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Diurnal expression of *Dnmt3b* mRNA in mouse liver is regulated by feeding and hepatic clockwork

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Abbreviations: 5-mC, 5-methyl-cytosine; 5-medC, 5-methyl-deoxycytidine; ANOVA, analysis of variance; BER, base excision repair; BMAL1, brain and muscle Arnt-like protein-1; cDNA, complementary DNA; CLOCK, circadian locomotor output cycles kaput; CRY, cryptochrome; DBP, D site of albumin promoter (albumin D-box) binding protein; DEC, differentiation of human embryo chondrocytes; Dnmt, DNA methyltransferase; dC, deoxycytidine; Gadd, growth arrest and DNA damage; ICF, immunodeficiency, centromeric instability and facial anomalies; L-*Bmal1* KO, liver-specific *Bmal1* knockout; LC/ESI-MS, liquid chromatography/electrospray ionizing mass spectrometry; Per, period; Rev-erb, reverse-erb receptor; Ror, RAR-related orphan receptor; RRE, Rev-Erb/ROR binding element; rRNA, ribosomal RNA; S.E.M, standard error of the mean; SF, scheduled feeding; TET, ten-eleven translocation; ZT, zeitgeber time

DNA methyltransferase 3B (DNMT3B) is critically involved in de novo DNA methylation and genomic stability, while the regulatory mechanism in liver is largely unknown. We previously reported that diurnal variation occurs in the mRNA expression of *Dnmt3b* in adult mouse liver. The aim of this study was to determine the mechanism underlying the diurnal expression pattern. The highest level and the lowest level of *Dnmt3b* mRNA expression were confirmed to occur at dawn and in the afternoon, respectively, and the expression pattern of *Dnmt3b* closely coincided with that of *Bmal1*. Since the diurnal pattern of *Dnmt3b* mRNA expression developed at weaning and scheduled feeding to separate the feeding cycle from the light/dark cycle led to a phase-shift in the expression, it could be assumed that feeding plays a critical role as an entrainment signal. In liver-specific *Bmal1* knockout (L-*Bmal1* KO) mice, L-*Bmal1* deficiency resulted in significantly higher levels of *Dnmt3b* at all measured time points, and the time when the expression was the lowest in wild-type mice was shifted to earlier. Investigation of global DNA methylation revealed a temporal decrease of 5-methyl-cytosine percentage in the genome of wild-type mice in late afternoon. By contrast, no such decrease in 5-methyl-cytosine percentage was detected in L-*Bmal1* KO mice, suggesting that altered *Dnmt3b* expression affects the DNA methylation state. Taken together, the results suggest that the feeding and hepatic clockwork generated by the clock genes, including *Bmal1*, regulate the diurnal variation in *Dnmt3b* mRNA expression and the consequent dynamic changes in global DNA methylation.

Introduction

DNA methylation is an essential epigenetic machinery that suppresses gene expression via transcription silencing at gene promoters and repetitive sequences and is considered to be important not only in early development[1,2] but also in the pathogenesis of diseases, such as cancer, diabetes and stress disorder in adults.[1,3,4] The cytosine that is present in abundance in CpG islands of genomic DNA can be converted to 5-methyl-cytosine (5-mC) by DNA methyltransferase (DNMT). Three subtypes of DNMT, DNMT1, DNMT3A and DNMT3B, have been found to be indispensable for conversion of the cytosine in genomic DNA to 5-mC.[2] Dnmt1 plays a central role in the transmission of methylation pattern from mother strand to daughter strand, whereas DNMT3A and DNMT3B are thought to be responsible for de novo DNA methylation. Overexpression of DNMT3B, but not of DNMT3A, in adult $Apc^{Min/+}$ mice, a murine model of intestinal neoplasia, causes methylation and transcriptional silencing of tumor suppressor genes and promotes tumorigenesis.[5] In humans, mutations in *Dnmt3b* account for 60–70% of

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the cases of ICF (immunodeficiency, centromeric instability and facial anomalies) syndrome, a rare autosomal recessive disease.[6,7] Marked hypomethylation and decondensation of pericentromeric heterochromatin on specific chromosomes have been reported in adult patients with ICF syndrome,[2,6,7] suggesting that *Dnmt3b* plays a critical role in DNA methylation and genomic stability in adults as well as in embryos.

The balance between methylation and demethylation determines DNA methylation status.[8] Recent studies demonstrated the ten-eleven translocation (TET) family proteins, TET1–3, as the key molecules involved in active DNA demethylation.[9] The growth arrest and DNA damage (Gadd) 45 family proteins have also been reported to be associated with active demethylation.[10,11] The DNA methylation state at specific loci in the brain, such as the *Bdnf* and *Fgf1* promoter loci, can be changed within 4 h by active demethylation catalyzed by TET1 and/or GADD45B.[11,12] Although the methylation state of genomic DNA was generally regarded as capable of changing over time but not in a very flexible manner, the results of these studies clearly indicated that the machinery of active demethylation allows cells to change the DNA methylation state of their genome rather easily.

In the liver, DNA hyper- and hypo-methylation are often associated with the onset of adult-onset diseases[1,3,4] and life events that adversely affect DNA methylation, such as exposure of environmental pollutants, dietary habits and drug intakes, may increase susceptibility to adult-onset diseases.[3] Some agents are known to alter the DNA methylation state by changing the level of expression of *Dnmt* and/or by modifying the enzymatic activity of DNMT.[3,13] Transcription of *Dnmt3a* and *Dnmt3b* is reported to be regulated by the ubiquitous transcription factors specific protein 1 (Sp1) and Sp3.[14] However, the regulatory mechanisms of expression of these enzymes are not fully elucidated. We previously reported that *Dnmts* mRNA expression in adult mouse liver can vary with diet and according to gender, in combination with chronic arsenic exposure.[15] We have further reported a dynamic change in *Dnmt3b* mRNA expression in mouse liver between the morning and the afternoon.[15] These findings suggest a novel regulatory mechanism of *Dnmt3b* linked to clockwork.

Clockwork seems to be driven by a limited number of core molecules.[16,17] Two of the core molecules, CLOCK and BMAL1 form a heterodimer, which binds to E-box motifs and activates transcriptional expression of two other core circadian pacemakers, *Period (Per)* and *Cryptochrome (Cry)*,[18] by means of the histone acetyltransferase activity of CLOCK protein.[19] The newly formed heterodimer complex of PER and CRY negatively regulates transcriptional activation of the CLOCK/BMAL1 heterodimer by recruiting histone deacetylase to the *Per1* E-box site.[20] In addition to these molecules, several other circadian oscillators, including *Rev-erb*α,[21] *Ror*α[22-24] and *Dbp*,[25] have been demonstrated to play an important role in modifying the feedback loop formed by CLOCK/BMAL1 and PER/CRY.

