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Chapter 6

Epigenetic Modulation of Circadian Rhythms: *Bmal1* Gene Regulation

Tatsunosuke Tomita and Yoshiaki Onishi

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Abstract

Circadian rhythms that function in behaviour and physiology have adaptive significance for living organisms from bacteria to humans and reflect the presence of a biological clock. The engine of circadian rhythms is a transcription-translation feedback loop that is fine-tuned by epigenetic regulation in higher eukaryotes. We elucidated the chromatin structure of the *Bmal1* gene, a critical component of the mammalian clock system, and have continued to investigate transcriptional regulation including DNA methylation. Various ailments including metabolic diseases can disrupt circadian rhythms, and many human diseases are associated with altered DNA methylation. Therefore, regulated circadian rhythms are important for human health. Here, we summarise the importance of epigenetic clock gene regulation, including DNA methylation of the *Bmal1* gene, from the viewpoint of relationships to diseases.

Keywords: molecular clock, transcriptional mechanism, cytosine methylation, chromatin, cancer, metabolic syndrome

1. Introduction

Circadian rhythms function in most living organisms and govern many behavioural and biochemical processes with 24-h periodicity regardless of changes in the cellular environment. This is closely associated with the natural rhythm of the sun, which provides light and heat with 24-h periodicity. The master clock that generates circadian rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and is governed by blue-light sensing in eyes. Peripheral organs also contain molecular clocks. These biological clocks control all aspects of physiology such as sleep-wake cycles, body temperature, hormone secretion, blood pressure and metabolism [1]. Biological clocks oscillate via a mechanism

based on interlocking transcriptional-translational feedback loops that have both positive and negative elements. The circadian oscillator orchestrates the rhythmic mRNA expression and output of hundreds or thousands of clock-controlled genes (CCG) that temporally coordinate many cellular functions [2]. Circadian transcriptional regulators are apparently involved in the initial stages of RNA polymerase II recruitment and initiation, as well as the histone modifications associated with these events to set the stage for gene expression [3]. The methylation of cytosine on CpG dinucleotides, 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. About 43% of all protein-encoding genes in mice exhibit circadian rhythms of mRNA abundance somewhere in the body, largely in an organ-specific manner [4]. The temporal coordination of cellular functions is lost when circadian rhythms are disrupted by age, the environment or genetic mutation, with deleterious effects on health. For instance, the adrenal steroid hormone glucocorticoid that controls various physiological processes, such as metabolism, the immune response, cardiovascular activity and brain function, is under the control of the circadian clock [5], implying that several diseases are closely associated with disrupted circadian rhythms.

2. Transcriptional mechanism of the circadian clock

2.1. Basic regulation of circadian transcription

The engine of the mammalian molecular clock consists of a transcription-translation feedback loop initiated by the transcription factor BMAL1-CLOCK heterodimer. BMAL1 and CLOCK have paralogs, known as BMAL2 and NPAS2, respectively. Heterodimers such as BMAL1-CLOCK bind to E-box enhancer sequences and activate the transcription of three *Per* (*Per1*, *Per2* and *Per3*) and two *Cry* (*Cry1* and *Cry2*) genes. The PER and CRY proteins subsequently repress the transcription at their own promoters through negative feedback by acting on the BMAL1-CLOCK heterodimer. The cellular circadian clock mediates the rhythmic output of the hundreds or thousands of CCG transcripts that are regulated by transcription factors or coregulators with rhythmic abundance that is a part of the cellular circadian clock [3]. The prominent transcription factors activated by BMAL1-CLOCK are REV-ERB α and β, which bind to ROREs, as well as DBP and E4BP4, which bind to D-boxes. E-box motifs contain a core CANNTG sequence, which is recognised by a basic helix-loop-helix (bHLH) domain that contains transcription factors. BMAL1-CLOCK binds tandem E boxes spaced 6 or 7 nucleotides (nt) apart with high affinity [6]. The bHLH containing the oncoprotein Myc also binds to E-boxes and directly activates the expression of multiple repressors of the clock, including *Rev-erbα* and *Rev-erbβ* [7]. In addition, USF1 binds to the E-box motifs of *Dbp*, *Per1* and *Per2* [8]. The RORE motif comprises an AT-rich sequence preceding a core (G/A) GGTCA motif. ROR and REV-ERB, respectively, activate and repress the transcription of genes by binding to ROREs [9]. They co-ordinately maintain robust circadian expression of core clock proteins, such as BMAL1. D-boxes are variants of basic leucine-zipper (bZIP) motifs and are 9- or 10-bp palindromes of two GTAA (C/T) half-site sequences [10]. The D-box motif is bound by the proline- and acidic

