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# Deep Insight Section

# The contribution of circadian rhythms to cancer formation and mortality

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## Abstract

Cellular circadian clocks represent a coordinated system of gene expression stimulated by both environmental and physiological cues that induces and maintains the rhythmicity of many metabolic processes. Circadian clocks confer the important benefit of anticipation of rhythmic environmental variation which serves to improve the health and survival of the organism. When disruption of these circadian patterns of gene expression occurs due to alterations in the physical (light/dark) and behavioral (feeding/sleeping) environments and/or due to genetic variation in the DNA sequence of clock component and clock regulated genes, negative health consequences can arise. One such consequence appears to be increased risk for cancer development. Circadian disruption has been associated with higher rates of tumorigenesis, faster tumor growth, and increased cancer severity in humans and animal models. Tumor formation is also associated with circadian disruption within the affected cells, and metabolic processes of the cancer host tend to lose their rhythmicity as the cancer becomes more severe. In addition, response to cancer treatment has been shown to have a time-dependent component in certain individuals. Knowledge of the type of circadian disruptions that induce or result from cancer can allow for temporally augmented treatments of cancer, ultimately making cancer treatments more effective and less harmful.

## Introduction

Circadian rhythms refer to daily organismal fluctuations that occur consistently within a 24-hour cycle and correspond with anticipated changes in the environment. These rhythms, which include sleeping patterns, oscillation of gene expression, and varied secretion of hormones, are present in nearly all organisms (Bell-Pedersen et al., 2005; Kondratov et al., 2007). They have likely evolved because of their ability to confer the benefit of anticipation. When a specific type of stimulus is encountered repeatedly and consistently, organismal activity can begin to predict the stimulus and prepare for it, aiding in survival (Bell-Pedersen et al., 2005; Ko and Takahashi, 2006). These rhythms continue to approximate 24 hour cycles even in the absence of timed environmental stimuli (Kondratov et al., 2007).

A circadian clock is a transcriptional timing mechanism that is present ubiquitously in mammalian cells such that isolated cells can maintain oscillatory activity and function (Kondratov et al., 2007). This mechanism is governed by multiple highly conserved positive and negative feedback loops whose cycles approximate 24 hours (see figure 1). In the positive component of the feedback loop, BMAL1 forms a heterodimer with CLOCK, which then binds to E-box elements in promoter regions to drive the transcription of both *bmal1* and *clock* genes (Bell-Pedersen et al., 2005; Ko and Takahashi, 2006; Kondratov et al., 2007; Stow and Gumz, 2011). In addition, the BMAL1/CLOCK dimer drives transcription of the period (*per*) and cryptochrome *(cry*) gene families, retinoic acid orphan receptor alpha (*rorα*), and nuclear receptor subfamily 1 group D (*nr1d2* or *rev-erbα*) (Ko and Takahashi, 2006; Kondratov et al., 2007; Stow and Gumz, 2011). PER and CRY proteins then create the negative feedback loop by forming a complex which acts to inhibit the transcription mediated by the BMAL1/CLOCK dimer, ultimately resulting in downregulation of their own

production (Kondratov et al., 2007; Stow and Gumz, 2011). RORA and REV-ERBα interact with ROR elements to enhance and inhibit *bmal1* expression, respectively (Kondratov et al., 2007; Stow and Gumz, 2011). In addition, the timeless (*tim*) gene has been speculated to play a role in the maintenance of circadian rhythms as well, although its function may be more important in altering rhythms based on external stimuli (Kondratov et al., 2007; Engelen et al., 2013). The interplay of these feedback loops ultimately leads to consistent, daily cycles of gene/protein expression that can be maintained within each cell (Ko and Takahashi, 2006; Kondratov et al., 2007; Stow and Gumz, 2011).

Nearly all cells have a clock mechanism, but the suprachiasmatic nucleus (SCN) or "master clock" acts to set the central clock in the brain by aligning its rhythms with light signals registered by the retina and transmitted through the retinohypothalamic tract (Bell-Pedersen et al., 2005; Ko and Takahashi, 2006; Kondratov et al., 2007). While the SCN contributes to the synchronization of cellular circadian mechanisms in the peripheral tissues of the body, neurohumoral signals driven in large part by food intake are thought to be the primary entrainers of peripheral clocks (Bell-Pedersen et al., 2005; Kondratov et al., 2007). Although multiple stimuli and activities can entrain the circadian system in lieu of light, light is commonly used to experimentally induce circadian disruption (Bell-Pedersen et al., 2005; Kondratov et al., 2007).

## Cellular mechanisms linking circadian rhythms to cancer

Multiple studies have provided compelling evidence that both central and peripheral circadian clocks regulate many energy homeostatic functions, including insulin sensitivity, endocrine regulation, satiety signaling, cellular proliferation, and cellular substrate metabolism. Thus, disruption of the circadian clock (e.g., via dyssynchrony between light/dark cycles and sleep/wake/feeding behavior) has the potential to induce a host of disease states, including cancer. To examine whether circadian disruption can induce or exacerbate the

development of cancer at the cellular level, several studies have examined and/or manipulated the expression of core circadian and related genes in human cell models of cancer. *Clock*, *Bmal1*, *Cry1*, and *Per* family gene expression have all been shown to be upregulated when host cells are exposed to radiation, and PER family proteins appear to play a role in DNA damage control and tumor suppression (Fu et al., 2002; Gery et al., 2006; Rana and Mahmood, 2010).

