Abstract: The circadian clock is an evolutionarily conserved time-keeping system that coordinates the physiology of the organism with daily changes in the environment. A growing body of evidence gradually leads to the conception that virtually all aspects of the biochemical, physiological, and behavioral functions of the animal are linked to circadian regulation. Moreover, proper synchronization of various processes through the activity of circadian components is important for the well-being of many organisms, including humans. The focus of this review is the circadian control of an organism’s response to genotoxic stress, which is a major contributor to life-threatening human pathologies such as cancer and cardiovascular disease. (Circ Res. 2010;106:68-78.)

Key Words: circadian rhythms ♦ cell cycle ♦ genotoxic stress ♦ oxidative stress ♦ carcinogenesis ♦ cardiovascular ♦ chronotherapy

The structural and molecular organization of the mammalian circadian system has been the subject of extensive studies, which are reviewed in detail elsewhere. It has been shown that the clock is operative in virtually every cell and tissue and that it is generated by a network of transcription/translation feedback loops (Figure 1). The positive components of the loop are the transcription factors CLOCK and BMAL1. In the form of a heterodimer, they drive the rhythmic expression of numerous genes through E-box elements in their promoter regions. Among the transcriptional targets of the CLOCK/BMAL1 complex are the Period (Per1 and Per2) and Cryptochrome (Cry1 and Cry2) genes. PER and CRY proteins function as negative components of the circadian loop by inhibiting CLOCK/BMAL1-mediated transactivation. The Bmal1 gene is also regulated by 2 of its transcriptional targets, the nuclear receptors REV-ERBα and RORα (retinoic acid receptor–related orphan receptor α), which function respectively as a repressor or an activator of Bmal1 transcription by competing for the RORE (RORα response element) in its promoter. In addition to the core components of circadian oscillatory machinery, the CLOCK/BMAL1 complex drives the rhythmic expression of numerous output genes harboring E-box sequences in their promoters, allowing for daily variations in cellular, metabolic and physiological functions. The results of global temporal transcriptional profiling of various tissues using a microarray hybridization approach have estimated that as much as 10% of mammalian transcriptome oscillate in a circadian manner. In addition to direct transcriptional regulation, this oscillation is achieved at posttranscriptional level through the regulation of RNA stability. Recent data have implicated microRNAs...
BMAL1 functional activity results in oscillatory pattern of expression of numerous clock-controlled genes. As a result, the expression/activity of core clock genes and clock-controlled genes oscillates with a period close to 24 hours. Reprinted from Antoch et al with permission.

and RNA-binding proteins as essential contributors to circadian output rhythmicity.\textsuperscript{7–9} Mechanistically, CLOCK/BMAL1-mediated transactivation is associated with rhythmic changes in histone acetylation,\textsuperscript{10,11} which suggests that, as in many other systems, chromatin remodeling constitutes an important regulatory step governing the circadian clock machinery. The CLOCK protein itself possesses an intrinsic acetyltransferase activity specific for histones\textsuperscript{12} and for its dimerization partner BMAL1.\textsuperscript{13} Acetyltransferase activity of CLOCK is counterbalanced by sirtuin 1, which deacetylates circadian proteins PER2 and, presumably, BMAL1.\textsuperscript{14,15}

Besides direct transcriptional control, clock proteins are regulated at the posttranslational level through various modifications. Both positive and negative components of the mammalian transcriptional feedback loops are subject to posttranslational modifications, which determine their stability, nuclear/cytoplasm distribution, and functional activity (reviewed elsewhere\textsuperscript{16,17}). It is generally believed that these modifications (particularly for proteins representing the negative arm of the loop) introduce a temporal delay in imposing transcriptional repression, which is necessary to produce a period close to 24 hours.\textsuperscript{16} Such a complex multilevel regulation of the molecular clock ensures high plasticity of the system enabling fast adaptation of an organism to its constantly changing environment (reviewed elsewhere\textsuperscript{18}).

Here, we review recent data highlighting the role of circadian proteins in modulating response to genotoxic stress at the cellular and organismal levels. We describe recently discovered connections between the circadian proteins and key regulators of the cell cycle and cell cycle checkpoint control, oxidative stress, and carcinogenesis. Because the majority of our knowledge came from studies of mice with mutations or targeted disruption of individual circadian proteins, we summarize this information in the Table, which provides a concise reference to the text of the review. Finally, we discuss the involvement of the circadian control of cell cycle and stress response in physiology of cardiovascular system and potential translational applications of these new findings.

