Research Article

Epigenetic regulation of the circadian clock: role of 5-aza-2′-deoxycytidine

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We have been investigating transcriptional regulation of the BMAL1 gene, a critical component of the mammalian clock system including DNA methylation. Here, a more detailed analysis of the regulation of DNA methylation of BMAL1 proceeded in RPMI8402 lymphoma cells. We found that CpG islands in the BMAL1 and the PER2 promoters were hyper- and hypomethylated, respectively and that 5-aza-2′-deoxycytidine (aza-dC) not only enhanced PER2 gene expression but also PER2 oscillation within 24 h in RPMI8402 cells. That is, such hypermethylation of CpG islands in the BMAL1 promoter restricted PER2 expression which was recovered by aza-dC within 1 day in these cells. These results suggest that the circadian clock system can be recovered through BMAL1 expression induced by aza-dC within a day. The RPIB9 promoter of RPMI8402 cells, which is a methylation hotspot in lymphoblastic leukemia, was also hypermethylated and aza-dC gradually recovered RPIB9 expression in 3 days. In addition, methylation-specific PCR revealed a different degree of aza-dC-induced methylation release between BMAL1 and RPIB9. These results suggest that the aza-dC-induced recovery of gene expression from DNA methylation is dependent on a gene, for example the rapid response to demethylation by the circadian system, and thus, is of importance to clinical strategies for treating cancer.

Introduction

Circadian rhythms function in most living organisms and govern many behavioral and biochemical processes with 24-h periodicity, regardless of changes in the cellular environment. The master clock that generates circadian rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The master clock is governed by blue-light sensing in the eye and it controls all the aspects of physiology such as sleep-wake cycles, body temperature, hormone secretion, blood pressure and metabolism [1]. The molecular mechanism of the circadian oscillator is based on interlocked transcriptional/translational feedback loops that have both positive and negative elements. The circadian oscillator orchestrates the output of the rhythmic mRNA expression of typically hundreds or thousands of clock-controlled genes (CCGs) that are mediated by transcription factors or coregulators with rhythmic abundance. Whereas post-transcriptional regulation contributes to the rhythmic transcription of mature abundant mRNA, transcriptional regulation remains the dominant determinant of the rhythmic transcriptome [2].

Transcriptional regulation initially requires the coordinated control of chromatin and the genome structure [3]. In general, genetic information is packed into the chromatin structure, and the nucleosome is the most basic unit of the chromatin structure; it determines the large-scale chromatin structure as a building block and influences transcription. Eukaryotic promoter regions are thought to have inactive states, assured by the tendency of nucleosomes to inhibit transcription by protecting protein–DNA interaction. Therefore, chromatin remodeling and loosening the nucleosomal barrier including histone tail modifications are key steps in circadian modifications. For example, rhythmic BMAL1/CLOCK binding, histone H3 Lys4 trimethylation (H3K4me3) and Lys9 acetylation are required as well as rhythmic H3 abundance

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at the start site for Dbp transcription [4]. In addition, protein complexes containing clock proteins such as PER contain various interactive partners with known catalytic activity towards chromatin [5,6].

The methylation of cytosines on CpG dinucleotides [7], which is also epigenetic regulation of gene expression, either directly interferes with the binding of transcriptional regulators, or indirectly inactivates a gene by modulating chromatin to a repressive structure. Light-induced DNA methylation is dynamic at specific promoters that correspond to circadian gene expression in the SCN [8] and altered DNA methylation is associated with many human diseases. Clock gene methylation is highly prevalent in dementia with Lewy bodies (DLB), a disorder that is similar to Parkinson’s disease [9], in which the NPAS2 promoter is hypomethylated [10]. DNA methylation is also prevalent in various types of cancer and clock genes influence tumorigenesis; for example the methylation of clock gene promoters such as CLOCK [11] and PERs [12-15] contribute to cancer progression. Many tumor suppressors and oncogenes are under circadian control and Per genes function as tumor suppressors [16].

