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Daily rhythms are retained both in spontaneously developed sarcomas and in xenografts grown in immunocompromised SCID mice

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Abstract

The circadian clock generates and regulates many daily physiological, metabolic and behavioral rhythms as well as acute responses to various types of stresses including those induced by anticancer treatment. It has been proposed that modulatory function of the clock may be used for improving the therapeutic efficacy of established anti-cancer treatments. In order to rationally exploit this mechanism, more information is needed to fully characterize the functional status of the molecular clock in tumors of different cellular origin; however, the data describing tumor clocks are still inconsistent. Here we tested the status of clock in two models of tumors derived from connective tissue: sarcomas spontaneously developed in p53-deficient mice and human fibrosarcoma cells grown as xenografts in immunocompromised severe combined immunodeficient (SCID) mice. We show that both types of tumors retain a functional clock, which is synchronized in phase with normal tissues. We also show that spontaneously developed tumors are not only oscillating in the context of an organism where they receive hormonal and metabolic signals but continue oscillating *ex vivo* in tissue explants demonstrating that tumors have functional clocks capable of timing all their functions. We also provide evidence that similar to liver, tumors can be synchronized by food availability independent of the central pacemaker in the suprachiasmatic nuclei (SCN). These data provide the basis for the design of anticancer therapies that take into account the circadian metabolic and physiological patterns of both the tumor and normal tissues.

Keywords

Circadian clock; sarcoma; xenografts; p53^{-/-}; *Per2*-driven luciferase; *Bmal1*-driven luciferase; *in vivo* imaging; food restriction; tissue explants

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DECLARATION OF INTEREST

Authors declare no conflicts to disclose.

INTRODUCTION

The circadian clock generates and regulates many daily physiological and behavioral rhythms with a period of 24 h at the cell, tissue and organism levels. Processes such as sleep, wakefulness, immune activity, hormone levels, metabolism, tumor growth, apoptosis and cellular proliferation display circadian variations (Takahashi et al., 2008). In mammals, the central pacemaker is located at the suprachiasmatic nuclei (SCN), which is synchronized to the 24 h environmental light–dark cycle mainly via the light input received by photoreceptors of the retina that is directly transmitted via the retinohypothalamic tract (RHT) to the SCN. Moreover, clocks are known to be present in virtually all individual cells in the organism (Balsalobre et al., 1998). Under normal conditions multiple peripheral clocks are synchronized by the SCN via neural and hormonal cues and rhythms such as feeding-fasting behavior and rest activity cycles are in synchrony (Dibner et al., 2010). However, peripheral clocks can be uncoupled from the central pacemaker in response to other synchronizing signals (Mohawk et al., 2012). Several studies have shown that food availability is an SCN-independent synchronizing cue for the liver and, as a consequence, restricting food administration to the rest period of the circadian cycle alters the phase relationship between liver and SCN (Damiola, 2000; Hara et al., 2001; Stokkan et al., 2001).

At the molecular level, the circadian clock consists of tightly controlled transcriptional activation/repression of positive and negative components of the feedback loops. The positive component comprises the heterodimer formed by CLOCK (or its closest paralog NPAS2) and *BMAL1* proteins and this heterodimer activates the transcription of genes involved in the negative component of the loop, *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*). After transcription of the genes, the PER and CRY proteins dimerize and are translocated back to the nucleus where they repress the activity of the positive components, creating a negative feedback loop. In addition, there is another loop in which CLOCK/BMAL1 activates the transcription of REV-ERB α and ROR α . REV-ERB α and ROR α proteins can bind to the promoter of *Bmal1* and respectively activate or repress its transcription. One full cycle of oscillation lasts for about 24 h which is due to the delays between transcription, translation and subsequent transcriptional repression (Partch et al., 2013). The CLOCK/BMAL1 complex regulates the expression of multiple clock-controlled genes either directly or indirectly through other transcription factors.

Among the clock-controlled genes are the ones regulating cell cycle, apoptosis, DNA damage and genotoxic stress response including the one induced by anticancer treatment (Antoch & Kondratov, 2013). Previous work performed in animal models demonstrated that side effects of anticancer treatment can be minimized by manipulating the schedule of drug administration and adjusting it to times of the day associated with lower toxicity, a concept implicated in clinical practice as chronotherapy (Levi & Schibler, 2007; Lévi et al., 2010). Initial observations of successful use of chronotherapeutic approach were further extended by demonstrating that drug-induced toxicity in tumor-free mice correlate with the functional status of the major circadian regulator, CLOCK/BMAL1 transcriptional complex (Gorbacheva et al., 2005). Furthermore, drug-induced toxicity *in vivo* can be reduced by treatment with pharmacological activators of CLOCK/BMAL1 functional activity as it has

