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DNA methylation of the *BMAL1* promoter

R. Satou [a], N. Sugihara [a], Y. Ishizuka [a], T. Matsukubo [a], Y. Onishi [b,*]

[a] Department of Epidemiology and Public Health, Tokyo Dental College, Japan
[b] Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Japan

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ABSTRACT

We previously analyzed transcriptional regulation of the *BMAL1* gene, a critical component of the mammalian clock system and found that the *BMAL1* gene is expressed with circadian oscillation and that its regulatory region is located in hypomethylated CpG islands with an open chromatin structure. Here, we found that the *BMAL1* gene is not expressed with circadian oscillation in CPT-K cells because the CpG islands located in the *BMAL1* promoter are hypermethylated and that 5-aza-2′-deoxycytidine (aza-dC) recovered *BMAL1* expression. In contrast, CpG islands in the *PER2* promoter were hypomethylated, the *PER2* gene was expressed and aza-dC enhanced *PER2* gene expression in CPT-K cells. Reporter gene assays showed that intracellular transcriptional machinery for the *BMAL1* gene is active, suggesting that *BMAL1* inactivation is caused by DNA methylation and not by malfunctional promoter activity. Incubating CPT-K cells with aza-dC also increased *CRY1* expression, whereas *CLOCK* expression was not altered and the *CRY1* promoter was unmethylated. These results suggest that aza-dC induces *BMAL1* expression via DNA demethylation in the *BMAL1* promoter and enhances *PER2* and *CRY1* transcription. Finally, aza-dC recovered the circadian oscillation of *BMAL1* transcription. These results suggest that DNA methylation of the *BMAL1* gene is critical for interfering with circadian rhythms.

1. Introduction

Circadian rhythms function in most living organisms and govern many behavioral and biochemical processes. The circadian clock operates robust rhythms coupled with changes in the cellular environment. The master clock that generates circadian rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus where it controls all aspects of physiology such as sleep-wake cycles, body temperature, hormone secretion, blood pressure and metabolism. Circadian clock coordination of such physiological aspects is essential to optimize metabolic responses and strengthen inherent homeostatic regulatory mechanisms [1]. The molecular mechanism of the circadian oscillator is based on interlocked transcriptional and translational feedback loops that have both positive and negative elements. Among the core clock genes, *BMAL1* expression that is closely associated with circadian rhythms oscillates in the SCN and in peripheral clock cells [2]. The mammalian core clock proteins BMAL1 and CLOCK heterodimerize, bind to E-boxes and activate the transcription of *PERs* and *CRYs*. The core clock proteins PERs and CRYs heterodimerize, associate with other partners in the nucleus and repress BMAL:CLOCK-driven activation, thus generating a negative autoregulatory feedback loop [1]. The BMAL:CLOCK heterodimer also binds to E-boxes in many clock-controlled genes and dictates their expression. Thus, circadian dysfunction is considered to contribute to the incidence and severity of a wide range of clinical and pathological conditions including sleep disorders, cancer, depression, metabolic syndrome and inflammation [3].

The methylation of DNA is a major epigenetic modification in multicellular organisms. DNA is methylated mostly on CpG dinucleotides (CpG islands) in humans [4] and this results in transcriptional repression either by interfering with transcription factor binding or by including a repressive chromatin structure [5]. Altered DNA methylation is associated with many human diseases and it is a hallmark of cancer. Clock gene methylation is highly prevalent in dementia with Lewy bodies (DLB), a disorder that is similar to Parkinson's disease [6]. In addition, the promoters of the crucial clock genes, *CRY1* and *NPAS2* are regulated by DNA methylation and the *NPAS2* promoter is hypomethylated in patients with Parkinson's disease [7]. Clock genes influence tumorigenesis and the methylation of clock gene promoters such as *CLOCK* [8] and *PERs* [9–12] contributes to the progression of cancer. The *BMAL1* gene is also transcriptionally silenced by the hypermethylation of CpG islands in its promoter in hematological malignancies [13].