### The Circadian Clock and Cell Proliferation

As the picture of circadian molecular machinery operation begins to clarify, numerous studies have been initiated trying to mechanistically link circadian clocks to other important biological processes. One such process is the cell cycle. Similarities in the duration of circadian and cell cycles (at least in fibroblasts grown in culture) provoked numerous speculations about a possible interconnection between these 2 processes.\textsuperscript{19} Indeed, temporal expression profiling of mRNA from different tissues has revealed a 24-hour oscillatory pattern for numerous key regulators of cell proliferation. Some of them, such as c-myc, cyclin D1 (Ccnd1), and Weel, appeared to be under direct transcriptional control of the CLOCK/BMAL1 complex.\textsuperscript{20,21} Others, such as the important cell cycle regulator p21 (Cip1/Waf1/Cdkn1a), which does not possess an E-box element in its regulatory region, are controlled indirectly via CLOCK/BMAL1-mediated transcriptional regulation of the orphan nuclear receptor Rev-Erb\textsubscript{a}.\textsuperscript{22}

Consistent with daily variations in the expression of cell cycle-related genes, experiments performed in regenerating liver after partial hepatectomy have demonstrated that entry
into mitosis of proliferating hepatocytes is gated by the circadian clock.21 Whereas normal mice display strong diurnal variations in the timing of mitosis, arrhythmic Cry-deficient mice show significant impairment in hepatocyte proliferation. Mechanistically, this defect has been ascribed to constant high levels of the WEE1 kinase, an important regulator of the G2/M progression, in the tissues of Cry-deficient mice.22 Analysis of another circadian mutant model, Bmal1 knockout mice, also revealed defects in hepatocyte proliferation; however, in this case, the delay has been attributed to a different pathway and involves second-order targets of CLOCK/BMAL1 transcriptional activity. It has been shown that a deficiency in Bmal1 results in an imbalance in the expression levels of 2 of its direct transcriptional targets, Rev-Erb and Ror. This imbalance results in upregulation of p21 and a subsequent delay in progression through the G1 phase of the cell cycle.22

An intriguing example of the physiological consequences of high levels of p21 expression in tissues of Bmal1-deficient mice has been demonstrated recently by Lin et al.23 Using a genomic approach, the authors discovered that several clock and clock-controlled genes are differentially expressed during the hair growth cycles with enhanced circadian expression during progression from telogen (a quiescent phase of the hair growth cycle) to anagen (a growth phase of the cycle). Analysis of Clock/Clock and Bmal1−/− mice has demonstrated that although the duration of the entire hair growth cycle in these mutants is not altered, they display a prominent delay in anagen progression. This delay correlates with a significant upregulation of p21, a decrease in phosphorylation of retinoblastoma protein and lack of mitotic cells in hair follicle precursor cells. All these changes are consistent with previously described block in the G1 phase of the cell cycle in Bmal1-deficient cells. Hence, the circadian clock (or the activity of some of its core components) can modulate other cycling processes with a much longer than 24-hour duration. These recent findings further broaden our view on the multi-faceted functional roles of core clock proteins, implicating them in the modulation of noncircadian cycling processes, such as hair growth cycles, via cell cycle control.24

Although the interconnection between the circadian clock and cell proliferation is well established now, it is important to keep in mind that even in vitro, the duration of the cell cycle may vary from several hours (in cancer cell lines) to several days (in primary fibroblasts at later passages). In vivo, the timing of cell division may vary even more, lasting from several minutes (in developing embryos) to several days and probably even months and years, depending on cell type. Therefore, it is unlikely that the function of the circadian clock is critical for regulation of cell proliferation in all cell types. Consistent with this, there are no embryonic defects associated with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17 Some of these pathologies, at least in part, can be related to deregulation of cell proliferation. The only exception to this is animals with a deficiency of core circadian components; all circadian mutant mice are born normal and most of the defects are developed later in life.17

<table>
<thead>
<tr>
<th>Circadian Gene</th>
<th>Carcinogenesis</th>
<th>Aging</th>
<th>Response to Genotoxic Stress</th>
<th>Cardiovascular</th>
<th>Control of Proliferation</th>
<th>Cell Cycle Checkpoint Control</th>
<th>Expression in Human Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periods</td>
<td>Higher rate after exposure to ionizing radiation³⁵; higher rate on ApoE−/− background⁴⁶-⁵⁰</td>
<td>Accelerated after ionizing radiation³⁵</td>
<td>Increased sensitivity²⁰</td>
<td>Increased vascular senescence³³</td>
<td>Regulation of proliferation in tumor cells⁶⁰,⁶³,⁶⁴</td>
<td>PER1 interacts with ATM and Chk2 kinases³⁴</td>
<td>Per1 and Per2 are decreased in breast tumors²⁵ and hepatocellular carcinoma³⁶; Per1 decreased in colorectal tumors³⁷</td>
</tr>
<tr>
<td>Cryochrome</td>
<td>Lower rate on p53−/− background⁷⁶</td>
<td>ND</td>
<td>Increased resistance (acute response to high dose of ionizing radiation)³⁷ or no effect (long-term effects after low dose of ionizing radiation)³⁷</td>
<td>ND</td>
<td>G2/M progression in hepatocytes²¹</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clock</td>
<td>No effect after ionizing radiation⁷³</td>
<td>Accelerated after ionizing radiation⁷³</td>
<td>Increased sensitivity⁸¹</td>
<td>Regulation of thrombogenesis⁶⁴</td>
<td>Decreased rate of MEF proliferation⁷³,⁹⁶</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bmal1</td>
<td>ND</td>
<td>Accelerated⁰⁰</td>
<td>Increased sensitivity⁸¹</td>
<td>Vascular disease⁵¹; regulation of thrombogenesis⁶⁴</td>
<td>Delay in progression through G1 in hepatocytes⁷²; modulation of hair growth cycles²³</td>
<td>p53-dependent G1 growth arrest³⁸</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table. Effects of Mutations of Circadian Genes on Response to Genotoxic Stress, Cell Proliferation, Carcinogenesis, and Aging
It is known that proliferating and nonproliferating cells differ in the way they metabolize glucose. This phenomenon, known as the Warburg effect, was originally described in cancer cells, which represent actively proliferating cells. Normally, in the presence of oxygen, quiescent cells use highly effective oxidative phosphorylation, producing approximately 36 molecules of ATP by oxidizing one molecule of glucose. Proliferating cells use anaerobic glycolysis, generating only 4 molecules of ATP and lactate, which is toxic and is excreted from dividing cells. It has been proposed that such an “inefficient” way of glucose metabolism is necessary to fulfill other metabolic needs of proliferating cells, in which glucose is used as a source of carbon for synthesis of lipids, nucleic acids and other macromolecules. Therefore, cell proliferation depends on the availability of extracellular glucose and its transport into the cell, as well as on the export and inactivation of lactate. Because serum glucose level, glucose tolerance and insulin sensitivity display daily variations, the availability of glucose to proliferating cells will most likely vary during the day. It is reasonable to hypothesize that the circadian clock may gate certain phases of the cell cycle to synchronize daily metabolic cycles of glucose with times when it is highly required for cell proliferation. Interestingly, the cell division cycle in yeasts is gated by the metabolic cycle through Rad53/Chk2 kinase, which is a checkpoint kinase controlled by the circadian clock in mammals. Thus, similar to yeast cycles, the physiological significance of circadian control of the cell cycle is to synchronize the metabolic and DNA replication cycles, which is necessary for maintaining the integrity of the genome. To perform this function in the most efficient way, circadian proteins have to be involved in the control of cell cycle checkpoints.