Amongst the core clock genes, BMAL1 expression oscillates in the SCN and in peripheral clock cells, this is closely associated with circadian rhythms [17]. The hypermethylation of CpG islands in the promoter of BMAL1 transcriptionally silences its expression in hematological malignancies [18,19]. We previously found that REV-ERB orphan nuclear receptors (ROREs), which are recognition motifs for ROR and REV-ERB orphan nuclear receptors and critical elements for BMAL1 oscillatory transcription [20], are embedded in a unique GC-rich open chromatin structure, with which a nuclear matrix like structure at the 3′-flanking region co-operates to regulate BMAL1 transcription [21,22]. We also found that DNA demethylation of the BMAL1 promoter in CPT-K cells enhances BMAL1, and then PER2 and CRY1 transcription, and finally circadian functions is recovered [19]. The present study further investigates the effects of DNA demethylation in detail.

Materials and methods

Chemicals

The premix reagent for real-time quantitative PCR was SYBR(R) Premix Ex Taq (TM) II (Tli RNaseH Plus) from Takara Bio (Shiga, Japan). Reverse transcription proceeded using the PrimeScript™ RT Reagent Kit with gDNA Eraser from Takara Bio, according to the manufacturer’s instructions. D-luciferin potassium salt was purchased from Wako (Osaka, Japan). All other chemicals were of reagent grade and used without further purification.

Cell culture

RPMI8402 cells [23] were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and a mixture of penicillin and streptomycin in a humidified incubator at 37°C under a 5% CO₂ atmosphere.

CpG methylation analysis

We identified CpG islands in PER2, BMAL1, and RPIB9 promoters using the algorithm at www.urogene.org/methprimer [24]. Methylation was analyzed as a modification generated using EpiTect Bisulftite from Qiagen (Hilden, Germany) according to the manufacturer’s instructions, followed by PCR cloning and sequencing. The primer sequences were as follows: BMAL1: 5′-GTGTGGTTGCGGTTTAGTG-3′ and 5′-CACATCAACAAAAATTCTTC-3′; PER2: 5′-GGTGTGGTTATATTTTTTTTTTGTTG-3′ and 5′-CCACACACCCCAAAAACCTTCC-3′; RPIB9: 5′-GTGAGTGTAGAGATTGATTAGTTGG-3′ and 5′-AACCACCATCACTCCACACCACCCTC-3′.

Promoter assay

A luciferase reporter gene plasmid containing the PER2 promoter [25] and the internal control plasmid, pRL-CMV from Promega (Madison, WI, U.S.A.) were transfected into RPMI8402 cells using Lipofectamine and Plus reagents (Thermo) according to the manufacturer’s instructions. Reporter luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) as described [26]. Transcriptional activities were normalized relative to Renilla luciferase activities.

Real-time quantitative RT-PCR

Reverse transcription of total RNA in each cell lysate was performed using PrimeScript™ RT Reagent Kit described above according to the manufacturer’s instructions. These obtained reactants were applied to real-time quantitative PCR. The real-time quantitative PCR is performed with LightCycler® Nano(R) from Roche (Basel, Switzerland) and SYBR(R) Premix Ex Taq (TM) II (Tli RNaseH Plus; Takara Bio) as described [23]. The primer sequences were as follows: PER2: 5′-TGATTGAAACCCAGTGCTCGT-3′ and 5′-CTCCATGGGTTGATGAAGCTTG-3′;
Results

Aza-dC releases methylation of BMAL1 CpG islands

We previously reported that ROEs in the BMAL1 promoter are embedded in a unique GC-rich open chromatin structure under CpG island hypomethylation, which is important for circadian transcription [21,22]. On the other hand, CpG islands in the BMAL1 promoter of some cancer cell lines are hypermethylated and the BMAL1 gene is transcriptionally silenced [19].

