The Clock Proteins, Aging, and Tumorigenesis

R.V. KONDRATOV* AND M.P. ANTOCH†

*BGES Department, Cleveland State University, Cleveland, Ohio 44115; †Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263

Many aspects of mammalian physiology and behavior are driven by an intrinsic timekeeping system that has an important role in synchronizing various biological processes within an organism and coordinating them with the environment. It is believed that deregulation of this coordination may cause the development of various pathologies. However, recent studies using mice deficient in individual components of the circadian system clearly demonstrated more complex interaction of the circadian system with various biological processes. The growing amount of evidence suggests that in addition to their roles in the core clock mechanism, some of the components of the molecular oscillator are involved in modulation of such diverse physiological processes as response to genotoxic stress, regulation of the cell cycle, aging, and carcinogenesis. These new data provide a mechanistic link between deregulation of the circadian system and/or some of its core components and the development of various pathologies, suggesting novel strategies for the disease treatment and prevention.

INTRODUCTION

Circadian Rhythms and Human Disease

The circadian clock is a universal intrinsic timekeeping system, which regulates many vital physiological and biochemical processes. In mammals, it is organized as a hierarchical network of molecular clocks that are operative in all tissues, with the master clock residing in the hypothalamic suprachiasmatic nucleus (SCN). The master clock is synchronized with the environment by daily changes in the light/dark cycles and transmits information regarding its phase to multiple tissue-specific clocks (Schibler and Sassone-Corsi 2002). Precise coordination of numerous tissue-specific clocks with each other and with the environment, as well as their proper functioning at the cellular level, is recognized now as important factors of an organism's well being. In humans, the malfunctioning of the circadian clock apparatus has been linked to various pathological syndromes, including sleep and mood disorders, jet lag, tumorigenesis (for review, see Kondratov et al. 2007; Sahar and Sassone-Corsi 2007), and, more recently, to hypertension and type-2 diabetes (Woon et al. 2007). Detailed phenotypic analysis of mice with targeted disruption or mutations in core clock genes further advanced our current view on the role of circadian proteins in pathophysiology. Thus, the growing amount of evidence suggests that core clock proteins may have distinct noncircadian functions important for maintaining tissue homeostasis under normal and stress conditions, and their malfunction may contribute to disease development (Ko and Takahashi 2006; Kondratov et al. 2007). Here, we review recent data from our and other laboratories that address complex interaction of mammalian circadian system with such fundamental biological processes as aging and tumorigenesis.

CLOCK/BMAL1 Transcriptional Complex as the Major Regulator of Circadian Output and a Modulator of the Response to Genotoxic Stress

The current view of the molecular circadian machinery underlying daily oscillations in physiology, metabolism,

and behavior describes the CLOCK/BMAL1 transcriptional complex as the major regulator of both core clock function and circadian output. CLOCK and BMAL1 are two basic helix-loop-helix (bHLH)-PAS domain transcription factors that in a form of heterodimer activate expression of target genes through the E-box enhancer elements in their promoter regions. Among the genes induced by CLOCK/BMAL1 are those encoding their own repressors, PERIODS (PERs) and CRYPTOCHROMES (CRYs), the regulators of *Bmal1* transcription (REV-ERB? and ROR?) as well as multiple clock-controlled genes (CCGs). The expression of CCGs is achieved either directly or indirectly through the activity of the E box containing noncircadian transcription factors (Gachon et al. 2006).

Among other vital physiological processes, the circadian system has been implicated in modulation of an organism's response to various genotoxic treatments. The initial observations that an organism's response to chemotherapeutic drugs and radiation varies depending on time of administration justified the use of chronotherapy in cancer patients (Levi 2001). Several successful clinical trials have conclusively demonstrated that the therapeutic index of any given treatment (the ratio of tumor response to treatment to the amount of damage caused to normal tissues) could be significantly improved by proper timing (Hrushesky 1985; Giacchetti 2002; Kobayashi et al. 2002). Recent studies performed on mice with targeted disruption of individual clock genes provide the mechanistic background for these initial observations.