This study attempted to determine the mechanism that underlies generation of the diurnal expression of *Dnmt3b* mRNA in mouse liver. To investigate the mechanism, we (1) compared the *Dnmt3b* transcription pattern to the transcription patterns of circadian pacemaker genes, (2) conducted an experiment to analyze the ontogeny of diurnal *Dnmt3b* expression, (3) conducted an experiment to determine whether feeding is responsible for the entrainment of *Dnmt3b* expression, (4) conducted an experiment to test whether intrinsic clock genes in liver control the expression pattern of *Dnmt3b* mRNA using liver-specific *Bmal1* knockout (L-*Bmal1* KO) mice and (5) measured 5-mC in L-*Bmal1* KO and wild-type mice to determine whether the diurnal *Dnmt3b* change affects the DNA methylation state in adult liver.

Results

Diurnal variations in *Dnmt* mRNA expression and comparison to the patterns of circadian pacemaker genes. At zeitgeber times (ZTs) of 0, 4, 8, 12 and 16 h, 7-week-old mice of both sexes were sacrificed, and the gene expression patterns of *Dnmt1, Dnmt3a, Dnmt3b, Bmal1, Clock, Per1, Per2* and *Cry* were examined. Although there was no significant diurnal variation in the *Dnmt1* or *3a* mRNA levels (**Fig. 1A and B**), a clear diurnal variation was observed in *Dnmt3b* mRNA expression in both sexes. The highest and the lowest *Dnmt3b* mRNA expression levels were confirmed at ZT 0 and ZT 8, respectively, and the lowest level was less than 20% of the peak level (**Fig. 1C**). Comparison with typical circadian clock genes revealed an obvious similarity between the diurnal patterns of *Dnmt3b* and *Bmal1* expression (**Fig. 1C and D**), whereas the daily patterns of expression of other circadian pacemakers, e.g.., *Clock, Per1, Per2* and *Cry,* did not seem to be very similar to the pattern of *Dnmt3b* expression (**Fig. 1E–H**).

To investigate possible sex differences in *Dnmt3b* expression, gonad-intact 11-week-old males and females were sacrificed when the *Dnmt3b* mRNA level was the highest (ZT 0) or the lowest (ZT 8). There were differences between the *Dnmt3b* mRNA level at ZT 0 and ZT 8 in both sexes, and at ZT 0 the *Dnmt3b* mRNA level was about 1.6 times higher, and significantly higher in the females than in the males (**Fig. S1**). Gonadectomized groups were established by performing orchiectomy and ovariectomy at 7 weeks of age, and after waiting 4 weeks for their sex steroid hormones to become deleted, at 11 weeks of age they were sacrificed at ZT 0 or ZT 8. The patterns observed in the gonadectomized mice were very similar to the patterns observed in gonad-intact mice (**Fig. S1**), indicating that the differences in hormonal milieu created by different gonads in adults is unrelated either to the diurnal pattern of *Dnmt3b* transcription or to the sex difference in *Dnmt3b* mRNA level observed at ZT 0.

Differences between *Dnmt* and *Bmal1* mRNA expression at ZT 0 and ZT 8 during development. Male mice at 1, 3, 5 and 9 weeks of age were sacrificed at either ZT 0 or ZT 8 and mRNA levels in the liver were examined. The *Dnmt1* and *Dnmt3a* mRNA levels decreased with age, but there was no significant difference between levels at ZT 0 and 8 at any age (**Fig. 2A and B**). The pattern of *Dnmt3b* mRNA expression being high at ZT 0 and low at ZT 8 that was observed in adults was also observed at 3, 5 and 9 weeks of age (**Fig. 2C**), but the pattern of expression at 1 week of age was the opposite, i.e., high at ZT 8 and low at ZT 0 (**Fig. 2C**). Similar to *Dnmt3b* mRNA expression, *Bmal1* mRNA expression was found to be high at ZT 0 and low at ZT 8 at 3, 5 and 9 weeks of age (**Fig. 2D**), but at 1 week