A survey of cells with hypermethylated CpG islands in the BMAL1 promoter found that the human lymphoblastic leukemia cell line, RPMI8402, has methylated CpG islands. The bisulphite genomic sequencing of six individual clones indicated that the BMAL1 promoter in RPMI8402 cells is hypermethylated in CpG islands (Figure 1A). We used RT-PCR to study the effects of 5-aza-2'-deoxycytidine (aza-dC) on suppressed BMAL1 transcription in RPMI8402 cells to clarify its relationship with promoter methylation. Demethylation of the CpG islands in the promoter using aza-dC induced the transcription of BMAL1 13- and 9-fold at 1 and 3 days, respectively, in RPMI8402.
cells (Figure 1B). These results suggested that hypermethylation of the promoter CpG islands represses BMAL1 transcription in RPMI8402 cells and that the demethylation CpG islands in the promoter byaza-dC enhanced BMAL1 transcription within 1 day.

**PER2 gene expression profile in RPMI8402 cells**

We investigated the gene expression ofPER2, a circadian clock component. We applied bisulphite sequencing to investigate the methylation profile of thePER2 promoter as described above forBMAL1. The sequence results of six individual clones showed that a maximum of four cytosine bases were methylated in the CpG islands of thePER2 promoter region (Figure 2A), indicating that the region is hypomethylated and quite different from that of theBMAL1 promoter region. We also studied the effects ofaza-dC onPER2 promoter activity and transcription (Figure 2B,C, respectively).PER2 promoter activity was 3.5- and 3.8-fold enhanced on days 1 and 3 compared with day 0 (Figure 2B), suggesting that the expressedBMAL1 affected activation. The amount ofPER2 transcripts was 2.0- and 2.3-fold enhanced on days 1 and 3 compared with day 0 (Figure 2C), indicating an increase over the basal value. These results indicated that although the CpG region of thePER2 promoter was hypomethylated, azadC enhancedPER2 transcription in RPMI8402 cells.

**Aza-dC recovers circadian oscillation of the PER2 gene**

The expression ofBMAL1 was restricted in RPMI8402 cells as described above and theBMAL1 function released byaza-dC reflected activities of other genes, such asPER2 transcription. We therefore investigated the oscillation ofPER2 transcription using aPER2-Luc real-time reporter assay system. In the absence ofaza-dC, the reporter proceeded with the first induction, but the oscillation then became damped (Figure 2D, gray line). In the presence ofaza-dC, the reporter oscillated robustly for over 5 days (period length: 23.3 h; Figure 2D, red line). These results imply thataza-dC can regenerate endogenous circadian rhythms through restoration ofBMAL1 expression andPER2 induction in RPMI8402 cells.
Figure 2. Aza-dC activates PER2 gene expression at the level of transcription

(A) Hypomethylation of DNA in PER2 promoter. Genomic sequence of RPMI8402 cells was analyzed after modification with bisulphite. Vertical lines, CpG sites in PER2 promoter region. Filled and unfilled squares, methylated and unmethylated CpG sites, respectively. Arrow, transcription start site; unfilled boxes D (A) and D (B), DBP-binding sites; unfilled box E', non-canonical E-box.  

(B) Aza-dC activates PER2 promoter within 1 day. Transcription assays proceeded using construct containing PER2 promoter with 2.5 μM aza-dC. Normalized expression levels were calculated relative to luciferase activities in cells incubated without aza-dC. Values are means ± SE of triplicate assays. *P<0.05; Student’s t test.  

(C) Aza-dC enhances PER2 transcripts within 1 day. RPMI8402 cells were incubated with 2.5 μM aza-dC for indicated days, and then RNA was analyzed using qRT-PCR. Levels of RNA were normalized to those of ACTIN expression, and value for cells incubated without aza-dC was set at 1. Values are means ± SE of triplicate assays. *P<0.05; Student’s t test.  

(D) Aza-dC recovers transcriptional oscillation of PER2. RPMI8402 cells transfected with PER2 reporter plasmid were stimulated with 50% FBS for 2 h, and then bioluminescence was measured in presence of 2.5 μM aza-dC. Detrended fit curves are representative of at least three independent experiments (control, gray; aza-dC, red).

