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Pharmacological modulators of the circadian clock as potential therapeutic drugs: focus on genotoxic/anticancer therapy

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Abstract

The circadian clock is an evolutionary conserved intrinsic time-keeping mechanism that controls daily variations in multiple biological processes. One important process that is modulated by the circadian clock is an organism's response to genotoxic stress, such as that induced by anticancer drug and radiation treatments. Numerous observations made in animal models have convincingly demonstrated that drug-induced toxicity display prominent daily variations, therefore undesirable side effects could be significantly reduced by administration of drugs at specific times when they are better tolerated. In some cases, these critical times of the day coincide with increased sensitivity of tumor cells allowing for a greater therapeutic index. Despite encouraging results of chronomodulated therapies, our knowledge of molecular mechanisms underlying these observations remains sketchy. Here we review recent progress in deciphering mechanistic links between circadian and stress response pathways with a focus on how these findings could be applied to anticancer clinical practice. We discuss the potential for using high throughput screen to identify small molecules that can modulate basic parameters of the entire circadian machinery as well as functional activity of its individual components. We also describe the discovery of several small molecules that can pharmacologically modulate clock and that have a potential to be developed into therapeutic drugs. We believe that translational applications of clock-targeting pharmaceuticals are two-fold: they may be developed into drugs to treat circadian-related disorders or used in combination with existing therapeutic strategies to improve therapeutic index of a given genotoxic treatment via the intrinsic clock mechanism.

Molecular clocks in mammals

It is well recognized now that the circadian clock regulates almost every important biological process, including sleep-wake cycle, body temperature, metabolism as well as acute responses to stress (Antoch and Kondratov 2011; Chen and McKnight 2007; Rutter et al. 2002; Sack et al. 2007). A major function of the circadian system is to ensure temporal synchronization of various physiological, behavioral and metabolic processes within an organism and between an organism and its environment in order to achieve optimal performance. Disruption of proper synchronization results in development of various

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pathological conditions, including depression and bipolar disease (McClung 2007), sleep (Ptacek et al. 2007), metabolic (Gimble et al.) and cardiovascular disorders (Paschos and FitzGerald 2010). Several epidemiological studies have demonstrated an increased risk of cardiovascular disease, diabetes and cancer associated with abnormal working schedules resulting in desynchronization between the internal clock and the environment (shift-work, frequent travels across time zones, etc) (Salhab and Mokbel 2006; Szosland 2010; Wang et al. 2011). Furthermore, studies of animals deficient in individual components of the circadian machinery have identified numerous gene-specific pathologies, including metabolic defects, cancer and accelerated aging (Kondratov et al. 2007; Takahashi et al. 2008).

During the past 15 years following the cloning of the first mammalian circadian gene, *Clock*, (Antoch et al. 1997; King et al. 1997) enormous progress has been made in deciphering molecular details of clock operation. These advances are summarized is several excellent reviews addressing various aspects of circadian regulatory circuits in different species (Advances in Genetics 2011). Here we will give a brief outline of major mechanisms involved in generation of circadian rhythmicity at the cellular level in order to introduce key players and justify their potential use as perspective therapeutic targets.

At the molecular level, the circadian clock is comprised of a network of transcriptional and translational feedback loops that drive 24-hr-based oscillations in RNA and protein abundance of key clock components (Lowrey et al. 2011). At the core of the major circadian loop are two bHLH-PAS domain transcription factors CLOCK and BMAL1 that form a heterodimer to drive rhythmic expression of several genes harboring E-box elements in their promoter region. The negative arm of this loop includes three *Period* (*Per1*, *Per2* and *Per3*) and two *Crypto-chrome* (*Cry1* and *Cry2*) genes that inhibit CLOCK/BMAL1-driven transcriptional activation. A second loop involves rhythmic regulation of *Bmal1* gene transcription by two nuclear receptors, REV-ERBα (NR1D1) and RORα, both of which are transcriptional targets of CLOCK/BMAL1, and function respectively as a repressor and an activator competing for the same regulatory ROR element in the promoter of the *Bmal1* gene. In addition, the CLOCK/BMAL1 dimer regulates transcription of multiple clock-control genes with E-box regulatory elements in their promoter regions. Importantly, some of these CLOCK/BMAL1 targets in turn encode transcription factors (such as DBP, TEF, HLF, E4BP4), which work as transcriptional activators or repressors through a different binding element (D-box) (Schrem et al. 2004). As a result of this multi-level transcriptional regulation, as much as 10% of mammalian transcriptome display rhythmicity at the mRNA level (Panda et al. 2002). In mammals, the molecular clocks are operative in virtually all tissues thereby affecting a wide range of physiological and metabolic processes in a tissue-specific manner (Duguay and Cermakian 2009). Notably, the list of clock-controlled genes includes many key regulators of cell cycle, DNA repair and genotoxic stress response, and circadian oscillations in their concentration and/or activity would be expected to modulate sensitivity to stress and control cell cycle progression under normal and stress conditions (Kondratov and Antoch 2007).