We previously found that ROREs, which are recognition motifs for ROR and REV-ERB orphan nuclear receptors and critical elements for *BMAL1* oscillatory transcription [14], are embedded in

* Corresponding author. Address: Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba, Ibaraki 305-8566, Japan. Fax: +81 29 861 6402.
E-mail address: y-onishi@aist.go.jp (Y. Onishi).

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a unique GC-rich open chromatin structure, with which a nuclear matrix-like structure at the 3′-flanking region cooperates to regulate *BMAL1* transcription [15,16]. Although *BMAL1* transcription is silenced through DNA methylation of its promoter in tumor cells, how the DNA methylation of this promoter affects circadian rhythms remains obscure. Here, we investigated how DNA methylation of the *BMAL1*, *PER2* and *CRY1* promoters affects *BMAL1* transcription in CPT-K cells. We also analyzed the levels to which other clock genes are transcribed and investigated the effect of the aza-dC-induced DNA demethylation of the *BMAL1* promoter on clock gene transcription. We then characterized the effects of aza-dC on the rhythmic transcription of *BMAL1*.

2. Materials and methods

2.1. Cell culture

Cells stably expressing the luciferase reporter gene driven by the *BMAL1* promoter region (−197 to +27) were derived from CPT-K cells [17]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin and streptomycin in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

2.2. Reverse transcriptase-polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) proceeded as described [16] using the following primer sets: *BMAL1*, 5′-AGGACTTCCCCTCTACCTGCTC-3′ and 5′-AACTACATGAGAATGCAGTCGTC-3′; *PER2*, 5′-TGATTGAAACCCCAGTGCTCGT-3′ and 5′-CTCCATGGGTTGATGAAGCTGG-3′; *CRY1*, 5′-AGAACAGATCCCAATGGAGAC-3′ and 5′-ATTAGAAGGTACTGATGCCAG-3′; *CLOCK*, 5′-TACAACGCACACATAGGCCATC-3′ and 5′-ATACCCTATTATGGGTGGTGC-3′; *ACTIN*, 5′-TACGCCAACACAGTGCTGTCTG-3′ and 5′-TTTTCTGCGCAAGTTAGGTTTTGTC-3′. The PCR fragments were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

2.3. Real-time quantitative PCR

Real-time quantitative PCR proceeded using LightCycler (Roche) with Light Cycler-FastStart DNA Master SYBR Green I kits (Roche) and the same primer sets described above. An authentic template comprised PCR products cloned into the pGEM-T Easy vector (Promega). Relative expression levels were evaluated using Light Cycler software version 3.5.

2.4. CpG methylation analysis

We identified CpG islands in *BMAL1*, *PER2* and *CRY1* promoters using the algorithm at www.urogene.org/methprimer [18]. Methylation was analyzed as a modification generated using EpiTect Bisulfite (Qiagen) according to the manufacturer's instructions, followed by PCR cloning and sequencing. We also analyzed the CRY1 promoter using methylation-specific PCR for bisulfite-treated DNA and the methylated primer sequences, 5′-TATTATTGGTTTTTGAAGGAAATCG-3′ and 5′-CTACGTTCCAAAAAATAAAAAAACG-3′ the unmethylated primer sequences, 5′-TATTATTGGTTTTTGAAGGAAATTG-3′ and 5′-ACATTCCAAAAAATAAAAAAACAAA-3′.

2.5. Transient reporter gene assay

Luciferase reporter gene plasmids and the internal control plasmid, pRL-CMV (Promega) were transfected with or without *BMAL1*- and *CLOCK*-expression vectors [19] into CPT-K cells. Luciferase assays proceeded using the Dual Luciferase Reporter Assay System (Promega) as described [20]. Transcriptional activities were normalized relative to *Renilla* luciferase activities.

