Epigenetic Inactivation of the Circadian Clock Gene BMAL1 in Hematologic Malignancies

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Abstract
Disruption of circadian rhythms, daily oscillations in biological processes that are regulated by an endogenous clock, has been linked to tumorigenesis. Normal and malignant tissues often show asynchronies in cell proliferation and metabolic rhythms. Cancer chronotherapy takes biological time into account to improve the therapy. However, alterations of the circadian clock machinery genes have rarely been reported in human cancer. Herein, we show that the BMAL1 gene, a core component of the circadian clock, is transcriptionally silenced by promoter CpG island hypermethylation in hematologic malignancies, such as diffuse large B-cell lymphoma and acute lymphocytic and myeloid leukemias. We also describe how BMAL1 reintroduction in hypermethylated leukemia/lymphoma cells causes growth inhibition in colony assays and nude mice, whereas BMAL1 depletion by RNA interference in unmethylated cells enhances tumor growth. We also show that BMAL1 epigenetic inactivation impairs the characteristic circadian clock expression pattern of genes such as C-MYC, catalase, and p300 in association with a loss of BMAL1 occupancy in their respective promoters. Furthermore, the DNA hypermethylation–associated loss of BMAL1 also prevents the recruitment of its natural partner, the CLOCK protein, to their common targets, further enhancing the perturbed circadian rhythm of the malignant cells. These findings suggest that BMAL1 epigenetic inactivation contributes to the development of hematologic malignancies by disrupting the cellular circadian clock.

Introduction
Circadian rhythms are the daily oscillations of many biological processes driven by endogenous clocks. In humans, the central pacemaker of the circadian clock is located in the suprachiasmatic nucleus of the anterior hypothalamus. One of the discoveries that has deeply affected the field of circadian biology is the discovery that molecular clockwork similar to that present in suprachiasmatic nucleus neurons exists in all peripheral tissues studied (1–3). Circadian oscillations even occur in established cell lines, such as cultured fibroblasts, in which the endogenous clock system needs a simple serum shock to be synchronized (4). Disruption of the circadian rhythms has a profound influence on human health and has been linked to several major diseases, among them cancer. In this latter area, it has also been shown that the proliferation of tumor cells follows autonomous circadian patterns that are out of phase with nontumor cells (5, 6). This latter observation has been explored for therapeutic purposes in chronotherapy, which takes into account biological time to improve the efficacy of cancer therapy by administering the therapy at a specific time of the day (7–10). Most of the evidence supports the idea that the circadian clock has a tumor suppressor role at the systemic, cellular, and molecular levels. In peripheral tissue cells, the circadian clock controls cell proliferation and apoptosis by regulating the expression of circadian-controlled genes, such as C-MYC, MDM2, p53, Cyclin D1, and GADD45a (7, 11). Indeed, microarray data show that up to 10% of genes in different tissues are regulated by the circadian clock system (12, 13). This precise regulation is accomplished by molecular clockwork machinery composed of several interacting genes. To date, eight core circadian genes have been identified: Casein kinase 1e (CK1e), Cryptochrome1 (Cry1), Cryptochrome2 (Cry2), Period1 (Per1), Period2 (Per2), Period3 (Per3), Clock, and BMAL1 (brain and muscle ARNT-like protein, Arntl, MOP3). Most important, Per2-mutant mice are cancer prone, have an attenuated p53 DNA-damage response, and overexpress C-MYC (14), whereas Per2 and Per1 ectopic expression results in growth inhibition (15, 16). Even more recently, preliminary reports suggest that the Period genes may undergo aberrant DNA methylation events in human tumors (17, 18).

A central player in the circadian clock molecular machinery is BMAL1, a member of the basic helix-loop-helix-PAS domain–containing transcription factor family (19). In a complex with another major member of the pathway, CLOCK, which possesses intrinsic histone acetyltransferase activity (20), BMAL1 regulates expression of many genes by binding to E-boxes located in their respective promoters. BMAL1-deficient mice show early aging and age-related pathologies (21). These phenotypes partly resemble those observed when defects in progeroid syndrome genes, such as Lamin A/C and Werner, are present (22). Because the Lamin A/C and WRN genes undergo DNA methylation-associated silencing in human cancer (23, 24), and because there is increasing evidence that disrupted circadian clocks affect human tumorigenesis (11, 25), we examined whether epigenetic silencing of BMAL1 was involved in the development of human malignancies.