been shown for organic selenium compounds (Hu et al., 2011). Together, these data provide a proof-of-principle for potential use of clock-targeting pharmaceuticals for reducing damaging effects to normal tissues. However, in order to fully explore therapeutic potential of the modulatory function of the circadian clock in cancer therapy, it is important to characterize its functional status in tumors and to understand whether sensitivity of tumors can be modulated through the circadian mechanism similar to normal tissues. Although this information is critical for future translational applications directed for the improvement of therapeutic indexes of existing regimens, the data on tumor clocks remain very limited and inconsistent. Here, we tested the functional status of clock in two models of tumors derived from connective tissue: sarcomas spontaneously developed in p53-deficient mice and human fibrosarcoma cells grown as xenografts in immunocompromised severe combined immunodeficient (SCID) mice. We show that both types of tumors retain a functional clock, which under normal conditions (12:12 light: dark cycle and food available *ad libitum*) is synchronized in phase with other peripheral tissues. Moreover, similar to liver, circadian gene expression in xenografts can be uncoupled from the SCN-derived synchronization signal under restricted feeding conditions. Together, these data suggest that development of clock-targeting therapeutic strategies should take into account circadian mechanism in tumors and that tumor clocks are amenable for manipulation. This knowledge is critical for refining future approaches for improving the therapeutic index of cancer treatment.

MATERIALS AND METHODS

Animals

$p^{53-/-}$ mice on a C57B1/6J background were purchased from Jackson Laboratories (Bar Harbor, ME). PER2::LUC mice were obtained from Dr Takahashi. To generate *Per2*-Luc reporter line on p^{53-} deficient background, PER2::LUC female mice were crossed to $p^{53-/-}$ male mice to obtain heterozygous F1 mice, which were then inter-crossed to obtain homozygous F2 mice. Animals were genotyped for the presence of PER2::LUC fusion and p^{53-} deficiency according to standard protocols (<http://jaxmice.jax.org/protocolsdb>). After weaning, PER2::LUC/ $p^{53-/-}$ mice were group-housed at 12 h light:12 h dark cycle (LD12:12) with food and water *ad libitum* in a sound attenuated room, at a temperature of 23 ± 1 °C. Animals were weekly observed until tumor appearance was visually detected or dramatic loss of weight, indicative of tumor appearance, happened. SCID mice were obtained from RPCI colony (C.B-Igh-1bIcrTac-Prkdcscid/Ros, Buffalo, NY). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Roswell Park Cancer Institute and are in accordance with the international ethics standards (Portaluppi et al., 2010).

Wheel-running behavior recording

PER2::LUC/ $p^{53-/-}$ were individually housed in cages equipped with a running wheel. Activity data were collected as previously described (Antoch et al., 1997; Vitaterna et al., 1994). A group of mice were synchronized to 12 h light:12 h dark cycle (LD12:12). Another group was synchronized to LD12:12 for 7 days and then were released into constant darkness (DD). Actograms were plotted using ClockLab software (Actimetrics Inc, Evanston, IL).

Cell culture and real-time luciferase recording

HT1080 cells were purchased from ATCC and were maintained in DMEM supplemented with 10% fetal bovine serum. Lentiviruses expressing *Bmal1*-Luc or *Per2*-Luc (generously provided by Dr S. Kay) were generated using Invitrogen ViraPower™ HiPerform™ Promoterless Gateway® Expression System (Grand Island, NY) according to manufacturer's protocol as described by Liu et al. (2008). HT1080 cells were plated on 24-well plates at 50% confluency. Viral stocks were added at 1:10 dilution for 3 days after which media was replaced with fresh media containing 3 µg/ml Blasticidin. After 2 weeks of selection, individual clones were picked up and tested for luciferase expression. Clones with the highest luciferase signal per µg of protein were used for further analysis.

For *in vitro* luminescence imaging cells were plated on 35 mm dish at 4×10^5 cells/plate in DMEM and allowed adhering overnight. Next day cells were treated with 100 nM dexamethasone for 2 h, then media was replaced with recording media (DMEM without phenol red supplemented with 0.35 g/L Sodium Bicarbonate, 5% FBS, 10 mM HEPES, pH 7.2, 3.5 g/L D-Glucose, 100 U/mL penicillin-100 µg/mL streptomycin, and 0.1 mM Beetle Luciferin (Promega, Madison, WI) as described by Yamazaki & Takahashi (2005). Bioluminescence was recorded continuously; data were analyzed by Actimetrics software (Actimetrics, Wilmette, IL) and presented as photon counts per second. Baseline correction was calculated using a 24-hour average.

For the real-time monitoring of circadian profile of *Per2*-driven luciferase expression in explant cultures of normal tissues and spontaneously developed sarcomas, PER2::Luc mice were sacrificed one hour before lights-off and tissues were prepared for several days of bioluminescence real-time measurement as previously described by Yoo et al. (2004) and Yamazaki & Takahashi (2005). Bioluminescence was measured for several cycles using a LumiCycle (Actimetrics, Wilmette, IL). LumiCycle software (Actimetrics Inc., Wilmette, IL) was used to analyze the data. The circadian period was calculated performing a periodogram analysis to three consecutive cycles. The peak phase was determined from the first peak of *Per2-luc* expression.