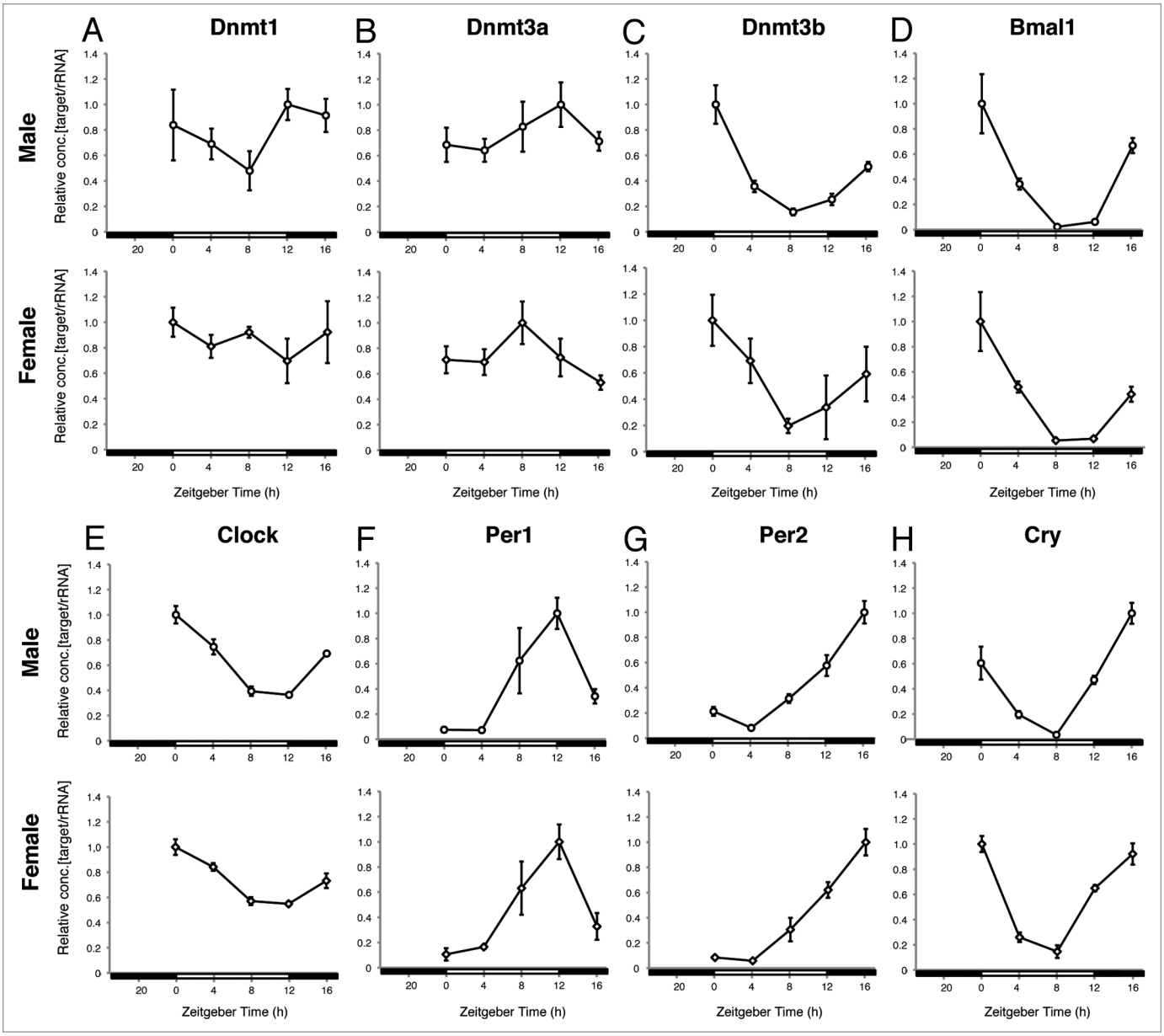

Figure 1. Diurnal mRNA expression of *Dnmts* and circadian clock-related genes in the liver. The mRNA expressions of *Dnmt1* (A), *3a* (B), *3b* (C) and circadian clock-related genes such as *Bmal1* (D), *Clock* (E), *Per1* (F), *Per2* (G) and *Cry1* (H) were examined in livers of male and female mice sacrificed at ZT 0, 4, 8, 12 and 16. n = 3 at each time point. Highest mRNA level in each figure was set as 1 and relative concentrations are shown.

of age, there was no difference between expression of ZT 0 and ZT 8. These results indicate that adult-like patterns of diurnal variation in the expression of *Dnmt3b* and *Bmal1* mRNA emerge sometime between 1 and 3 weeks of age.

Alteration of the patterns of *Dnmt3b* and *Bmal1* expression by scheduled feeding. Based on the result showing that the adult-like diurnal variation in *Dnmt3b* mRNA expression was first detected at 3 weeks of age, we hypothesized that the ontogeny of the adult-like pattern of *Dnmt3b* mRNA expression is associated with the start of weaning, which, in mice, is generally between 2 and 3 weeks after birth.[26,27] If eating chow acts as an entrainment signal for diurnal variation in *Dnmt3b* mRNA expression in the liver, scheduled feeding (SF) during daytime alone, which is intended to separate the feeding cycle from the light/dark cycle, should lead to a phase-shift in the *Dnmt3b* mRNA expression pattern. We therefore compared *Dnmt3b* mRNA expressions at ZT 0, 4, 8, 12 and 16 in an ad-lib fed group and SF group. In the SF group, the male mice were allowed to adapt for 6 d to a feeding schedule in which they were given access to food only from ZT 5 to ZT 9. In the SF group, the lowest *Dnmt3b* mRNA level and the highest *Dnmt3b* mRNA level shifted to ZT 4 and ZT 8, respectively, which were just before the start of feeding and just after the start of feeding, respectively (**Fig. 3A**). Thus, the diurnal pattern of *Dnmt3b* expression undergoes a phase-shift in response to SF. Similar to the results reported in a previous study,[28] SF induced a phase-shift in the diurnal pattern

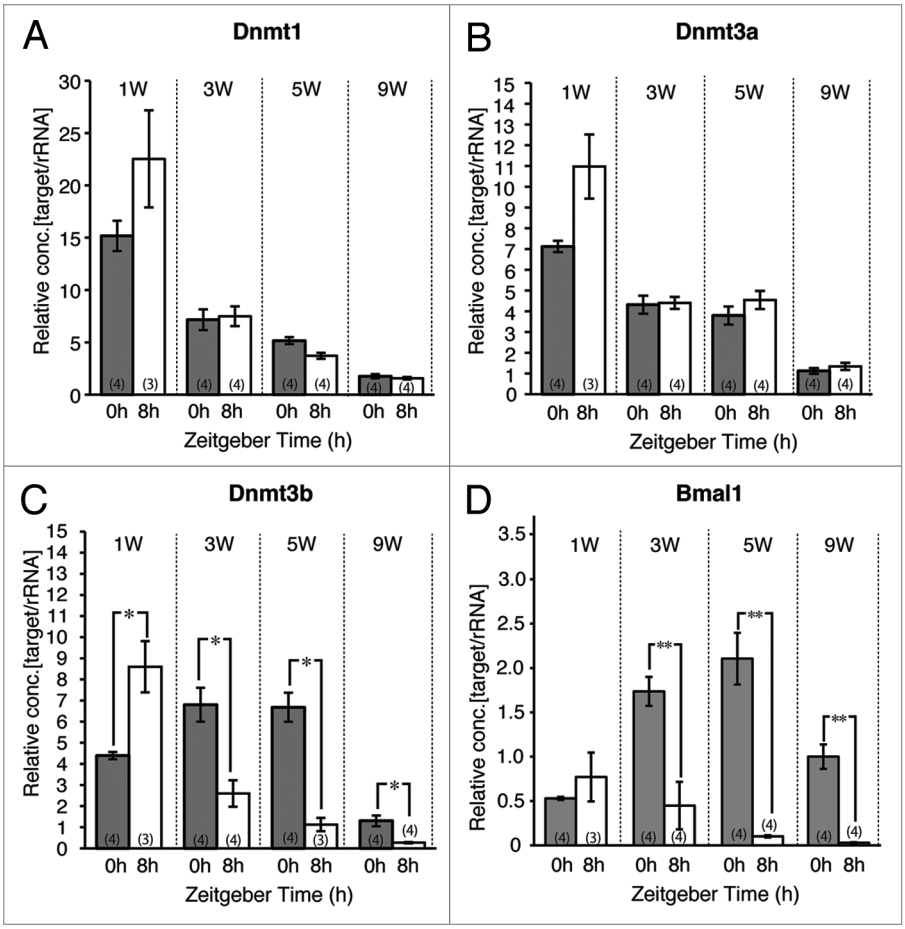

Figure 2. *Dnmts* and *Bmal1* mRNA expression at ZT 0 and 8 in the liver during postnatal development. There were no significant differences between ZT 0 and 8 through postnatal development in mRNA expressions of *Dnmt1* (A) and *Dnmt3a* (B). Significant decrease of *Dnmt3b* mRNA in postnatal 3, 5, and 9 weeks at ZT 8 vs. ZT 0, although significant increase was found in postnatal 1 week (C). Significant increase of *Bmal1* mRNA in postnatal 3, 5, and 9 weeks at ZT 8 vs. ZT 0 (D). The expression level in ZT 0 at postnatal 9 weeks was set as 1 and relative concentrations are shown. Numbers used in each group is shown in parentheses. *p < 0.05, **p < 0.01 (Student's t-test).