The key role of major circadian proteins in genotoxic stress response was demonstrated by testing the sensitivity of wild-type, *Clock* mutant, *Bmal1*^{-/-} knockout, and *Cry1*^{-/-}*Cry2*^{-/-} double-knockout animals to toxicity induced by the chemotherapeutic drug cyclophosphamide. Wild-type mice display a robust daily rhythm in sensitivity to the drug. Importantly, morbidity and mortality associated with treatment were at their highest levels when cyclophosphamide was administered at the times of day corresponding to minimal functional activity of the CLOCK/BMAL1 complex and the lowest at the peak times of its activity. Consistently, animals with

mutations or targeted disruption of the *Clock* or *Bmal1* genes that are characterized with constant low levels of CLOCK/BMAL1 transcriptional activity (*Clock/Clock* and *Bmal1^{-/-}*) showed high levels of drug sensitivity at all times tested. Moreover, animals with constant high levels of CLOCK/BMAL1 functional activity due to the lack of circadian repressors (*Cryptochrome* double-knockout animals) were extremely resistant to the treatment (Gorbacheva et al. 2005). These data suggest that drug sensitivity is affected by the functional status of major circadian *trans*-activation complex, which translates into different gene expression patterns of its direct and indirect targets. Indeed, as shown recently, many drug metabolizing and detoxification enzymes as well as a number of key regulators of cell cycle progression and genotoxic stress responses display circadian patterns of expression at either the mRNA or protein level (Fu et al. 2002; Panda et al. 2002; Storch et al. 2002; Gachon et al. 2006). Moreover, some of them, such as the *Wee1* kinase and *c-Myc* transcription factor, have been identified as direct transcriptional targets of the circadian CLOCK/BMAL1 *trans*-activation complex (Fu et al. 2002; Matsuo et al. 2003). As expected, the expression pattern of many of these genes is altered in tissues of circadian mutant mice or in cells with modulated expression of circadian proteins (Gauger and Sancar 2005; Miller et al. 2007). It has been proposed that the abnormal response to genotoxic stress due to the changes in the abundance and/or activity of the key regulators of cell cycle and apoptosis underlies a cancer-prone phenotype reported for mice with a mutation in the *Per2* gene (Fu et al. 2002).

The differential sensitivity of various circadian mutant mice to drug-induced toxicity provided the first example for contrasting physiological phenotypes displayed by behaviorally arrhythmic animals and suggested a more complex interconnection between the circadian system and physiology. Detailed analysis of animals and cells deficient in individual core clock components further confirmed the complexity and the multilevel character of circadian modulation of stress response.

It was thus shown that a deficiency in BMAL1 (positive component of the circadian transcriptional feedback loop) sometimes results in the up-regulation of CLOCK/BMAL1 transcriptional targets, suggesting that CLOCK/BMAL1 may also work as a transcriptional repressor (Kondratov et al. 2006b). Importantly, while serving as a repressor, CLOCK/BMAL1 can interfere with the ability of other transcription factors to activate their targets, thus attenuating a DNA-damage response. Hence, the circadian clock may act as a master switch that under normal conditions permits or restricts activation of various signaling pathways. Consistently, the deficiency of individual clock components will result in deregulation of inducibility of stress response pathways, which may cause the development of various pathologies, including premature aging and tumorigenesis (see below).

In addition to transcription-based regulation, core circadian proteins PERIOD1 and TIMELESS interact with components of the cell cycle checkpoint system such as ATM/Chk2 and ATR/Chk1, respectively, and are necessary for activation of Chk1 and Chk2 by DNA damage (for

review, see Kondratov and Antoch 2007). Importantly, radiation-induced ATM activation followed by ATM-dependent phosphorylation of CHK2 is impaired in cells with specific suppression of PER1 with small interfering RNA (siRNA). These data suggest that PER1 may act either as a cofactor for the activation of ATM or as an adapter protein for recruiting ATM substrates. In addition, modulation of PER1 expression alters both the steady-state expression levels and IR-dependent induction of several cell cycle checkpoint-related genes (Gery et al. 2006).