The Circadian Clock and Cell Cycle Checkpoints

DNA damage initiates a cascade of intracellular events that may result in the blocking of the cell cycle progression at specific phases – checkpoint cell cycle arrest. DNA damage is recognized by sensor proteins; the signal is then transmitted to checkpoint kinases, which in turn activate or repress proteins important for transition from one stage of the cell cycle to another (G1/S or G2/M) or for progression through the S phase of the cycle (reviewed elsewhere). The way the circadian clock is involved in the checkpoint control has been extensively studied and the results have been summarized in several recent reviews; therefore, here, we just briefly describe the major findings.

In addition to CLOCK/BMAL1-mediated transcriptional control of important cell cycle regulators, more recent data revealed other points of intersection between the 2 regulatory systems. Thus, the circadian protein PER1 interacts with the 2 critical components of cellular stress response pathways, the ATM (ataxia telangiectasia mutated) and Chk2 kinases, whereas another circadian protein, the mammalian homolog of Drosophila timeless (TIM) interacts with the cell cycle checkpoint proteins Chk1, ATR (ataxia telangiectasia and Rad3-related), and the ATR-interacting protein ATRIP. These interactions, which are necessary for the activation of Chk1 and Chk2 by DNA damage, represent a novel and previously unrecognized mechanism of the circadian modu-

**Figure 2.** Interconnection between the circadian and cell proliferation cycles. The cell growth occurring during the G1 phase of the cell cycle depends on the availability of various extracellular growth and metabolic factors, some of which are controlled by the circadian oscillators in other tissues. When cells progress through the cell cycle, they pass several checkpoints (G1/S, S, G2/M) when the integrity of genetic information and other parameters are monitored. Circadian proteins are involved in regulation of all checkpoints either through the interaction with the checkpoint proteins or by controlling expression of corresponding genes.

nts. Normal cells also need to monitor the integrity of genetic material to be able to successfully proliferate. The circadian clock may be involved in controlling the accomplishment of all these requirements as well. Multiple levels of regulation of the cell cycle by the circadian clock are summarized in Figure 2.

What is the biological significance of the coupling of the circadian and cell cycles? In lower organisms, the answer is somewhat obvious; indeed, cells need to restrict DNA synthesis to a specific time of day to reduce/repair DNA lesions induced by UV light and to synchronize metabolic and mitotic cycles. The physiological meaning of circadian control of cell proliferation in higher organisms such as mammals is less apparent. In adult organisms, cell proliferation is restricted to very few cell types: the epithelium of the skin and gastrointestinal tract, hematopoietic cells in the bone marrow and lymphoid organs, and germ cells in the reproductive organs. Most other tissues remain in a differentiated nonproliferating state. In mammals, exposure to UV light is restricted to the skin, and skin epithelium is one of the most actively proliferating tissues. This suggests that the phases of the cell cycle in keratinocytes may be gated by the circadian clock. If future studies establish such a correlation, it would be important to test whether the coupling of the circadian and cell cycles in mammals is affected by the exposure to sunlight in a way, similar to lower organisms.

The major source of DNA damage for other types of proliferating cells, as well as for quiescent cells, are reactive oxygen and nitrogen species (reactive oxygen species [ROS] and reactive nitrogen species [RNS], respectively). Below, we address the potential role of the circadian clock in regulation of ROS homeostasis; here we would like to discuss another possible link between the circadian clock and the cell cycle, which has not been addressed so far.