of *Bmal1* expression, with the lowest expression occurring at ZT 4 and the highest expression at ZT 16 (**Fig. 3B**). There were significant differences between the groups regarding *Dnmt3b* and *Bmal1* expression at all time points (**Fig. 3A and B**). These results demonstrate that feeding is a key factor in entraining the diurnal expression pattern of both *Dnmt3b* and *Bmal1*.

Effect of L-*Bmal1* KO on the level and pattern of *Dnmt3b* mRNA expression. Since BMAL1 is one of the core circadian oscillators, gene targeting destruction of tissue-specific *Bmal1* expressions by the Cre-loxP system induces circadian dysfunction in several tissues.[29-31] To determine whether L-*Bmal1* KO affects the diurnal expression of *Dnmt3b* mRNA, *Dnmt* expression levels at ZTs 4, 10, 16 and 22 were compared in L-*Bmal1* KO mice and wild-type mice. A slight but significant increase in *Dnmt1* mRNA expression was seen at ZT 10 as a result of L-*Bmal1* KO (**Fig. 4A**), but there were no significant differences between the groups in *Dnmt3a* mRNA expressions at any time point (**Fig. 4B**). The most dramatic change as a result of L-*Bmal1* KO was in *Dnmt3b* expression (**Fig. 4C**). *Dnmt3b* mRNA levels of the L-*Bmal1* KO group were significantly higher than in the wild-type group at all time points and, at ZT 10, the *Dnmt3b* mRNA level in the L-*Bmal1* KO mice reached more than 10 times the level in the wild-type mice. The time point when the level of expression was lowest had shifted in the L-*Bmal1* KO mice. Although the lowest level occurred at ZT 10 in wild-type mice, it occurred at ZT 4 in the L-*Bmal1* KO mice, indicating that not only increased mRNA level but also the phase-shift in expression pattern in *Dnmt3b* were induced by L-*Bmal1* KO. Finally, *Bmal1* mRNA levels were measured to confirm *Bmal1* deficiency in the liver of L-*Bmal1* KO mice, and they were found to be extremely low at ZT 4, 16 and 22 (**Fig. 4D**).

Binding of circadian oscillators on the regulatory regions of *Dnmt3b* gene. The results of experiments using L-*Bmal1* KO mice clearly showed the linkage between BMAL1 and the expression of *Dnmt3b*. Since the *Dnmt3b* promoter region contains putative E-box consensus sequences, which mediate transactivation by CLOCK/BMAL1 heterodimer, we investigated whether BMAL1 binds to these E-boxes by ChIP assay. When the DNA sequence containing E-box in *Per1* promoter was examined as a positive control, we found that the efficiency of DNA recovery by anti-BMAL1 antibody was significantly increased at ZT 8 (**Fig. S2A**), as previously reported.[20] Then, we tested whether BMAL1 can bind to six different putative E-box consensus sequences localized in the *Dnmt3b* promoter. However, we detected no significant increase in DNA recovery, indicating that BMAL1 does not bind to the consensus sequences having a homology to E-box in *Dnmt3b* promoter. We also examined the involvement of REV-ERB/ROR binding elements (RREs) in genomic loci associated with *Dnmt3b*. RRE is another oscillator sequence: binding of REV-ERB α/β suppress and binding of ROR α/β/γ enhance transcription.[21-24] As for ChIP assay using anti-REV-ERBα antibody, we used a known RRE sequence in *Bmal1* promoter as a positive control (**Fig. S2B**). The efficiency of DNA recovery was significantly increased at ZT 8, as previously reported.[32] For *Dnmt3b*, we examined the DNA recovery of two RREs localized in the promoter region and five RREs in gene body (**Fig. S2B**). However, we did not find time-dependent changes of DNA recovery between ZTs (**Fig. S2B**), indicating the possibility that REV-ERBα does not play a critical role in diurnal variation of *Dnmt3b* mRNA. Further arguments about this notion are considered in the Discussion section.

Diurnal variation in 5-mC as a percentage of the total cytosine in liver genomic DNA and the effect of L-*Bmal1* KO on the 5-mC percentage. In order to investigate whether the diurnal changes in *Dnmt3b* expression affects global DNA methylation, we accurately measured non-methylated cytidine and 5-methyldeoxycytidine (5-medC) levels in liver genomic DNA by a highly reliable method that we established previously[15] and calculated 5-mC as a percentage of total cytosine. Comparing the 5-mC percentages at ZTs 0, 4, 8, 12, 16 and 20 showed that the lowest level that was significantly different from the level at ZT 0 occurred at ZT 12 (**Fig. 5A**). In addition, the 5-mC percentage at ZT 12 tended to be lower than at ZT 4 and at ZT 8 (p = 0.07 and 0.08, respectively). Thus, the 5-mC level seemed to follow the variation of *Dnmt3b* expression with a certain time lag. The 5-mC level was changed by 11.2% between the highest and lowest time points in a day. We also analyzed 5-mC percentages in wild-type and L-*Bmal1* KO mice sacrificed at ZTs 4, 10, 16 and 22. In the wild-type mice, 5-mC as a percentage of total cytosine was significantly lower at ZT 10 than at the other time points (**Fig. 5B**). By contrast, the time point-specific decline in 5-mC percentage seen in wild-type mice at ZT 10 was not observed in L-*Bmal1* KO mice (**Fig. 5B**). Recent studies have discovered an active DNA demethylation pathway starting with hydroxylation of 5-mC by TET family proteins and enabling fast DNA demethylation.[9] The analyses of mRNA levels of *Tet1*, *2* and *3* showed no significant diurnal variation (**Fig. S3**). These results support the notion that the changes in 5-mC level are attributable to diurnal changes in methylation by DNMT3B but not to diurnal changes in demethylation.