2.6. Real-time reporter gene assays

Real-time reporter gene assays proceeded as described [15]. Cells that stably expressed reporter genes were stimulated with 50% FBS for 2 h and then incubated with DMEM containing 0.1 mM luciferin (Promega), 25 mM HEPES (pH 7.2) and 10% FBS. Bioluminescence was measured and integrated for 1 min at 10-min intervals using a Kronos AB-2500 (ATTO). Data were detrended by subtracting a best fit line followed by subsequent fitting to a sine wave to determine the length of the circadian period as described [21].

3. Results

3.1. Hypermethylation of BMAL1 CpG islands in CPT-K cells

The *BMAL1* gene is transcriptionally silenced via the hypermethylation of promoter CpG islands in cancer cell lines [13] and we previously reported that ROREs in the *BMAL1* promoter are embedded in a unique GC-rich open chromatin structure under CpG island hypomethylation, which is important for circadian transcription [15,16]. Thus we surveyed cell lines with hypermethylated *BMAL1* promoters to evaluate how DNA methylation of the *BMAL1* promoter affects the circadian clock system. We found that the human lymphoblastic leukemia cell line, CPT-K, expressed very low levels of the *BMAL1* gene (Fig. 1A). Bisulfite genomic sequencing of five individual clones indicated that the *BMAL1* promoter in CPT-K cells is extremely hypermethylated in CpG islands (Fig. 1B). To clarify the relationship between promoter methylation and *BMAL1* expression, we demethylated CPT-K cells using 5-aza-2′-deoxycytidine (aza-dC) and determined *BMAL1* transcription by RT-PCR. Fig. 1A shows that significant levels of *BMAL1* transcription was were in CPT-K cells, suggesting that hypermethylation of the promoter CpG islands repressed *BMAL1* transcription in CPT-K cells.

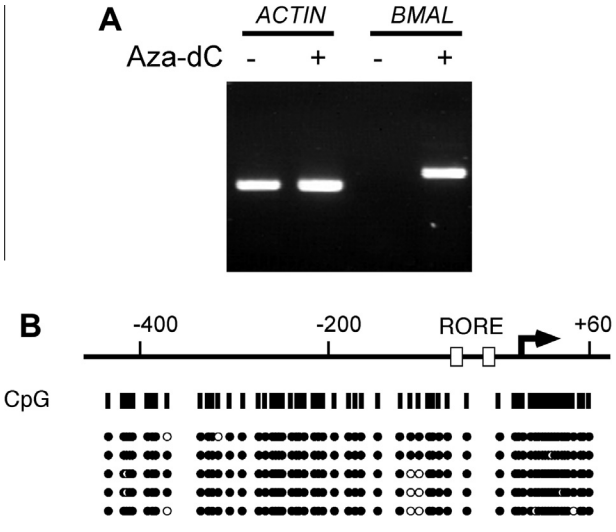

Fig. 1. Decreased expression and DNA hypermethylation of the *BMAL1* gene in CPT-K cells. (A) *BMAL1* is minimally expressed in CTP-K cells but induced by aza-dC. CPT-K cells were incubated with or without 2.5 μM aza-dC for 2 days, and then RNA in harvested cells was analyzed by RT-PCR. (B) DNA hypermethylation in *BMAL1* promoter. *BMAL1* promoter sequence was modified with bisulfite and then CpG islands were analyzed. Vertical lines, CpG sites in *BMAL1* promoter region. Filled and open circles, methylated and unmethylated CpG sites, respectively. Arrow and open boxes in map, transcription start site and two recognition motifs for ROR and REV-ERB orphan nuclear receptors (RORE).