Both positive and negative regulators of the major circadian loop are subject to various posttranslational modifications (phosphorylation, sumoylation, ubiquitination, acetylation),

which are important for functional activity, nuclear/cytoplasmic shuttling and stability of clock proteins. A number of enzymes have been associated with specific modifications of clock proteins and many of them are now considered as integral clock components. These chemical modifications generate a delay in CRY/PER-mediated repression to establish the ~ 24 h rhythms and provide fine tuning of the system (reviewed in (Kojima et al. 2011). The complexity of the entire system is further amplified by the involvement of post-transcriptional and epigenetic regulatory mechanisms (reviewed in (Lowrey et al. 2011) that together with transcriptional and posttranslational mechanisms are integrated into a multi-faceted and tightly regulated timing system that renders robustness and precision under constant conditions, and provides the necessary plasticity to effectively respond to environmental changes for better adaptation.

One important process that is modulated by the circadian clock is an organism's response to genotoxic stress, such as that induced by anticancer drug and radiation treatments. Pharmacological drugs, UV light and ionizing radiation are exogenous DNA damaging agents, which together with endogenous reactive oxygen species (ROS), collapsed replicative forks and spontaneous lesions of DNA (i.e. cytosine deamination) represent major causes of DNA damages. Under normal circumstances, mammalian cells and tissues may be exposed to DNA damage caused mainly by endogenous factors and to a certain extent, by UV light. Under unusual conditions, i.e. in the course of cancer treatment, various tissues within an organism are exposed to high doses of genotoxic agents. Both chemotherapy and radiation remain major therapeutic approaches directed towards elimination of tumor cells; unfortunately both approaches are non-specific and do not spare normal cells and tissues causing debilitating side effects.

Numerous animal model observations have convincingly demonstrated that drug-induced toxicity displays prominent daily variations; therefore undesirable side effects could be significantly reduced by administration of drugs at specific times when they are better tolerated. In some cases, these critical times of the day coincide with increased sensitivity of tumor cells allowing for a greater therapeutic index (reviewed in (Levi et al. 2010). The results of several clinical trials have confirmed the advantage of chronomodulated therapy over conventional regimens (Innominato et al. 2010). Unfortunately, despite encouraging results, chronotherapy has not yet become a general clinical practice, explained in part for the following reasons. The vast majorities of observations are of a descriptive nature and lack mechanistic explanation of the findings. Additionally, one might expect that chronomodulated therapy will require treatments at non-conventional times such as the night hours, which would require introducing significant changes in established working schedules of medical personnel. An alternative approach to overcome the latter problem would be to develop pharmaceuticals that reset the molecular clock in sensitive tissues to achieve higher resistance and therefore to allow for a greater therapeutic index. The rationale behind this approach was supported by studies of mice with genetic disruption of either positive or negative components of the circadian transcriptional feedback loop that displayed opposing responses to toxicity induced by chemotherapeutic drug cyclophosphamide suggesting that *in vivo* responses to genotoxic stresses can be modulated by the functional status of core clock components (Gorbacheva et al. 2005).

Circadian proteins as modulators of DNA damage (genotoxic stress) responses

Following exposure to DNA damaging agents, the cell has multiple response options. The cell may undergo growth arrest allowing for DNA repair, and if the damage is eliminated, the cell may return to its original normal state. If the cell fails to repair the DNA damage, it can be eliminated through apoptosis; alternatively cell proliferation before elimination of DNA mutations potentially leads to neoplasia and tumor development. Finally, the cell may respond by initiating the program of senescence (irreversible growth arrest). The DNA damaging response option depends on the type of tissue as well as on many extra- and intracellular factors. Recent data suggest that circadian proteins may be involved in this decision-making process. Below, we discuss new findings highlighting the role of circadian proteins at all steps following DNA damage including cell cycle regulation and checkpoint controls, DNA repair and senescence emphasizing the importance of circadian proteins as novel therapeutic targets.

1. The circadian clock in cell cycle and checkpoint control

Circadian gating of the cell cycle was observed decades ago in unicellular organisms (Edmunds and Funch 1969) and was proposed as a mechanism to prevent DNA replication at times of high exposure to UV light to protect genome from the accumulation of UV-induced mutations. Mechanistic links between the circadian clock and the cell cycle have been extensively investigated and major findings are summarized in several recent reviews (Khapre et al. 2010; Borgs et al. 2009). Here we will focus on the experimental evidences of the involvement of clock proteins in regulation of cell cycle progression after DNA damage.