Tumor cells inoculation and *in vivo* bioluminescence imaging

Bmal1-Luc and *Per2*-Luc expressing HT1080 cells were inoculated subcutaneously into left and right flanks, respectively, of SCID) male mice at 5×10^6 cells/site. After the tumors reached 5 mm bioluminescence, imaging was performed using IVIS 50 imaging system Caliper LifeSciences (Xenogen Corp., Alameda, CA) at two time points, ZT2 and ZT14. Mice were injected i.p. with firefly D-Luciferin (150 mg/kg, Caliper Life Sciences, Alameda, CA), anesthetized with isoflurane and imaged 15 min later using 10 s integration time and medium binning. Data were quantified as the sum of photon flux within the region of interest using Living Image software (Xenogen Corp., Alameda, CA). The same procedure was used to measure daily profiles of *Per2*-driven luciferase in normal tissues and tumors of PER2::LUC-*p*^{53-/-} mice with the exception that each individual mouse was profiled every 4 h.

Isolation of total RNA and real-time PCR

Total RNA from liver and inoculated tumors was isolated using Trizol[®] Reagent (Life Technologies, Grand Island, NY) and purified using Qiagen RNeasy kit (Valencia, CA) following manufacturer's protocols. Quantity and quality of the RNA was assessed with the Nanodrop[®] ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). RT-qPCR analysis was performed with total RNA using the ABI Prism 7700 and TaqMan EZ RT-PCR core reagent kit (Applied Biosystems, Grand Island, NY) with pre-made mouse-specific TaqMan gene Expression Assays for *Bmal1* (Mm00500226_m1), *Per2* (Mm00478113_m1) and 18S *rRNA* (Mm03928990_g1), which was used as the reference gene. Relative gene expression was calculated using $\Delta\Delta C_t$ method as previously described (Panda et al., 2002).

Tumor histology

Tissues were fixed in 10% neutral formalin for 24 h, and then transferred to 70% ethanol. Samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathological examination was performed using Zeiss AxioImager A1 with Axiocam MRc digital camera (Thornwood, NY). In determining the diagnosis the guidelines of Bethesda classification was used (Morse et al., 2002). Pictures were taken using Zeiss AxioImager A1 with Axiocam MRc digital camera.

Statistical analyses

Significance of the rhythmicity of the *in vivo mPer2*-driven luciferase expression profiles was determined with the CircWave v1.4 software using a linear harmonic regression fit (<http://webpage2.woelmuis.nl/downloads.htm>; R.A. Hut, University of Groningen, NL; Comas et al., 2008; Lincoln et al., 2006). For other statistical analysis Statistix 7.0 (Analytical Software, Tallahassee, FL) was used. Independent *t* tests were used for comparison of two groups of data. Values of $p < 0.05$ were considered significant.

RESULTS

Spontaneously developed sarcomas in $p^{53-/-}$ mice show rhythmic circadian profile of *Per2* gene expression both *in vivo* and *ex vivo*

To evaluate the functionality of the molecular clock in tumors we used *Per2*-Luciferase reporter mice (PER2::LUC) (Yoo et al., 2004) crossed to tumor-prone $p^{53-/-}$ mice, which develop sarcomas and lymphomas early in life (Donehower et al., 1992). The presence of circadian-driven luciferase in this model allows for simultaneous accessing of daily variations in clock function both in normal tissues and in spontaneously developed tumors. Importantly, the locomotor activity assay shows that tumor-bearing mice retain their ability to synchronize to the light–dark cycle and remain rhythmic under constant darkness conditions indicating that presence of tumors does not affect the function of the central clock in the SCN (Supplementary Figure 1A and B). To access the functional status of the clock in normal tissues and tumors we monitored daily temporal profiles of *Per2*-driven luciferase in livers and salivary glands (normal tissues that are amenable for *in vivo* imaging) and spontaneously developed visible solid tumors using the Xenogen IVIS Imaging System

(Perkin Elmer, Walham, MA). Imaging of each individual animal was performed at six different time points every four hours throughout a day. As shown in Figure 1(A) and (B), young tumor-free PER2::LUC-*p*^{53-/-} mice display significant *mPer2*-luciferase circadian profile with a peak of expression in the middle of the night.

This profile was retained in normal tissues of tumor-bearing mice. Importantly, *Per2*-luciferase expression was rhythmic in solid tumor with the phase of expression similar to normal tissues (Figure 1C and D) but with reduced amplitude (2 to 4-fold). Histological analysis performed after completion of the experiment confirmed that all solid tumors that were profiled represent spontaneously developed sarcomas (Supplementary Figure 2). Thus, spontaneously developed sarcomas retain rhythmic expression of circadian genes, which is in phase with the oscillations found in normal tissues.