Materials and Methods
Human cancer cell lines, primary tumor samples, and normal lymphocytes. The 41 human cancer cell lines examined in this study were
obtained from the American Type Culture Collection and the German Collection of Microorganisms and Cell Cultures. The cell lines represented 11 types of malignancy (leukemia, lymphoma, glioma, neuroblastoma, osteosarcoma and breast, colon, gastric, prostate, thyroid, and endometrial carcinomas). The described cell banks performed cell line authentication using DNA fingerprinting by short tandem repeat analysis fewer than 6 mo after receipt or resuscitation. Cell lines were maintained in appropriate media and treated with 1 μ 5-aza-2'-deoxycytidine (Sigma) for 48 h to achieve demethylation. Synchronization of cells was accomplished by serum shock (4). Briefly, cells were grown to confluence in 150-mm tissue culture dishes in RPMI 1640 media supplemented with 10% FCS, followed by culture for 2 d with starvation medium (0.5% serum). At time t = 0, the medium was exchanged for 50% serum in RPMI 1640; after 2 h, the medium was replaced with serum-free RPMI 1640. At the indicated times (0, 4, 8, 12, 16, and 20 h), the dishes were prepared for immediate RNA extraction and chromatin immunoprecipitation (ChIP) assay. One hundred twenty-six primary hematologic malignancies and 100 normal lymphocyte samples from healthy donors were obtained from the Cancer Epigenetics and Biology Program Tissue Bank.

DNA methylation analysis of the BMAL1 gene. We determined the BMAL1 CpG island methylation status by PCR analysis of bisulfite-modified genomic DNA. First, methylation status was analyzed by bisulfite genomic sequencing of the CpG island. The primers used were 5'-TTA AAT GAT and methylation-specific PCR (white arrows) primers shown. Results are shown of bisulfite genomic sequencing of eight individual clones in normal lymphocytes (NL1 and NL2) and cell lines (RAJI, AKATA, A3.01, JIJOYE, FARAGE, MOLT-4, and MRC5). Presence of a methylated (■) or unmethylated cytosine (□) is shown. Bottom, a representative methylation-specific PCR for the BMAL1 gene is also shown. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. In vitro methylated DNA (IVD) is used as a positive control for methylated DNA.
TGG TGG TAG GAA A-3' (sense) and 5'-TAA CCT ACT TTC TCC CCC TAA ATA-3' (antisense). The second analysis used methylation-specific PCR with primers specific to either the methylated or modified unmethylated DNA. Primer sequences for the methylated reaction were 5'-TAA ATC GGT TTG CGT TTC-3' (sense) and 5'-CGA ATA CCT ACC TCT GCC CG-3' (antisense), and for the unmethylated reaction were 5'-GGT AAA TTG GTT GTT GTT TT-3' (sense) and 5'-CCC AAA AAA CAT CTA CCT CCC A-3' (antisense).

**BMAL1 RNA and protein analysis by conventional reverse transcription-PCR, Western blot, and immunofluorescence.** RNA was isolated by using TRIZOL (Life Technologies). Two micrograms of RNA were reverse transcribed using SuperScript II reverse transcriptase (Life Technologies/Bethesda Research Laboratories), and amplified using specific primers for BMAL1 (forward, 5'-GCG TCG GGA TAA AAT GAA CA-3'; reverse, 5'-CTT CCC TCG GTC ACA ACA TA-3'). PCR was performed for 25 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s). Western blots and immunofluorescence experiments were carried out using the BMAL1 antibody H-170 (Santa Cruz Biotechnology).  

**BMAL1 transfection and small interfering RNA assay.** The BMAL1 expression vector pcDNA3-BMAL1 was constructed by cloning the cDNA corresponding to the BMAL1 gene from MRC5 cell line into the pcDNA3 vector (Invitrogen) and confirmed by sequencing. For transfection experiments, we used the pcDNA3 vector containing the BMAL1 gene or pcDNA3 empty vector. Transfection was performed by electroporating 10⁷ cells in 0.8 mL PBS with 40 μg of the vector at 250 V and 975 uF. Electroporated cells were washed with PBS and seeded with 10⁶ cells mL⁻¹ in fresh medium containing 20% fetal bovine serum. Transfected cells were selected by adding G418 (600 μg mL⁻¹). MOLT4 cells were nucleofected with 1.5 μg of BMAL1 and control small interfering RNA (Qiagen) using the Cell Line Nucleofector kit L (Amaxa Biosystems). Cells were harvested 1 to 2 d after electroporation.