Normal cell cycle progression requires several control checkpoints that serve as a surveillance mechanism of DNA lesions induced by endogenous (stalled replication folk, excessive production of ROS, etc.) and exogenous (DNA damaging drugs, UV light, ionizing radiation) factors. This mechanism provides cells with time to repair the damage, which is critical for maintaining the genome integrity and promote cell survival. In many tumors this pathway is de-regulated allowing for uncontrolled proliferation of tumor cells with multiple DNA lesions and leading to aberrant mitosis and cell death through the mechanism known as mitotic catastrophe (Galluzzi et al. 2007). Mitotic catastrophe has been reported as a prominent response of tumor cells to different anticancer drugs (Mansilla et al. 2006).

In normal cells, genotoxic treatments mainly target rapidly dividing cells (bone marrow, intestinal epithelium and hair follicles), resulting in common side effects, such as myelosuppression, mucositis and alopecia. All of these tissues are known to harbor functional clocks, and for some, daily variations in the distribution between different phases of the cell cycle have been described (Geyfman and Andersen; Hoogerwerf 2006; Mendez-Ferrer et al. 2009). Therefore, regulation of cell cycle checkpoints by the circadian clock may contribute to protection of normal tissues.

The sensing of DNA damage that results in cell cycle arrest is mediated by two important checkpoint protein kinases, ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) (reviewed in (Smith et al. 2010)). ATM is activated in response to DNA double-strand breaks, and phosphorylates numerous key regulators of cell cycle progression, including CHK2 kinase. It has been reported that circadian protein PER1 can interact with ATM/CHK2 complex and that this interaction is stimulated by ionizing radiation. Importantly, IR treated cells with siRNA suppression of PER1 are impaired in ATM activation and ATM-dependent phosphorylation of CHK2. Accordingly, the down regulation of *Per1* in human tumor cell lines makes them more resistant to anticancer drugs (Gery and Koeffler 2010).

Another interesting interconnection between the components of the circadian clock and cell cycle regulators involves the *Drosophila* homolog TIMELESS (TIM). Although the role of TIM in mammalian circadian clock is not well-defined, its association with core circadian proteins PER and CRY have been reported (Barnes et al. 2003; Field et al. 2000). Notably, human TIM interacts with the cell cycle checkpoint proteins CHK1, ATR and the ATR small subunit ATRIP and this interaction is stimulated by treatment with DNA damaging agents such as hydroxyurea and UV light. Moreover, down-regulation of TIM by siRNA results in reduced ATR-dependent phosphorylation of CHK1 both under normal and stress conditions (Unsal-Kacmaz et al. 2005). In complex with its non-circadian partner TIPIN (TIM interacting protein) TIM is involved in regulation of DNA replication and cell cycle progression (Gotter 2003). Functional analyses also revealed the importance of TIM/TIPIN complex in proper checkpoint control after DNA damage (reviewed in (Sancar et al. 2010).

Together, these data identified several components of the circadian clock that can modulate activity of cell cycle regulators under stress conditions and therefore can be viewed as potential targets for pharmacological manipulations directed towards alleviating cellular defects caused by DNA damage.

2. Circadian control of DNA repair

The first response of a mammalian cell after DNA damage is detected and proliferation has temporarily been restricted, is to repair lesions. In mammals, there are five major systems of DNA repair: nucleotide-excision repair (NER) and base-excision repair (BER) are responsible for the repair of the single strand brakes and specific lesions to base/nucleotide; homologues recombination (HR) and non-homologous end joining (NHEJ) are responsible for the repair of double strand brakes; and mismatch repair deals with insertions, deletions or A-G, T-C mismatches. Recent work has established a clear connection between core circadian proteins CRYs and NER.

CRY proteins belong to the family of cryptochrome/photolyases. Most likely, all member of these family evolved from a common ancestor CPD photolyase, an enzyme, which removes UV light-induced cyclobutane pyrimidine dimers from DNA. A common evolutional origin suggests functional interaction between the circadian clock and DNA repair systems. Indeed, in plants CPD-photolyases interact with and regulate the activity of CLOCK/BMAL1 complex similar to mammalian CRYs; moreover, they are able to compensate *Cry*-deficiency and restore circadian oscillation of gene expression in cultured cells and in the

liver (Chaves et al. 2011). Mammalian CRYs on the contrary, do not possess a photolyase-like activity and the removal of UV-damaged nucleotides in mammals depends solely on NER.

It has been demonstrated that NER of a UV photoproduct displays daily oscillations in the mouse brain and liver with a maximum and minimum values at ZT6 and ZT18, and ZT10 and ZT22 for the brain and liver respectively (Kang et al. 2010; Kang et al. 2009). Interestingly, in both tissues the maximum of NER activity coincided with the light phase of the cycle, which may reflect the adaptation to UV in the sunlight. In the brain, NER activity also coincides with daily oscillation in the levels of ROS resulting from the brain metabolic activity (Kondratova et al. 2010). This is not surprising given the fact that although the UV light and ROS produce different types of lesions (two major products of DNA oxidation are 8-oxyguanine and thymine glycol), they both are removed by the NER system.