It is known that proliferating and nonproliferating cells differ in the way they metabolize glucose. This phenomenon, known as the Warburg effect, was originally described in cancer cells, which represent actively proliferating cells. Normally, in the presence of oxygen, quiescent cells use highly effective oxidative phosphorylation, producing approximately 36 molecules of ATP by oxidizing one molecule of glucose. Proliferating cells use anaerobic glycolysis, generating only 4 molecules of ATP and lactate, which is toxic and is excreted from dividing cells. It has been proposed that such an “inefficient” way of glucose metabolism is necessary to fulfill other metabolic needs of proliferating cells, in which glucose is used as a source of carbon for synthesis of lipids, nucleic acids and other macromolecules. Therefore, cell proliferation depends on the availability of extracellular glucose and its transport into the cell, as well as on the export and inactivation of lactate. Because serum glucose level, glucose tolerance and insulin sensitivity display daily variations, the availability of glucose to proliferating cells will most likely vary during the day. It is reasonable to hypothesize that the circadian clock may gate certain phases of the cell cycle to synchronize daily metabolic cycles of glucose with times when it is highly required for cell proliferation. Interestingly, the cell division cycle in yeasts is gated by the metabolic cycle through Rad53/Chk2 kinase, which is a checkpoint kinase controlled by the circadian clock in mammals. Thus, similar to yeast cycles, the physiological significance of circadian control of the cell cycle is to synchronize the metabolic and DNA replication cycles, which is necessary for maintaining the integrity of the genome. To perform this function in the most efficient way, circadian proteins have to be involved in the control of cell cycle checkpoints.

The Circadian Clock and Cell Cycle Checkpoints

DNA damage initiates a cascade of intracellular events that may result in the blocking of the cell cycle progression at specific phases – checkpoint cell cycle arrest. DNA damage is recognized by sensor proteins; the signal is then transmitted to checkpoint kinases, which in turn activate or repress proteins important for transition from one stage of the cell cycle to another (G1/S or G2/M) or for progression through the S phase of the cycle (reviewed elsewhere). The way the circadian clock is involved in the checkpoint control has been extensively studied and the results have been summarized in several recent reviews; therefore, here, we just briefly describe the major findings.

In addition to CLOCK/BMAL1-mediated transcriptional control of important cell cycle regulators, more recent data revealed other points of intersection between the 2 regulatory systems. Thus, the circadian protein PER1 interacts with the 2 critical components of cellular stress response pathways, the ATM (ataxia telangiectasia mutated) and Chk2 kinases, whereas another circadian protein, the mammalian homolog of Drosophila timeless (TIM) interacts with the cell cycle checkpoint proteins Chk1, ATR (ataxia telangiectasia and Rad3-related), and the ATR-interacting protein ATRIP. These interactions, which are necessary for the activation of Chk1 and Chk2 by DNA damage, represent a novel and previously unrecognized mechanism of the circadian modu-
lation of cell proliferation and response to genotoxic stress (reviewed elsewhere). Another important finding connecting circadian proteins and checkpoint control came from a large-scale genetic screening of human cells for novel regulators of the p53 tumor suppressor pathway. p53 tumor suppressor protein is a key mediator of cellular response to genotoxic stress. DNA damage leads to activation of the ATM/ATR, CHK1/2 cascade, which in turn activates p53. Activation of p53 is achieved by increased stability and post translational modifications of the p53 protein and leads to transcriptional activation of a large set of target genes, which in turn causes cell cycle arrest or apoptosis. A short hairpin RNA–based screening identified that BMAL1 is necessary for p53-dependent growth arrest. Consistently, cells in which BMAL1 was suppressed by specific short hairpin RNA were unable to undergo growth arrest on p53 activation induced by DNA damage. In contrast to the in vivo mouse data, which link Bmal1-dependent delay in G1 progression to upregulation of p21, radiation-induced growth arrest in Bmal1-deficient human cells correlated with the decrease in levels of p53 and p21. These differences, which may reflect either interspecies variations in the regulation of expression of p21 between mice and humans or differences between the in vitro versus in vivo conditions used, should be clarified by further investigations.

Recent data have demonstrated that the link between the circadian clock and DNA damage response is mutual and that DNA damaging agents can affect circadian parameters. Thus, exposure of Rat-1 cultured fibroblasts to ionizing radiation induces a shift in the phase of oscillation in expression of circadian genes. However, in contrast to chemical synchronization signals, such as dexamethasone or forskolin, which cause both phase advances and phase delays, ionizing radiation induces exclusively phase advances. This suggests that the mechanism of radiation-induced phase resetting could be different from the one induced by chemical agents. The fact that it is significantly impaired in fibroblasts isolated from patients with ataxia telangiectasia and Nijmegen breaking syndrome points toward the possible involvement of ATM/ATR-mediated signaling. Notably, in addition to synchronized cultured cells, ionizing radiation can reset behavioral rhythms in mice, suggesting that this effect is universal both for the central oscillator in the suprachiasmatic nuclei and for peripheral clocks.