Aza-dC recovers RPIB9 expression

The RPIB9 (RUNDC3B) gene is a candidate of a biomarker in lymphoid malignancy which possibly serves as a mediator between Rap2 and the MAPK signaling cascade [28]. Studies have indicated that the RPIB9 gene is methylated in acute myelogenous leukemia (AML) and malignant B cells and that aza-dC enhances the expression of this gene [29,30]. We examined the methylation status of RPIB9 in RPMI8402. The DNA sequences of six individual clones (Figure 3A) showed that CpG islands in the RPIB9 promoter region are hypermethylated. We applied real-time quantitative RT-PCR to determine the effects of aza-dC on RPIB9 transcription. Figure 3B shows that RPIB9 expression gradually increased 1.8- and 2.5-fold on days 1 and 3, respectively, after aza-dC demethylation compared with the amount of mRNA on day 0. These results indicate that aza-dC gradually changed the transcriptional profile of RPIB9 and that this profile is quite different from those of clock genes.
Aza-dC releases methylation in BMAL1 and RPIB9 promoters at different rates

We compared the total genomic content of methylated dCTP with and without aza-dC to determine the effects of aza-dC on methylcytosine in the genomic DNA of RPMI8402 cells. Figure 4A shows that approximately 1% of the total dCTP was methylated in the absence of aza-dC, which was consistent with previous findings [31,32] whereas the amount of methylated dCTP was reduced by approximately half within 1 day in the presence of aza-dC.

We then quantitated the unmethylated ratios of CpG islands in the promoter regions of both BMAL1 and RPIB9 in the presence and absence of aza-dC. Figure 4B shows that the islands in both BMAL1 and RPIB9 promoters were methylated without aza-dC, because their unmethylated ratios were quite low at 0.04 and 0.003, respectively. However, aza-dC demethylated almost all CpG islands in the BMAL1 promoter (Figure 4B, black bars) and approximately 70% of those in the RPIB9 promoter within 1 day (Figure 4B, white bars). These results indicate that the rate of demethylation of the CpG islands was slower in RPIB9 than in BMAL1, suggesting that the demethylation mechanisms differ amongst these genes.

Discussion

Changes in the methylation of clock gene DNA cause the misregulation of various critical cell physiological processes that can lead to diseases such as various types of cancers [33]. For example, the circadian oscillation of gene expression is aberrant in leukemic cells, as BMAL1, PER1, and PER2 are down-regulated in patients with chronic lymphocytic leukemia [34]. Disrupted CRY2 and PER2 are associated with non-Hodgkin’s lymphoma [18] and the initiation and/or progression of AML [35], respectively, and CpG islands of PER3 are highly methylated in all patients with chronic myelogenous leukemia [36]. Taniguchi et al. [18] and we reported that BMAL1 is epigenetically inactivated in hematologically malignant cells [19]. The findings of these reports together indicate that hematopoietic cell malignancies are associated with down-regulation of the circadian clock, used in this cell lines. We found hypermethylated CpG islands in the BMAL1 promoter and repressed BMAL1 expression (Figure 1) and hypomethylated CpG islands in the PER2 promoter of human lymphoblastic RPMI8402 cells (Figure 2A). The expression of BMAL1 (Figure 1B) and PER2 (Figure 2B,C) as well as oscillation or function (Figure 2D) were recovered by aza-dC which is
Figure 4. Effects of aza-dC on methylation of genome in RPMI8402 cells

(A) Methylcytosine of genomic DNA in RPMI8402 cell. Amounts of methylcytosine were determined using anti-methylcytosine antibody as described in ‘Materials and methods’ section and indicated as methylated dCTP ratios by weight in genomic DNA (%). Values are means ± SE of triplicate assays. *P<0.05; Student’s t test. (B) Demethylation in BMAL1 completes earlier than that in RPIB9. Filled and unfilled boxes show demethylation ratios of BMAL1 and RPIB9, respectively. Genomic DNA prepared from RPMI8402 was incubated with 2.5 μM aza-dC for indicated days, modified with bisulfite and analyzed using methylation-specific PCR. Value for cells incubated without aza-dC for 3 days was set at 1. Values are means ± SE of triplicate assays.*P<0.05; Student’s t test.