It has been previously demonstrated that individual peripheral tissues contain circadian oscillators capable of persistent rhythmicity for up to 20 days independent of SCN input (Yoo et al., 2004). In order to test whether this characteristic is retained in tumors, we monitored *Per2*-luciferase expression *ex vivo* in explant cultures of normal tissues (liver, spleen and lung) and tumors using real-time luciferase recording. After entraining animals to a LD12:12, peripheral tissues (liver, lung, spleen) and tumors were dissected one hour before lights-off and placed in cultures containing media supplemented with luciferin, and luminescence was measured for at least four cycles (Figure 2). Similar to what has been previously reported (Yoo et al., 2004), explanted liver, lung and spleen display prominent circadian oscillation in *Per2* gene expression with a peak in the dark phase of the cycle. Interestingly, both the phase and the amplitude of oscillation in normal tissues were identical between tumor-free and tumor-bearing animals. Consistent with our *in vivo* data, explanted sarcomas show rhythmic expression of *Per2* gene with a peak phase similar to liver, lung and spleen but with reduced amplitude. Thus, spontaneously developed sarcomas are not only oscillating in the context of an organism but also in isolation suggesting that these tumors retain a functional clock.

Human fibrosarcoma HT1080 cells retain functional clock, which could be synchronized both *in vitro* and *in vivo*

One of the concerns in the interpretation of the spontaneous tumors data is that tissue explants represent a mixture of cells and the oscillation may originate from normal stromal cells rather than from tumor cells. Also, the conclusions obtained in the first set of experiments are solely based on the PER2-LUC reporter construct and the *Per2* gene expression is known to be influenced by temperature cycles of the mouse itself (Buhr et al., 2010). In addition, the procedure of preparation of the explants itself (e.g. slicing tissue, placing explants into cold Hank's balance salt solution or culture media) can reset the clock and phase relationship between different tissues cannot be accurately measured. To address these issues, we used another model – human fibrosarcoma HT1080 cells stably expressing luciferase under control of either *Bmal1* or *Per2* promoters. Real-time bioluminescence recording showed that both *Bmal1*-Luc and *Per2*-Luc exhibit cell autonomous oscillations after synchronization by dexamethasone (Figure 3). As expected, the oscillations are 180-degree out of phase. Since HT1080 cells can be grown as xenografts in

immunocompromised mice, they represent an ideal model for testing the potential ability of their clock to oscillate in the context of an organism and to evaluate the phase of expression of *Bmal1* and *Per2* promoters in implanted tumors with respect to normal tissues. To explore this, each individual SCID mouse was subcutaneously injected with *Bmal1*-Luc expressing HT1080 cells in the left flank and *Per2*-Luc expressing HT1080 cells in the right flank. After tumor sizes reached 0.5–1 cm, *in vivo* imaging was performed at ZT2 and ZT14. These time points were chosen as known peak times for the expression of *Bmal1* and *Per2*, respectively, in peripheral tissues (Preitner et al., 2002). Figure 4 shows representative images of two individual animals performed at ZT2 and ZT14. As it can be observed, both genes display prominent oscillation in their expression with the corresponding peaks and troughs that are characteristic of normal tissues (i.e. liver, Figure 1).

HT1080 transplanted tumors can be synchronized by food availability independent from the SCN

It has been long shown that the glucocorticoid hormone analog dexamethasone induces circadian gene expression in fibroblasts *in vitro* and phase shifts in peripheral tissues, including liver, *in vivo* but not in the SCN (Balsalobre et al., 2000b). These results suggested that glucocorticoid hormones, similar to restricted feeding, could uncouple peripheral oscillators from the master clock in the SCN. Since HT1080 reporter cells can be synchronized by dexamethasone in culture (Figure 3), we hypothesize that when transplanted to SCID mice, HT1080 cells may respond to synchronization by food availability similar to the liver. To test this prediction, *Bmal1*-Luc and *Per2*-Luc expressing HT1080 cells were inoculated following the same protocol as the previous experiment. In the first (control) group, food was available *ad libitum* whereas the second group was maintained on restricted feeding schedule (food available at lights-on time only) starting from 2 weeks prior to tumor inoculation. Differences in feeding schedule had no effect on the rate of tumor growth and both groups of animals were imaged at the same time at ZT2 and ZT14. Consistent with our previous experiment (Figure 4), mice on *ad libitum* food schedule showed prominent oscillation of both circadian promoters that are out of phase with each other with *Per2*-driven signal increasing and *Bmal1*-driven luciferase signal decreasing in the same animal at ZT14 compared to ZT2 (Figure 5A). In contrast, animals that were maintained on restricted feeding schedule demonstrated a shift in the phase of both *Bmal1* and *Per2* genes expression (Figure 5B). More detailed quantitative analysis performed using RT-PCR on tumor tissue extracts collected at ZT2 and ZT14 at the completion of the experiments confirmed the imaging data. Thus, it showed that normal condition (food available *ad libitum*) tumor xenografts display significant oscillation in *Per2* and *Bmal1* genes expression, which is reduced in the amplitude compared to normal tissues (liver) but retain the same phase of oscillation (Figure 5C). Restricted feeding reverses the phases of *Bmal1* and *Per2* genes expression both in liver and in tumor xenografts, such that *Per2*-driven luciferase expression is decreased whereas *Bmal1*-driven signal is increased at ZT14 compared to ZT2. These data clearly demonstrate the ability of tumor clock to be manipulated by external signal (feeding schedule).