Transfection of a *Per1* overexpression vector into cultured human colon cancer cells exposed to ionizing radiation led to greater DNA damage-induced apoptosis than cells transfected with an empty vector (Gery et al., 2006). In contrast, small interfering RNA (siRNA) inhibition of endogenous *Per1* expression led to less DNA damage-induced apoptosis, creating conditions which could readily allow for the replication of damaged cells and tumor formation, and revealing that even endogenous levels of PER1 have an oncostatic effect (Gery et al., 2006). Similar in vitro effects were seen when a *Per2* expression plasmid was transfected into human leukemia cells, with increased PER2 causing an increase in cell growth arrest, apoptosis, and an upregulation of the tumor suppressing protein p53 while siRNA knockdown of *Per2* led to a downregulation of p53 (Sun et al., 2010).

While PER1 and PER2 have shown evidence of oncostatic effects, surprisingly, CRY1 may have an oncogenic effect. Contrary to some of the previous findings examining *Cry* gene family expression in cancerous tissue, Yu et al. found *Cry1* mRNA overexpression in 8 of 10 paired tissue samples from patients with colorectal cancer (Yu et al., 2013).

Additionally, they reported high CRY1 expression in 101 of 168 paired samples, low expression in the other 67, and a correlation between the cancer stage and CRY1 expression, with more severe diagnoses being associated with higher CRY1 expression levels (Yu et al., 2013). To explore the role of CRY1 in tumorigenesis, *Cry1* was overexpressed in a human colorectal cancer cell line, and showed that cells transfected with a *Cry1*-expressing plasmid demonstrated significantly more cellular growth, proliferation, and increased migration capacity than those transfected with an inert control (Yu et al., 2013). In addition, siRNA knockdown of endogenous *Cry1* expression inhibited cell colony formation and reduced migration capacity (Yu et al., 2013). These studies point to a putative non-circadian function of *Cry1* in cancer etiology.

*Bmal1* also appears to play a role in cancer risk and oncogenesis.

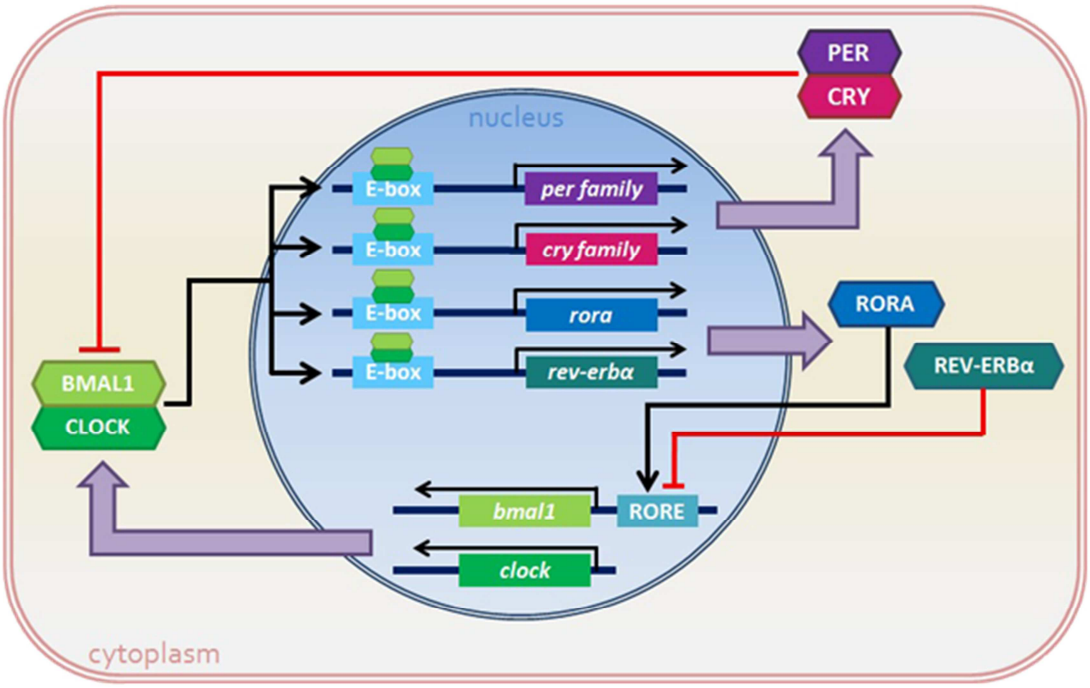


**Figure 1. The BMAL1/CLOCK heterodimer drives the transcription of several genes.** PER and CRY family proteins form heterodimers that inhibit the actions of the BMAL1/CLOCK heterodimer. RORA and REV-ERBα act to activate and inhibit *bmal1* transcription, respectively. Adapted from Stow and Gumz, 2011 (JASN).