In addition to UV- or oxidative stress-induced lesions, NER system is also capable of removing intra-strand diadducts caused by treatment with cisplatin compounds (cisplatin-d(GpG) and cisplatin-(GpXpG)). Cisplatin is a chemotherapeutic drug widely used to treat various types of cancers, including sarcomas, some carcinomas (i.e. small cell lung and ovarian cancers), lymphomas, and germ cell tumors (Kelland 2007). The repair of cisplatin-induced DNA damage displays daily oscillations in liver extracts with maximum and minimum activity at ZT10 and ZT22 respectively (Kang et al. 2010). Interestingly, NER activity appeared to be constant in testis, an organ that does not demonstrate prominent circadian oscillation and it is constitutively high in the livers of *Cry*-deficient mice. The latter result indicates that this repair system is activated by the disruption of clock through the *Cry*-deficiency, and suggests that the circadian clock down-regulates the activity of nucleotide excision repair at certain times of the day.

Mammalian NER is formed by six core repair factors: XPA, XPC, XPF, XPG, RPA and TFIIH. It has been demonstrated that circadian oscillations in NER activity correlates with the oscillation in protein expression levels of only one of these factors, Xeroderma pigmentosum A (XPA) suggesting that XPA is responsible for daily fluctuations in repair activity. Indeed, supplementation of liver lysates isolated at ZT18, when daily repair activity is at its minimum, with XPA restores the level of NER to that observed in the extracts isolated at ZT6. Direct measurement of *Xpa* transcript and protein levels in the liver has showed that both exhibit prominent daily oscillations. The mRNA for *Xpa* peaks at the time of maximum activity of CLOCK/BMAL1 suggesting direct regulation of *Xpa* gene expression by major circadian transactivation complex. In agreement with this, *Xpa* transcript is constitutively high in tissues of *Cry*-deficient mice and does not display oscillations in testis. Thus, the circadian clock regulates nucleotide excision repair in different tissues, most likely, through CLOCK/BMAL1-dependent control of *Xpa* gene expression (Kang et al. 2010).

Circadian regulation of NER was also detected in the mouse skin. Importantly, development of skin tumors after exposure to carcinogens strongly depends on time of exposure and directly correlates with oscillations in NER activity (Gaddameedhi et al. 2011). Together, these findings indicate a physiological significance for circadian regulation of DNA repair.

They also underscore the central role of CLOCK/BMAL1 functional activity in modulating cellular response to DNA damage and predict that in contrast to *Cry*-deficient mice, nucleotide excision repair may be significantly reduced in tissues of *Bmal1* knockout animals due to constant low levels of CLOCK/BMAL1-dependent transcriptional activity.

Since skin is the only mammal tissue that is exposed to light (including DNA damaging UV light), it raises the question of functional significance of the circadian control of NER in other tissues. One possibility is that this functional link is just a relic of the activity that had been advantageous at certain stage of evolution. Alternatively, NER may be involved in protecting cells from oxidative stress as it utilizes similar mechanisms of repair of oxidative lesions. Regardless of the answer, this newly discovered link between the circadian clock and DNA repair system provides an invaluable tool for therapeutic applications.

It is noteworthy that the above-described interactions of PER and TIM with ATM and ATR respectively may also affect ATM- and ATR-mediated homologous recombination, which is another mechanism of DNA repair in mammals. Although direct control of a double-strand brake (DSB) repair by the circadian clock has not been demonstrated yet, and the involvement of checkpoint kinases ATM and ATR in these processes is not fully understood (Smith et al. 2010), the fact of their interaction with core circadian proteins PER and TIM respectively allows suggesting their potential involvement in this process.

Indirect evidence for clock control of DSB repair comes from a recent study directed to identification of proteins that regulate checkpoint function, sensitivity to mitomycin C and efficiency of homologous recombination. The list of 24 strongest candidates includes CLOCK protein; moreover, in subsequent experiments in cells with laser-induced DNA damage, CLOCK was one out of just three proteins that co-localize with γ-H2AX, a well-known marker of DSB sites (Cotta-Ramusino et al. 2011). The latter discovery radically alters the perception of CLOCK protein as exclusively a circadian transcriptional regulator and suggests its involvement in the control of genotoxic stress response not only through transcriptional regulation of target genes but also through a transcription-independent mechanism. One possibility is that DBS DNA repair, which requires chromatin modifications, utilizes the intrinsic HAT activity of CLOCK protein (Doi et al. 2006). Alternatively, CLOCK may recruit other chromatin modifying or repair enzymes to the sites of DNA damage. For example, SIRT1, a deacetylase which is a well-known regulator of stress response (Rajendran et al. 2011) specifically interacts with CLOCK/BMAL1 complex (Nakahata et al. 2009; Ramsey et al. 2009) suggesting that CLOCK may be necessary for recruitment of SIRT1 to the sites of DNA lesions.