3.2. Hypomethylation of PER2 CpG islands in CPT-K cells

PER genes are thought to be tumor suppressors [22] and considerable evidence supports the notion that the hypermethylation of CpG islands in *PER* promoters is associated with tumorigenesis [9–12]. We found *PER2* transcripts in CPT-K cells (Fig. 2A) and genomic methylation analysis showed that a CpG island in the *PER2* promoter remained hypomethylated (Fig. 2B), indicating that *PER2* was transcribed in these cells. The activation of *PER2* transcription was increased twofold in CPT-K cells incubated with aza-dC (Fig. 2A) despite the *PER2* promoter having hypomethylated CpG islands. Transient reporter assays showed that the *PER2* promoter activity increase induced by the exogenous expression of *BMAL1* and *CLOCK* (Fig. 2C) was comparable to the amount of aza-dC-enhanced *PER2* transcription in RT-PCR (Fig. 2A). These data suggest that aza-dC activates *BMAL1* transcription in CPT-K cells, which subsequently activates *PER2* transcription. Compared with the promoter-less construct, the significant activity of the *BAML1* promoter construct in CPT-K cells was suppressed by the exogenous expression of *BMAL1* and *CLOCK* (Fig. 2C). The suppression of *BMAL1* promoter activity by BMAL1:CLOCK was consistent with previous findings [23], suggesting that the reporter gene driven by the *BMAL1* promoter is active. Taken together, these results suggest that the transcriptional machinery for *BMAL1* in CPT-K cells is functional except for methylation of the *BMAL1* promoter.

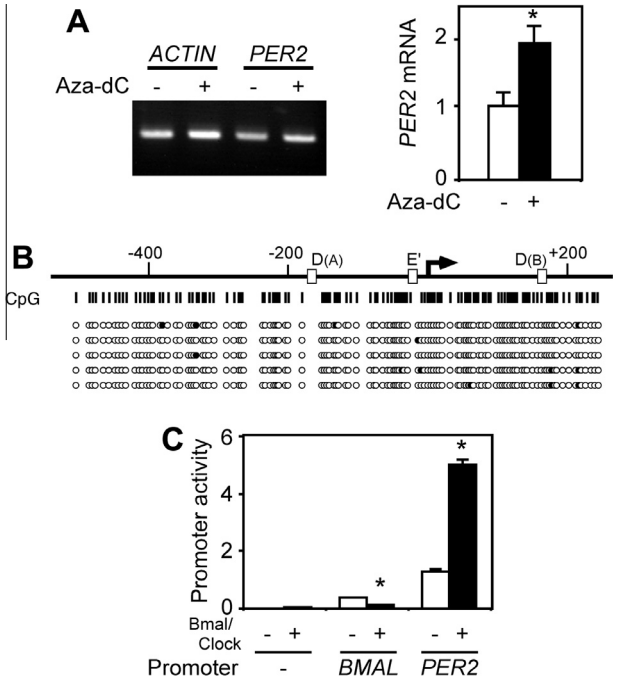

Fig. 2. *PER2* gene is abundantly expressed and DNA in *PER2* promoter is hypomethylated in CPT-K cells. (A) *PER2* is abundantly expressed in CTP-K cells that were incubated with or without 2.5 μM aza-dC for 2 days. We analyzed RNA in harvested cells using RT-PCR. Levels of RNA were normalized to those of *ACTIN* expression, and value in cells incubated without aza-dC was set at 1. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$; Student's *t* test. (B) Hypomethylation of DNA in *PER2* promoter. Genomic sequence of CPT-K cells was analyzed after modification with bisulfite. Vertical lines, CpG sites in *PER2* promoter region. Filled and open circles, methylated and unmethylated CpG sites, respectively. Arrow, transcription start site; open boxes D (A) and D (B), D-boxes (DBP-binding sites); open box E', non-canonical E-box. (C) Transcriptional machinery for *BMAL1* gene is functional. Transcriptional assays proceeded using constructs containing *BMAL1* or *PER2* promoters. *BMAL1* and *CLOCK* expression plasmids were also introduced into CPT-K cells. Normalized expression levels were calculated relative to luciferase activities of *PER2* reporter transfectants. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$; Student's *t* test.