In a complementary study, similar responses have been described in fibroblasts treated with either the radiomimetic drug methyl methane sulfonate or with exposure to UV light, in which the only phase advances were induced by both DNA damaging treatments. However, unlike in the study by Oklejewicz at al, which reports no changes in clock protein levels resulting from exposure to γ radiation, methyl methane sulfonate–, and UV-mediated responses have been mechanistically linked to degradation of the PER2 protein and subsequent upregulation of the Bmal1 promoter. Further studies are needed to determine whether these differences in fact reflect the diversity in signaling pathways linking clock proteins and DNA damage response. It is also important to note that the response of the circadian clock to genotoxic stress is likely to be evolutionarily conserved. Earlier studies have shown that in Neurospora, the PRD-4/Chk2 protein is involved both in circadian regulation (through direct interaction with FRQ) and in checkpoint control, findings that are now extended to higher eukaryotes.

The Circadian Clock and Oxidative Stress
ROS/RNS are highly reactive molecules. The major site of ROS production in the cell is the mitochondrial respiratory chain, which, in some tissues, accounts for more than 90% of total oxidants generated. Other important sources include the activities of enzymes such as NADPH oxidases, cyclooxygenases, peroxisomal enzymes, and the cytochrome P450 system. RNS are generated by nitric oxide synthases. Because ROS/RNS serve as important regulators of cellular metabolism, proliferation, signal transduction, and gene expression, they are implicated in control of many physiological processes such as inflammation, ventilation, blood pressure control and others (reviewed elsewhere). However, the increase in ROS levels causes significant damage to biological macromolecules and cellular structures. Normally, levels of ROS/RNS are determined by a balance between their production through the above-described mechanisms and their elimination through the activity of the antioxidant system consisting of superoxide dismutases, catalases, peroxiredoxins, and glutathione and thioredoxin systems. Shifting a balance toward excessive production of ROS, results in oxidative stress, the major source of DNA damage in mammalian cells. It is believed that oxidative stress plays a major role in the pathogenesis of a variety of human diseases, including cancer.

The increased level of carcinogenesis in several mouse models of chronic oxidative stress strongly argues in support of this view. Oxidative stress has also been linked to pathologies in cardiovascular system, particularly to atherosclerosis, kidney disease, hypertension and coronary artery disease. Therefore, it is critically important to maintain ROS/RNS levels within the normal physiological range; as a result, intracellular and extracellular levels of ROS/RNS are tightly controlled (reviewed elsewhere).

Although no direct measurements of temporal fluctuations in generation of ROS/RNS have been reported, it is reasonable to believe that they may arise from daily variations in metabolism governed by the circadian clock. Daily fluctuations in body temperature, which may be viewed as a general indicator of gross metabolic rate, provide an indirect evidence for a possible link between the circadian clock and mitochondrial ROS production.

Periodic variations in levels of ROS imply that different tissues are challenged by different amounts of oxidants during the day. Therefore, it would be beneficial to counterbalance these variations by periodic changes in the activity of the antioxidant defense system. Obviously, the circadian clock is the best candidate for the regulation of daily variations in antioxidant defense, by providing an adequate environment for the expression and enzymatic activity of antioxidant and metabolic proteins. In agreement with this, daily and circadian oscillations of the expression and activity
of pro- and antioxidant enzymes and low-molecular weight compounds have been reported.\textsuperscript{49} Furthermore, the core clock transcriptional factor BMAL1 directly controls ROS homeostasis. Deficiency in Bmal1 leads to chronic oxidative stress and accelerated aging syndrome in mice.\textsuperscript{50} Oxidative stress has also been implicated in the cardiovascular pathology observed in other circadian mutant mice.\textsuperscript{51-54} It is important to note that the circadian and redox control systems are tightly interconnected. Although the circadian clock is implicated in the control of metabolism, its own activity can be regulated by the components of the cellular redox system.\textsuperscript{55}

Oscillations in the production of ROS and in the control of their intracellular levels provide one of the rationales for circadian and cellular clocks connection. Indeed, as has already been mentioned, the restriction of DNA replication to the respiratory phase of the metabolic cycle allows yeast to reduce the rate of spontaneous mutation.\textsuperscript{56} It would be of interest to test whether higher eukaryotes use a similar mechanism enabling them to protect DNA from oxidative challenge during the most critical part of cellular division.

The Circadian Clock and Carcinogenesis

Results of several epidemiological studies involving shift workers (such as nurses or flight attendants) have shown that abnormal working schedules correlate with an elevated risk for several types of cancer, ie, breast and prostate (reviewed elsewhere\textsuperscript{56}). These data argue in favor of a functional interconnection between the defects in the circadian system and carcinogenesis. Indeed, experiments performed in mice show that the disruption of normal rhythmicity either by surgical ablation of the suprachiasmatic nuclei or by chronic exposure to frequent changes in the light:dark cycle result in the accelerated growth of implanted tumors.\textsuperscript{57} The availability of mice with deficiencies in particular components of the molecular clock through genetic knockout or mutation provides a great opportunity for studying the role of the circadian system in the development of tumors.