DISCUSSION

It is well established that successful and less debilitating anti-cancer treatment relies on a subtle balance between the drug-induced damage to tumors and drug-induced toxicity to normal tissues. Therefore, finding novel approaches for selective modulation of stress response between normal and malignant cells could significantly improve therapeutic indexes of existing strategies. In this respect, the components of the molecular clock machinery represent attractive therapeutic targets. Previous work showed that clocks are present in every mammalian tissue where they orchestrate rhythmic expression of hundreds of genes involved in multiple cellular processes including those that are related to various aspects of cancer treatment (Antoch & Kondratov, 2013). It is believed that these oscillations underlie circadian variations in drug metabolism, therapeutic efficacy and tolerability (Lévi et al., 2010; Ortiz-Tudela et al., 2013).

The major question that yet remains unanswered is whether tumors retained functional clocks and, if so, whether they are functioning in synchrony with normal tissues and whether they are amenable for manipulations. Recent studies addressing the expression levels of clock proteins in different types of tumors have suggested that the circadian clock of tumor cells may be disrupted (Yu & Weaver, 2011). However, the data remain inconsistent, and as a result, inconclusive. Whereas some studies show a down-regulation of clock gene expression compared to normal surrounding tissue (Cao et al., 2009; Gery et al., 2006; Hsu et al., 2012; Krugluger et al., 2007; Lin et al., 2008; Mostafaie et al., 2009; Pogue-Geile et al., 2006; Relles et al., 2013; Winter et al., 2007; Xia et al., 2010; Yeh et al., 2005), others report up-regulation of clock gene expression in certain tumors (Geusz et al., 2010; Sato et al., 2009; Yu et al., 2013). Tokunaga and colleagues showed that in ovarian cancer some clock genes are down-regulated (*Per1*, *Per2*, *Cry2*, *Clock* and *CK1ε*) whereas others are up-regulated (*Cry1*, *Per3* and *Bmal1*) compared to normal ovaries (Tokunaga et al., 2008). Although these studies give some insight into the relationship of the circadian clock of different tumor types compared to normal tissue, they remain correlative as it is still not clear whether they represent the cause or the consequence of malignant tumor development. In addition, the functionality of tumor clock (i.e. the ability to sustain rhythmicity in gene expression and the ability to respond to changing environment) has not been tested yet.

Since tumors often lose pathways that are either detrimental or neutral for their survival and growth, it has been generally accepted that the functionality of tumor clocks is likely to be disrupted and this may contribute to deregulation of clock-controlled aspects of cell cycle, apoptosis, migration, differentiation in favor of tumor progression (Yu & Weaver, 2011). However, our data show that two types of tumors of connective tissue origin (spontaneously developed sarcomas in *p⁵³-/-* mutant mice and HT1080 human fibrosarcoma cells grown in SCID mice) display significant rhythmic profiles of *Per2*- and *Bmal1*-driven luciferase expression with the peaks and troughs coinciding with normal tissues. Although reduced in amplitude, *Per2*-driven luciferase recorded from sarcoma explants *ex vivo* displays oscillations that are as robust as those found in explants of normal tissues and the peak phase occurs, similar to liver, lung and spleen, in the dark phase suggesting that like in other peripheral tissues (Yoo et al., 2004), these clocks are self-sustained.

Another important finding that comes out of our study is that molecular clocks in tumors can be manipulated in the context of an organism. This is an important result that opens new avenues for translational applications. Hence, it suggests that therapeutic efficacy of anti-cancer treatment may be improved not only by clock-dependent protection of normal tissues but also through clock-dependent regulation of tumor-specific functions (efficiency of drug delivery, sensitivity, etc.). Interesting initial steps in this direction has been reported recently in application to photodynamic therapy (PDT). Thus, it has been shown that intratumoral accumulation of photosensitizers conjugated with anti-VEGF antibody in transplanted Lewis lung carcinoma and sarcoma-180 followed circadian pattern of VEGF expression (Gamaleia et al., 2012). As a consequence, PDT efficacy was higher when delivered at time of daily peak in VEGF expression. Our data also further supports the idea of potential value of clock-targeting pharmaceuticals in combinational anti-cancer therapies by applying it to clock components of tumors.

One of the confines of our study is that it is limited to tumors of connective tissue origin, which initially possess robust molecular clock both in tissues *in vivo* and in isolation. Thus, normal fibroblasts can be synchronized by variety of treatments, including glucocorticoids (Balsalobre et al., 2000a), glucose (Hirota et al., 2002), temperature (Sladek & Sumova, 2013), insulin (Tahara et al., 2011) and others. At this point it is not clear whether the idea of functional clocks in tumors applies to other tumor types. Recent studies of mammary epithelial cells have demonstrated robust oscillation in normal cells and the loss of rhythmic gene expression in cancer cell lines (Rossetti et al., 2012). It is important to note that these studies are limited to cell culture and the ability of breast tumor cells to be entrained in the context of an organism has not been tested.