Analysis of the expression pattern of clock genes in tumor cells also revealed significant differences. Thus, expression of *Per1* is down-regulated in colon cancer (Krugluger et al. 2007), breast cancers (Chen et al. 2005) and endometrial carcinoma (Yeh et al. 2005) partly due to gene inactivation by promoter methylation. It has been suggested that down-regulation of *Per1* may benefit the survival of cancer cells, thus promoting carcinogenesis (Chen et al. 2005). Consistent with this, expression of exogenous PER1 dramatically reduces the growth of several tested cancer cell lines, presumably by increasing the rate of apoptosis and inducing cell cycle arrest (Gery et al. 2006). Finally, as was shown recently, intratumoral delivery of another *Period* gene, *Per2*, inhibits tumorigenesis in C57BL/6 mice (Hua et al. 2007).

Taken together, these data establish a strong molecular link between the clock proteins, on the one hand, and response to DNA-damaging agents, cell cycle regulation, and carcinogenesis, on the other hand.

Premature Aging in BMAL1-deficient Mice

Detailed analysis of mice deficient in various components of the molecular clock revealed a variety of pathological changes, which sometimes are specific for each mutation, and the list of these pathologies is constantly growing. One of the most striking phenotypes has been described in BMAL1-deficient animals, which display various metabolic defects (Rudic et al. 2004), severe joint ankylosis (Bunger et al. 2005), and premature aging (Kondratov et al. 2006a). BMAL1 (brain and muscle ARNT-like protein 1, also known as MOP3, ARNT3, or ARNTL1) belongs to the family of bHLH-PAS domain transcription factors. In complex with its partner, CLOCK, it regulates expression of target genes. Targeted disruption of BMAL1 results in an immediate loss of behavioral rhythmicity, suggesting that BMAL1 is a core component of the mammalian circadian clock (Bunger et al. 2000).

In addition to its well-established role in circadian mechanisms, a growing amount of recent data suggests that BMAL1 is also directly involved in regulation of a variety of physiological processes. This evidence came from the detailed phenotypic characterization of *Bmal1^{-/-}* mice performed in several laboratories, implying BMAL1 in the regulation of adipogenesis (Shimba et al. 2005), glucose and fat metabolism (Rudic et al. 2004), blood pressure, and heart rate (Curtis et al. 2007). In addition, both male and female mice are infertile (Kennaway 2005) and develop idiopathic calcification and ossification of joints later in age (Bunger et al. 2005). The molecular mechanisms leading to the development of these patholo-

gies are still unclear; however, it is believed that observed deregulation in the endocrine system and increased sensitivity to DNA-damaging agents may contribute to overall pathogenesis (Gorbacheva et al. 2005).

Recently, we have demonstrated that *Bmal1*^{-/-} mice have several phenotypic changes, which may be summarized as segmental progeria (Kondratov et al. 2006a). Being born with an expected ratio and indistinguishable from their wild-type littermates, at the age of 16–18 weeks, BMAL1-deficient animals start showing signs of growth retardation. As a result, at the age of 35–40 weeks, the knockout animals morphologically resemble aged mice and die when they are 37.0 ± 12.1 weeks old (Kondratov et al. 2006a).

The progeria-like phenotype in *Bmal1*^{-/-} mice is accompanied with many known features of normal and premature aging in animals. The most prominent among them are sarcopenia (age-related reduction of a muscle mass due to a decrease in the number of muscle fibers and their diameter), osteoporosis (reduction of a bone mass), kidney and spleen shrinkage, significant reduction of visceral and subcutaneous fat, and changes in blood cell composition (increased amount of neutrophils and monocytes and decreased amount of lymphocytes). In addition, *Bmal1*^{-/-} mice develop various forms of cataracts and cornea inflammation and lose elasticity of skin and hair growth ability early in age (Kondratov et al. 2006a).