The transcriptional activity of CLOCK/BMAL1 complex can also be important for DNA repair-associated chromatin modifications. Indeed, expression of *Tip60*, a member of MIST family of histone acetylases that is involved in DSB repair in yeast (Sun et al. 2010), is directly regulated by CLOCK/BMAL1 via circadian E-box elements in its promoter (Miyamoto et al. 2008). Regardless of the exact mechanisms, these new findings define the core circadian protein CLOCK as a regulator of several mechanisms of DNA repair that is induced by various genotoxic agents, and warrants additional investigation (Kang and Sancar 2009).

3. Circadian clock and senescence

There is growing evidence that deficiency in certain circadian proteins leads to initiation of the senescence program. Indeed, *Bmal1−/−* and *Clock/Clock* mice develop a phenotype of premature aging, the former naturally in life (Kondratov et al. 2006), whereas the latter after challenge by ionizing radiation (Antoch et al. 2008). In agreement with the development of premature aging, increased amount of senescent cells are detected in vasculature of mice with a mutation in the *Per2* gene (Wang et al. 2008) and in the liver, lung and spleen of *Bmal1*-deficient mice (Khapre et al. 2011). Most likely, accumulation of senescent cells in circadian mutants is associated with stress-induced rather than with replicative senescence. At this moment it is unclear if an increase in senescence is caused by a deficiency in a specific clock protein(s) or by desynchronization of cellular metabolic processes that is induced by deregulation of clock. Most likely both processes contribute to development of senescence as it is observed in different circadian mutants although the severity of the phenotype varies. Stress-induced senescence has been proposed as one of the mechanisms for tumor suppression (Campisi 2005) and it is likely that regression in size in many tumors in response to chemotherapy results from activation of their senescence program. In this respect, involvement of some circadian proteins in development of senescent phenotype provides additional argument in support of their therapeutic potential.

It is important to emphasize though, that many senescent cells retain their metabolic activity and can secrete many factors affecting an organism's physiology including those that promote tumorigenesis. Such a dual role of senescence in promoting both tumor suppression and tumor development may depend on conditions (normal versus stress-induced) as well as type of tissue and may explain an existing controversy regarding the role of clock proteins in tumorigenesis. Indeed, mice with mutations in circadian protein PER2 were reported to display a cancer-prone phenotype resulting from decrease in *p53*-dependent apoptosis following exposure to ionizing radiation (Fu et al. 2002). At the same time, *Clock* mutant mice respond to ionizing radiation by accelerating their aging and do not develop tumors (Antoch et al. 2008), whereas the deficiency in both CRY proteins rescues tumor-prone phenotype of *p53*-null mice (Ozturk et al. 2009). It is possible that, depending on the type of circadian deficiency and the methods used to induce tumors in experimental mouse models, exposure to genotoxic agents and activation of senescent program in normal cells can stimulate tumor growth and at the same time suppress growth of transformed cells.

In summary, recently established interaction between the components of molecular clock, cell cycle regulation, genotoxic stress response and tumorigenesis opens novel perspectives both in anti-cancer treatment and tumor prevention. More studies are needed to refine molecular mechanisms of clock-mediated regulation of stress response pathways and to resolve multiple contradictions currently existing in the field. However, the very fact of the established cross-talk among these metabolic processes underscores the importance of circadian proteins as targets for therapeutic applications.

Search for pharmacological modulators of circadian clock by high-throughput chemical screen

High-throughput screening of libraries of small organic molecules is one of the most effective tools for the discovery of bioactive compounds. The pioneer work of Balsalobre et al (Balsalobre et al. 1998), which demonstrated that cultured cells could display rhythms in circadian gene expression after short treatment by high serum concentration, initiated a cascade of experiments performed in different laboratories that resulted in the identification of several compounds that could affect circadian function. Thus, circadian oscillation in cultured cells can be induced by the glucocorticoid receptor agonist, dexamethazone (Balsalobre et al. 2000a), by the activator of adenylate cyclase, forskolin (Yagita and Okamura 2000), phor-bol-12-myristate-13-acetate (PMA), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, calcium ionophore calcimycin (Balsalobre et al. 2000b), endothelin (Yagita et al. 2001), glucose (Hirota et al. 2002) and pros-taglandin E2 (Tsuchiya et al. 2005). Furthermore, several intracellular small molecules such as NAD (Nakahata et al. 2009; Ramsey et al. 2009), heme (Raghuram et al. 2007; Yin et al. 2007) and cAMP (O'Neill et al. 2008) can function as circadian modulators. The differences in rhythm inducing properties, which was revealed by comparative quantitative analysis of ten individual signaling compounds (Izumo et al. 2006) indicated that all of them likely exert their action through different pathways. Together, these work provided a proof-of-principle for performing large-scale screens in a cell-based assay to identify more specific chemicals that can modulate regulators of the circadian oscillator.