an anticancer drug [37]. These findings imply an association between the anticancer function of aza-dC and induction of the tumor-suppressor function of PER2 [16]. These also indicate that methylation of the BMAL1 promoter is a key factor in the oscillation of the clock gene, PER2. The down-regulation of clock genes might cause the up-regulation of typical oncogenes such as c-Myc and Cyclin-D1 [34] that are both under the control of circadian genes. Therefore, these genes are likely to be aberrant in malignant cells with defective clock genes. On the other hand, recent studies have shown that disruption of the core circadian clock in a mouse model of AML causes antileukemic effects in AML (that is, BMAL1 and CLOCK are necessary for AML cell growth) [38], suggesting that the mechanisms of aberrant clock gene expression in leukemia are highly complex. Further investigation at different stages of malignancy or in various types of leukemia are required to determine the function of clock genes in leukemia.

The most common epigenetic modification is DNA methylation, which is a covalent chemical modification that plays a crucial role in numerous biological processes. Generally, although approximately 70% of CpG sequences in the entire mouse and human genomes are methylated, CpG islands in promoter sequences are methylated at a relatively lower level. However, CpG islands of the promoter regions are frequently hypermethylated and expression of the corresponding gene is damped in tumor cells, a situation that is rather tumor type specific [39]. This implies that
methylation status would be a good biomarker of malignant stage in specific tissues and cells. The non-CpG methylation of DNA can regulate gene expression through affecting the binding of transcription factors [40]. Bisulphite-based methods are the most prevalent means of distinguishing between cytosine and 5′-methylcytosine in epigenetic studies. For example, bisulphite-sequencing, combined bisulphite restriction analysis (COBRA), methylation specific PCR and pyrosequencing can identify the methylation status of a specific sequence at the level of a single CpG. On the other hand, quantitative analysis of global DNA methylation is difficult because a limitation is that bisulphite-based methods are inherently prone to variability due by DNA degradation caused by the required acidic conditions [41]. We therefore quantitated global cytosine methylation using an immunochemoical approach that does not require either bisulphite or methylation-sensitive enzymes, suggesting that DNA degradation is minimized and that this method of quantitation is highly accurate. Assays using anti-methylcytosine antibody (Figure 4A) indicated a 1% global methylation rate in the genomic DNA of RPMI8420 cells, which was consistent with previous findings [31,32] and that aza-dC reduced this by approximately 0.5%, indicating the release of DNA methylation. Such information could serve as a biomarker of cancer prognosis.

The level of DNA methylation within a ±1 kb region surrounding the transcription start site closely correlated with gene repression [42]. The hypermethylation of CpG islands surrounding the transcription start site (Figures 1 and 3) indicated that the BMAL1 and RPIB9 genes are repressed, which is in agreement with the above. The methylation of DNA promotes stable nucleosome positioning of methylated CpG dinucleotides in the minor groove in proximity to the histone octamer complex [43] and those in the major groove influence nucleosome dynamics towards a more open structure [44]. We previously described an open chromatin structure in the promoter region of BMAL1 with hypomethylated CpG [21,22], suggesting a lesser effect of DNA methylation on nucleosome positioning at the BMAL1 promoter region. DNA methylation affects the binding dynamics of transcription factors and knocking out DNA methyltransferases increases the number of binding events of the transcription factor NRF1 [45]. Besides, the methylation of CpG adjacent to the core Sp1 motif decreases the Sp1/Sp3 binding [46], which might be related to the repression of BMAL1 transcription by DNA methylation because many putative Sp1 binding motifs are located around the BMAL1 promoter [47]. The mechanism of the repression by DNA methylation remains unclear and further studies are required.