3.3. Circadian clock system in CPT-K cells

Circadian oscillation of *BMAL1* transcription was absent in CPT-K cells (gray in Fig. 4). CPT-K cells expressed abundant amounts of the *CLOCK* gene regardless of aza-dC (Fig. 3), suggesting that the transcriptional activator function of the BMAL1:CLOCK heterodimer is dependent on the amount of BMAL1. Aza-dC activated *BMAL1* transcription (Figs. 1A and 3). Similar to the transcription of *PER2*, that of *CRY1* was activated about twofold by aza-dC in CPT-K cells (Fig. 3), suggesting that *CRY1* transcription was activated via *BMAL1* expression induced by aza-dC. Fig. 3B shows that the *CRY1* promoter was unmethylated, supporting the notion that aza-dC activates *BMAL1* transcription in CPT-K cells, which subsequently results in activated *CRY1* transcription. These results implied that aza-dC can recover the circadian oscillation of *BMAL1* transcription in CPT-K cells. We examined this notion using cells that stably expressed reporter genes derived from CPT-K cells that had been incubated with aza-dC. Real-time reporter assays indicated that aza-dC caused the circadian oscillation of *BMAL1* transcription in these cells (Fig. 4). Previously, we reported that level of *BMAL1* expression affects the circadian oscillation using NIH3T3-derived stable cells [24]. These findings suggest that DNA methylation in the *BMAL1* gene is a key phenomenon that disrupts circadian rhythms.

4. Discussion

The present study found epigenetic inactivation or DNA methylation of the *BMAL1* promoter in CPT-K cells. Taniguchi et al. found *BMAL1* hypermethylation in hematological malignancies, but not in solid tumors [13]. Notably, *BMAL1* is hypermethylated in CPT-K cells that are derived from acute lymphoblastic leukemia [25] and hypomethylated in HSG cells derived from solid tumors [16]. NIH3T3 cells also have a hypomethylated *BMAL1* promoter and show the circadian transcription [15], and we previously showed that level of *BMAL1* expression affects the circadian oscillation using NIH3T3-derived stable cells [24]. Loss of *BMAL1* in mice results in immediate and complete loss of circadian rhythmicity [26]. These indicate the importance of the amount of *BMAL1* expression on circadian rhythms. Because of *BMAL1* gene inactivation, the circadian oscillation of *BMAL1* transcription was absent in the line that were derived from CPT-K cells; however circadian

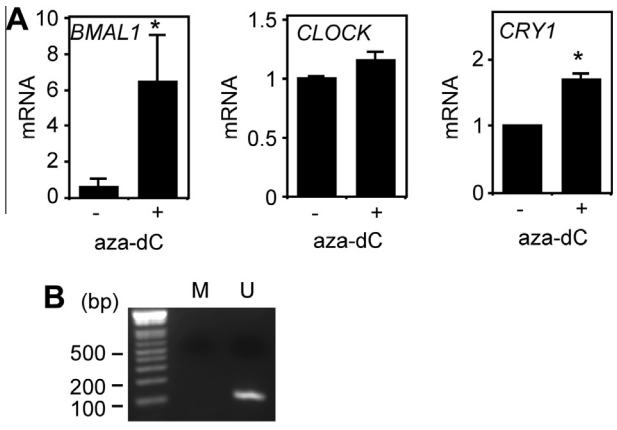

Fig. 3. Aza-dC enhances *CRY1* transcription. (A) Effect of aza-dC on transcription. CPT-K cells were incubated with or without 2.5 μM aza-dC for 2 days, and then RNA in harvested cells was analyzed by RT-PCR. Levels of RNA were normalized to those of *ACTIN* expression, and value in cells incubated without aza-dC was set at 1. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$; Student's *t* test. (B) Analysis of *CRY1* promoter in CPT-K cells. M and U, methylation- and unmethylation-specific PCR primers.