amino acid-rich bZIP (PAR-bZIP) transcription factor family, including DBP, E4BP4, HLF and TEF [11, 12]. A combination of three binding elements, E-boxes, RORES and D-boxes, coordinates CCG transcription. **Figure 1** shows that most core clock proteins including BMAL1, CLOCK, PER, CRY, REB-ERB, ROR and E4BP4 bind to many thousands of sites in the genome in a circadian manner [13].

2.2. Epigenetic mechanism: effect of chromatin structure

Transcriptional regulation initially requires the coordinated control of chromatin and the genome structure [3]. In general, genetic information is packed into the chromatin structure, of which the nucleosome is the most basic unit; 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 remodelling and loosening of the nucleosomal barrier including histone tail modifications are key steps in circadian modifications followed by sequence-specific, transcription factor binding that regulates gene expressions [14]. Distinct chromatin states are determined by unique histone post-translational modifications. First, histone acetylation levels fluctuate rhythmically at clock gene promoters and enhancers. Specifically, acetylated histone H3 at Lys27 (H3K27ac), a marker of active enhancers, and H3 at Lys9 (H3K9ac) are rhythmic and positively correlate with clock gene expression. For example, rhythmic BMAL1-CLOCK binding and H3K9ac are required as well as rhythmic histone H3 abundance at the start site for *Dbp* transcription [15]. Complexes of clock proteins such as PER contain various interactive partners with known catalytic activity towards chromatin [16, 17]. The acetylation of histone H3 (at Lys9 and Lys14) at *Per1*, *Per2* and *Cry1* and of H4 at *Per1* during the transcriptional activation phase has been identified [18, 19]. Rhythmic histone acetylation at clock loci is largely mediated by p300 and CBP histone

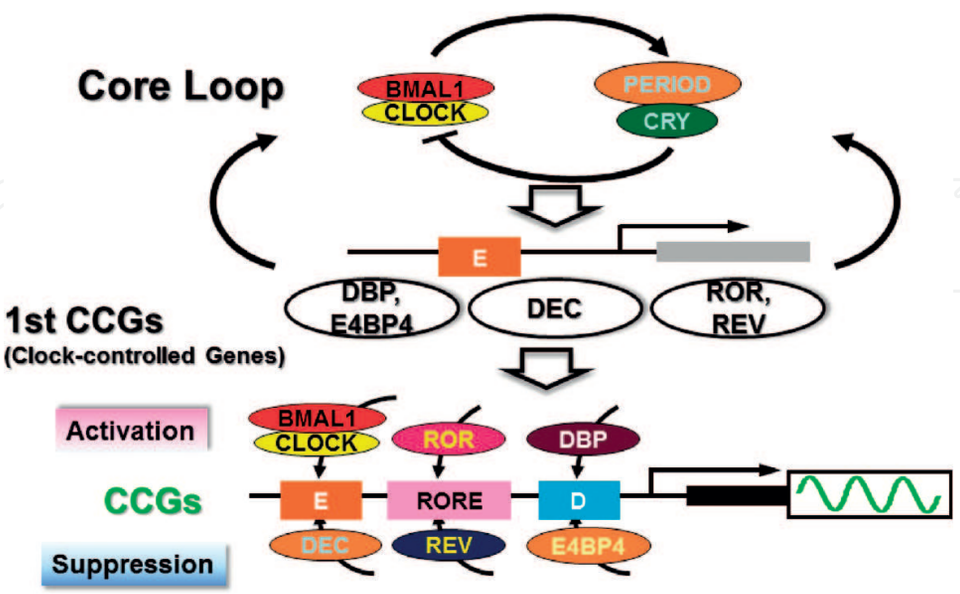

Figure 1. Hierarchical regulation mechanism of circadian transcription. E, RORE and D indicate transcription factor recognition sites: E-box, RORE and D-box, respectively.