**Colony formation/cell viability assays and mouse xenograft model.** Colony formation on methylcellulose medium (StemCell Technologies) was assayed. Transfected cells were added to a medium containing 80% methylcellulose and 20% conditioned medium from RAJI cultures and 600 μg mL⁻¹ G418. The mixture was then placed in a six-well plate and incubated for 15 d. Colonies containing >20 cells were considered positive. Cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Six-week-old female athymic nude mice nu/nu (Harlan Sprague-Dawley) were used for RAJI tumor xenografts. Ten specimens were used. Both flanks of each animal were injected s.c. with 10⁷ cells in a volume of 200 μL of PBS. The right flank was always used for BMAL1-transfected cells and the left for empty vector control cells. Tumor development at the site of injection was measured daily.

**Quantitative reverse transcription-PCR.** Two micrograms of total RNA were converted to cDNA with the ThermoScriptTM RT-PCR System (Invitrogen) using Oligo-dT as primer. PCR amplifications were performed in 96-well optical plates in a volume of 20 μL. We used 0.2 μg of cDNA, 5 pmol of each primer, and 10 μL of 2× SYBRGreen PCR Master Mix (Applied Biosystems). Three measurements were taken and the amounts estimated by extrapolation from a standard curve. Expression values were normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase and used as an endogenous control. PCR reactions were run and analyzed using the Prism 7700 Sequence Detection System (Applied Biosystems). Primers are shown in Supplementary Table S1.

**Quantitative ChIP.** The ChIP assay was performed with the same BMAL1 antibody as used for Western blotting and the antibodies against CLOCK (H-276, Santa Cruz Biotechnology), and acetylated histones H3 and H4 (Upstate Biotechnologies). Chromatin was sheared to an average length of 0.25-1 Kb. PCR amplifications were performed in 96-well optical plates in a volume of 20 μL. We used 0.2 μg of immunoprecipitated DNA, 5 pmol of each primer, and 10 μL of 2× SYBRGreen PCR Master Mix (Applied Biosystems). Three measurements were made and the amounts estimated by extrapolation from a standard curve. PCR reactions were run and analyzed using the Prism 7700 Sequence Detection System (Applied Biosystems). Primers are shown in Supplementary Table S1.

**Results and Discussion**

BMAL1 has a 2,000 bp CpG island with a 8.6% CpG content around its transcription start site (Fig. 1). We first determined...
the BMAL1 CpG island DNA methylation status of a panel of 41 human cancer cell lines from 11 tumor types by bisulfite genomic sequencing of multiple clones and methylation-specific PCR (Fig. 1 and Supplementary Fig. S1). Strikingly, BMAL1 CpG island hypermethylation was only found in hematologic malignancies (Fig. 1 and Supplementary Fig. S1). It was present in non-Hodgkin lymphoma cell lines (60%, 3 of 5) and in A3.01 cells derived from an acute lymphocytic leukemia patient.
(ALL; Fig. 1 and Supplementary Fig. S1). However, it was unmethylated in all solid tumor cell lines (Supplementary Fig. S1). The hypermethylation event was cancer specific because all normal peripheral lymphocytes analyzed by methylation-specific PCR (n = 100) were completely unmethylated at the BMAL1 CpG island (Fig. 1 and Supplementary Fig. S2). BMAL1 unmethylated cell lines, such as MOLT-4 (leukemia), FARAGE (lymphoma), and MRC-5 (fibroblasts), strongly expressed BMAL1 transcript and protein (Fig. 2A and B). Most importantly, lymphoma cell lines with BMAL1 CpG island hypermethylation, such as RAJI, AKATA, and JIJOYE, did not express BMAL1 transcript and protein (Fig. 2A and B). Western blot analysis of nuclear extracts showed the nuclear localization of the BMAL1 protein in CpG island unmethylated cells, and its absence in BMAL1 hypermethylated cell lines (Supplementary Fig. S3). We established a further link between BMAL1 CpG island hypermethylation and its gene silencing by the treatment of the methylated cell lines with a DNA demethylating agent. The treatment of the BMAL1-hypermethylated RAJI, AKATA, and JIJOYE lymphoma cell lines with the demethylating drug 5-aza-2′-deoxycytidine restored the

![Figure 4](cancerres.aacrjournals.org/content/69/21/8451/F4.large.jpg)