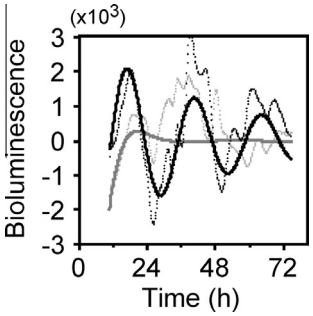

Fig. 4. Effect of aza-dC on transcriptional oscillation of *BMAL1*. Cells with stable gene expression derived from CPT-K cells were incubated with 2.5 μM aza-dC for 2 days, stimulated with 50% FBS for 2 h and then bioluminescence was measured. Detrended fit curves are representative of at least three independent experiments (control, gray; aza-dC, black). Dots, raw values; lines, fit curve data.

oscillation was restored by the demethylating agent, aza-dC in the present study. *BMAL1* is also hypermethylated in RPMI 8402 cells from which CPT-K cells were isolated by camptothecin resistance (data not shown). Unfortunately continuous *BMAL1* expression did not restore circadian oscillation in CPT-K cells (data not shown). McDearmon et al. reported that constitutive *BMAL1* expression in brain restores the circadian oscillation but not in muscle, indicating that *BMAL1* has distinct tissue-specific regulation and functions [27]. Therefore, to establish the negative feedback loop system and restore circadian oscillation in CPT-K cells, the tissue-specific regulation of *BMAL1* expression may be required, which is introduced enodogenously by aza-dC. The *PER* genes are epigenetically silenced not only in leukemia [10], but also in other types of cancer such as that of the lung [12], cervix [11] and breast [9], indicating that PERs act as tumor suppressors [22]. Hypermethylation in the *CLOCK* promoter reduces the risk of breast cancer [8] and the nature of *CRY1* promoter methylation has significant prognostic impact in chronic lymphocystic leukemia [28]. These findings indicate that the disruption of circadian rhythm influences tumor development and we identified epigenetic silencing of the *BMAL1* gene along with expression of the clock genes, *PER2*, *CLOCK* and *CRY1* in CPT-K cells. The expression profiles of clock genes in tumor cells should be elucidated from the perspective of their roles in cancer.

The following core circadian genes are associated with circadian rhythms in peripheral tissues: casein kinase 1 ε (*CSNK1E*), cryptochromes (*CRY1* and *CRY2*), periods (*PER1*, *PER2* and *PER3*), *CLOCK* and *BMAL1*. The *BMAL1* gene plays a central role in circadian systems and its rhythmic expression is circadian. We previously showed that the *BMAL1* promoter is a unique open chromatin structure with hypomethylated CpG islands that are important for circadian transcription [15,16]. Therefore, the methylation status of the *BMAL1* promoter is critical for the circadian system and for evaluating how the *BMAL1* promoter is methylated. Because the *BMAL1* promoter is basically hypomethylated, the methyltransferases DNMT3a and DNMT3b might be mainly responsible for introducing cytosine methylation *de novo* at unmethylated CpG sites in the promoter, but the precise mechanism remains unclear [5]. In addition, as the stoichiometric relationship among components is critical for the robustness of circadian rhythms [29], the methylation status of other clock gene promoters should also be determined.

Circadian rhythms regulate many physiological processes in humans. Their disruption profoundly influences health and they have been linked to several major diseases. For instance, the adrenal steroid hormone glucocorticoid that controls various physiological process such as metabolism, the immune response, cardiovascular activity and brain function, is under the control of the circadian clock. This implies that several diseases are closely associated with disrupted circadian rhythms [30]. Several recent reports have described relationships between DNA methylation of the clock genes and diseases other than cancer such as dementia [6], Parkinson's disease [7] and obesity [31]. Furthermore, DNA methylation might contribute to the developmental expression of clock genes [32]. These lines of evidence suggest that the DNA methylation of the clock genes, in particular, *BMAL1* is a key player in the disruption of circadian rhythms that are closely associated with various diseases.