Figure 4B shows that recovery from DNA methylation by aza-dC differs between the BMAL1 and RPIB9 genes, suggesting that the release of methylation depends on the locus/gene or sequence. Taken together with the quantitative results of global methylation (Figure 4A), the demethylation rate of BMAL1 was essentially comparable with that of other genes, whereas that in RPIB9 was slower (Figure 4B), suggesting that methylation status is DNA site specific. One of the most important issues regarding DNA methylation is how the machinery is directed towards and maintains specific sequences in the genome. One answer might be the PML-RAT fusion protein in leukemia, which induces DNA hypermethylation and gene silencing at specific target promoters [48]. siRNA-mediated, RNA-directed DNA methylation is a stepwise process initiated by dsRNAs that recruit DNMT to catalyze the de novo DNA methylation of specific regions [49]. Therefore, although the susceptibility of individual CpG islands to de novo methylation might intrinsically differ, the mechanism remains obscure. The methylation of CpG is strictly regulated and stable, and changes in methylation profiles are associated with diseases including cancer, indicating close relationships amongst biological function, DNA methylation sites and the mechanism of methylation. Taken together, these findings imply that methylation is specific to gene function and an early response to the aza-dC demethylation of sites in BMAL1 might be functionally important for adaptation to environmental changes. The information herein provides novel insights into clock gene function that should affect the clinical treatment and diagnosis of diseases.

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Author contribution

T.T. performed the cell culture, real-time quantitative RT-PCR and real-time reporter gene assays. R.K. quantitatively analysed DNA methylation. Y.O. conceived the study, participated in its designing and carried out the methylation analysis. All authors read and approved the final manuscript.

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Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Abbreviations
aza-dC, 5-aza-2'-deoxyctydine; DMEM, Dulbecco's modified Eagle's medium; RORE, ROR and REV-ERB orphan nuclear receptor responsive element; SCN, suprachiasmatic nucleus.