*Bmal1* deficient human cells are less likely to undergo cell cycle arrest due to DNA damage, and knockdown caused by *Bmal1* targeting small hairpin RNAs (shRNAs) reveal a potential modulatory effect of p53 on p21, a process which may be involved in DNA-damage induced apoptosis (Mullenders et al., 2009; Rana and Mahmood, 2010). In murine colon cancer cells, shRNA *bmal1* knockdown produced similar results, including increased cell growth, decreased apoptosis, as well as downregulation of *Per* family genes and upregulation of *rev-erbα* (Zeng et al., 2010). The protein DBC1 (deleted in breast cancer 1), which is speculated to be associated with tumorigenesis, has been shown to regulate and increase REV-ERBα expression and stability as well as modulate its transcription repression activity on *Bmal1* (Chini CC et al., 2013; Chini EN et al., 2013). In human embryonic kidney cells, the tandem transfection of *Dbc1* and *Rev-erbα* plasmids resulted in lower *Bmal1* transcription and cell cycle arrest than the transfection of either plasmid alone or a control plasmid (Chini EN et al., 2013). Independent of the interaction of DBC1 with circadian genes, overexpression of DBC1 has been reported in several different cancers and is correlated with poorer prognoses in breast carcinoma, lymphoma, colorectal cancer, gastric carcinoma, and others (Cha et al., 2009; Lee et al., 2011; Kim et al., 2012; Park et al., 2013; Zhang et al., 2013). The downstream effects of DBC1 on REV-ERBα and subsequently on *Bmal1* can lead to disruption of typical protein oscillations, which could be a factor in the physiological effects on tumor growth associated with the deletion of DBC1 (Chini EN et al., 2013).

## Circadian gene dysregulation in cancerous tissue

The relationship between circadian rhythms and cancer is also seen at the cellular level when comparing cancerous to non-cancerous human tissue. Both circadian rhythmicity and core circadian gene expression appear to be disrupted in cancerous cells. Several circadian genes have been shown to be correlated with poor prognosis when overexpressed in various cancers, including *Cry1* in colorectal cancer and *Timeless* in lung cancer (Rana and Mahmood, 2010; Yoshida et al., 2013; Yu et al., 2013). A series of studies using tissue from 30-70 patients with subcortical gliomas showed abnormal expression patterns of critical circadian genes in high-grade versus low-grade gliomas and high-grade gliomas compared to surrounding non-glioma tissue, in which higher levels of CLOCK and lower levels of PER1, PER2, CRY1, CRY2 and corresponding mRNA were reported (Xia et al., 2010; Luo et al., 2012; Chen et al., 2013). Tissue samples from 32 patient skin biopsies revealed lower mRNA levels of the same circadian genes excluding *Cry2* that were seen in of both malignant melanoma and nonmalignant nevus tumors compared to surrounding skin tissue (Lengyel et al., 2013). Downregulation of PER1 was also shown in tissue collected from 38 patients who had been diagnosed but not yet treated for buccal squamous cell carcinoma;

this effect was significantly correlated with increased risk of metastasis and clinical stage, where more severe risk and diagnosis corresponded with more PER1 downregulation (Zhao et al., 2013). Nevertheless, the studies described above have their limitations since they all collected their human tissue samples from a single three hour period and did not categorize tissue by collection time (Zhao et al., 2013). While these studies do not measure the target genes over time due to practical considerations of subject burden, since they all compare the cancerous tissue to non-cancerous tissue collected at the same time from the same patient, the compared samples do provide important information related to protein abundance of circadian components and cancer risk.

Recently, a study by Chen et al. used 55 samples of breast cancer and paired non-cancerous tissue collected from 5 different time points from 10am until 8pm and reported differential expression of at least one PER protein for all type II or type III breast cancer tissue samples compared to their paired control tissue (Chen et al., 2005). The overall expression of the samples was not lower as they were in the previous studies, which could be due to variability in collection time points, although the fact that distinct cell populations of the same cancer tissue expressed different PER expression could indicate heterogeneity even within breast cancer cell populations (Chen et al., 2005). An abnormal pattern of circadian gene expression was also observed in tissue from 38 patients with diagnosed but not treated colorectal carcinoma collected across 6 time points between 10am and 10pm; PER2 immunostaining showed a heterogeneous expression pattern in the cancerous tissue compared to the homogeneous expression seen in paired non-cancerous tissues, with a trend of decreased PER2 expression in cancerous versus non-cancerous tissue (Wang et al., 2011). A study by Lin et al. examined mRNA expression of several circadian genes, including *Per* and *Cry* family genes, as well as *Clock*, *Bmal1*, and *Timeless*, and revealed significant decreases in levels of *Per*, *Cry2*, and *Timeless* expression in hepatocellular carcinoma samples collected from 46 patients in one of five time points from 8am to 6pm (Lin et al., 2008). Heterogeneous immunostaining patterns were also apparent in many of the cancerous as opposed to paired non-cancerous tissue samples, and the number of genes whose expression was disrupted was positively correlated with tumor size (Lin et al., 2008). These studies reveal that cancerous tissues show both disrupted and less homogenous expression of circadian genes than their non-cancerous counterparts, but cannot convey causal relationships between these factors.