**Figure 4.** BMAL1 as an inhibitor of tumor cell growth. A, effect of BMAL1 transfection on the *in vitro* growth of RAJI cells. Top, Western blot of BMAL1 in empty vector and BMAL1-transfected RAJI cells. The BMAL1 unmethylated cell line MOLT-4 is shown as a positive control for BMAL1 expression. Bottom, the plot shows the monitoring over time of the number of cells after transfecting the cell line RAJI with BMAL1. A decrease of cell viability, determined by the MTT assay, upon BMAL1 transfection is observed. B, colony formation assay. Left, example of the colony-focus assay after a 2-wk selection with G418. Right, quantification of the colony formation reduction upon BMAL1 transfection. C, effect of BMAL1 transfection on the growth of RAJI cells in nude mice. Shown are female athymic nude mice 45 d after injection of 10⁷ RAJI cells. Note the large tumor on the left flank, corresponding to empty vector cells, and the small mass on the opposite flank, corresponding to BMAL1-transfected cells. Tumor volume was monitored over time and tumor weight determined at the time of sacrifice. D, effect of BMAL1 depletion on cell growth in the BMAL1-unmethylated MOLT-4 leukemia cell line. Left, BMAL1 expression upon RNA interference monitored by RT-PCR (top) and Western blot (bottom). Right, effect of BMAL1 reduction on the *in vitro* growth of MOLT-4 cells at 48 h determined by the MTT assay. *, Student’s *t* test *P* = 0.0429.
expression of the BMAL1 RNA transcript and protein (Fig. 2A and B). The recent finding that BMAL1 (which its other alternative name is MOP3) is critical for the development of mature B-cells (26) reinforces our observation of a specific BMAL1 CpG island hypermethylation profile that is restricted to hematologic malignancies.

The observed epigenetic disruption of BMAL1 in lymphoma and leukemia cells was not just an in vitro cell culture phenomenon. We extended our BMAL1 CpG island methylation analysis to 126 patients with hematologic malignancies, including 71 diffuse large B-cell lymphomas, 24 ALLs, and 26 acute myelogenous leukemias (AML). We observed that BMAL1 CpG island hypermethylation was a relatively common event in diffuse large B-cell lymphomas (19.7%, 14 of 71), Burkitt lymphomas (100%, 5 of 5), ALLs (33.3%, 8 of 24), and AMLs (19.2%, 5 of 26). Illustrative examples of the primary data from hematologic malignancies obtained by methylation-specific PCR are shown in Fig. 3A. We confirmed the BMAL1 CpG island methylation status detected by methylation-specific PCR using bisulfite genomic sequencing of multiple clones in eight primary samples (two methylated AMLs, two methylated ALLs, two unmethylated AMLs, and two unmethylated ALLs; Fig. 3B). All normal peripheral lymphocytes analyzed by methylation-specific PCR (n = 100) were completely unmethylated at the BMAL1 CpG island (Supplementary Fig. S2).

From a cancer biology standpoint, we next wanted to determine whether epigenetic inactivation blocked growth suppression in those lymphomas with BMAL1-methylation–associated silencing. We adopted a double approach to addressing this matter. First, we transfected BMAL1 in a lymphoma cell line with BMAL1 hypermethylation, RAJI. Upon restoration of BMAL1 expression, as shown by Western blot (Fig. 4A), RAJI cells experienced reduced cell growth according to the MTT assay (Fig. 4B). We further showed the growth-inhibitory features of BMAL1 reintroduction in colony-focus assays (Fig. 4C). We observed that BMAL1 re-expression revealed tumor-suppressor activity, whereby there was a marked 50.2% lower colony-formation density with

![Figure 5. Loss of circadian rhythmicity of target genes upon epigenetic silencing of BMAL1. A, quantitative RT-PCR analysis of the expression of C-MYC, catalase, and p300 in synchronized RAJI and MRC5 cells. The expression of C-MYC, catalase, and p300 retained a circadian rhythm in the BMAL1-unmethylated and BMAL1-expressing MRC-5 cells, whereas a constant level of expression of the three genes without any rhythmicity was observed in the BMAL1-methylated and silenced RAJI cells. B, determination by quantitative ChIP of BMAL1 and CLOCK1 occupancy and histone H3 and H4 acetylation at the 5′-end promoter regions of the C-MYC, catalase, and p300 genes in RAJI and MRC5 cells. Time points of 0, 8, and 12 h are shown. The flow of BMAL1 and CLOCK recruitment and histone H3 and H4 acetylation recapitulates the rhythmicity of expression of the target genes in MRC5 cells, whereas minimal changes are observed in BMAL1 hypermethylated-RAJI cells. C, recruitment of BMAL1 to its target genes in BMAL1-transfected RAJI cells.](8452fig05.png)
respect to the empty vector (Fig. 4B). We next tested the ability of BMAL1-transfected RAJI lymphoma cells to form tumors in nude mice compared with empty vector–transfected cells (Fig. 4C). Cells transfected with the empty vector formed tumors rapidly, but cells infected with the BMAL1 expression vector had much lower tumorigenicity (Fig. 4C). At the time of sacrifice, tumors were 17 times larger in mice with the empty vector than in the BMAL1-transfected xenografts (Mann-Whitney U test; P = 0.0018; Fig. 4C). Next, we also knocked down BMAL1 expression by RNA interference in an expressing leukemia cell line unmethylated at the BMAL1 CpG island, MOLT-4. We observed that reduced BMAL1 expression (Fig. 4D) was associated with increased cell growth (Student’s t test P = 0.0429; Fig. 4D).