Another important question that still remains unanswered is whether the robustness of tumor clock decreases with tumor progression. The inverse regulation (effect of cancer progression on an organism's circadian function) is well-documented in cancer patients showing altered circadian variation of corticosterone, cortisol, melatonin, prolactin, temperature or rest-activity rhythms characterized by decrease in amplitude, changes in the period and phase of activity, etc. (Chevalier et al., 2003; Grutsch et al., 2011; Innominato et al., 2009a, 2009b; Lévi et al., 2007; Mormont & Lévi, 1997; Mormont et al., 2000; Sephton & Spiegel, 2003). In our study, development of spontaneous tumors in mice did not affect wheel-running activity rhythm under LD12:12 or DD conditions (Supplementary Figure 1), which is in line with previously reported data (Geusz et al., 2010). It also had no effect on robustness of tissue-specific clocks, which showed very similar pattern of oscillation in tumor-free and tumor-bearing animals; however, it is still not clear whether the lack of the effect is due to presumably early stages of tumorigenesis, at which mice were tested or it represents the general phenomenon.

Taken together, our results open up a new spectrum of possibilities to develop new therapies where clock-targeting pharmaceutical compounds are directed at manipulation of the tumor clock instead of normal tissues. Further research is needed to investigate whether tumors sensitivity to drugs can be manipulated by circadian clock. For now, our work establishes a new vision of the role of circadian clock in tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Antoch, M.; Kondratov, R. Pharmacological modulators of the circadian clock as potential therapeutic drugs: Focus on genotoxic/anticancer therapy. In: Kramer, A.; Mellow, M., editors. *Circadian clocks*. Berlin-Heidelberg: Springer; 2013. p. 289-309.
- Antoch MP, Song E-J, Chang A-M, et al. Functional identification of the mouse circadian clock gene by transgenic BAC rescue. *Cell*. 1997; 89:655–67. [PubMed: 9160756]
- Balsalobre A, Brown SA, Marcacci L, et al. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 2000a; 289:2344–7. [PubMed: 11009419]
- Balsalobre A, Brown SA, Marcacci L, et al. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 2000b; 289:2344–7. [PubMed: 11009419]
- Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*. 1998; 93:929–37. [PubMed: 9635423]
- Buhr ED, Yoo SH, Takahashi JS. Temperature as a universal resetting cue for mammalian circadian oscillators. *Science*. 2010; 330:379–85. [PubMed: 20947768]
- Cao Q, Gery S, Dashti A, et al. A role for the clock gene *Per1* in prostate cancer. *Cancer Res*. 2009; 69:7619–25. [PubMed: 19752089]
- Chevalier V, Mormont M-C, Curé H, Chollet P. Assessment of circadian rhythms by actimetry in healthy subjects and patients with advanced colorectal cancer. *Oncol Rep*. 2003; 10:733–7. [PubMed: 12684651]
- Comas M, Beersma DG, Hut RA, Daan S. Circadian phase resetting in response to light-dark and dark-light transitions. *J Biol Rhythms*. 2008; 23:425–34. [PubMed: 18838608]
- Damiola F. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Develop*. 2000; 14:2950–61. [PubMed: 11114885]
- Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Ann Rev Physiol*. 2010; 72:517–49. [PubMed: 20148687]
- Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*. 1992; 356:215–21. [PubMed: 1552940]
- Gamaleia NF, Lisnyak IA, Shishko ED, et al. Chronobiological approaches to antiangiogenic photodynamic therapy of tumors: The first experimental evaluation. *Exp Oncol*. 2012; 34:364–6. [PubMed: 23302997]
- Gery S, Komatsu N, Baldjyan L, et al. The circadian gene *Per1* plays an important role in cell growth and DNA damage control in human cancer cells. *Mol Cell*. 2006; 22:375–82. [PubMed: 16678109]
- Geusz ME, Blakely KT, Hiler DJ, Jamasbi RJ. Elevated mPer1 gene expression in tumor stroma imaged through bioluminescence. *Int J Cancer*. 2010; 126:620–30. [PubMed: 19637242]
- Gorbacheva VY, Kondratov RV, Zhang R, et al. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc Natl Acad Sci USA*. 2005; 102:3407–12. [PubMed: 15689397]