## Transgenic mouse models

As convincing as the accumulation of human and cellular data may be, animal models can provide even more insight by allowing for genetic manipulations and near complete control of environmental factors. Transgenic mouse models with functional disruptions of circadian genes have many phenotypes that parallel the pathologies seen in humans experiencing consistent circadian disruptions. *Clock* dominant negative Δ19 mutant mice show hypoinsulinemia, hyperglycemia, and obesity, consistent with the high risk of diabetes and obesity seen in humans (Kondratov et al., 2007). *Bmal1*$^{-/-}$ mice show premature aging, sterility, metabolic defects, and shorter average lifespan than their wild-type counterparts (Kondratov et al., 2007). Interestingly, mice with only one copy of *Bmal1* (*Bmal1*$^{+/-}$) exhibit increased spontaneous tumors as well as an increased sensitivity to tumor formation after irradiation (Lee et al., 2010). *Per2*$^{-/-}$ mice show a decrease in DNA damage response along with higher rates of tumor formation from exposure to low levels of ionizing radiation, which coincides with the human data showing a downregulation of *Per* expression in cancerous versus non-cancerous tissue (Kondratov et al., 2007; Rana and Mahmood, 2010). In addition, m*Per2*$^{m/m}$ homozygous mutant mice have higher mortality rates and are naturally cancer prone (Fu et al., 2002). The m*Per2*$^{m/m}$ mice also have higher sensitivity to the effects of radiation, including a more than ten-fold increase in the likelihood of cancer induction, and show resistance to radiation induced-apoptosis, further emphasizing the importance of a functional PER2 in tumor suppression (Fu et al., 2002). Knockout mice lacking both *Per1* and *Per2* or both *Cry1* and *Cry2* were shown to have higher rates of spontaneous tumor formation, higher rates of tumorigenesis after irradiation, and increased tumor formation and growth when exposed to continuous, alternating phase advances and delays (Lee et al., 2010). However, *Per1*$^{-/-}$, *Cry1*$^{-/-}$, *Cry2*$^{-/-}$ and *Rev-erbα*$^{-/-}$ mice do not have strong phenotypes, with *Per1*$^{-/-}$ mice showing increased drug sensitivity but little else, both *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice exhibiting high bone mass but no metabolic or tumorigenesis abnormalities, and *Rev-erbα*$^{-/-}$ mice being phenotypically indistinguishable from wild-type mice (Kondratov et al., 2007; Rana and Mahmood, 2010).

## Surgical and environmental circadian disruption in animal models

Though transgenic models can shed light on gene function, total knockout models have limitations in that all tissues are lacking a specific gene, and the organism has undergone the entirety of its development without that gene. Because of these possible confounding factors, several studies have employed environmentally or surgically induced circadian disruption rather than genetic interventions and have demonstrated that such manipulations can influence morbidity and cancer sensitivity. Rats exposed to continuous or increased

daily light rather than the typical 12 hour light/dark cycle have shown increased spontaneous tumorigenesis, decreased lifespan, as well as more infectious diseases, abdominal obesity, hyperglycemia, and hyper-cholesterolemia (Vinogradova et al., 2009; Bukalev et al., 2012). Mice with ablated SCN regions showed completely disrupted rest/activity patterns, along with phase shifts and blunted amplitudes of processes that typically showcase 24-hour rhythmicity, such as body temperature, serum corticosterone, and circulating lymphocyte count (Filipski and Lévi, 2009). Additionally, subjection to chronic jet lag via alterations in light/dark timing disrupted rest/activity, body temperature, and serum corticosterone patterns, similar to SCN lesions (Filipski et al., 2004). Moreover, jet lag conditions caused a marked disruption of the typical circadian expression of all the examined genes, including *bmal1*, *cry1*, *per2*, and *rev-erbα* mRNA in liver tissue, and increased the risk of development of several cancers (Filipski et al., 2005; Lee et al., 2010).

In order to focus on the effects of these disruptive factors on tumor growth, xenografts of human cancer cells or induction of tumors by other means can be informative. Filipski et al. performed several experiments exploring various methods of circadian disruption and recovery, and their effects on tumor growth (Filipski and Levi, 2009). Destruction of the SCN after tumor inoculation produced accelerated growth in both slow-growing (pancreatic adenocarcinoma) and fast-growing (Glasgow osteosarcoma) tumors, doubling the size of the tumors compared to their sham lesioned counterparts by day 12 and 22, respectively (Filipski and Levi, 2009). In non-lesioned mice bearing tumors, meal timing was used as an entrainment factor, with a four hour feeding time at the beginning of the light phase causing a decrease in tumor growth for both types of tumors as compared to mice given access to food ad libitum (Filipski and Levi, 2009). Chronic jet-lag simulated by five 8-hour light advances caused an increase in mortality rates and nearly a 50% increase in Glasgow osteosarcoma tumor size by day 11 compared to mice exposed to normal light/dark cycles (Filipski et al., 2004; Filipski and Levi, 2009). Chronic jet lag also caused phase shifts and altered mRNA expression of *bmal1* and *per2* in the livers of tumor-bearing mice (Filipski et al., 2005). To investigate whether meal timing could mitigate the increased tumorigenesis effect cause by chronic jet lag, mice were exposed to the jet lag conditions but also given a consistent cycle of 12 hours access to food then 12 hours without access, which slowed tumor growth to rates to those similar to the non-jet lagged mice (Filipski and Levi, 2009). Meal timing also nearly restored *per2* and *rev-erbα* mRNA expression patterns but dampened those of bmal1 in the liver, and induced a more typical sinusoidal expression of *per2*, *bmal1*, and *rev-erbα* in the tumor tissue (Filipski and Levi, 2009).

Although a relationship between the disruption of specific circadian gene expression and disordered SCN function or light/dark hours has consistently been demonstrated, the exact nature of the relationship between circadian gene expression and oncogenesis is still unclear. To examine if the expression of a single circadian gene could induce the tumorigenic effects observed in light-induced circadian phase shifts, Yu et al. examined the overexpression of a specific circadian gene, *Cry1*, in a xenografted tumor (Yu et al., 2013). Nine mice were administered bilateral subcutaneous injections of human colorectal cancer cells, with one side being injected with cells that overexpressed *Cry1* and the other maintaining endogenous levels of *Cry1* expression (Yu et al., 2013). After four weeks, tumors overexpressing *Cry1* were over 50% larger and heavier than tumors originating from wildtype *Cry1* (Yu et al., 2013). Another study subcutaneously injected murine colon cancer cell lines that had either normal or downregulated *bmal1* expression into the axilla of mice to induce tumor growth (Zeng et al., 2010). Tumors derived from cell lines with downregulated bmal1 grew faster than those with normal *bmal1* expression, with the *bmal1* suppressed tumor growing to twice the size of the control tumor after 31 days (Zeng et al., 2010). More experiments utilizing this tumor-specific/gene-specific abnormal expression model would be informative to the oncogenic and oncostatic properties for all circadian genes.