acetyltransferases (HAT) [15, 19], and CLOCK itself might also have intrinsic HAT activity [20]. Levels of histone acetylation are also regulated by histone deacetylases (HDAC) as well as by HAT. Several HDAC are important in the control of circadian histone acetylation. For example, REV-ERBα represses transcription in part by recruiting the co-repressor complexes NCoR and/or SMRT to ROREs [21]. One major mechanism of transcriptional repression mediated by CRY and PER is the direct recruitment of the Sin3 complex, which contains HDAC1 and HDAC2 [17]. Another co-repressor complex containing HDAC1 and HDAC2 subunits, NuRD, binds PER-CRY and deacetylates nearby histones, thereby represses clock genes [22]. Sirtuins are another class of HDAC involved in the core clock mechanism that associate with the BMAL-CLOCK heterodimer, and levels of their common cofactor, nicotinamide adenine dinucleotide, are under tight circadian control in many physiological systems [23, 24]. In addition to being acetylated, lysine side chains can be methylated by methyltransferases, and their deacetylation often precedes and facilitates an acetylation-methylation switch. Histone H3 at Lys9 methylation (H3K9me) promotes heterochromatin formation and transcriptional repression. Rhythmic H3K9me near circadian E boxes is mediated by SUV39 methyltransferase and is antiphase to H3K9ac rhythms in the mouse liver [15]. The di- and trimethylation of H3 at Lys27 also proceed at *Per1* and *Per2* during the repressive phase [25]. The circadian clock regulates global transcriptional integrity and chromatin status by regulating RNA polymerase II, because circadian transcription is clustered in phase and accompanied by circadian control of RNA polymerase II recruitment and initiation [26]. The above individual mechanism is governed by the three-dimensional (3D) architecture of chromatin and its critical contributions to long-distance cis-acting mechanisms of gene regulation [27]. Regulatory elements such as enhancers, silencers and insulators built up functional 3D architectures in the nucleus and manage the transcription factory with specific properties [28]. Several looping factors, such as components of the Mediator complex, interact with clock transcription factors [29]. Deletion of one of the factors important for looping, Smc3, causes major disruptions to the clock [30]. Recently, the detailed 3D multi-loop aggregate/rosette chromatin architecture and functional dynamics have been revealed [31, 32], and this may explain how physiological functions are regulated with a tissue-specific rhythm in spite of the same core clock system. These results suggest that epigenetic regulation caused by the chromatin structure is important for circadian transcription, and further researches from the viewpoint of 3D chromatin structure are required to elucidate the physiological function with circadian rhythm in the tissue.

2.3. DNA methylation

The most common epigenetic modification is DNA methylation, which is a covalent chemical alteration that plays a crucial role in numerous biological processes. It occurs in mammals predominantly on cytosine residues in cytosine-guanine (CpG) dinucleotides, and tissue-specific genomic DNA methylation patterns play a fundamental role in establishing cell identity during differentiation. Generally, although about 70% of all CpG sequences in mouse and human genomes are methylated, CpG islands in promoter sequences are methylated at a relatively lower level [33]. Overall, DNA methylation exhibits no major rhythmic changes and the cellular function of DNA methylation depends on which gene is methylated. One of the most important issues regarding DNA methylation is how the machinery is directed towards

and maintains specific genomic sequences. One mechanism might be the PML-RAT fusion protein in leukaemia, which induces DNA hypermethylation and gene silencing at specific target promoters [34]. Another is siRNA-mediated, RNA-directed DNA methylation, which is a stepwise process initiated by dsRNA that recruits DNMT to catalyse the *de novo* DNA methylation of specific regions [35]. Therefore, the susceptibility of individual CpG islands to *de novo* methylation might intrinsically differ, but the mechanism remains obscure. In any event, CpG methylation is strictly regulated and stable, and changes in methylation profiles are associated with diseases, indicating close relationships among DNA methylation sites, the mechanism of methylation and biological functions.