Discussion

In this study, we showed that expression of one of the *Dnmt* subtypes, *Dnmt3b*, undergoes dynamic changes closely coinciding with those of *Bmal1* during the day in the adult liver. The diurnal pattern of *Dnmt3b* mRNA expression was shown to be common to both sexes and to be maintained in mice that had been subjected to ovariectomy and orchiectomy, to create models of menopause and andropause, respectively.[33,34] Although the sex of the animals affected the expression level of *Dnmt3b* at ZT 0, the difference between sexes was relatively small. These results indicate that the diurnal pattern of *Dnmt3b* mRNA expression in the liver is not specifically influenced by the hormonal milieu originated from the difference in gonads between the sexes and might be maintained stable in mature mice.

Comparison between *Dnmt3b* and *Bmal1* mRNA expression at ZT 0 and 8 at 1, 3, 5 and 9 weeks of age showed that the adult-like diurnal expression pattern of both genes, in which expression was high at ZT 0 and low at ZT 8, started at 3 weeks of age. The previous study on the ontogeny of circadian oscillations revealing that the adult-like diurnal expression pattern of *Bmal1* in rat liver starts 20 d after birth[35] coincides with our present result. The hepatic circadian clock has been found to be entrained by central signals[36-38] and peripheral signals of ingested nutrients.[39-42] In view of the similarities between the expression patterns of *Bmal1* and *Dnmt3b* observed in our developmental experiment and SF experiment, the entrainment signals that reset the diurnal expression of *Bmal1* may also regulate *Dnmt3b* mRNA expression in the liver, although the hierarchical relationships between central and peripheral signals for entrainment of *Dnmt3b* remains unknown at present.

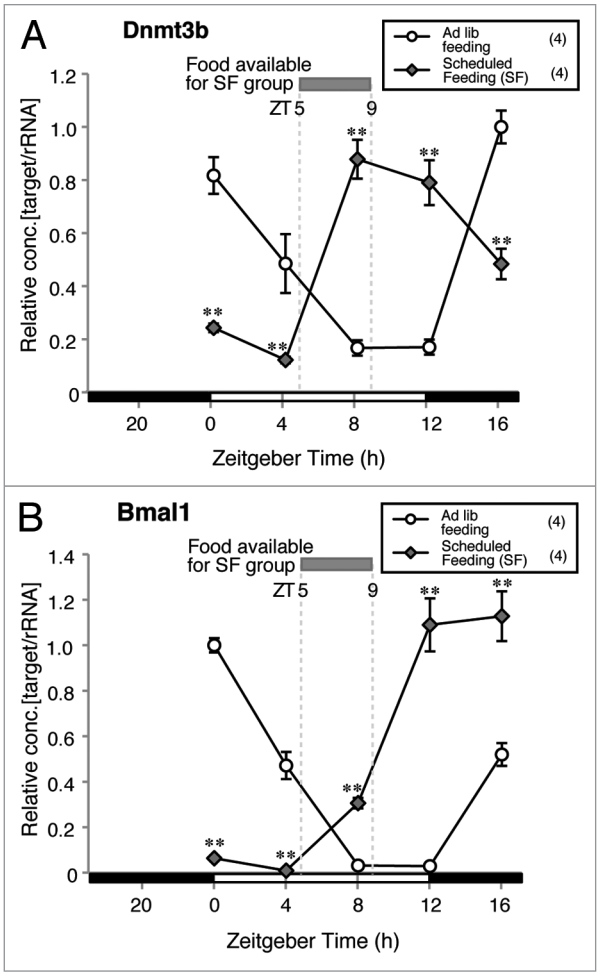

Figure 3. Effect of scheduled feeding on *Dnmt3b* and *Bmal1* mRNA expression patterns in the liver. Significant differences at all time points were found between groups in both *Dnmt3b* and *Bmal1* mRNA expressions (**p < 0.01 vs. ad lib fed group, Student's t-test). The expression level in ZT 16 for *Dnmt3b* and ZT 0 for *Bmal1* of ad lib fed group was set as 1 and relative concentrations are shown. Numbers used in each group is shown in parentheses.

To determine definitively whether endogenous clockwork in the liver regulates the diurnal expression pattern of *Dnmt3b*, we compared *Dnmt3b* mRNA expression in wild-type and L-*Bmal1* KO mice. The Dnmt3b mRNA levels of the L-*Bmal1* KO mice were significantly higher at all time points examined. The time of the lowest Dnmt3b mRNA level shifted from ZT 10 to ZT 4. These results demonstrate that the endogenous hepatic clockwork controlled by core clock genes including *Bmal1* is the driving force behind the diurnal rhythm of *Dnmt3b*. On the other hand, the results of ChIP assay using anti-BMAL1 and anti-REV-ERBα antibodies did not show binding of these proteins to their binding sites around *Dnmt3b*. We also could not find