- Grutsch J, Ferrans C, Wood P, et al. The association of quality of life with potentially remediable disruptions of circadian sleep/activity rhythms in patients with advanced lung cancer. *BMC Cancer*. 2011; 11:193. [PubMed: 21605390]
- Hara R, Wan K, Wakamatsu H, et al. Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells*. 2001; 6:269–78. [PubMed: 11260270]
- Hirota T, Okano T, Kokame K, et al. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem*. 2002; 277:44244–51. [PubMed: 12213820]
- Hsu C-M, Lin S-F, Lu C-T, et al. Altered expression of circadian clock genes in head and neck squamous cell carcinoma. *Tumor Biol*. 2012; 33:149–55.
- Hu Y, Spengler ML, Kuropatwinski KK, et al. Selenium is a modulator of circadian clock that protects mice from the toxicity of a chemotherapeutic drug via upregulation of the core clock protein, BMAL1. *Oncotarget*. 2011; 2:1279–90. [PubMed: 22249125]
- Innominato PF, Focan C, Gorlia T, et al. Circadian rhythm in rest and activity: A biological correlate of quality of life and a predictor of survival in patients with metastatic colorectal cancer. *Cancer Res*. 2009a; 69:4700–7. [PubMed: 19470769]
- Innominato PF, Mormont M-C, Rich TA, et al. Circadian disruption, fatigue, and anorexia clustering in advanced cancer patients: Implications for innovative therapeutic approaches. *Integr Cancer Ther*. 2009b; 8:361–70.
- Krugluger W, Brandstaetter A, Kállay E, et al. Regulation of genes of the circadian clock in human colon cancer: Reduced period-1 and dihydropyrimidine dehydrogenase transcription correlates in high-grade tumors. *Cancer Res*. 2007; 67:7917–22. [PubMed: 17699798]
- Lévi F, Filipski E, Iurisci I, et al. Cross-talks between circadian timing system and cell division cycle determine cancer biology and therapeutics. *Cold Spring Harbor Symp Quant Biol*. 2007; 72:465–75.
- Lévi F, Okyar A, Dulong S, et al. Circadian timing in cancer treatments. *Ann Rev Pharmacol Toxicol*. 2010; 50:377–421. [PubMed: 20055686]
- Levi F, Schibler U. Circadian rhythms: Mechanisms and therapeutic implications. *Ann Rev Pharmacol Toxicol*. 2007; 47:593–628. [PubMed: 17209800]
- Lin Y-M, Chang JH, Yeh K-T, et al. Disturbance of circadian gene expression in hepatocellular carcinoma. *Mol Carcinogen*. 2008; 47:925–33.
- Lincoln GA, Clarke IJ, Hut RA, Hazlerigg DG. Characterizing a mammalian circannual pacemaker. *Science*. 2006; 314:1941–4. [PubMed: 17185605]
- Liu AC, Tran HG, Zhang EE, et al. Redundant function of REV-ERBa and b and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genet*. 2008; 4:e1000023. [PubMed: 18454201]
- Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. *Ann Rev Neurosci*. 2012; 35:445–62. [PubMed: 22483041]
- Mormont MC, Lévi F. Circadian-system alterations during cancer processes: A review. *Int J Cancer*. 1997; 70:241–7. [PubMed: 9009166]
- Mormont MC, Waterhouse J, Bleuzen P, et al. Marked 24-h rest/activity rhythms are associated with better quality of life, better response, and longer survival in patients with metastatic colorectal cancer and good performance status. *Clin Cancer Res*. 2000; 6:3038–45. [PubMed: 10955782]
- Morse HC, Anver MR, Fredrickson TN, et al. Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood*. 2002; 100:246–58. [PubMed: 12070034]
- Mostafaie N, Kállay E, Sauerzapf E, et al. Correlated downregulation of estrogen receptor beta and the circadian clock gene Per1 in human colorectal cancer. *Mol Carcinogen*. 2009; 48:642–7.
- Ortiz-Tudela E, Mteyrek A, Ballesta A, et al. Cancer chronotherapeutics: Experimental, theoretical, and clinical aspects. In: Kramer A, Merrow M, editors. *Circadian clocks*. Berlin–Heidelberg: Springer; 2013. p. 261–88.
- Panda S, Antoch MP, Miller BH, et al. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*. 2002; 109:307–20. [PubMed: 12015981]
- Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol*. 2013; 24:90–9. [PubMed: 23916625]