## Circadian disruption in humans and the melatonin hypothesis

The importance of circadian rhythms in relation to various aspects of human health has been a topic of extensive research. Several studies have indicated that people with jobs causing "jet-lag" and disallowing typical sleep patterns, such as shift workers, pilots and flight attendants, are at a higher risk for health issues including heart disease, obesity, mood disorders, diabetes, gastrointestinal symptoms, and overall mortality (Healy and Waterhouse, 1995; Anisimov, 2003; Bray and Young, 2007; Kondratov et al., 2007; Pan et al., 2011; Schernhammer and Thompson, 2011). Similar results can be found for people with profound sleep disturbances not necessarily caused by occupation (Hublin et al., 2007; Kondratov et al., 2007; Adamantidis and de Lecea, 2008). Intriguingly, this increased risk extends to several cancers, but is most commonly seen in hormonally regulated cancers such as breast, prostate, ovarian, and thyroid cancer (Davis et al., 2001; Hansen, 2001; Anisimov, 2003; Schernhammer et al., 2003; Megdal et al., 2005; Anisimov, 2006; Kubo et al., 2006; Schernhammer et al., 2006; Blask, 2009; Hansen and Stevens, 2011; Poole et al., 2011; Monsees et al., 2012; Luo et al., 2013). In fact, the International Agency for Research on Cancer Working Group concluded that shift-work with circadian disruption is probably carcinogenic to humans (Straif et al., 2007). Mortality risk for cancer

patients increases when severe rest-activity pattern disruptions are present, and behavioral and molecular circadian disruptions are more frequent and prominent in later stages of cancer development (Mormont and Lévi, 1997; Mormont et al., 2000; Mormont and Waterhouse, 2002; Innominato et al., 2009; Innominato et al., 2012).

Certain cancer rates are higher in industrialized countries, and light pollution is thought to be a possible contributor to this phenomenon (Anisimov, 2006; Kloog et al., 2010; Spivey, 2010). This theory, known as the melatonin hypothesis, attributes increased cancer risk to decreased melatonin excretion due to the presence of light at night. Melatonin levels have a typical circadian rhythmicity, with the peak of circulating melatonin levels occurring in the middle of the night around 4am, a time at which natural light is rarely present and most humans are asleep (Healy and Waterhouse, 1995; Rohr and Herold, 2002; Stow and Gumz, 2011). However, with the advent of artificial light, sleep/wake and work cycles have become more malleable, and light exposure can easily occur during times that are atypical evolutionarily. It is important to note that melatonin production in the pineal gland can be suppressed by both natural and artificial light (Stevens et al., 1992; Rohr and Herold, 2002; Anisimov, 2003; Nakahara et al., 2003; Blask, 2009; Stow and Gumz, 2011), and plasma melatonin levels are often used as an indicator of circadian phase because of the interconnectedness of the pineal gland and the SCN. Anomalies in melatonin cycles are considered symptomatic of circadian disruption (Healy and Waterhouse, 1995; Rohr and Herold, 2002; Nakahara et al., 2003).

While suppression of melatonin occurs as a result of exposure to visible light, melatonin synthesis remains rhythmic in the absence of light. Therefore, if melatonin is a protective factor in tumorigenesis, then populations exposed to less light should have lower cancer risks. Indeed, Alaska, Canada, and Greenland had less than half the global incidence of breast cancer based on epidemiological data collected from 1968-1988, where the darkness during the winter months may have bestowed a protective element (Erren and Piekarski, 1999). Unfortunately, this protective element may be declining, as breast cancer rates have continued to rise since the previous data was collected (Fredslund and Bonefeld-Jørgensen, 2012). While still lower than global incidence rates, epidemiological data from the range of 1988-2008 in Greenland, Canada, Arctic Russia, and Alaska have shown a consistent trend of increasing breast cancer incidence (Fredslund and Bonefeld-Jørgensen, 2012). Changing environmental factors, including increased exposure to light at night through TV and computer screen time and outdoor high intensity output lamps, may be contributing to the increase in breast cancer rates (Pauley, 2004).

To substantiate the claim that levels of light at night exposure can lead to higher breast cancer incidence, a study by Kloog and colleagues used a composite image for nighttime light levels in 1996/1997 from the daily readings by U.S. satellites to assess nighttime light levels across 164 countries, and compared these levels to breast cancer rates reported by GLOBOCAN (Kloog et al., 2010). The investigators reported that higher light at night levels were associated with higher breast cancer incidence rates even after controlling for other risk factors, which corresponds with their previous findings in communities in Israel (Kloog et al., 2008; Kloog et al., 2010). In addition, intensity of light exposure in the bedroom during sleeping hours has been indicated to be a strong predictor of breast cancer while controlling for other known predictive factors (Anisimov, 2003; Blask, 2009; Kloog et al., 2011).