3. Transcriptional regulation of the *Bmal1* gene

Bmal1 was originally characterised due to its high expression levels in brain and muscle cells [36]. The activity of *Bmal1*$^{-/-}$ mice immediately becomes arrhythmic in constant darkness; therefore, BMAL1 is apparently an essential and non-redundant component of the mammalian clock [37]. Among the core clock genes, *BmalL1* expression oscillates in the SCN and in peripheral clock cells, in close association with circadian rhythms [38]. We evaluated the chromatin structure of the *Bmal1* gene and discovered a unique structure within the *Bmal1* promoter. The *Bmal1* promoter region comprises mainly a general nucleosome structure upstream of a 5′ SacI site, an open chromatin structure around RORE and a nuclear matrix-like structure at a 3′-flanking region (**Figure 2**). Oscillatory transcription of the *Bmal1* gene requires the chromatin structure to undergo rhythmic alterations *in vivo* at the region around the ROREs and at the 3′-flanking region in response to SAF-A binding, indicating cooperative alteration of the chromatin structure between the 3′-flanking region and the ROREs [39]. The methylation of DNA on CpG islands results in transcriptional repression either by interfering with transcription factor binding or by including a repressive chromatin structure [40]. The methylation of CpG adjacent to the core Sp1 motif decreases Sp1/Sp3 binding [41], which might be associated with the repression of *Bmal1* transcription by DNA methylation, because many putative Sp1-binding motifs are located around the *Bmal1* promoter. The level of DNA methylation within a ±1 kb region surrounding the transcription start site closely correlates with gene repression, and the promoter of clock genes including *Bmal1* is usually unmethylated [39]. However, the hypermethylation of CpG islands in the promoter of *Bmal1* transcriptionally silences its expression in haematological

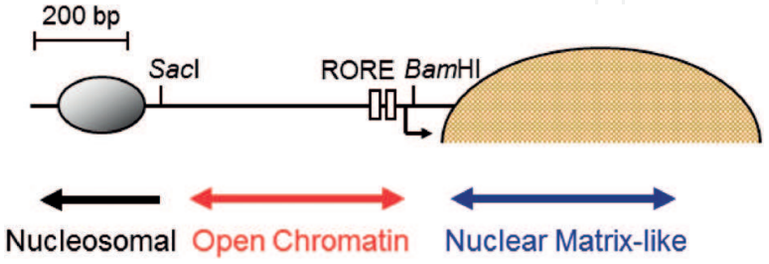

Figure 2. Chromatin structure of *Bmal1* promoter. Oval, unfilled boxes and arrow near *BamHI* indicate nucleosome, RORE and transcription start site, respectively. RORE: recognition motifs for retinoic acid receptor-related orphan receptor (ROR) and reverse Erb (REV-ERB) orphan nuclear receptors.

malignancies [42, 43]. Relationships between the DNA methylation of clock genes and diseases have been identified. The ROREs, which are critical elements for *Bmal1* oscillatory transcription [44], are embedded in a unique GC-rich open chromatin structure. We also found that DNA demethylation of the *Bmal1* promoter enhances *Bmal1*, and then *Per2* and *Cry1* transcription that function in the circadian oscillation of *Bmal1* transcription recover, suggesting that the circadian rhythm is restored [42, 43]. Furthermore, DNA methylation might contribute to the developmental expression of clock genes [45]. These lines of evidence suggest that the DNA methylation of clock genes, in particular, *Bmal1*, plays a key role in the disruption of circadian rhythms that are closely associated with various diseases.

We recently found that recovery from DNA methylation by 5-aza-2′-deoxycytidine (aza-dC) differs between the *Bmal1* and *Rpib9* genes, suggesting that the release of methylation depends on the locus/gene or sequence and that methylation status is specific to the DNA site [46]. Taken together, these findings imply that methylation is specific to gene function and that an early response to the aza-dC demethylation of sites in *Bmal1* might be functionally important for adaptation to environmental change.

4. Disease

Appropriate circadian gene expression is necessary for the normal cell development. That is, distorted clock gene expression leads to various diseases. This chapter focuses on cancers and some other diseases.

