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研究課題名（和文）Role of RNA methylation in the regulation of gene expression.

研究課題名（英文）Role of RNA methylation in the regulation of gene expression.

研究代表者

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研究成果の概要（和文）：アデノシン塩基のメチル化であるm6A は、mRNAの内部のメチル化として、転写後制御機構の重要な機構として、現在非常に注目を集めている。これまでに、m6AがRNAプロセシングや翻訳の過程を調節することが知られているが、どのような生理的機能をm6Aが担っているのかは、未だ不明である。今回の研究において、我々は、次の2点を探求する。第一は、m6Aの概日変動をするのかどうか、第二は、m6Aの脱メチル化酵素であるFtoやAlkbh5、また、メチル基転移酵素であるMettl3が、遺伝子発現にどのような役割を及ぼすのかの解明である。今回、我々は、概日リズムの生理学におけるm6Aの役割を検索した。

研究成果の概要（英文）：This proposal had two parts: 1, investigate circadian variations in the m6A methylation of messenger RNA; 2, investigate the roles of Fto, Alkbh5 and Mettl3 in the regulation of gene expression.
Our results for the first part have yielded significant insights on the link between the circadian clock and RNA methylation. We have identified transcripts that are highly methylated across the circadian cycles and that are currently investigated. Moreover, we have identified a significant group of transcripts whose methylation show circadian rhythms of m6A methylation, and are currently preparing a manuscript.For the second part, both Fto and Alkbh5 knock-out mice gave negative results, and no circadian phenotypes were detected. Therefore, we are currently breeding double knock-out mice. Mettl3 knock-out mice were embryonic lethal and conditional knock-out mice have been generated and are currently being mated with SCN-CRE and Alb-CRE to generate SCN and liver-specific KO mice.

研究分野：　systems biology

キーワード：　m6A　circadian

１．研究開始当初の背景

The physiological functions of the methylation of adenosine nucleotides at the N6 position (m6A) of mRNA is still unknown despite a tremendous interest in m6A research in recent years. M6A is known to be involved in the regulation of splicing, RNA processing and translation, but what are its physiological functions is still not well understood. Internal adenosines are methylated at specific RRACH consensus elements enriched in the 3'-UTR of many mRNAs by the methyltransferases Mettl3 and Mettl14. In turn, m6A can be erased by the demethylases Fto and Alkbh5. The biochemical functions of these genes are known, but what physiological processes they regulate is unknown.

Two years ago, we reported that m6A was critical for the function of the circadian clock, and identified m6A sites in clock gene transcripts (Fustin et al., 2013). What is the role of m6A in the control of clock gene expression, and the physiological significance of these m6A sites, however remained unknown.

The biggest hurdle in m6A research is that, so far, information originates from whole transcriptome RNA-seq data showing general roles of m6A in RNA processing and translation. m6A sites have been identified in many transcripts, but why these transcripts are methylated is unknown.

Our investigations attempt to resolve this problem by identifying m6A sites in transcript that are critical for the regulation of the circadian clock. The circadian clock is based on a transcription-translation feedback loop of exquisite precision, ticking in a self-sustained manner with a period of around 24 hours. Therefore, it can be considered as the perfect paradigm to investigate the role of selected m6A sites since relatively small transcript specific changes in RNA processing and/or translation will have overt consequences in the oscillations of core clock genes, and thus in the circadian behaviour of the animal.

２．研究の目的

This research had two purposes: 1, identify circadian changes in m6A methylation at the level of the whole transcriptome, and document which transcripts show such circadian rhythms of m6A; 2, investigate the role of Fto, Alkbh5 and Mettl3 in the control of circadian gene expression. These two points represent only the start of our investigations, as the transcripts identified in our screening will then be investigated to understand the role of m6A in the control of the expression of these genes, and how it is related to function of the circadian clock. The roles of the m6A demethylases/methylases Fto, Alkbh5 and Mettl3 will be first investigated by generating knock-out mice, but this as well is only a starting point.

３．研究の方法

Messenger m6A methylation levels from mRNA extracted from liver samples dissected every 4 hours during 24 hours were measured by m6A mRNA immunoprecipitation followed by RNA-seq and analyses were carried out by the Tuxedo protocol followed by MACS2 peak calling analysis. To visualize m6A peaks, the MACS2 command bdgcomp was used. Identification of m6A target sequences was carried out by MEME.

Candidate transcripts with significant m6A sites found at all time points were then selected for further analyses. These analyses were performed mainly using Western Blotting, quantitative real-time PCR (qPCR) to quantify how the expression of these candidates changed under conditions in which m6A methylation was inhibited, such as the under the methylation inhibitor deazaneplanocin A or when the m6A methylases Mettl3 and Mettl14 are silenced. To identify the intrinsic role of m6A, we have engineered in vitro transcribed luciferase reporter mRNAs that bear the destabilised luciferase coding sequence flanked by the 5'and the 3'UTR of the candidate transcripts to be investigated. M6-adenosines identified by our peak-calling analyses were mutated to uridines to investigate their importance in RNA stability and translation. These mRNAs were then transfected in wild-type Mouse embryonic fibroblasts (MEF) and luciferase was measured in real-time to appreciate the translation dynamics.

The same mutations were also generated in

MEFs at the genomic level by using CRISPR-Cas9 genomic editing, and their impact on the candidate gene expression was measured by qPCR. Genetic deletions of various sizes (-7 bp and -43 bp) of the locus corresponding to the highly methylated region in the 3'UTR of candidate transcripts was also engineered in mice using CRISPR-Cas9 using the same vector and guide RNA. Mice are currently being analysed.