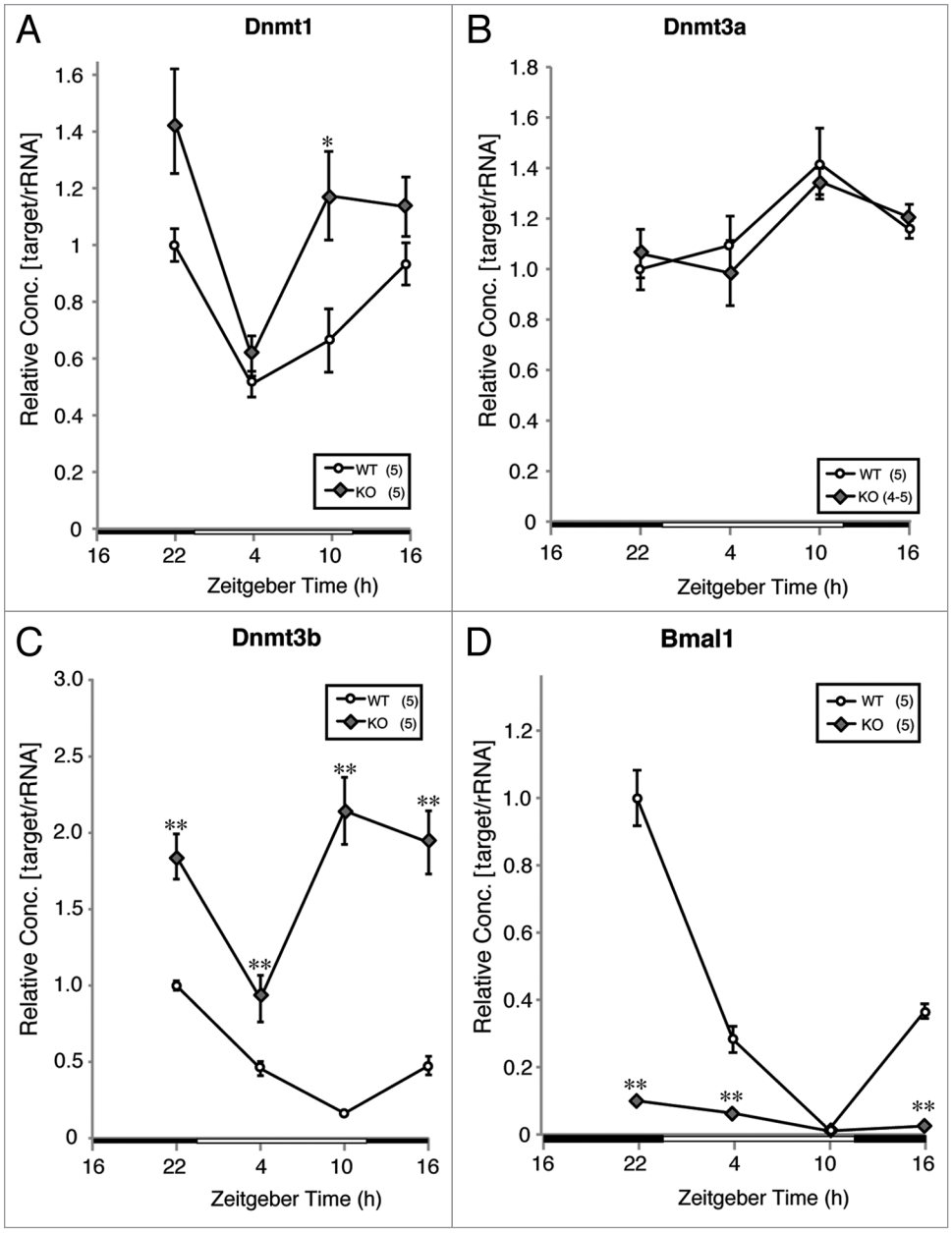

Figure 4. Effect of L-*Bmal1* KO on diurnal expressions of *Dnmts* and *Bmal1* mRNA in the liver. Slight but significant increase of *Dnmt1* mRNA expression was demonstrated in L-*Bmal1* knockout at ZT 10 (A, *p < 0.05, Student's t-test). There was no significant difference between groups in *Dnmt3a* mRNA expressions (B). Significant increases of *Dnmt3b* mRNA expressions at ZT 4, 10, 16 and 22 by L-*Bmal1* knockout was revealed [(C) **p < 0.01 at ZT 22, 10, 16 and *p < 0.05 at ZT 4, Student's t-test]. Significant reductions of *Bmal1* mRNA expressions at ZT 4, 16 and 22 by L-*Bmal1* knockout were confirmed [(D) **p < 0.01, Student's t-test]. The expression level in ZT 22 of wild-type was set as 1 and relative concentrations are shown. Numbers used in each group is shown in parentheses.

any BMAL1-binding site around *Dnmt3b* gene by searching the database of BMAL1-binding sites (CircaClock, http://circaclock.epfl.ch) and no specific reference of *Dnmt3b* in the gene lists appeared in the recent ChIP-seq analyses using anti-REV-ERB antibodies.[32,43,44] Since it is not feasible that *Dnmt3b* transcription is regulated by E-box and RREs, we are now assuming that *Dnmt3b* transcription is not controlled directly by core-clock genes such as *Bmal1* and *Rev-erbs* but, instead, by the secondary and higher-order clock genes. For example, DBP and E4BP4 bind to D-site and B-site, respectively, and work as intermediaries of clockwork.[25,45] The transcription of *Dnmt3b* might be affected by their transcriptional regulations. In addition, as it has been reported recently that *Per2* mRNA circadian oscillation is modulated by mRNA rhythmic degradation,[46] there is growing evidence that posttranscriptional regulation is important in the circadian oscillation of mRNA. Since it has been reported that *Dnmt3b* mRNA stabilization is regulated by RNA-binding proteins such as HuR[47] and microRNAs,[48,49] there is a possibility

that *Dnmt3b* mRNA receives posttranscriptional regulation along with the time of day. Further study based on these hypotheses will be required to unveil the mechanism of diurnal variation of *Dnmt3b* transcription.

By measuring the amounts of cytidine and 5-medC by high-performance liquid chromatography/electrospray ionizing mass spectrometry (LC/ESI-MS) using stable-isotope-labeled surrogates as internal standards,[15] this study revealed that the level of 5-mC in liver genomic DNA varies with the time of day. A previous study that assessed global DNA methylation by fluorescently labeled cytosine extension assay reported diurnal changes in global DNA methylation in humal blood.[50] The present study is the first to demonstrate a diurnal rhythm of global DNA methylation in the liver. Furthermore, the variation appeared to be coordinated with the diurnal variation in *Dnmt3b* expression. mRNA expressions of *Tet1–3*, the enzymes regulating active DNA demethylation, were not altered with time of day (**Fig. S3**), suggesting the possibility that the active methylation process contributes greatly to diurnal variation of global DNA methylation. In comparing 5-mC level between wild-type mice and L-*Bmal1* KO mice, wild-type mice showed the lowest 5-mC level at ZT 10, when *Dnmt3b* mRNA level was also the lowest. On the other hand, L-*Bmal1* KO mice showed higher degree of DNA methylation in parallel with increased *Dnmt3b* expression at ZT 10. These results suggest the possibility that DNMT3B in the adult liver dominantly controls the diurnal change in global DNA methylation state. In our previous study, we measured the 5-mC level of the liver of mice fed a methyl-deficient diet, which has been reported to induce DNA hypomethylation,[51] by the precise LC/ESI-MS method, and found that 5 mo of feeding with methyl-deficient diet reduced the 5-mC level by 3.6% in the liver genome of male mice.[15] This study demonstrated that the 5-mC level is changed by up to 11.2% in a day and the deviation is thought to be substantial. On the other hand, we could not find the quantitative difference of DNMT3B protein between ZT 0 and 8 by western blotting analysis (**Fig. S4**). The development of methods with better accuracy to detect subtle difference in DNMT3b expression levels, such as radioimmunoassay or enzyme-linked immunoassay, might be required to clarify whether DNMT3B protein level in liver varies within a day.