4.1. Cancer

Close relationships between clock gene expression and the initiation and progression of cancer are obvious from the findings of many studies. Clock gene expression is altered in many types of malignancies including breast, lung, haematopoietic, pancreatic and skin cancers. Clock genes are apt to be downregulated in many cancer types, as shown in **Table 1**. These phenomena imply that clock genes have some anti-tumour effects. The physiological disruption of circadian

Gene	Expression	Mechanism	Cancer type	DNA methylation	References
Per1	Downregulated	Apoptosis	Colon, lung, breast	—	[38]
Per2	Downregulated	Apoptosis	Lung, lymphocyte	—	[36, 37]
	Downregulated	MYC-downregulation	Lung, breast	—	[34, 36, 37]
	Downregulated	p53-upregulation	Lung, breast	—	[36, 37]
Cry2	Downregulated	Unknown	Breast	Hypermethylation	[39, 40]
Bmal1	Downregulated	p53 pathway	Pancreas	—	[35]
	Downregulated	p300, CAT activation	Leukaemia	Hypermethylation	[29, 33, 34]

Table 1. Clock genes and their possible functions for cancer suppression.

rhythms and the genetic loss of *Per2* or *Bmal1* promote tumorigenesis in lung cancer [47], and such disruptions are associated with upregulated *c-Myc* levels. The expression of *Bmal1* is suppressed in pancreatic cancer, and this gene activates the p53 tumour suppressor pathway, playing an important role in cancer suppression [48]. Fu et al. found that PER2 is an important factor for tumour suppression and the DNA damage response [49]. The overexpression of *Per1* or *Per2* can lead to the apoptosis of cancer cells [50, 51]. Mao et al. reported that *Cry2* expression is decreased in breast cancer, resulting in an altered methylation pattern in CpG islands [52]. The findings of another study support this observation, and CRY2 suppression is closely associated with risks for breast cancer [53]. From a mechanistic viewpoint, one of the main factors in such disrupted circadian gene expression might be MYC. According to a report by Altman et al., this gene directly activates REV-ERB, which suppresses *Bmal1*, and their constitutive expression suspends clock mechanisms [7]. These findings suggest that the appropriate expression of clock genes is necessary to maintain normal tissues. On the other hand, leukaemia stem cells in acute myeloid leukaemia (AML) have intact circadian expression. Furthermore, knockdown studies have shown that *Bmal1* and *Clock* are required for AML cell growth and that disrupted circadian rhythm machinery is an anti-leukaemic factor that leads to leukaemia stem cell differentiation [54]. In addition, upregulated *Clock* plays critical roles in the proliferation of colorectal carcinoma cells and the inhibition of apoptosis [55].

The roles of clock genes seem to differ among stages or tissues in patients with cancer. In addition to classical genetic mutations, the epigenetic landscapes of cancer cells are rather contorted. From an epigenetic perspective, clock genes functionally associate with histone modifying genes that are responsible for cancer progression and maintenance. Mixed lineage leukaemia (MLL) genes were originally discovered through detailed analyses of leukaemogenic rearrangement but they are now thought to be responsible for histone H3K4 methyltransferase activity and promoters of target gene transcription. Mutations of MLL genes literally trigger mixed lineage leukaemia and are necessary to maintain malignancy through aberrant epigenetic gene regulation [56]. The relationship between MLL genes and circadian rhythm maintenance through histone modification has been studied in detail. According to Katada et al., MLL1 has CLOCK-associated histone modifying activity, and it is necessary to generate circadian rhythms in fibroblasts [57]. Kim et al. found that MLL3 and 4 are factors that regulate circadian rhythmic homeostasis in the liver [58]. In addition, MLL3 contributes to circadian rhythm generation in mouse embryonic fibroblasts (MEFs) [59]. The histone modifying enzyme EZH2 is another histone-lysine N methyl transferase that is responsible for histone H3K27 methyl transfer. This modification results in transcription repression. *Ezh2* also promotes tumorigenesis by altering the expression of numerous tumour suppressor genes [60]. EZH2 interacts with CLOCK-BMAL1 complexes and is necessary for circadian rhythm maintenance [25]. Although CLOCK *per se* is not considered to be an oncogene, it might affect cancer cell proliferation if it is atypically expressed [55].