Environmental studies of light exposure during night hours may be confounded by the variability of light intensities and frequencies across countries and settings. Investigations of blindness and visual impairment have been performed to control for such environmental variability (Rohr and Herold, 2002). The possibility of blindness as a protective trait has been examined using public data available from the last five decades from national cancer registries, blindness registries, and national hospital discharge surveys. The data were derived from Norway, USA, Sweden, and Finland, collectively including over 50000 visually impaired patients (Hahn, 1991; Feychting et al., 1998; Verkasalo et al., 1999; Kliukiene et al., 2001; Rohr and Herold, 2002; Pukkala et al., 2006). Consistent with the melatonin hypothesis, these studies have reliably reported that women with near to total blindness have approximately half the incidence of breast cancer as compared to sighted women (Hahn, 1991; Feychting et al., 1998; Verkasalo et al., 1999; Kliukiene et al., 2001; Rohr and Herold, 2002; Pukkala et al., 2006).

While the above findings are consistent with the melatonin hypothesis, more direct evidence for the role of melatonin in cancer risk can be found in clinical and experimental data. In humans, decreasing melatonin levels have been suggested as markers for certain types of cancer, particularly those in which tumors are hormonally dependent (Karasek et al., 2000). In addition, lower melatonin levels in urine have been shown in women with breast cancer compared to matched controls, and diminished pineal gland function, which results in lower melatonin levels, has long been suggested to be involved in the promotion of breast cancer and other cancers (Cohen et al., 1978; Schernhammer and Hankinson, 2005). A study by Schernhammer and Hankinson examined the association of urinary melatonin and breast cancer risk by taking urinary samples from nearly 30000 cancer-free women who participated in the Nurse's Health Study from 1996-1999 (Schernhammer and Hankinson, 2005). A four-year follow-up revealed that the quarter with the lowest urinary melatonin had over twice as many cases of breast cancer than the quarter with the highest urinary melatonin concentration

(Schernhammer and Hankinson, 2005). A comprehensive summary of the various mechanisms by which melatonin may produce an oncostatic effects are presented by Srinivasan and colleagues (Srinivasan et al., 2008).

Melatonin has been shown to have anti-oncogenic effects in several human cancer cell lines. The effects of application of physiological levels of melatonin, pharmacological levels of melatonin, or a control diluent for 24 hours was examined in human breast and prostate cancer cell culture; physiological levels of melatonin were shown to decrease cell proliferation in these cell lines, which may be due to their modulation of the cell cycle length or inhibition of DNA synthesis (Cos et al., 2002; Jung-Hynes et al., 2011; Kostoglou-Athanassiou, 2013; Liu et al., 2013). In addition, physiological levels of melatonin were shown to stimulate multiple DNA repair systems and reduce the metastatic properties of human breast cancer cells (Sliwinski et al., 2007; Liu et al., 2013). At pharmacologically relevant levels, melatonin decreased cell proliferation in a variety of cancer cell type, including cervical cancer, gliomas, and ovarian cancer, and has also been shown to induce apoptosis in specific cancer cell lines without cytotoxicity to any non-cancerous cells (Kostoglou-Athanassiou, 2013; Rodriguez et al., 2013).

To examine the tumorigenic effects of "light at night" in animal models, Blask and colleagues exposed rats with human breast cancer xenografts to one of six different intensities of light during their normal dark phase, ranging from total darkness to bright light, and reported a dose-related tumor growth rate and blood-melatonin suppression, with more light at night corresponding to faster tumor growth rates and lower blood-melatonin levels (Blask et al., 2009). One noteworthy finding was that "dim light" (0,08μW/cm$^2$) caused less melatonin suppression but nearly equivalent levels of tumor growth as "bright light" (345μW/cm$^2$) at night (Blask et al., 2009), further supporting the importance of light at night and melatonin levels. In another study, blood collected at night from pre-menopausal women was perfused into in situ rat hepatomas or human breast cancer xenografts, resulting in inhibited signal transduction activity; this oncostatic effect was not present when the tumors were perfused with blood collected during the daytime or at night after 90 minutes of bright light exposure (Blask et al., 2005). Melatonin levels were five times higher in the blood collected at night without light exposure than the other blood collection conditions, and the introduction of a nonselective melatonin receptor antagonist blocked the tumor-suppressing effects, providing support for melatonin as the primary oncostatic factor in these studies (Blask et al., 2005).

In rats exposed to dim light (8,8μW/cm$^2$), near darkness (1-2,5μW/cm$^2$), or complete darkness (0μW/cm$^2$) and implanted with human breast cancer xenografts, latency-to-onset growth was recorded at 11 days for dim light exposure, 12 days for near darkness, and 15 days for complete darkness, demonstrating an increase in latency with decreasing levels of light at night (Dauchy et al., 2011). The same effect was observed in rats with hepatomas, reporting latency-to-onset at 5, 9, and 13 days with decreasing light at night (Dauchy et al., 2011). Blood melatonin levels in non-tumor bearing rats exposed to the varying degrees of light at night intensity were similar in dim light at night as in daytime, and a ten-fold increase of melatonin levels in near darkness compared to dim light was observed, which increased even further to normal levels in complete darkness (Dauchy et al., 2011). These findings expose a direct relationship between intensity of light at night and peak nighttime melatonin levels and provide evidence that disruption of melatonin expression can exacerbate tumor growth (Dauchy et al., 2011).