The first mechanistic link between the genetic disruption of the molecular clock and carcinogenesis came from the analysis of Per2 mutant mice. It was reported that these mice develop \(\gamma\) radiation-induced lymphomas at a higher rate than their wild-type littermates.\textsuperscript{20} Furthermore, crossing these mice with polyP formation-prone adenomatosis polyposis coli (Apc\textsuperscript{Min/+}) animals increases the frequency of formation of intestinal and colonic polyps in Apc\textsuperscript{Min/+}Per2\textsuperscript{m/m} mice compared to Apc\textsuperscript{Min/+}-58-60 Based on these findings it has been proposed that the PER2 protein functions as a tumor suppressor.\textsuperscript{20} It is notable though that these conclusions are based on studies done in mPer2\textsuperscript{m/m} mutant mice with a short deletion in the Per2 gene, which translates into a truncated protein.\textsuperscript{61} Therefore, at this point it is not possible to explicitly conclude whether the PER2 is in fact a tumor suppressor or whether the Per2\textsuperscript{m/m} mutation has oncogenic properties. This could be tested by comparing the phenotypes of Per2\textsuperscript{m/m} and Per2-null mice which do not express the PER2 protein.\textsuperscript{62}

In agreement with the proposed tumor suppressor function of PER2, downregulation of mPer2 expression with siRNA increases the proliferation of tumor cells\textsuperscript{60} whereas its over-expression inhibits cell growth and induces apoptosis in carcinoma cell lines.\textsuperscript{63} This was further supported by data showing that the intratumoral delivery of mPer2 has a significant antitumor effect in C57Bl/6J mice transplanted with Lewis lung carcinoma.\textsuperscript{64}

The accumulating data on the role of PER proteins in control of cell cycle and response to DNA damage prompted the investigation of the status of Period genes in tumors of cancer patients. Thus, several reports have demonstrated the reduced expression of Period genes in sporadic and familial breast tumors\textsuperscript{65} and hepatocellular carcinoma,\textsuperscript{66} and down-regulation of Per1 (but not Per2) in colorectal tumors.\textsuperscript{67} Some of these changes in expression occur through epigenetic mechanisms. Methylation of the promoter regions of \(h\text{Per1,} \ h\text{Per2, and} \ h\text{Per3}\) have been observed in breast cancer,\textsuperscript{68} whereas \(h\text{Per1,} \ h\text{Per2 and} \ h\text{Cry1}\) promoters were hypermethylated in endometrial cancers.\textsuperscript{69} Although potentially important, at this point the data remain correlatively and have to be taken with caution, because the observed genetic changes in the loci of the Period genes may reflect a general genomic instability characteristic of tumor cells.

Whereas the proposed cancer-prone phenotype of Per2 mutant mice was originally viewed as a strong argument in favor of the concept that the disruption of the circadian clock promotes carcinogenesis, it has been recently challenged by several studies performed in other circadian models, Clock/Clock mutant mice and Cry1\textsuperscript{−/−}/Cry2\textsuperscript{−/−} double knockout animals. The Clock mutation affects the transactivation properties of the CLOCK protein and is manifested by behavioral arrhythmicity, disruption of rhythmic pattern of gene expression and downregulation of CLOCK/BMAL1 transcriptional targets.\textsuperscript{70,71} Animals deficient in both Cry genes (Cry1\textsuperscript{−/−}/Cry2\textsuperscript{−/−}) are also behaviorally arrhythmic and demonstrate disruption in rhythmic pattern of gene expression as well; however, because the repressor function of the CRY proteins, their deficiency results in the upregulation of the CLOCK/BMAL1 transcriptional targets.\textsuperscript{72} Although in both animal models circadian rhythmicity is impaired, these mice do not develop spontaneous tumors.\textsuperscript{73,74} Unlike Per2 mutant mice, Clock/Clock animals do not show an increase in tumorigenesis when challenged by a low dose of ionizing radiation, a well-known carcinogen that is able to initiate and promote neoplastic progression.\textsuperscript{73} Instead, exposure of Clock/Clock mice to ionizing radiation results in the development of pathological conditions reminiscent of the premature aging phenotype previously described for Bmal1\textsuperscript{−/−} mice.\textsuperscript{50} Even more striking, when circadian Cry mutation was combined with p53 mutation, the early cancer onset characteristic of mice deficient in this important tumor suppressor protein was significantly delayed and the median lifespan was increased \(\approx 1.5\)-fold (from 19 to 28 weeks).\textsuperscript{75} The relationship between circadian proteins, shift work and carcinogenesis is summarized in Figure 3. The data suggest that disruption of the circadian clock per se does not affect the rate of carcinogenesis and that the increased incidence of tumor formation reported for Per2\textsuperscript{m/m} mice could result because of either oncogenic properties of the Per2 mutation or tumor suppressor properties of the PER2 protein. These
The circadian clock and carcinogenesis. According to epidemiological data, shift work increases the risk of tumor development, presumably through the disturbance of circadian synchronization. Consistent with this, exposure of mice to conditions initiating chronic jetlag increases the growth of transplanted tumors. Mice with mutation in the Period2 gene are arrhythmic and demonstrate higher incidence of lymphomas after exposure to low doses of γ radiation and higher incidence of colon carcinomas when crossed to cancer-prone ApcMin/+ animals. In contrast, arrhythmic Bmal1−/−, Cry1−/− Cry2−/−, and Clock/Clock mice are not cancer-prone. Moreover, the growth of transplanted tumors in Clock/Clock mice is reduced (Y Hu, MPA, unpublished data, 2009), and deficiency in CRY proteins delays the development of spontaneous tumors on a highly cancer prone p53−/− background. Therefore, individual circadian proteins play important but distinct roles in tumor formation and development, and the question regarding the interconnection between the disruption of the circadian clock and carcinogenesis still remains open.