Considering the altered methylation patterns of the promoter regions of clock genes, the features of epigenetic abnormalities of cancer cells comprise highly methylated CpG islands of specific genes accompanied by low methylation status of other genes [61]. Some studies have indicated that this phenomenon is true for clock genes. The *Cry2* promoter tends to be highly methylated in patients with breast cancer, resulting in lower *Cry2* expression compared

with controls [43]. Taniguchi et al. reported that CpG islands of the *Bmal1* promoter are hypermethylated in diffuse large B-cell lymphoma and in acute lymphocytic and myeloid leukaemia [42]. We also reported this phenomenon and that the methylation pattern of the *Per2* promoter region does not change in RPMI8402 cells [46]. The aberrant methylation pattern of the *Bmal1* promoter was restored, and the intrinsic rhythm was revived after 1 day of aza-dC treatment. These findings indicate that active mechanisms in leukaemia cells maintain the promoters of hypermethylated *Bmal1* gene status.

As noted above, many studies have emphasised close relationships between epigenetic modification and circadian clock genes in cancer proliferation and progression. However, the precise mechanisms seem highly complex and remain obscure. Further investigation is required to elucidate these mechanisms.

4.2. Other diseases

Circadian rhythms are also associated with diseases other than cancer through effects on the cardiovascular, renal, immune, endocrine, neuropsychiatric and metabolic systems [5, 62–67]. Many physiological processes cannot be harmonised when the intrinsic rhythm is aberrant and such dyssynchrony leads to many diseases.

Here, we consider neuropsychiatric disorders. Disrupted sleep-wake cycles, depression, Alzheimer's disease and mood disorders among neuropsychiatric disorders are notably linked to altered circadian rhythms. However, circadian epigenomics have received less consideration in studies of neuropsychiatric disorders compared with cancers.

Alzheimer's disease is an age-dependent neurodegenerative disorder that is associated with severe cognitive impairment, and its incidence is increasing, particularly in developed countries due to extended life spans. The typical clinical symptoms are disordered circadian rhythms and abnormal sleep patterns. Amyloid beta is a key molecule in this neurodegeneration [68], and it reportedly degrades BMAL1 protein [69]. The lack of this powerful rhythm generator disrupts circadian rhythms in many patients. Furthermore, the methylation rhythm of the *Bmal1* promoter changes in the neocortex of patients with this disease. These phenomena imply that the aberrant methylation of the *Bmal1* promoter and rapid BMAL1 degradation together affect behavioural changes or cognitive impairments. Furthermore, a methylome study of the neocortex of brains at autopsy revealed attenuated methylation rhythms in samples from patients with Alzheimer disease compared with controls [70]. The neocortex is very rare in terms of tissues with circadian methylation rhythms.

According to many studies, contorted clock gene expression patterns and mood disorders are closely associated in experimental animal models. Genetic experiments have found that CLOCK is a key factor in maniac states because *Clock* mutant mice (*Clock*Δ19) develop clear features [71–73] of mania, circadian rhythm disruption, hyperactivity and decreased sleep. The physiological features of these mutant mice include altered gene expression patterns and excited neurons due to upregulated dopamine content in the ventral tegmental area (VTA) [74]. Notably, knockdown of CLOCK in the VTA using RNA interference results in concomitant mania-like (hyperactivity and decreased anxiety) and depression-like behaviours in mice. Since patients with mania often experience depressive episodes, this knockdown mouse is a

more appropriate model of mania in humans. However, precisely how these CLOCK disruptions affect the upregulated dopamine content in the VTA remains obscure. The expression of monoamine oxidase A (MAOA), which inactivates monoamine neurotransmitters including dopamine, serotonin and norepinephrine, is regulated by circadian clock genes including *Bmal1*, *Npas2* and *Per2* [75]. However, in this mechanism, CLOCK, unlike NPAS2, does not work as a transcriptional activator. Therefore, the absence of CLOCK directly results in downregulated MAOA activity, and consequent dopamine upregulation cannot be concluded. Some other CLOCK functions including histone modification activity or an indirect action of CLOCK might be involved in dopamine upregulation, and investigations into this are underway.