The molecular mechanisms underlying the early aging phenotype of *Bmal1*^{-/-} mice are still unclear. Most likely, it results from the superposition of deficiencies in multiple pathways that are normally controlled by BMAL1, including the regulation of glucose and fat metabolism and homeostasis (Curtis et al. 2004; Shimba et al. 2005) and the modulation of the genotoxic stress response (Gorbacheva et al. 2005), as well as direct involvement of BMAL1 in the regulation of reactive oxygen and reactive nitrogen species (ROS/RNS) (Kondratov et al. 2006a). Age-related accumulation of oxidative damage in an organism has been long recognized as one of the major causes of aging and age-associated degenerative diseases (Balaban et al. 2005). Consistent with this view, the level of free radicals in several tissues of BMAL1-deficient animals appeared to be significantly higher than in the tissues of the age-matched wild-type littermates. Importantly, such age-dependent accumulation of ROS/RNS occurs in those tissues that demonstrate severe reduction in size (i.e., spleen and kidney). Moreover, suppression of BMAL1 levels by siRNA in a cell culture results in an increase of ROS concentration, suggesting a direct involvement of this core circadian protein in ROS homeostasis (Kondratov et al. 2006a).

Noteworthy, BMAL1 is highly conserved between species. Thus, the sequences of the zebra fish and human homologs of the protein are 84% identical, whereas mouse and human proteins are 99% identical. Consistent with this, BMAL1 has a similar functional role in molecular circadian oscillator in human, mouse, and fly (Bell-Pedersen et al. 2005). A recently reported 15% reduction in life span in flies with a mutation in the CYCLE protein (*Drosophila* homolog of the mammalian BMAL1) (Hendricks et al. 2003) suggests that the role of BMAL1 in aging is conserved through evolution as well.

Low Doses of Radiation Provoke Premature Aging in *Clock* Mutant Mice

Although animals with a mutation in the BMAL1 transcriptional partner CLOCK (*Clock* mutant mice) share some of the phenotypic characteristics of *Bmal1*-deficient animals (i.e., high sensitivity to drug-induced genotoxic stress) (Gorbacheva et al. 2005), they normally do not demonstrate any signs of premature aging. On the contrary, some of the phenotypes are completely opposite to those displayed by *Bmal1* knockout animals. Unlike *Bmal1*-deficient mice that show age-dependent loss of muscle and adipose tissue, *Clock* mutant animals develop obesity relatively early in their life. It is caused by various metabolic deregulations including hyperleptinemia, hyperlipidemia, hyperglycemia, and hypoinsulinemia (Turek et al. 2005).

Another circadian mutant that showed some of the features of the early aging are *Per2*^{m/m} mice (Fu et al. 2002; Lee 2006), although there are some important differences. First, in *Per2*^{m/m} mice, this phenotype is developed only after exposure of animals to a low dose of irradiation. Second, it is not that severe compared to BMAL1 deficiency and is manifested mainly by early hair graying. Finally, it is accompanied by a higher incidence of tumor formation (Fu et al. 2002). The latter finding supports previous epidemiological studies linking the disruption of the circadian system (due to abnormal working schedules and/or exposure to frequent changes in the light/dark regime) to tumorigenesis (Antoch et al. 2005; Levi and Schibler 2007). Because previous studies of *Clock* mutant mice did not identify either a higher incidence of spontaneous tumor development or premature aging, in order to explore the potential role of the CLOCK protein in these processes, we exposed wild-type and *Clock/Clock* mice to a low dose of ionizing radiation and monitored them for any changes in their gross appearance for 80 weeks following the treatment.