The results of animal studies may raise a question regarding the relevance of epidemiological data showing that circadian disruption increases the incidence of tumor formation. In fact, there is no real conflict between the data. As shown schematically in Figure 4, shift work and circadian properties may be unique for PER2 and may be unrelated to its circadian function. In any case, further studies elucidating the functional role of clock proteins in carcinogenesis are needed.

Another possible reason for an apparent discrepancy between epidemiological and genetic data arises from the complex nature of DNA damage response. After exposure to DNA damaging agents, the cell has several options for response (schematically shown in Figure 5). The cell may undergo growth arrest allowing for DNA repair, and if the damage is eliminated, the cell may return back to its original normal state. If the cell fails to repair the DNA damage, it can be eliminated through apoptosis or some other type of programmed cell death. At the same time, the cell can start proliferation before elimination of induced mutations, which will potentially lead to neoplasia and tumor development. Finally, the cell may respond by initiating the program of senescence (irreversible growth arrest) but may still remain metabolically active and keep producing various factors and occupy its position within a tissue. In this case, if significant DNA damage occurs, massive apoptosis may result in the disruption of tissue integrity. The decision on which pathway to select depends on the type of tissue as well as on many extra- and intracellular factors. It is very likely that circadian proteins may be involved in this decision-making process. There is some evidence that deficiency in certain circadian proteins favors the initiation of the senescence program. As mentioned previously, Bmal1−/− and Clock/Clock mice develop a phenotype of premature aging, the former naturally in life, whereas the latter after challenging by ionizing radiation. In addition, mice with a mutation in the Per2 gene have an increased amount of senescent cells in vasculature developing early in life. Because stress-induced senescence has been proposed as one of the mechanisms for tumor suppression, the delay in tumor development ob-
The identification of circadian proteins, dissecting the molecular details of clock operation and generation of mice with deficiencies in individual components of the molecular oscillator provided new avenues for studying the molecular links between the circadian system and response to genotoxic stress induced by anticancer therapy. The direct involvement of key circadian proteins in modulating the response to genotoxic stress was first demonstrated by testing the sensitivity of several circadian mutant mice to toxicity induced by the anticancer drug cyclophosphamide (CY). Although all mutant lines used in this study \( \text{Clock/Clock} \) and \( \text{Bmal1}^{-/-} \) and \( \text{Cry1}^{-/-}\text{Cry2}^{-/-} \) knockout mice share a similar behavioral phenotype (disruption of rhythmicity in locomotor activity), their response to CY was dramatically different. Importantly, these differences correlated with the functional status of the \( \text{CLOCK/BMAL1} \) transcriptional complex, with \( \text{Clock/Clock} \) and \( \text{Bmal1}^{-/-} \) mice being extremely sensitive and \( \text{Cry1}^{-/-}\text{Cry2}^{-/-} \) mice extremely resistant to CY-induced toxicity. The data suggest that the \( \text{CLOCK/BMAL1} \) complex may directly control the molecular determinants of drug sensitivity at the transcriptional level.

More recent work has investigated the effect of the circadian clock on nucleotide excision repair in mice. The mammalian excision repair system removes all DNA lesions, including ones that are produced by chemotherapeutic drugs, such as cisplatin. It has been found that nucleotide excision repair activity in the mouse cortex displays prominent circadian oscillations, peaking in the afternoon/early evening hours. These fluctuations correlated with circadian oscillations in the abundance of DNA damage recognition protein xeroderma pigmentosum A. Interestingly, the peak of repair activity coincides with the previously determined peak of the animal’s resistance to CY. Although it is still not clear whether higher nucleotide excision repair activity accounts for increased resistance to CY (there are no data that the type of lesions produced by CY might be repaired by this system in vivo), both findings form a background for important clinical applications and may be used for improving the therapeutic index of any given treatment. This implies that delivery of chemotherapeutic drugs at the time of the daily peak in nucleotide excision repair activity may reduce its toxicity to normal tissues (ie, cisplatin associated renal toxicity). However, to exploit such an approach, it would be important to find differences in the functional characteristics of the molecular clock in normal cells and tumors, which is still largely missing.

### The Circadian Clock, Genotoxic Stress, and Cardiovascular System

Although in this review we focused mainly on the role of the circadian clock in carcinogenesis and anticancer therapy, similar translational approaches may be equally relevant to

---

**Figure 5.** Circadian proteins and regulation of the cellular response to genotoxic stress. The DNA damage initiates a cascade of intracellular events, which can result in either reversible (repair of the damaged DNA) or irreversible (senescence, transformation, cell death) changes in cellular metabolism. The final outcome will depend on the type of the cell, various extracellular signals, and the functional status of the corresponding intracellular pathways. The circadian proteins control some important steps of various pathways: DNA repair (CRYs), senescence (BMAL1, CLOCK), transformation (CRYs, PERs), and apoptosis (PERs). Therefore, deficiency of a specific circadian protein, which can result from either genetic defect or as a consequence of systemic malfunction of circadian regulation, will affect particular pathway in which it is engaged. This, in turn, will determine final outcome of exposure to genotoxic stress. Arrows and T-inhibition symbols indicate positive and negative regulations, respectively.

served in \( \text{p53}^{-/-}\text{Cry1}^{-/-}\text{Cry2}^{-/-} \) mice may reflect a switch of the genotoxic stress response program toward senescence, which in the case of the compound mutant mice results from a deficiency of Cryptochromes.