Patients with depression frequently have insomnia and abnormal circadian rhythms that could reasonably relate to altered clock gene expression. Circadian clock gene expression has been compared between post-mortem brain samples from patients with major depressive disorder (MDD) and age-matched controls [76]. The findings showed abnormal clock gene phasing and decreased *Bmal1* and *Per2* oscillation in most brain regions of the patients. These findings provided direct evidence that clock gene expression is altered in the central nervous system of patients with MDD. On the other hand, depression states and anti-depressant effects are often tested in experimental animal models such as laboratory mice that are suspended by the tail or forced to swim to mimic short duration stress or exposed to social defeat to mimic chronic stress [77]. The volume of the hippocampus is reduced in both patients and in a model that develops depressive pathophysiology after exposure to chronic stress, and this volume is restored by administering anti-depressant medicine. Brain-derived neurotropic factor (BDNF) plays a very important role in the hippocampus as an anti-depressant and for adaptation to stress. Anti-depressants enhance BDNF expression in the mouse brain [78]; BDNF infused into the hippocampus has anti-depressant effects in behavioural mouse models of depression [78, 79] and the action of the anti-depressant desipramine is attenuated mice with a BDNF deletion in the forebrain [78, 80]. Expression of *Bdnf* gene is rhythmic in rat brain regions including hippocampus. However, its downregulation in an animal model of depression was due to the methylation status of the promoter region of the *Bdnf* gene [81]. Tsankova et al. found that the expression of two BDNF variants, Bdnf III and IV, is downregulated and that the promoter regions of corresponding variants are hypermethylated in laboratory mice exposed to defeat stress [82]. Furthermore, chronic imipramine administration increased histone acetylation on the corresponding promoters, and this downregulation was reversed. The findings of the above studies indicate that *Bdnf* gene expression is rhythmically maintained under normal conditions but is epigenetically regulated under stress. However, precisely how *Bdnf* expression is rhythmically maintained remains unclear and awaits further investigation.

5. Assays of *Bmal1* transcription modulators

The circadian clock controls the daily oscillations of gene expression and physiological function at the cellular level, indicating that the control of circadian rhythms at the cellular level is important for human health. After we elucidated the transcriptional mechanism of the non-redundant essential unique clock gene, *Bmal1*, we developed a circadian functional assay system that consists of luminescent reporter cells and the application of *Bmal1* findings. We found that

the minimal essential region of the *Bmal1* promoter for circadian transcription is embedded in an open chromatin structure, suggesting that this region can remain functional even when inserted into a reporter plasmid [39]. We then established stable reporter cell lines with which to analyse circadian clock function [83] and the effects of DNA methylation on circadian clock function [43]. **Figure 3** shows the application of these systems to further dissection of the molecular mechanisms underlying the mammalian circadian clock [46, 84, 85].

One of the most important findings was that altering the DNA configuration of the *Bmal1* promoter causes the epigenetic regulation of *Bmal1* circadian transcription [86]. Topoisomerase I (TOP1) is located at an intermediate region between two ROREs that are critical *cis*-elements of circadian transcription, which is required for transcriptional suppression in cooperation with the distal RORE. The DNA fragment between the ROREs, where the TOP1-binding site is located, behaved like a right-handed superhelical twist, and the modulation of TOP1 activity by the TOP1 inhibitor, camptothecin and *Top1* siRNA altered the footprint, indicating the modulation of the chromatin structure. These findings indicated that TOP1 modulates the chromatin structure of the *Bmal1* promoter, regulates the *Bmal1* transcription and influences the circadian period.