The biological significance of diurnal variation in global DNA methylation is yet unclear. In the present study we found higher *Dnmt3b* expression around the age younger than 5-weeks-old, when pup's organs rapidly grow with extensive cellular proliferation. Cell cycle progression is regulated by core clock molecules such as BMAL1/CLOCK complex and shows a diurnal pattern.[52] DNMT3B is reported to localize to centromeric/pericentromeric satellite repeats and play a role in maintaining chromosomal function, which is essential for accurate chromosome segregation during mitosis, through DNA methylation as well as histone modifications.[53] Although it has not yet been clarified whether DNA methylation and histone modifications show diurnal changes, the diurnal changes in *Dnmt3b* expression and level of 5-mC might be involved in centromeric/pericentromeric chromatin organization in clock-regulated cell cycle progression.

It is also not elucidated how the diurnal variation in *Dnmt3b* expression is involved in gene specific DNA methylation and hepatic function. Glucose homeostasis and lipid homeostasis in the liver exhibit a circadian pattern, which is known to at least in part be dependent on the diurnal variation in expression of metabolism-related genes.[54,55] The circadian rhythm of metabolism-related genes has been reported to be regulated by epigenetic histone modifications.[43] Since there is still no clear evidence linking the diurnal change in DNA methylation to the circadian gene expression and subsequent hepatic functions associated with metabolism, future studies measuring precise promoter methylation states in individual glucose and lipid homeostasis-related genes along with the time of day may lead to a better understanding of the link between the diurnal *Dnmt3b* variation and liver physiology. The results of the present study may also help to elucidate the mechanism by which clock disorders lead to metabolic abnormalities that have attracted attention as a result of epidemiological studies of shift workers.[56] Aberrant DNA methylation levels have been found to be associated with the onset and progression of adult-onset metabolic diseases.[3,4,13,56,57] Thus, we can speculate that the higher *Dnmt3b* expression and the temporarily higher DNA methylation state, as

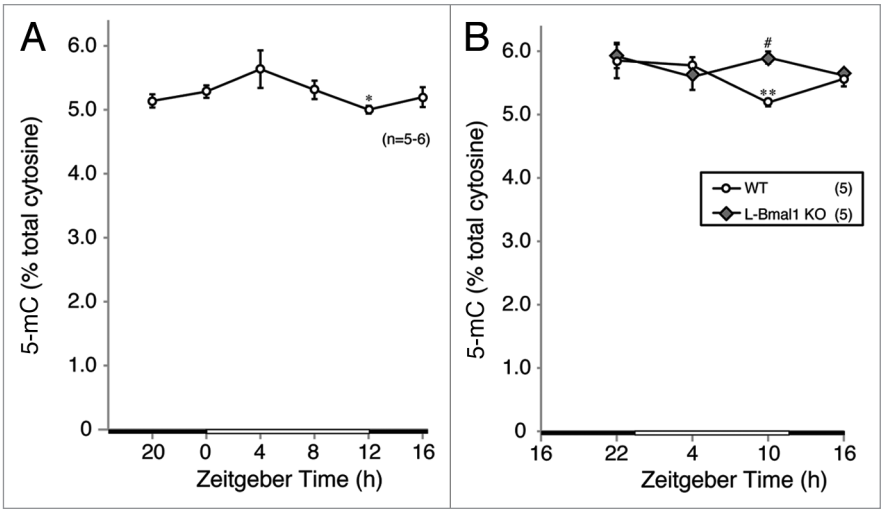

Figure 5. Diurnal change in global 5-mC in liver genomic DNA from L-*Bmal1* KO and wild-type mice. Comparison of 5-mC percentage at ZT 0, 4, 8, 12, 16 and 20. ZT 12 showed the lowest 5-mC percent value, being significantly different from that at ZT 0 [(A) *p < 0.05, Student's t-test]. In addition, 5-mC percent at ZT 12 showed decreased tendency when compared with ZT 4 and 8 (p = 0.07 and 0.08, respectively, Student's t-test). In (B) we compared 5-mC percentage in wild-type and L-*Bmal1* KO mice at ZT 4, 10, 16, 22. In wild-type mice, significantly lower 5-mC percent at ZT10 was found, compared with other time points (**p < 0.01 vs. ZT 4, p < 0.05 vs. ZT 16 and 22, Student's t-test). In L-*Bmal1* KO mice, the time point-specific decline of 5-mC percentage (found in wild-type mice at ZT 10) was not observed; instead, a significant increase in 5-mC percentage was found (#p < 0.001 vs. wild-type, Student's t-test).

a result of L-*Bmal1* KO, increase susceptibility to such diseases. Indeed, L-*Bmal1* KO mice have been reported to have abnormal glucose tolerance, possibly due to the impaired expression of glucokinase and glucose transporter-2, which regulate hepatic glucose homeostasis.[29] Importantly, the expression of these genes is known to be controlled by the methylation state of their promoters.[58,59] Although further study is required, the results of the present study suggest that overexpression of *Dnmt3b* has some role in the pathogenesis of metabolic diseases triggered by molecular clock dysfunction.

Materials and Methods

Mice. Except for L-*Bmal1* KO experiments, male and female C57BL/6J mice were purchased from CLEA Japan and were bred in National Institute for Environmental Studies (NIES). They were acclimatized to the environment for about 1 week prior to use. Throughout the experiment, the animals were maintained in a controlled environment at a temperature of 24 ± 1°C and humidity of 50 ± 10% and under a 12/12 h light/dark cycle (light, ZT 0–12; dark, ZT 12–24). Food (CE-2, CLEA) and water were available ad libitum unless otherwise indicated.

For L-*Bmal1* KO experiments, C57BL/6J mice with or without genetic modification were maintained at the School of Pharmacy, Nihon University at 23 ± 1°C with 50 ± 10% humidity and under a 12/12 h light dark cycle (light, ZT 0–12; dark, ZT 12–24). The Cre-loxP system was applied for creating L-*Bmal1* KO mice as described elsewhere.[31] Briefly, we constructed a conditional *Bmal1* (flox/flox) mice, which carry the conditional *Bmal1* allele containing exons 6–8 flanked by loxP sites using ES cells derived from C57BL/6J mice. To create the liver-specific Bmal1 excision, the mice expressing *Bmal1* (flox/flox) allele were crossed to the mice expressing a Cre transgene driven by the albumin promoter (Jackson Laboratories). Mice homozygous for the floxed allele and hemizygous for the Cre transgene *Bmal1* (flox/flox/Cre^{Alb}) were obtained by crossing *Bmal1* (flox/+/Cre^{Alb}) mice to *Bmal1* (flox/flox) mice. Littermates that were negative for Cre transgenes [*Bmal1*(flox/flox)] were used as controls.

Ethical statement. Except for L-*Bmal1* KO experiments, the mice were handled in a humane manner in accordance with the NIES guidelines. For L-*Bmal1* KO experiments, the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nihon University.