To investigate the role of m6A in the regulation of unknown alternatively spliced isoforms of our candidate transcripts, which were discovered in the course of our investigations, we measured the expression of these isoforms in multiple tissues by qPCR, and investigated how these isoforms were affected by subcutaneous injection of the methylation inhibitor deazaneplanocin A.

To investigate the function of these new isoforms, we cloned expression vectors for each isoform identified and stably transfected PER2::LUCIFERASE knock-in reporter MEFs by antibiotic selection, generating multiple monoclonal cell lines overexpressing the appropriate isoform. The function of these isoforms were investigated by real-time luciferase assay, which allowed us the follow the rhythmic expression of the core clock gene PER2 and the extract circadian parameters such as amplitude and period.

We also performed co-immunoprecipitation of each isoform to investigate their interaction with other clock proteins.

Since our main candidate transcript code for a kinase, we also investigated the phosphorylation of clock proteins by Phostag acrylamide gel electrophoresis, and performed isoform-dependent phosphoproteome and interactome analyses by LC-MS/MS in collaboration with Prof. Yasushi Ishihama from the Graduate School of Pharmaceutical Sciences.

4．研究成果

We are currently preparing a manuscript presenting our results. In brief, we have identified significant methylations sites in transcripts known to be critical for the function of the circadian clock, and shown that these m6A sites act as inhibitor of translation and also promote degradation of mRNA. We have identified rhythms in m6A is many transcripts that are coupled to transcriptional activity, as well as an overall trend for higher methylation during the early day.

From our m6A analyses, we have focused our downstream investigation on a specific candidate, here named Kinase A, known to be essential for the function of the circadian clock, that displayed high m6A levels at all time points during the day. When m6A methylation was inhibited by pharmacological agents or silencing of Mettl3 or Mettl14, Kinase A mRNA and protein increased. Moreover, two isoforms of unknown functions were discovered, one isoform being more sensitive to methylation inhibition. We then identified the function of these two isoforms and revealed they have opposite functions on the circadian clock. We showed that the two isoform of Kinase A phosphorylate different resides on a well-known circadian clock-related target protein. Moreover, we documented the phosphointeractome of each Kinase A isoforms, and revealed the different specificities of each isoform. This is the first time that the existence and physiological importance of the isoforms is reported.

To demonstrate that the m6A sites are critical for the post-transcriptional regulation of Kinase A expression, by CRISPR-Cas9 genomic editing we mutated putative m6A sites in the 3'-UTR of Kinase A in vitro and in vivo. For in vitro mutations, we used PER2::LUCIFERASE knock-in mouse embryonic fibroblasts since they can be easily used to monitor the circadian clock by real-time luciferase assay. When PER2::LUCIFERASE MEFs were mutated, resulting in homozygous mutants with putative m6A sites mutated to T, the circadian clock period became longer, associated with a decrease in Kinase A expression. This demonstrated that the highly methylated 3'-UTR of Kinase A is an important regulatory factor in the control of Kinase A expression. We also mutated the 3'-UTRby CRISPR-Cas9 in vivo and are currently analysing the mice.

The Fto and Alkbh5 knock-out mice were analyses for locomotor activity rhythms but unfortunately the results were negative, maybe because the lack of one is

compensated by the other. Therefore, we are pursuing these investigations with Fto/Alkbh5 double knock-out mice that are currently being bred in our laboratory.

Whole body Mettl3 knock-out was embryonic lethal. To continue our investigations, liver- and SCN-specific Mettl3 knock-out mice were generated and are currently being characterised. We will report the results in due time.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　2　件）
Doi M, Satoh F, Maekawa T, Nakamura Y, Fustin JM, Tainaka M, Hotta Y, Takahashi Y, Morimoto R, Takase K, Ito S, Sasano H, Okamura H. J Clin Endocrinol Metab.
Isoform-specific monoclonal antibodies against 3β-hydroxysteroid dehydrogenase/isomerase family provide markers for subclassification of human primary aldosteronism.
2014 Feb;99(2):E257-62.
doi: 10.1210/jc.2013-3279

Fustin JM, Doi M, Yamaguchi Y, Hida H, Nishimura S, Yoshida M, Isagawa T, Morioka MS, Kakeya H, Manabe I, Okamura H.
RNA-methylation-dependent RNA processing controls the speed of the circadian clock.
Cell. 2013 Nov 7;155(4):793-806.
doi: 10.1016/j.cell.2013.10.026.

〔学会発表〕（計　5　件）

Fustin JM
m6A mRNA methylation is coupled to transcription but enhance dynamic changes in mRNA levels.
TGSW2015, Symposium 28, Frontier Science on Transcription and Metabolism, Tsukuba, Sept 2015

Fustin JM
Epitranscriptome, the chemical modification on mRNA, in mammalian circadian rhythms
Molecular Biology Society of Japan 2014, Symposium 2S3: Diverse Gene Regulation Systems for Environmental Adaptation、Organizer: Yuichiro Watanabe. Yokohama, 25-27 Nov 2014.

Fustin JM
m6A shows its mettle: the birth of the circadian epitranscriptome
The 21st Annual Meeting of the Japanese Society for Chronobiology, Symposium S2-7. Fukuoka, 9-10 Nov. 2014

Fustin JM
The circadian epitranscriptome:RNA m6A methylation shows its mettle
MSD 7th SYMPHONY meeting. Tokyo, 14-15 Sep 2014

Fustin JM
RNA methylation shows its mettle
2014 Meeting of the Society for Research on Biological Rhythms, Slide session D 21. Montana, 14-18 June 2014

〔図書〕（計　0　件）

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〔その他〕
https://kyouindb.iimc.kyoto-u.ac.jp/e/lV8tI

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