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References

[1] S.M. Reppert, D.R. Weaver, Coordination of circadian timing in mammals, Nature 418 (2002) 935–941.
[2] M.K. Bunger, L.D. Wilbacher, S.M. Moran, C. Clendenin, L.A. Radcliffe, J.B. Hogenesch, M.C. Simon, J.S. Takahashi, C.A. Bradfield, Mop3 is an essential component of the master circadian pacemaker in mammals, Cell 103 (2000) 1009–1017.
[3] D.A. Bechtold, J.E. Gibbs, A.S. Loudon, Circadian dysfunction in disease, Trends Pharmacol. Sci. 31 (2010) 191–198.
[4] A. Bird, DNA methylation patterns and epigenetic memory, Genes Dev. 16 (2002) 6–21.
[5] R.J. Klose, A.P. Bird, Genomic DNA methylation: the mark and its mediators, Trends Biochem. Sci. 31 (2006) 89–97.
[6] H.C. Liu, C.J. Hu, Y.C. Tang, J.G. Chang, A pilot study for circadian gene disturbance in dementia patients, Neurosci. Lett. 435 (2008) 229–233.
[7] Q. Lin, H. Ding, Z. Zheng, Z. Gu, J. Ma, L. Chen, P. Chan, Y. Cai, Promoter methylation analysis of seven clock genes in Parkinson's disease, Neurosci. Lett. 507 (2012) 147–150.
[8] A.E. Hoffman, C.H. Yi, T. Zheng, R.G. Stevens, D. Leaderer, Y. Zhang, T.R. Holford, J. Hansen, J. Paulson, Y. Zhu, CLOCK in breast tumorigenesis: genetic, epigenetic and transcriptional profiling analyses, Cancer Res. 70 (2010) 1459–1468.
[9] S.T. Chen, K.B. Choo, M.F. Hou, K.T. Yeh, S.J. Kuo, J.G. Chang, Deregulated expression of the *PER1*, *PER2* and *PER3* genes in breast cancers, Carcinogenesis 26 (2005) 1241–1246.
[10] M.Y. Yang, J.G. Chang, P.M. Lin, K.P. Tang, Y.H. Chen, H.Y. Lin, T.C. Liu, H.H. Hsiao, Y.C. Liu, S.F. Lin, Downregulation of circadian clock genes in chronic myeloid leukemia: alternative methylation pattern of hPER3, Cancer Sci. 97 (2006) 1298–1307.
[11] M.C. Hsu, C.C. Huang, K.B. Choo, C.J. Huang, Uncoupling of promoter methylation and expression of Period1 in cervical cancer cells, Biochem. Biophys. Res. Commun. 360 (2007) 257–262.
[12] S. Gery, N. Komatsu, N. Kawamata, C.W. Miller, J. Desmond, R.K. Virk, A. Marchevsky, R. Mckenna, H. Taguchi, H.P. Koeffler, Epigenetic silencing of the candidate tumor suppressor gene *Per1* in non-small cell lung cancer, Clin. Cancer Res. 13 (2007) 1399–1404.
[13] H. Taniguchi, A.F. Fernandez, F. Setien, S. Ropero, E. Ballestar, A. Villanueva, H. Yamamoto, K. Imai, Y. Shinomura, M. Esteller, Epigenetic inactivation of the circadian clock gene *BMAL1* in hematologic malignancies, Cancer Res. 69 (2009) 8447–8454.
[14] H.R. Ueda, W. Chen, A. Adachi, H. Wakamatsu, S. Hayashi, T. Takasugi, M. Nagano, K. Nakahama, Y. Suzuki, S. Sugano, M. Iino, Y. Shigeyoshi, S. Hashimoto, A transcription factor response element for gene expression during circadian night, Nature 418 (2002) 534–539.
[15] Y. Onishi, S. Hanai, T. Ohno, Y. Hara, N. Ishida, Rhythmic SAF-A binding underlies circadian transcription of the *BMAL1* gene, Mol. Cell. Biol. 28 (2008) 3477–3488.
[16] Y. Onishi, HSG cells, a model in the submandibular clock, Biosci. Rep. 31 (2010) 57–62.
[17] Y. Onishi, Y. Kawano, Rhythmic binding of topoisomerase I impacts on the transcription of *BMAL1* and circadian period, Nucleic Acids Res. 40 (2012) 9482–9492.
[18] L.C. Li, R. Dahiya, MethPrimer: designing primers for methylation PCRs, Bioinformatics 18 (2002) 1427–1431.
[19] T. Ohno, Y. Onishi, N. Ishida, The negative transcription factor E4BP4 is associated with circadian clock protein PERIOD2, Biochem. Biophys. Res. Commun. 354 (2007) 1010–1015.
[20] Y. Onishi, Y. Wada-Kiyama, R. Kiyama, Expression-dependent perturbation of nucleosomal phases at HS2 of the human β-LCR: possible correlation with periodic bent DNA, J. Mol. Biol. 284 (1998) 989–1004.
[21] Y. Onishi, K. Oishi, Y. Kawano, Y. Yamazaki, The harmala alkaloid, harmine is a modulator of circadian *BMAL1* transcription, Biosci. Rep. 32 (2011) 45–52.
[22] C.C. Lee, Tumor suppression by the mammalian Period genes, Cancer Causes Control 17 (2006) 525–530.