References
1 Ruby, N.F., Brennan, T.J., Xie, X., Cao, V., Franken, P., Heller, H.C. et al. (2002) Role of melanopsin in circadian responses to light. Science 298, 2211–2213
2 Luck, S., Thurley, K., Thaben, P.F. and Westmark, P.O. (2014) Rhythmic degradation explains and unifies circadian transcriptome and proteome data. Cell Rep. 9, 741–751
3 Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K. et al. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 338, 349–354
4 Ripperger, J.A. and Schibler, U. (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat. Genet. 38, 369–374
5 Brown, S.A., Ripperger, J., Kadener, S., Fleury-Diela, F., Vilbois, F., Rosbash, M. et al. (2005) PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science 308, 693–696
6 Duong, H.A. and Weitz, C.J. (2014) Temporal orchestration of repressive chromatin modifiers by circadian clock period complexes. Nat. Struct. Mol. Biol. 21, 126–132
7 Bird, A.P. and Wolfe, A.P. (1999) Methylation-induced repression-belts, braces, and chromatin. Cell 99, 451–454
8 Azzi, A., Dallmann, R., Casserly, A., Rehaura, H., Patrignani, A., Maier, B. et al. (2014) Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat. Neurosci. 17, 377–382
9 Liu, H.C., Hu, C.J., Tang, Y.C. and Chang, J.G. (2008) A pilot study for circadian gene disturbance in dementia patients. Neurosci. Lett. 435, 229–233
10 Lin, Q., Ding, H., Zheng, Z., Gu, Z., Ma, J., Chen, L. et al. (2012) Promoter methylation analysis of seven clock genes in Parkinson’s disease. Neurosci. Lett. 507, 147–150
11 Hoffman, A.E., Yi, C.H., Zheng, T., Stevens, R.G., Leaderer, D., Zhang, Y. et al. (2010) CLOCK in breast tumorigenesis: genetic, epigenetic, and transcriptional profiling analyses. Cancer Res. 70, 1459–1468
12 Chen, S.T., Chao, K.B., Hou, M.F., Yeh, K.T., Kuo, S.J. and Chang, J.G. (2005) Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. Carcinogenesis 26, 1241–1246
13 Yang, M.Y., Chang, J.G., Lin, P.M., Tang, K.P., Chen, Y.H., Lin, H.Y. et al. (2006) Downregulation of circadian clock genes in chronic myeloid leukemia: alternative methylation pattern of hPER3. Cancer Sci. 97, 1298–1307
14 Hsu, M.C., Huang, C.C., Chao, K.B. and Huang, C.J. (2007) Uncoupling of promoter methylation and expression of Period1 in cervical cancer cells. Biochem. Biophys. Res. Commun. 360, 257–262
15 Gery, S., Komatsu, N., Kawamata, N., Miller, C.W., Desmond, J., Virk, R.K. et al. (2007) Epigenetic silencing of the candidate tumor suppressor gene Per1 in non-small cell lung cancer. Clin. Cancer Res. 13, 1399–1404
16 Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell 111, 41–50
17 Burger, M.K., Wilbacher, L.D., Moran, S.M., Cledenin, C., Radcliffe, L.A., Hogenesch, J.B. et al. (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009–1017
18 Taniguchi, H., Fernández, A.F., Setién, F., Ropero, S., Ballestar, E., Villanueva, A. et al. (2009) Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies. Cancer Res. 69, 8447–8454
19 Sato, R., Sugihara, N., Ishizuka, Y., Matsukubo, T. and Onishi, Y. (2013) DNA methylation of the BMAL1 promoter. Biochem. Biophys. Res. Commun. 440, 449–453
20 Ueda, H.R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T. et al. (2002) A transcription factor response element for gene expression during circadian night. Nature 418, 534–539
21 Onishi, Y., Hanai, S., Ohno, T., Hara, Y. and Ishida, N. (2008) Rhythmic SAF-A binding underlies circadian transcription of the Bmal1 gene. Mol. Cell. Biol. 28, 3477–3488
22 Onishi, Y. (2010) HSG cells, a model in the submandibular clock. Biosci. Rep. 30, 57–62
23 Onishi, Y. and Kawano, Y. (2012) Rhythmic binding of Topoisomerase I impacts on the transcription of Bmal1 and circadian period. Nucleic Acids Res. 40, 9482–9492
24 Li, L.C. and Dahiya, R. (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics 18, 1427–1431
25 Ohno, T., Onishi, Y. and Ishida, N. (2007) A novel E4BP4 element drives circadian expression of mPeriod2. Nucleic Acids Res. 35, 648–655
26 Onishi, Y. and Kiyama, R. (2001) Enhancer activity of HS2 of the human β-LCR is modulated by distance from the key nucleosome. Nucleic Acids Res. 29, 3448–3457
27 Ogawa, Y., Kawano, Y., Yamazaki, Y. and Onishi, Y. (2014) Shikonin shortens the circadian period: possible involvement of Top2 inhibition. Biochem. Biophys. Res. Commun. 443, 339–343
28 Burmeister, D.W., Smith, E.H., Cristel, R.T., McKay, S.D., Shi, H., Arthur, G.L. et al. (2017) The expression of RUNC3B is associated with promoter methylation in lymphoid malignancies. Hematol. Oncol. 35, 25–33