1. Small molecules affecting circadian parameters in cultured cells

Two types of experimental approaches have been reported so far. The first one is based on a real-time recording of a circadian reporter activity in cells with synchronized circadian rhythms (by serum shock, dexamethasone or forskolin). In this experimental setup, chemical compounds are tested for their effect on basic circadian parameters (circadian period, amplitude and phase of rhythmicity). This screening paradigm was first tested in a small-scale screen of 1,280 structurally diverse chemicals present in commercially available Library of Pharmacologically Active Compounds (LOPAC, Sigma). It resulted in identification of small molecule inhibitors of glycogen synthase kinase 3β (GSK-3β) that mediated a shortening of the period of circadian oscillation in osteosarcoma U2OS cells stably expressing *Bmal1*-Luc reporter (Hirota et al. 2008). In mammals, GSK-3β has been previously identified as a kinase that directly phosphorylates several core clock proteins including PER2, CRY2, REV-ERBα, CLOCK and BMAL1 (Harada et al. 2005; Iitaka et al. 2005; Sahar et al.; Yin et al. 2006; Spengler et al. 2009), which leads to their degradation (in case of CRY2, CLOCK and BMAL1), increased nuclear translocation (PER2) or stabilization (REV-ERBα).

Further development of this approach was applied to a circadian screen of ~120,000 uncharacterized compounds, and resulted in identification of a small molecule (named longdaysin) that potently lengthened the circadian period in a variety of cultured cells and in explants of mouse suprachiasmatic nucleus (SCN), the site of central pacemaker in mammals (Hirota et al. 2010). Importantly, long-daysin also affected the circadian period *in*

vivo in transgenic zebrafish expressing circadian reporter. The combination of pharmacological, mass spectrometry and siRNA-mediated gene suppression approaches revealed that longdaysin targets several protein kinases, CK1δ, CKIα and ERK2. Whereas CK1δ and ERK have been identified previously as clock-regulating kinases, the role of CK1α in circadian control was unknown. It appeared that CK1α directly phosphorylated PER1 protein and similar to CK1δ promotes its degradation (Hirota et al. 2010).

In addition to several smaller screens that specifically focused on the effects of protein kinase inhibitors (Isojima et al. 2009; Yagita et al. 2009), the above-described large-scale screens mainly identified compounds affecting period of circadian oscillations. A recent novel screen targeted other circadian parameters, such as amplitude of rhythmicity (Chen et al. 2012). This study was performed on immortalized mouse fibroblast cells derived from *Per2::lucSV* reporter mice, synchronized by forskolin treatment, and involved testing of ~200,000 synthetic small molecules. Several hits were identified from these screens that in addition to period shortening caused a significant increase in the amplitude of oscillation, which correlated with an increase in expression of two clock output genes, *Dbp* and *RevErbα*. An interesting class of compounds, which have not been previously characterized, mediated an acute induction of *Per2*-driven luciferase signal followed by significant phase delay of oscillation, which was somewhat similar to the effect of forskolin in SCN slices (O'Neill et al. 2008). Further analysis revealed that these chemicals indeed induced intracellular cAMP levels. Their effect of circadian oscillation appeared to be very complex, which likely reflects the fact that cAMP is involved in regulation of numerous pathways.

Together, the above-described screens identified a number of compounds belonging to different chemical classes that affected circadian oscillatory parameters including several molecules with unknown biological function. The fact that they display diverse activities and affect different circadian parameters suggests multiple molecular mechanisms involved. These compounds have served as important tools for probing different regulatory mechanisms involved in circadian regulation and have already led to identification of novel players in circadian circuit, such as CK1α (Hirota et al. 2010) with a potential to discover more. They also have a potential to be developed into drugs for treating circadian-related pathologies. Thus, many of circadian disorders are associated with dampened clock, as for example, ones related to *Clock*Δ*19* mutation (Marcheva et al. 2010; Vitaterna et al. 2006). In this respect, the small molecules identified by these screens and tested for their ability to restore amplitude of oscillation in fibroblasts as well as in pituitary and SCN derived from *Clock*/+ mice, represent perspective prototype drugs (Chen et al. 2012). In addition, the kinase regulators affecting circadian function via control of the phosphorylation status of PER have a potential for further development for treatment of pathologies such as Familial Sleep Phase Syndrome (FASPS) (Vanselow et al. 2006).

2. Screen for small molecules affecting the functionality of CLOCK/BMAL1 transcriptional complex

In addition to their roles as components of a molecular circadian oscillator, many (if not all) core clock proteins have been ascribed clock-independent physiological functions (Yu and Weaver 2011). The impairment of any of these functions in experimental systems leads to