Sampling of liver along with time points from adult mice. Seven-week-old male and female mice (n = 3 at each time point) were killed by decapitation either at ZT 0, 4, 8, 12 and 16. For obtaining total RNA, ten mg of liver was collected and homogenized with Qiagen RLT buffer containing 1% β-mercaptoethanol by a microhomogenizing system (Micro Smash MS-100, TOMY, JAPAN) at 3,000 rpm for 30 sec immediately after sampling. For obtaining genomic DNA, 100 mg of liver was collected from each individual and stored at -80°C until extraction.

Gonadectomy and sampling. Twelve males and 12 females at 7 weeks of age were orchidectomized and ovariectomized under ether anesthesia, respectively. They were raised for additional 4 weeks until autopsy in order to establish lower gonadal sex hormone levels. These mice were decapitated at ZT 0 and 8 (n = 6 at each time point) and livers were collected. Male and female mice at 11 weeks of age were used as non-manipulated gonad-intact controls. Homogenate of liver was made as the same method as mentioned above.

Sampling from developing mice. Adult male and female were cohabitated as 1 by 1 to obtain newborn pups. Vaginal plug and delivery was checked every day to estimate and confirm the birthday of pups. One, three, five and nine weeks after birth, mice were sacrificed at either ZT 0 or 8 (n = 3–4 at each time point) and homogenate of liver was made as the same method as mentioned above.

Sampling from adult mice under scheduled feeding. In the SF group, food was available only during ZT 5 to 9 for 6 d without any alteration of light regimen. On the other hand, in ad lib feeding group, feeding availability was not limited. Mice in each group were killed by decapitation either at ZT 0, 4, 8, 12 and 16. Homogenate of liver was made as the same method as mentioned above.

RNA extraction and measurement of mRNA expressions. Total RNA was extracted by RNeasy Mini Kit (QIAGEN KK) following the manufacturer's instruction. Using 100 ng total RNA as template, cDNA was synthesized by reverse transcriptase XL (Takara Bio Inc.). Expression of target genes and rRNA (rRNA) was quantified by real-time PCR on LightCycler instrument (Roche Diagnostics) as described previously.[60] Amplification in experimental samples during the log linear phase was compared with the standard curve from the dilution series of a control cDNA using LightCycler quantification software (Version 3.5). The control cDNA to make a standard curve was prepared from the livers of C57BL/6 mice. The primer sequences and annealing temperatures used for real-time PCR are shown in **Table 1**.

Measurements of global DNA methylation. For 5-medC measurements, genomic DNA was prepared from the liver tissue by phenol/chloroform extraction. DNA hydrolysis to nucleosides was performed according to the previous report.[15] Briefly, 1 μg of DNA was denatured by heating at 98°C for 3 min. The solution was mixed with two units of nuclease P1 (Wako) and incubated at 45°C for 2 h in 10 mM ammonium acetate. Next, the solution was supplemented with 0.002 units of phosphodiesterase I (Worthington Biochemical Corp.) and incubated at 37°C for 2 h in 100mM ammonium bicarbonate, and then 0.5 units of alkaline phosphatase (Promega) was added and incubation was continued at 37°C for an additional 1 h.

LC–MS analyses were performed by using LC/MS-2010A mass spectrometer with an ESI ionization probe (Shimadzu) according to the previous report[15] with minor modification. Deoxycytidine (dC) and 5-medC were separated by using a reversed-phase column (Atlantis dC18, 2.1 × 150 mm, 5 μm, Waters) in gradient mode with methanol (2–20%) -10 mM ammonium acetate (v/v) at a total flow rate of 0.2 ml/min. The internal standards ($^{13}C_9$, $^{15}N_3$-2'-deoxycytidine, $^{13}C_{10}$, $^{15}N_2$-5-methyl-2'-deoxycytidine, 100 ng each) were added to 15 μl of the hydrolyzed sample, and the mixture was diluted to 500 μl with H_2O. A 10 μl volume of the mixture was injected into LC–MS, and dC and 5-medC were analyzed on a selected-ion monitoring (SIM) mode. The

SIM m/z of dC and 5-medC was 228.1 and 242.1, respectively. The SIM m/z of the stable-isotope labeled dC and 5-medC was 240.1 and 254.1, respectively. Quantification of 5-mC percent in total cytosine was calculated from integration peak areas of 5-medC relative to global cytidine (5-medC + dC).

Statistical analyses. Data was expressed as mean ± S.E.M. Samples number used was indicated in parentheses. In **Figure 2A–D**, the difference between ZTs was analyzed by unpaired Student's t-test by Microsoft Excel (MS-Excel 2008 for Mac, Microsoft). In **Figures 3–5**, the differences between groups and among time points were analyzed by unpaired Student's t-test.

Table 1. Primer sequences and PCR conditions for amplification

Targets	Primer sequences (5'- 3')	Annealing temperature (°C)
Dnmt1	CCA AGC TCC GGA CCC TGG ATG TGT	64
	CGA GGC CGG TAG TAG TCA CAG TAG	
Dnmt3a	GCA CCT ATG GGC TGC TGC GAA GAC G	64
	CTG CCT CCA ATC ACC AGG TCG AAT G	
Dnmt3b	GTC TGC ACA CCA GAG ACC AGA G	64
	TCA GAG CCA TTC CCA TCA TCT AC	
Bmal1	ATG CAG AAC ACC AAG GAA GG	64
	CCA TCC TTA GCA CGG TGA GT	
Clock	CAA AAT GTC ACG AGC ACT TAA TGC	64
	ATA TCC ACT GCT GGC CTT TGG	
Per1	ACA GCA GCC ACG GTT CTC	64
	GCT GCC ACA GTC CAC ACA	
Per2	CAA CAC AGA CGA CAG ATC A	64
	TCC TGG TCC TCC TTC AAC AC	
Cry1	CTC GGG TGA GGA GGT TTT CTT	64
	GAC TTC CTC TAC CGA GAG CTT CAA	
18S rRNA	TAC CAC ATC CAA GGA AGG CAG	64
	TGC CCT CCA ATG GAT CCT C	

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author's Contributions

Conceived and designed the experiments: F.M. and K.N. Performed the experiments: F.M., S.S., S.T., T. Sano, T. Suzuki, M.O., J.B., T.E. and K.N. Analyzed the data: F.M., S.T., M.O. and J.B. Contributed reagents/materials/analysis tools: F.M., S.S., T. Sano, Y.O. and K.N. Wrote the paper: F.M. and K.N.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/21539

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