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29 Taylor, K.H., Pena-Hernandez, K.E., Davis, J.W., Arthur, G.L., Duff, D.J., Shi, H. et al. (2007) Large-scale CpG methylation analysis identifies novel candidate genes and reveals methylation hotspots in acute lymphoblastic leukemia. Cancer Res. 67, 2617–2625
30 Wang, M.X., Wang, H.Y., Zhao, X., Srilatha, N., Zheng, D., Shi, H. et al. (2010) Molecular detection of B-cell neoplasms by specific DNA methylation biomarkers. Int. J. Clin. Exp. Pathol. 3, 265–279
31 Chowdhury, B., Cho, I.H., Hahn, N. and Iruayaj, J. (2014) Quantification of 5-methylcytosine, 5-hydroxymethylcytosine and 5-carboxycytosine from the blood of cancer patients by an enzyme-based immunosassay. Anal. Chim. Acta 852, 212–217
32 Wu, J., Xu, Y., Mo, D., Huang, P., Sun, R., Huang, L. et al. (2014) Kaposi’s sarcoma-associated herpesvirus (KSHV) vIL-6 promotes cell proliferation and migration by upregulating DNMT1 via STAT3 activation. PLoS ONE 9, e93478
33 Masri, S., Kinouchi, K. and Sassone-Corsi, P. (2015) Circadian clocks, epigenetics, and cancer. Curr. Opin. Oncol. 27, 50–56
34 Rana, S., Munawar, M., Shahid, A., Malik, M., Ullah, H., Fatima, W. et al. (2014) Deregulated expression of circadian clock and clock-controlled cell cycle genes in chronic lymphocytic leukemia. Mol. Biol. Rep. 41, 95–103
35 Gery, S., Gombart, A.F., Yi, W.S., Koeffler, C., Hofmann, W.K. and Koeffler, H.P. (2005) Transcription profiling of C/EBP targets identifies Per2 as a gene implicated in myeloid leukemia. Blood 106, 2827–2836
36 Yang, X., Wood, P.A., Ansell, C. and Hrushesky, W.J. (2009) Circadian time-dependent tumor suppressor function of period genes. Integr. Cancer Ther. 8, 309–316
37 Limonta, M., Colombo, T., Damia, G., Catapano, C.V., Conter, V., Gervasoni, M. et al. (1993) Cytotoxic activity and mechanism of action of 5-aza-2’-deoxycytidine in human CML cells. Leuk. Res. 17, 977–982
38 Puram, R.V., Kowalczyk, M.S., de Boer, C.G., Schneider, R.K., Miller, P.G., McConkey, M. et al. (2016) Core circadian clock genes regulate leukemia stem cells in AML. Cell 165, 303–316
39 Costello, J.F., Frühwald, M.C., Smiraglia, D.J., Rush, L.J., Robertson, G.P., Gao, X. et al. (2000) Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat. Genet. 24, 132–138
40 Jin, J., Lian, T., Gu, C., Yu, K., Gao, Y. and Su, X.D. (2016) The effects of cytosine methylation on general transcription factors. Sci. Rep. 6, 29119
41 Tanaka, K., Tainaka, K., Urnemoto, T., Nomura, A. and Okamoto, A. (2007) An osmium-DNA interstrand complex: application to facile DNA methylation analysis. J. Am. Chem. Soc. 129, 14511–14517
42 Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J. et al. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462, 315–322
43 Collins, C.K., Waddell, P.J. and Anderson, J.N. (2013) Effects of DNA methylation on nucleosome stability. Nucleic Acids Res. 41, 2918–2931
44 Jimenez-Useche, I., Ke, J., Tian, Y., Shim, D., Howell, S.C., Qiu, X. et al. (2013) DNA methylation regulated nucleosome dynamics. Sci. Rep. 3, 2121
45 Domcke, S., Bardet, A.F., Adrian Gino, P., Hardt, D., Burger, L. and Schübel, D. (2015) Competition between DNA methylation and transcription factors determines binding of NRF1. Nature 528, 575–579
46 Zhu, W.G., Srinivasan, K., Dai, Z., Duan, W., Druhan, L.J., Ding, H. et al. (2003) Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. Mol. Cell. Biol. 23, 4056–4065
47 Yu, W., Nomura, M. and Ikeda, M. (2002) Interacting feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER2. Biochem. Biophys. Res. Commun. 290, 933–941
48 Di Croce, L., Raker, V.A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M. et al. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 295, 1079–1082
49 Zaratiegui, M., Irvine, D.V. and Martienssen, R.A. (2007) Noncoding RNAs and gene silencing. Cell 128, 763–776