### Chronotherapy

Although the data on the role of clock proteins in carcinogenesis remain contradictory, their emerging role in modulating stress response, which is now well established, may have important clinical applications. The concept of chronotherapy emerged from empirical observations showing that the therapeutic index for many anticancer drugs tested in clinical practice. The overall skepticism resulted mainly from the superficial empirical nature of the data, which lacked a clear mechanistic foundation. Furthermore, the results of a recent large randomized trial involving patients with metastatic colorectal cancer showed significant gender-related differences. Thus, chronomodulated delivery of the drug extended median survival for men only whereas for females conventional schedules appeared to be more beneficial.

The effective scheduling of chemotherapeutic agents in patients with advanced colorectal cancer optimally combines the clinical advantage of higher response rate and patients' overall quality of life against the increased risk of severe toxicity, which is associated with increased cumulative dose of chemotherapy. The latter can be realized by chronic administration of doxorubicin/cisplatin to patients with advanced colorectal cancer. This scheduling, in the case of the compound mutant mice results from a deficiency of a specific circadian protein, which can result from either genetic defect or as a consequence of systemic malfunction of circadian regulation, will affect particular pathway in which it is engaged. This, in turn, will determine final outcome of exposure to genotoxic stress. Arrows and T-inhibition symbols indicate positive and negative regulations, respectively.
the pathology of the cardiovascular system. Indeed, molecular clocks are functional in the heart and vasculature, and many fundamental cardiovascular processes, such as blood pressure, display prominent circadian variations.\textsuperscript{5,4,84} An emerging interconnection between the circadian clock and oxidative stress, cellular senescence and apoptosis\textsuperscript{85–88} brings new aspect because all 3 are linked to cardiovascular pathology. When describing a link between the circadian clock and oxidative stress we have already mentioned its relation to development of cardiovascular disease. Indeed, circadian variations in antioxidant defenses were reported for rat heart, and increased oxidative stress has been correlated with the beginning of the active phase of daily cycle.\textsuperscript{89} It is tempting to speculate that if this is true for humans then daily variations in myocardial infarction\textsuperscript{90} could be attributed to clock-regulated levels of ROS. Additionally, it has been recently shown that the core clock component PER2 is an important mediator of vascular senescence, angiogenesis and endothelial function,\textsuperscript{53,91} all of which are involved in pathophysiology of the cardiovascular system.

In addition to representing a risk factor for cardiovascular disease, core circadian components might be important for the repair of cardiovascular tissue. The process of regeneration of blood vessels depends on proliferation of endothelial and smooth muscle cells, both of which harbor functional clock.\textsuperscript{54} Accordingly, the circadian proteins might be involved in regulation of the cell cycle checkpoints in a way similar to other tissues (ie, liver) and thus contribute to tissue repair. Recent report demonstrating the presence of functional circadian clock in cultured bone marrow–derived mouse and human mesenchymal stem cell provides the rationale for potential exploiting of the circadian machinery to facilitate heart repair.\textsuperscript{92,93}

Another important translational application of the components of the circadian machinery is based on the growing evidence for tissue-specificity of clock-mediated regulation of cellular functions. As discussed above, one of the major limitations of anticancer therapy is its low specificity resulting in severe damage to normal cells and tissues. Spectra and extent of tissue damage is specific for each drug. One of the oldest and still widely used anticancer drugs is doxorubicin (Adriamycin). Cardiac toxicity caused by doxorubicin is a serious dose-limiting factor in clinic. Doxorubicin-induced cardiotoxicity in rats depends on time of drug administration and is presumably linked to daily variations of the mitochondrial function and generation of ROS.\textsuperscript{84} At the same time, the antitumor effect of the drug is based on its function as an inhibitor of DNA topoisomerases. This provides an opportunity to increase therapeutic index by optimizing the timing of treatment. These examples clearly demonstrate that in addition to anticancer therapy, chronomodulated drug delivery might be exploited in treatment of cardiovascular diseases as well.

Conclusions

It is becoming more and more evident that circadian proteins play important roles in the processes of cell proliferation and control of response to genotoxic stress both at the cellular and organismal levels. The molecular systems and pathways affected by the circadian clock include extracellular signals, checkpoint control, cell proliferation, and DNA damage response, highlighting their importance and potential translational applications. Although, during recent years, significant progress has been made in our understanding of the mutual connection of the molecular clocks and cell death/proliferation, future studies are needed to establish detailed mechanistic links between these key regulatory pathways.

Sources of Funding

This work was supported by NIH grants GM5550926 and CA110522 (to M.P.A.) and American Heart Association Grant 0835155N (to R.V.K.).

Disclosures

None.

References


Circadian Proteins and Genotoxic Stress Response
Marina P. Antoch and Roman V. Kondratov

Circ Res. 2010;106:68-78
doi: 10.1161/CIRCRESAHA.109.207076

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/1/68

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/