development of various pathologies that often are related to various human diseases. Therefore, the identification of functional small molecule regulators of individual clock proteins that may not be necessarily linked to their circadian function may provide a more specific therapeutic drug. Such an approach has been recently used in a screen for modulators of CLOCK/BMAL1 transcriptional activity (Hu et al. 2011). The rationale for this approach was based on a previously published work that linked the acute response of genotoxic treatment in different circadian mutant mice with the functional status of the major circadian regulator, CLOCK/BMAL1 transcriptional complex (Gorbacheva et al. 2005). These studies have demonstrated that the different types of circadian mutants (*Clock* mutant mice, *Bmal1* knockout and *Cry* double-knockout mice) although all behaviorally arrhythmic, displayed an opposite response to toxicity induced by the chemotherapeutic drug cyclophosphamide (CY). The animals with a deficiency in circadian activators (CLOCK and BMAL1), which results in constant low levels of clock-controlled gene expression, were extremely sensitive to CY-induced toxicity; whereas mice with deficiency in circadian repressors CRYs, which results in constant high levels of CLOCK/BMAL1-mediated transcription, were very resistant to the treatment. These data highlight the importance of identifying the specific components of the circadian mechanism that are being targeted by circadian-modifiers in order to elicit the desired therapeutic response and indicate that for many therapeutic applications it is important to recognize not only the fact of circadian disruption, but to identify deficiency of which component caused this disruption. This data also allowed to define circadian transcriptional activators as potential targets for pharmacological modulation aimed at protecting normal tissues from damage induced by genotoxic treatments.

A small-scale screen for modulators of CLOCK/BMAL1-dependent transactivation was performed in a readout system based on mouse fibrosarcoma L929 cells expressing high levels of endogenous CLOCK and BMAL1 and stably expressing *Per1*-driven luciferase reporter. Two commercially available libraries, LOPAC (Sigma, 1200 compounds) and Spectrum (library of 2000 natural compounds, MicroSource Discovery, Inc), were used to screen for activators and inhibitors of CLOCK/BMAL1-mediated expression of *Per1* gene (Antoch and Chernov 2009). Importantly, this screen identified several known regulators of circadian function such as glucocorticoids, 2-methoxyestradiol, forskolin, PKC and p38 MAPK inhibitors as well as some hits identified by circadian-driven approach (Hirota et al. 2008), all of which validated the feasibility of the approach. It also identified several chemicals that have not been previously linked to circadian function including the organic selenium compound, L-methyl Selenocysteine (MSC) (Hu et al. 2011).

Selenium is an essential trace element that has two major clinical applications: tumor prevention and protection against DNA damage induced by anti-cancer therapy. Studies in cell-based model systems, as well as several clinical trials, have conclusively demonstrated that selenium supplementation ameliorates radiation-induced mucositis in mice treated with fractionated doses of ionizing radiation (Gehrisch and Dorr 2007) as well as radiation-induced diarrhea in treatment of patients with cervical and uterine cancers (Muecke et al. 2010). It has been determined that the observed increase in *Per1*-driven luciferase in cells treated with MSC is caused by selenium-mediated transcriptional up-regulation of the *Bmal1*

promoter resulting in increase in BMAL1 protein and presumably of the transactivation potential of CLOCK/BMAL1 complex. Mechanistically, the effect of selenium was attributed to its ability to prevent binding of Glucose-inducible gene 1 (*Tieg1*), an Sp1 family transcription repressor involved in *Bmal1* regulation (Hirota et al. 2007), to Sp1-binding sites in *Bmal1* promoter (Hu et al. 2011). Importantly, the effect of selenium on BMAL1 protein abundance was detected not only in cells, but also *in vivo* in mice that receive the compound either through a single injection or systemically via gavage or selenium-supplemented diet. Interestingly, the in vivo effect of selenium was found to be tissue-specific in that selenium-induced changes in BMAL1 were detected in the liver, but not in the SCN; consistent with this, no changes in circadian behavioral parameters were detected. This finding is reminiscent with characteristics of small molecules identified in circadian-based screens. Originally identified as circadian modulators in synchronized fibroblasts, they often display tissue-specific variations when tested in explants derived from different tissues (Chen et al. 2012). From the therapeutic standpoint, this may present a huge advantage rather than a weakness as it allows for modulation of response to genotoxic treatments in a tissue-specific manner without disturbing the central clock.

Notably, through the upregulation of BMAL1 selenium administration alleviated CY-induced toxicity in drug-sensitive *Clock* mutant mice as displayed by increase in their survival rate and decreased levels of myelosuppression. In contrast, selenium failed to produce these ameliorating effects in mice with genetic disruption of the *Bmal1* gene, thus confirming that the rescuing effect of selenium *in vivo* is mediated, to a large extend, through BMAL1. Together, these findings provide a plausible mechanism behind tissue protective effects of selenium by linking it to circadian regulation of gene expression, and suggest that selenium is capable of tuning circadian transcriptional machinery to the higher activity, which is associated with maximum resistance by upregulating BMAL1 expression.