To examine the mediating effects of melatonin administration on tumor development, intestinal tumors were induced in rats by giving them five injections of 1,2-dimethylhydrazine weekly for six months with or without a corresponding nighttime injection of melatonin (Anisimov et al., 1997). Of the 21 rats given the carcinogen alone, all developed at least one tumor with an average of 3,8 tumors per rat, and of the 21 given melatonin as well as the carcinogen, only 14 developed any tumors and had an average of 1,5 tumors per rat (Anisimov et al., 1997). In another study, tumors in the vagina and uterine cervix were induced in mice by applying the same carcinogen intravaginally twice a week for 3 hours each with or without the five nighttime injections of melatonin per week (Anisimov et al., 2000). Of the 20 mice exposed solely to the carcinogen, 10 tumors developed in the vagina and uterine cervix, 8 of which were classified as malignant, and of the 20 rats given melatonin along with the carcinogen, only 5 developed tumors with none in the uterine cervix, and all were benign (Anisimov et al., 2000). To examine the joint and independent effects of testosterone and melatonin, rats were given an intragastric dose of the carcinogen dimethylbenzathracene to induce the appearance of mammary tumors; ovariectomies were performed on rats with tumors that developed within 12 weeks, and rats were treated with testosterone only, a combination of testosterone and melatonin, or untreated (Cos et al., 2006). Mice treated with solely testosterone showed steep increases in the number and growth rate of tumors, and these effects were mitigated by the administration of melatonin, returning both values to similar levels as the untreated rats (Cos et al., 2006). In addition, the melatonin group showed the highest survival rates of any of the groups (Cos et al., 2006).

## Melatonin treatment and anticancer effects

Melatonin has been studied for its effectiveness in cancer treatments and is especially appealing because of its endogenous presence and lack of adverse side effects (Vijayalaxmi et al., 2002; Jung and Ahmad, 2006). Several non-randomized studies lacking placebo controls have shown potential for the usage of melatonin in addition to other anticancer treatments, demonstrating increased measures of quality of life, decreased anxiety, and potential for response to anticancer treatment in patients who were previously unresponsive without melatonin (Jung and Ahmad, 2006). A small study conducted by Lissoni and colleagues examined 63 patients with non-small lung cancer who did not respond to cisplatin treatment and thus had no other viable treatment options (Lissoni et al., 1992). The patients were randomly assigned to a group receiving only supportive care, which included steroid and anticonvulsant agents, or a daily evening dose of 10 mg of melatonin; patients treated with melatonin showed higher survival rates and performance status (Lissoni et al., 1992). A similar study headed by Lissoni supplemented 50 patients with unresectable brain metastases with either supportive care alone, or supportive care and a daily 20 mg dose of melatonin in the evening (Lissoni et al., 1994). The mean survival times were 5 months with supportive care alone and 9 months with melatonin added, with a mean progression free period of 6 months and 3 months, respectively (Lissoni et al., 1994). In addition, the melatonin group suffered from fewer steroid-induced complications and showed improved quality of life (Lissoni et al., 1994). Another study of 60 patients with non-small lung cancer showed increased survival when given interleukin and melatonin versus a chemotherapy treatment of cisplatin and etoposide (Barni et al., 1995). In a larger study with 250 patients with metastatic solid tumors from lung cancer, face or neck cancer, breast cancer, or gastrointestinal tract neoplasms, patients were treated, with a chemotherapy regime based on their cancer type, with or without 20 mg of melatonin each evening (Lissoni et al., 1999). Patients given melatonin in the evenings had a clinical response rate of 34%, while only 15% of those without melatonin treatment achieved a clinical response, and the groups showed a one year survival rate of over 50% and less than 25%, respectively (Lissoni et al., 1999). In addition, the average progression-free time was approximately 4 months for the group receiving chemotherapy alone and was 9 months for the melatonin group, and the majority of chemotherapy-related toxicities had less than half the frequency in the melatonin and chemotherapy versus the chemotherapy alone group (Lissoni et al., 1999). Similar levels of increased chemotherapy tolerance, higher survival, and increased response were also found in a five-year study of 100 patients with non-small lung cancer (Lissoni et al., 2003). Other studies have reported consistent results of increased survival, tolerance, progression-free time, as well as reductions in anxiety and pain (Vijayalaxmi et al., 2002). Overall, the collection of data on melatonin use in tandem with current cancer treatment appears to be very positive, with minimal additional risk.

## Circadian rhythmicity of response to anticancer drugs

In order to examine the effect of drug administration timing, animals entrained to a 12 hour light/dark cycle were divided into one of several groups representing different circadian times of drug administration. Differential rates of survival, tumor growth, activity, maximum dose, weight loss, leukocyte count, etc., were observed among the timed treatment groups, which was attributed to circadian variations in tolerability, efficacy, and side effects (Blumenthal et al., 1999). The most beneficial time points of lowest toxicity or highest efficacy observed in animal studies can then be extrapolated to chronotherapeutic administration of chemotherapy for human patients and can be optimized through computational modeling (Lévi et al., 2010). Chronotherapy, a method of chronomodulated drug administration that accounts for the interaction of cellular circadian rhythms and the corresponding changes in efficacy and tolerance of anti-cancer drugs, ideally allows for greater survival while maintaining or potentially decreasing the intensity and number of side effects (Librodo et al., 2012). Currently, at least thirty anticancer drugs have demonstrated up to a ten-fold tolerability variance depending on time of day administered, most in which the highest tolerability time point corresponded with the highest anti-tumor efficacy (Lévi, 1996; Lévi et al., 2010). The increase in drug efficacy at specific circadian time points continues even when the cancerous cells exhibit disrupted or completely ablated circadian organization (Lévi et al., 2010). These anti-cancer effects in rodents help to inform cancer treatment strategies that can be used in humans.