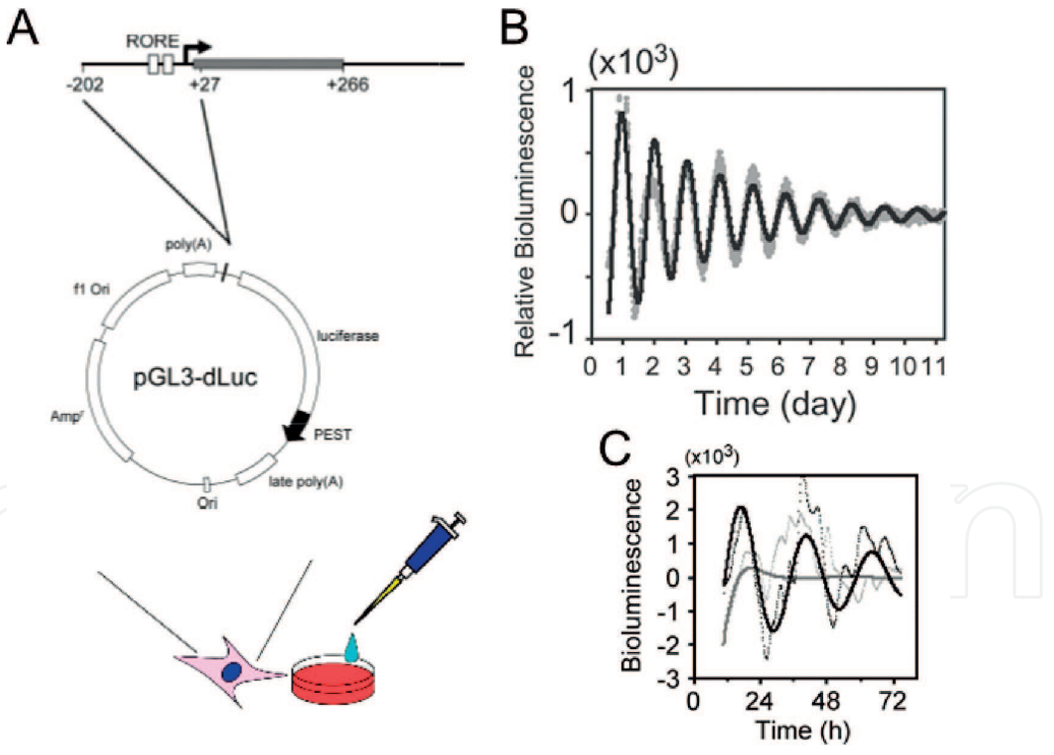

Figure 3. Monitoring cellular circadian clock system using stable reporter cell line. (A) Monitoring method. Promoter region (−202 to +27) of Bmal1 was inserted into pGL3-dluc and used to create cell lines with stable, real-time reporter gene to evaluate cellular circadian clock system. (B) Circadian oscillation monitored using host NIH3T3 cells. (C) Circadian oscillation monitored using host CPT-K cells with hypermethylated Bmal1 promoter region. 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, grey; aza-dC, black). Dots, raw values; lines, fit curve data.

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Another important finding was the epigenetic inactivation or DNA methylation of the *Bmal1* promoter. The methylation status of the *Bmal1* promoter is critical for the circadian system. Because the *Bmal1* gene is inactivated by the DNA hypermethylation of its promoter, the circadian oscillation of *Bmal1* transcription was absent in the haematological malignant cells. The demethylating agent aza-dC restored circadian oscillation, whereas continuous *Bmal1* expression did not. Because BMAL1 protein has distinct tissue-specific regulation and functions [87], tissue-specific regulation of BMAL1 expression might be required, and this can be introduced endogenously by aza-dC to establish the negative feedback loop system and restore circadian oscillation. 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 [40]. The methylation of DNA contributes to the expression of clock genes [45] in addition to *Bmal1*, a key player in the disruption of circadian rhythms.

6. Conclusion

Circadian rhythms control all aspects of physiology. When they are disrupted by changes in clock gene expression, various critical intracellular physiological processes become dysregulated, and this can lead to diseases that are induced partly by epigenetic effects including DNA methyation. The pathologies that are closely associated with disrupted circadian rhythms include cancer [88], dementia [89], Parkinson's disease [90] and obesity [91]. Among the clock genes, *Bmal1* is unique because the loss of BMAL1 protein in mice results in immediate and complete loss of circadian rhythmicity [33], indicating the importance of a specific amount of BMAL1 expression for circadian rhythms. In addition, DNA methylation of the *Bmal1* promoter disrupts the circadian system even when the *Per* and *Cry* gene promoters are unmethylated, indicating that the *Bmal1* gene is functionally important [43]. Epigenetic regulation, especially DNA methylation status, is specific to DNA sites and gene functions. Therefore, the finding that the epigenetic transcriptional regulation of *Bmal1* is functionally important for adaptation to environmental changes provides novel insights into clock gene functions that should affect the clinical diagnosis and treatment of diseases. Therefore, modulators of *Bmal1* transcription are needed for the human health.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author details

Tatsunosuke Tomita and Yoshiaki Onishi*

*Address all correspondence to: y-onishi@aist.go.jp

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), DBT-AIST International Laboratory for Advanced Biomedicine (DAILAB), Tsukuba, Japan

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