As we reported recently, both wild-type and *Clock/Clock* mice showed no differences in their acute responses to 4 Gy of total body irradiation. However, starting at about 20 weeks after the treatment, *Clock* mutant mice begin to show signs of higher morbidity manifested by progressive weight loss, kyphosis, development of cataracts and eye inflammation, early hair graying, and alopecia. As a result, the survival rate of *Clock/Clock* mice is significantly reduced compared to similarly treated wild-type mice, and during their life span, they developed a phenotype reminiscent to premature aging in BMAL1-deficient animals (Fig. 1). Detailed necropsy performed on animals of both genotypes suggested that the reduction in body weight in *Clock/Clock* mice resulted from shrinkage of several major organs (liver, spleen, and kidney) as well as from the loss of the adipose tissue. However, no differences in the incidence of tumors were detected in animals of both genotypes (M. Antoch et al., in prep.). Together, these data conclusively demonstrate that although under normal conditions *Clock/Clock* mice do not display signs of premature aging, exposure to genotoxic treatment such as ionizing radiation accelerates the aging program without affecting carcinogenesis (M. Antoch et al., in prep.).

Figure 1. Premature aging in *Bmal1*^{-/-} and *Clock/Clock* mice. (A) Gross appearance of 1-year-old wild-type (WT) and BMAL1-deficient mice (Kondratov et al. 2006a). (B) Gross appearance of wild-type (WT) and *Clock/Clock* mutant mice 80 weeks after exposure to 4 Gy of total body irradiation (M. Antoch et al., in prep.).

This observation is supported by gene expression experiments demonstrating that the *Clock* mutation results in down-regulation of several proliferative genes and up-regulation of genes associated with growth arrest and apoptosis (Miller et al. 2007; M. Antoch et al., in prep.) predicting less cancer-prone phenotype. Consistent with this, “clock-less” mice lacking both *Cryptochrome* genes are indistinguishable from their wild-type littermates in their response to genotoxic treatments (Gauger and Sancar 2005; Gorbacheva et al. 2005). They do not show signs of premature aging or a higher rate of tumorigenesis even after exposure to ionizing radiation (Gauger and Sancar 2005).

The unique progeria-like phenotype described in BMAL1-deficient animals, radiation-induced aging in *Clock/Clock* mice, or cancer-prone phenotype in *Per2*^{m/m} mutant mice raises an important question on the causal links of these phenotypes with the disruption of the circadian function. The growing list of diverse and sometimes even contrasting phenotypes displayed by behaviorally arrhythmic circadian mutant mice suggests that in addition to their involvement in the molecular clock mechanism, clock proteins may have important roles in maintaining normal tissue homeostasis, and these roles may be distinct from their circadian function (for review, see Kondratov et al. 2007). This becomes particular obvious in comparing the phenotypes of *Bmal1*^{-/-} and *Clock/Clock* mice.

Indeed, both CLOCK and BMAL1 are required for the formation of an active transcriptional complex that has a central role in circadian-regulated processes. Consistent with this, both *Bmal1*^{-/-} and *Clock/Clock* mice demonstrate disruption of circadian rhythmicity in behavior and

gene expression (Vitaterna et al. 1994; Bunger et al. 2000), as well as some similar changes in metabolic pathways (Rudic et al. 2004). However, in addition to this, they develop a variety of unique phenotypic characteristics. Thus, *Clock* mutants are obesity-prone, whereas the BMAL1-deficient animals show a “lean” phenotype and defects in adipogenesis (Shimba et al. 2005). In addition, only *Bmal1*^{-/-} mice normally display premature aging (Kondratov et al. 2006a). These differences may have several explanations. Thus, *Clock* mutant mice still express functional protein with reduced *trans*-activation properties and intact transcriptional repressor function (Gekakis et al. 1998; Kondratov et al. 2006b), which makes this model different from the conventional knockout. Because mice with a complete lack of the CLOCK protein have been generated only recently (DeBruyne et al. 2006) and are still not fully characterized, a detailed phenotypic comparison of *Clock*^{-/-} and *Bmal1*^{-/-} mice is required to fully address the role of the CLOCK/BMAL1 transcriptional complex and its individual components in maintaining normal tissue homeostasis. In addition, both CLOCK and BMAL1 may interact with unique partners forming the complexes, whose functional activity is independent from the CLOCK/BMAL1 dimer. It has been shown that NPAS2, the closest homolog of CLOCK, which has different tissue specificity of expression, can partially compensate CLOCK deficiency (DeBruyne et al. 2007).

