse body temperature rhythm was capable of synchronizing the phase of Tsco expression in wild-type cells. In contrast, the temperature entrainment of Tsco was no longer observed in the presence of TRK35 inhibitor. These results demonstrate that the regulation of Tsco expression through TRK35-PTRE pathway is important for the thermal entrainment of the circadian clock.

Based on the results from **Chapters 1–4**, I have, for the first time, experimentally demonstrated the physiological contribution of post-transcriptional regulation of protein expression to temperature entrainment of the circadian clock by identifying the temperature-dependent response of the post-transcriptional regulatory element in the UTR of the thermosensitive clock gene.

（続紙 ２ ）

（論文審査の結果の要旨）

生物時計は、地球の自転に伴う昼夜の規則正しい環境変動に体内の代謝応答等を同調させる、生命にとって最も根源的な「時間」の仕組みである。これまでの時間生物学分野では、「明と暗」「寒と熱」のような、二値化されたシグナルへの転写を介した生物時計リセット機構に着目した検討がなされてきた。一方、昼夜の照度変化や概日体温変動にみられる体内時計同調シグナルは二値化では決して表現できない連続的かつ緩やかな刺激変化である。特に、温度は臓器を越え全身に伝播することから、概日体温変動は全身の末梢臓器の位相を調律する重要な時刻情報である。漸次的インプットを介した体内時計の位相調節機構の解明に向け、漸次的温度変化の体内時計遺伝子転写・翻訳フィードバック機構への入力を理解することは極めて重要な課題である。このような中、井ノ上 雄一氏は本論文の第一章において、哺乳類の体内時計制御遺伝子のひとつである*Tsco*遺伝子mRNAの非翻訳領域にRNA結合タンパク質結合配列PTREが存在すること、PTREへのRNA結合タンパク質の集積が生理的微小温度変化に伴い増加することを明らかにした。第二章では、当該微小温度変化による*Tsco* mRNA発現変化を伴わないTscoタンパク質発現増加を明らかにし、PTRE欠損培養細胞では温度依存的Tsco発現増加が認められないことを証明した。さらに第三章では、温度非感受性ルシフェラーゼを用いたPTRE機能レポーター細胞株を樹立し、化合物ライブラリースクリーニングによる上流因子TRK35の同定に成功した。第四章では、当該TRK35およびPTREの漸次的温度変化を介した体内時計位相調節機構における役割を、生体マウスの日内体温変動を模した温度サイクル下における細胞時計エントレインメントアッセイにより証明した。これら一連の研究成果は、連続的かつ緩やかに変化する体温という物理的変化が生物時計の位相を調律する分子的仕組みを明らかにしたものである。ヒトにおいても体温変化の変調に伴う健康障害の一つとして臓器間の概日時計機能の内的脱同調が報告されており、本研究は全身の細胞に備わる概日時計の温度エントレインメント機構の解明を通じて医学・薬学に貢献する重要な知見をもたらしたといえる。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、２０２２年２月１８日に、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、（令和７年３月２３日までの間）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：　　年　　月　　日以降