Several clinical trials have been performed in an attempt to highlight the potential advantages of chronotherapy versus conventional drug administration, particularly using the antimetabolite 5-fluorouracil in the treatment of colorectal cancer. A phase I trial of 5-fluorouracil administered with 1-folic acid utilized programmable pumps to deliver five days of timed infusions, lasting from 10pm to 10am and peaking at 4am, to several groups of patients with metastatic colorectal cancer (Garufi et al., 1997). The doses were increased with each group given this treatment to determine the maximum tolerated dose, and the timed treatment revealed low toxicity and promisingly high efficacy rates for both previously treated and untreated patients (Garufi et al., 1997).

A phase II trial utilizing a similar drug administration schedule with the addition of oxaliplatin showed that the chronomodulated drug administration allowed for higher drug doses, resulted in higher rates of progression free and overall survival time, and caused fewer severe side effects (e.g., stomatitis) compared to patients given constant rates of infusion (Lévi et al., 1994). Additional randomized phase II trials comparing chronomodulated administration and constant administration have shown that groups with advanced colorectal cancer subjected to chronomodulated drug infusion had up to a 20% increase in objective response rate, increased tolerance, severely decreased frequencies of dose-limiting toxicity effects, and fewer patients dropping out from treatment when compared to conventional flat-rate infusions (Buroker et al., 1994; Lévi et al., 1994; Focan et al., 2000; Librodo et al., 2012). A meta-analysis performed on five studies comparing these two treatment plans in advanced colorectal cancer showed that chronomodulated drug administration provided a significant increase in overall survival rates while maintaining similar frequencies and intensities of side effects (Liao et al., 2010). In addition, a study of 77 patients with metastatic colorectal cancer who had previously shown to be resistant to treatments involving 5-fluorouracil, oxaliplatin, and leucovorin were treated with these drugs in a chronomodulated schedule, and were able to halt disease progression for a median time of 5,5 months in 61 of the patients (Gholam et al., 2006).

To further investigate the potential benefits of chronomodulated versus conventional flat-rate administration, phase III trials have been conducted. A trial of 554 previously untreated patients with colorectal cancer were randomly assigned to be given either a conventional 2-day flat rate infusion or a chronomodulated 4-day infusion of fluorouracil, oxaliplatin, and leucovorin (Giacchetti et al., 2006). Sex appeared as an important factor in the efficacy of chronomodulated versus conventional treatment; men showed a median survival rate of 21 months versus 18 months and a 2-year survival rate of 44% versus 34% for chronomodulated versus conventional treatment, respectively, while women showed a median survival rate of 16 months versus 19 months, and a 2-year survival rate of 27% versus 41% (Giacchetti et al., 2006). While treatment-related toxicities were similar both between treatment groups and sexes, the overall trend shows that chronomodulated treatment was beneficial in men and was potentially detrimental in women (Giacchetti et al., 2006). A meta-analysis including two other phase III trials and totaling 842 patients comparing the same two schedules of drug administration concluded that chronomodulated administration of anti-cancer drugs is safe and beneficial for males but not females, which may partially be a result of the majority of the relevant animal testing being performed on male rodents (Giacchetti et al., 2012).

Because of heterogeneity among patients, the individualization of treatment plans has the potential to be very efficacious. Patients can have differing activity schedules, tumor growth rates, and discrepancies between tumor and other cell cycles which all can be monitored and used to determine the most effective and least risky treatment plan (Bernard et al., 2010). Mathematical simulations of individualized treatments based on these patient characteristics have shown methods to optimize tumor cell death while minimizing non-tumor cell death by exploiting the differing circadian profile of the tumor cells compared to the patient's other cells, and have even suggested 29 hour treatment intervals as a possible method to maintain efficacy while minimizing risk due to individual variations in optimal administration time (Bernard et al., 2010). Investigation of individualized timed treatment is especially important since the most effective and most lethal 24 hour interval schedule for a patient can be separated by only a few hours, with larger variability of treatment efficacy and toxicity being seen among women (Bernard et al., 2010). Still, the inclusion of a time-dependent aspect of drug administration should be considered since it can allow for more effective and less harmful treatments for cancer in some individuals.

## Conclusion

Evidence from epidemiologic studies, cellular studies, rodent studies, and clinical trials all indicate that the genesis and proliferation of cancer can result from circadian abnormalities and be altered by circadian-based treatments. Genetically or environmentally disrupted circadian processes seem to counteract the naturally inhibitive properties of circadian gene expression and melatonin on oncogenesis present in both rodents

and humans with properly aligned rhythms. This connection is expressed such that circadian disruption appears to both induce tumorigenesis and cause poorer outcomes from cancer, whereas maintained rhythms seem to inhibit oncogenesis and increase survival rates. Because circadian rhythms span across many levels of complexity within an organism, they must be understood on multiple levels from circadian genes to behavior. Cancer treatment or prevention may be modified at one or more of these levels, and it should be understood that circadian variables are both present within the patient and are able to be manipulated by the environment when trying to determine an effective treatment plan.

Currently, the clinical trials using chronotherapy that have been conducted to date have produced some promising and some seemingly inconsistent results. However, when examining the results of clinical trials, baseline gender, cancer, and circadian differences must be taken into account which may resolve perceived inconsistencies. It could be that chronomodulated delivery of drugs is only preferable in hormonally

regulated cancers, useful in males but not females, or only advantageous when the patient's circadian rhythms are intact. Although many unknowns remain in circadian rhythms and cancer interaction, it seems clear that there is a circadian component to both the induction and progression of several types of cancer, and this compilation of information suggests that cancer prevention and treatment should include circadian aspects in order to be maximally effective.

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