[23] W. Yu, M. Nomura, M. Ikeda, Interactivating feedback loops within the mammalian clock: *BMAL1* is negatively autoregulated and upregulated by *CRY1*, *CRY2*, and *PER2*, Biochem. Biophys. Res. Commun. 290 (2002) 933–941.

[24] Y. Onishi, Y. Kawano, Y. Yamazaki, Lycorine, a candidate for the control of period length in mammalian cells, Cell. Physiol. Biochem. 29 (2012) 407–416.

[25] T. Andoh, K. Ishii, Y. Suzuki, Y. Ikegami, Y. Kusunoki, Y. Takemoto, K. Okada, Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I, Proc. Natl. Acad. Sci. USA 84 (1987) 5565–5569.

[26] M.K. Bunger, L.D. Wilsbacher, S.M. Moran, C. Clendenin, L.A. Radcliffe, J.B. Hogenesch, M.C. Simon, J.S. Takahashi, C.A. Bradfield, Mop3 is an essential component of the master circadian pacemaker in mammals, Cell 103 (2000) 1009–1017.

[27] E.L. McDearmon, K.L. Patel, C.H. Ko, J.A. Walisser, A.C. Schook, J.L. Chong, L.D. Wilsbacher, E.J. Song, H.-K. Hong, C.A. Bradfield, J.S. Takahashi, Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice, Science 314 (2006) 1304–1308.

[28] M. Hanoun, L. Eisele, M. Suzuki, J.M. Greally, A. Huttmann, S. Aydin, R. Scholtysik, L. Klein-Hitpass, U. Duhrsen, J. Durig, Epigenetic silencing of the circadian clock gene *CRY1* is associated with an indolent clinical course in chronic lymphocytic leukemia, PLoS One 7 (2012) e34347.

[29] Y. Lee, R. Chen, H.M. Lee, C. Lee, Stoichiometric relationship among clock proteins determines robustness of circadian rhythms, J. Biol. Chem. 286 (2011) 7033–7042.

[30] S. Chung, G.H. Son, K. Kim, Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications, Biochim. Biophys. Acta 2011 (1812) 581–591.

[31] F.I. Milagro, P. Gomez-Abellan, J. Campion, J.A. Martinez, J.M. Ordovas, M. Garaulet, CLOCK, PER2 and BMAL1 DNA methylation: association with obesity and metabolic syndrome characteristics and monounsaturated fat intake, Chronobiol. Int. 29 (2012) 1180–1194.

[32] Y. Ji, Y. Qin, H. Shu, X. Li, Methylation analyses on promoters of *mPer1*, *mPer2*, and *mCry1* during perinatal development, Biochem. Biophys. Res. Commun. 391 (2010) 1742–1747.