Finally, we addressed the functional impact of BMAL1 epigenetic silencing in the molecular clockwork of the cells. One of the breakthroughs in the field of circadian biology in the past 10 years has been the recognition that intrinsic oscillators are present in most peripheral tissues (1–3). It is conceivable that many millions of years ago, most cells were sensitive to light-dark cycles, and furthermore, we know that most eukaryotic cells undergo division in culture with a periodicity of 1 day. Thus, it has been proposed that the cell cycle might itself be a vestigial circadian clock (7, 11). This concept is particularly interesting because the expression of several mammalian cell cycle genes, such as C-MYC (11, 14, 25), is regulated in a circadian manner. Examples of other genes whose control features a circadian rhythm of expression include the metabolic enzyme catalase and histone acetyltransferase p300 (27, 28). We wondered whether the promoter CpG island methylation–associated silencing of BMAL1 could mediate the putative loss of the normal circadian expression of these genes in transformed cells.

To address this issue, we synchronized MRC-5 (BMAL1 unmethylated), RAJI (BMAL1 hypermethylated), and AKATA (BMAL1 hypermethylated) cells by serum shock, as described in Materials and Methods, and at the times indicated in Fig. 5A, the cell culture dishes were prepared for immediate RNA extraction or ChIP assay. We observed that the expression of C-MYC, catalase, and p300 retained a circadian rhythm in the BMAL1-unmethylated and BMAL1-expressing M5C-5 cells (Fig. 5A), whereas a constant level of expression of the three genes without any rhythmicity was observed in the BMAL1-methylated and silenced RAJI (Fig. 5A) and AKATA (Supplementary Fig. S4 and B). Most important, the ChIP experiments in RAJI cells showed that the BMAL1 protein (itself a transcription factor) and its natural partner, the CLOCK protein (with intrinsic histone acetyltransferase activity), were recruited to the 5′-regulatory regions of the C-MYC, catalase, and p300 genes coincidently with their peaks of circadian expression in MRC-5 cells (Fig. 5B). However, in sharp contrast, BMAL1 and CLOCK did not occupy the described promoters in RAJI cells (Fig. 5B). Furthermore, a histone modification pattern characterized by histone H3 and H4 hyperacetylation and compatible with active transcription and an open chromatin status (29, 30) was observed to coincide with the circadian peaks of expression and the recruitment of BMAL1 and CLOCK for the C-MYC, catalase, and p300 in M5C-5 cells (Fig. 5B). BMAL1-transfected RAJI cells recovered BMAL1 and CLOCK occupancy in the 5′-regions of these genes in association with an enrichment of the hyperacetylated histone forms (Fig. 5C and Supplementary Fig. S4C). The absence of BMAL1 from the 5′-end regions of the three genes in the untransfected RAJI cells obviously relates to the BMAL1 methylation–associated silencing, but CLOCK is unmethylated (Supplementary Fig. S5A) and expressed (Supplementary Fig. S5B) in RAJI cells. Thus, it is possible that the methylation-associated loss of BMAL1 prevents the BMAL1-mediated recruitment of CLOCK to its target promoter genes (31).

Overall, our results indicate that the BMAL1 gene, a major component of the endogenous clock of our cells, undergoes promoter CpG island methylation–mediated silencing in hematologic malignancies, where it might prevent the development of the physiologic circadian rhythm. These findings partially explain the observed autonomous circadian patterns of transformed cells, suggest a chromatin-based mechanistic explanation for the lack of rhythmic expression of clock-target genes in tumors, and might be useful for guiding the design of successful chronotherapy strategies for the treatment of disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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