Accelerated aging normally observed in BMAL1-deficient mice and provoked by a low dose of radiation in *Per2* and *Clock/Clock* mutants supports a recently proposed model describing the complex role that the entire circadian system and its core components has under normal and various stress conditions (Kondratov et al. 2007). According to this model, in addition to pathological changes resulting from systemically deregulated circadian function (Fig. 2A), the deficiency of a particular circadian protein may result in the development of a unique set of pathological changes (Fig. 2B). At the same time, because the expression and activities of all components of molecular clock machinery are interrelated, the deficiency of a particular circadian protein will affect the activity of others as well. Therefore, in addition to primary pathology, these animals will have an increased risk for development of secondary pathologies caused by a deficiency of other circadian proteins. In addition, mutations of circadian proteins may affect their interaction with the environment, in which case pathological changes that are not displayed normally will be provoked by environmental stress (Fig. 2C). Accelerated aging of *Clock/Clock* mice after exposure to ionizing radiation provides an excellent example for such scenario.

CONCLUDING REMARKS

The results obtained in our and other laboratories during the past several years clearly demonstrate the importance of the circadian system in mammalian and human physiology. The spectra of pathological changes described in mice with a deficiency of individual components of the molecular clock only partially overlap. Most of the observed pathologies are unique for each individual

Figure 2. Role of the circadian system, circadian proteins, and organism/environment interactions in development of pathologies. (A) Deficiency in any circadian protein results in deregulation of the circadian system and desynchronization of multiple tissue-specific clocks with each other and with the environment, thus causing various pathological changes. (B) Individual circadian proteins have unique roles in maintaining normal tissue homeostasis that may be relatively independent from their roles in the molecular circadian oscillator. Impaired activity of a particular circadian protein (in this example, BMAL1) will result in a specific primary pathology (Pathology 2). At the same time, since BMAL1 is one of the core components of molecular clock, its deficiency will also affect entire circadian function, leading to a variety of secondary pathologies. (C) Improper interaction of an organism with the environment may affect the expression and/or activity of circadian proteins and induce specific pathologies without directly affecting clock function. (Reprinted, with permission from Kondratov et al. 2007 [© Elsevier].)

mutant, suggesting that at least some of them develop not as a result of circadian deregulation (because, in this case, they will be identical in all circadian mutants), but as a result of a deficiency in specific noncircadian function of an individual circadian protein.

Most importantly, these results provide molecular mechanisms explaining the increased risk of various diseases in shift workers and frequent time zone travelers. Traditionally, it was proposed that abnormal working schedules result in desynchronization of the tissue-specific molecular clocks within an organism and within an

environment, which interferes with an organ's functions through a yet unknown mechanism. However, recent data suggest that exposure to frequent changes in the light/dark cycle may affect the expression of a particular circadian gene, which will cause the changes in the expression and/or activity of other molecular clock components. For example, the functional activity of BMAL1 depends on its expression (regulated by PERs, CRYs, and REV-ERB β proteins) and posttranslational modifications (regulated by CLOCK and CRY proteins). At the same time, BMAL1 is implicated in the control of ROS levels; therefore, any disturbance of the circadian system may have an effect on the activity of BMAL1, which, in turn, through ROS or other mechanisms, will change tissue homeostasis and increase risk of disease.

Taken together, the emerging data of the involvement of circadian proteins in the regulation of many processes in mammalian organisms demonstrate the dichotomy in biological consequences of disruption of the circadian clock with respect to many diseases, including aging and cancer. All of these findings make the circadian proteins perspective targets for the development of novel therapeutic approaches.

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R. V. Kondratov and M. P. Antoch

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