Although the exact mechanism, by which the increase in CLOCK/BMAL1 activity ameliorates CY-induced toxicity, is not fully understood, this work presents the first example of protection of normal tissue from drug-induced damage through the components of the molecular clock. Based on known targets of CY-induced toxicity, one could predict that an important factor in determining the *in vivo* drug response and host survival in clinical therapy is CLOCK/BMAL1-dependent modulation of the lymphocyte survival/recovery rate. Consistent with this, studies with *Bmal1*$^{-/-}$ mice revealed the involvement of BMAL1 in differentiation of pre-B to mature B cells, although direct molecular targets are still not known (Sun et al. 2006). Another potential mechanism may involve BMAL1-dependent regulation of ROS homeostasis (Kondratov et al. 2006), which would protect against excessive accumulation of ROS in response to genotoxic stress and thereby ameliorate drug-induced tissue damage. However, regardless of a precise molecular mechanism, the reported ability of selenium to modulate activity of circadian transcriptional complex, without affecting central clock, opens new possibilities for clinical applications. If previously clock-targeting pharmaceuticals were considered mostly as resetting agents (with the goal to reset molecular clocks in drug- and radiation-sensitive tissues to times of higher resistance to genotoxic treatment), selenium compounds demonstrate the ability to minimize the

damaging effects of genotoxic treatments by a constant up regulation of circadian transcriptional activators in a tissue-specific manner.

Another example of the therapeutic value of drugs targeting CLOCK/BMAL1 functional activity came from series of studies in *Cry*-deficient mice. It has been found that disruption of the circadian clock by a *Cry* mutation in *p53*-null background makes them more sensitive to UV light-induced apoptosis (Ozturk et al. 2009). Mechanistically, this increase accounted for CLOCK/BMAL1-mediated upregulation of *p73*-dependent apoptosis. It was shown that in the absence of *p53* (the primary tumor suppressor) (Lowe et al. 1994), down-regulation of *Cry* enhances expression of another member of the *p53* family, *p73* (Stiewe 2007), and subsequently enhances UV-mediated apoptosis, elimination of damaged cells and reduces risk of cancer. Up-regulation of *p73* in the absence of *Cry* correlates with increased levels of *Early growth response 1* (*Egr1*) gene, which works as a positive activator of *p73* (Yu et al. 2007), and which itself is directly regulated by CLOCK/BMAL1 transcriptional complex. Consistent with this, *Egr1* levels are constantly elevated in *Cry*-deficient cells and this up-regulation is reversed by down-regulation of BMAL1 (Lee and Sancar 2011). Chromatin immunoprecipitation experiments have also demonstrated that BMAL1 binds the *Egr1* promoter and that although both positive (EGR1) and negative (C-EBPα) regulators of *p73* are present on *p73* promoter, only EGR1 remains bound upon exposure to UV light (Lee and Sancar 2011). Importantly, when tumor xenografts induced by oncogenically transformed *p53*−/− and *p53*−/−*Cry*−/− cells were treated with oxaliplatin, a chemotherapeutic drug widely used to treat many forms of metastatic cancers, it suppressed tumor growth in *p53*−/−*Cry*−/− tumors but display no therapeutic effect on the growth of *p53*−/− tumors. Together, these data provide a plausible mechanism for the sensitization of tumor cells that are often deficient in *p53* function to cytotoxic drugs through activation of *p73*-dependent apoptotic program mediated by CLOCK/BMAL1 circadian regulators (Lee and Sancar 2011).

Concluding remarks

In conclusion, we would like to emphasize that cancer treatment involves frequent use of highly toxic compounds that commonly induce severe adverse effects resulting in reduced efficacy of therapy, creating risks of acquisition of additional diseases and reducing quality of life of cancer patients. In this respect, clock-targeting pharmaceuticals represent a huge potential that is still under-estimated and therefore not yet developed. However, to fully exploit circadian mechanism for increasing therapeutic index of anticancer treatment, it is important to define the functional status of clock proteins in tumor cells and tumors. Whereas the functionality of molecular clocks in normal tissues has been extensively studied and recently resulted in significant breakthroughs, our knowledge of circadian status of tumor cells and tumors are still sporadic and often controversial. There is growing evidence that at least in part, the controversy may arise from the fact that circadian proteins play different roles in normal and tumor cells. Thus, both CLOCK and BMAL1 are positive regulators of the cell cycle in normal cells such as hepatocytes (Grechez-Cassiau et al. 2008), hair follicles (Lin et al. 2009) and embryonic fibroblasts (Miller et al. 2007). At the same time, it has been reported that BMAL1 is necessary for *p53*-dependent growth arrest in human tumor cell lines in response to DNA damage. Accordingly, suppression of *Bmal1*

decreases induction of *p21*, impairs growth arrest and sensitizes tumor cells to DNA damaging agents (Mullenders et al. 2009). Consistent with its role as a mediator of growth arrest, BMAL1 is epigenetically silenced in several hematological malignancies, which may contribute to tumor growth (Taniguchi et al. 2009). These controversial data indicate that more work is required to better understand mechanistic differences in circadian modulation of stress response pathways in normal and tumor cells. However, even the first examples of such differential regulation are encouraging as they suggest the potential to develop therapeutic approaches that target an individual clock component that elicits both an in increased resistance of normal cells and increased sensitivity of tumors.

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