- Pogue-Geile KL, Lyons-Weiler J, Whitcomb DC. Molecular overlap of fly circadian rhythms and human pancreatic cancer. *Cancer Lett.* 2006; 243:55–7. [PubMed: 16451817]
- Portaluppi F, Smolensky MH, Touitou Y. Ethics and methods for biological rhythm research on animals and human beings. *Chronobiol Int.* 2010; 27:1911–29. [PubMed: 20969531]
- Preitner N, Damiola F, Luis Lopez M, et al. The orphan nuclear receptor REV-ERB $\pm$ controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell.* 2002; 110:251–60. [PubMed: 12150932]
- Relles D, Sendecki J, Chipitsyna G, et al. Circadian gene expression and clinicopathologic correlates in pancreatic cancer. *J Gastrointest Surg.* 2013; 17:443–50. [PubMed: 23254314]
- Rossetti S, Esposito J, Corlazzoli F, et al. Entrainment of breast (cancer) epithelial cells detects distinct circadian oscillation patterns for clock and hormone receptor genes. *Cell Cycle.* 2012; 11:350–60. [PubMed: 22193044]
- Sato F, Nagata C, Liu Y, et al. PERIOD1 is an anti-apoptotic factor in human pancreatic and hepatic cancer cells. *J Biochem.* 2009; 146:833–8. [PubMed: 19675098]
- Septon S, Spiegel D. Circadian disruption in cancer: A neuroendocrine-immune pathway from stress to disease? *Brain Behav Immunity.* 2003; 17:321–8.
- Sladek M, Sumova A. Entrainment of spontaneously hypertensive rat fibroblasts by temperature cycles. *PLoS One.* 2013; 8:e77010. [PubMed: 24116198]
- Stokkan K-A, Yamazaki S, Tei H, et al. Entrainment of the circadian clock in the liver by feeding. *Science.* 2001; 291:490–3. [PubMed: 11161204]
- Tahara Y, Otsuka M, Fuse Y, et al. Refeeding after fasting elicits insulin-dependent regulation of *Per2* and *Rev-erbalpha* with shifts in the liver clock. *J Biol Rhythms.* 2011; 26:230–40. [PubMed: 21628550]
- Takahashi JS, Hong H-K, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: Implications for physiology and disease. *Nat Rev Genet.* 2008; 9:764–75. [PubMed: 18802415]
- Tokunaga H, Takebayashi Y, Utsunomiya H, et al. Clinicopathological significance of circadian rhythm-related gene expression levels in patients with epithelial ovarian cancer. *Acta Obstet Gynecol Scandinavica.* 2008; 87:1060–70.
- Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science (New York, NY).* 1994; 264:719–25.
- Winter SL, Bosnyan-Collins L, Pinnaduwage D, Andrulis IL. Expression of the circadian clock genes *Per1* and *Per2* in sporadic and familial breast tumors. *Neoplasia.* 2007; 9:797–800. [PubMed: 17971899]
- Xia H-C, Niu Z-F, Ma H, et al. Deregulated expression of the *Per1* and *Per2* in human gliomas. *Can J Neurolog Sci.* 2010; 37:365–70.
- Yamazaki S, Takahashi JS. Real-time luminescence reporting of circadian gene expression in mammals. *Method Enzymol.* 2005; 393:288–301.
- Yeh K-T, Yang M-Y, Liu T-C, et al. Abnormal expression of period 1 (PER1) in endometrial carcinoma. *J Pathol.* 2005; 206:111–20. [PubMed: 15809976]
- Yoo S-H, Yamazaki S, Lowrey PL, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA.* 2004; 101:5339–46. [PubMed: 14963227]
- Yu EA, Weaver DR. Disrupting the circadian clock: Gene-specific effects on aging, cancer, and other phenotypes. *Aging.* 2011; 3:479–93. [PubMed: 21566258]
- Yu H, Meng X, Wu J, et al. Cryptochrome 1 overexpression correlates with tumor progression and poor prognosis in patients with colorectal cancer. *PLoS One.* 2013; 8:1–10.

FIGURE 1.

Rhythmic *Per2*-driven luciferase circadian profiles measured *in vivo*. (A) Representative example of series of images of *Per2*-driven luciferase of a non-tumor bearing mouse imaged throughout a day. (B) Quantification of the *mPer2*-luciferase reporter circadian activity profile measured *in vivo* in non-tumor bearing mice. Statistical significance of the circadian oscillations obtained was determined with the CircWave v1.4 software. Salivary glands and liver display significant *Per2*-luciferase circadian profile with a peak of expression in the middle of the night (salivary glands $p < 0.001$; liver $p < 0.001$). For both tissues, two sines (fundamental wave and the first harmonic) contributed significantly to the explained variance. Values represent mean ($n = 8$) $\pm$ SEM. (C) Representative example of series of images of *Per2*-driven luciferase of a sarcoma-bearing mouse imaged throughout a day. (D) Circadian profiles measured in tumor-bearing mice in normal liver, normal salivary glands and tumor. The three tissues analyzed display a significant *Per2*-luciferase circadian profile with a peak of expression at midnight (salivary glands $p < 0.001$; liver $p < 0.001$; tumor $p < 0.05$). In the circadian curves obtained from tumor-bearing animals, only the fundamental wave significantly contributed to the explained variance. Values represent mean ($n = 7$) $\pm$ SEM.

FIGURE 2.

Circadian profiles of *Per2*-driven luciferase from explanted tissues monitored *ex-vivo*. (A) Representative examples of rhythmic *mPer2*-driven luciferase records measured over several cycles from explant cultures of normal tissues (liver, lung, spleen) in non-tumor bearing and tumor-bearing mice as well as in sarcoma tumors. (B) Quantification of period of the *Per2*-driven luciferase records in the different tissues. In non-tumor bearing mice, the period of the oscillation of tissues measured over the first three cycles is close to 24 h. Tumor-bearing mice display a period in normal tissues not significantly different from those obtained in non-tumor bearing animals. (C) Quantification of peak phase of *mPer2*-driven luciferase records in different tissues. Explanted pieces of tissue of liver, spleen and lung from non-tumor and tumor-bearing mice peak in the dark phase. Sarcoma peak phase occurs, similar to the peripheral tissues measured in the dark phase. Values represent mean ($n = 11$) $\pm$ SEM.

FIGURE 3.

HT1080 cells are synchronized in circadian phase by dexamethasone treatment. Real-time luciferase recording of HT1080 cells stably expressing *Bmal1*-Luc (A) or *Per2*-Luc (B) promoters. Cells were plated on 35 mm dishes at ~50% confluency and allowed to adhere overnight. After a 2 h treatment with 100 nM dexamethasone media was replaced with luciferase recording media and placed in Lumicycle. HT1080 cells demonstrate at least two cycles of circadian oscillation of *Bmal1*- and *Per2*-driven luciferase, which are 180-degree out of phase.

FIGURE 4.

HT1080 xenografts are synchronized in phase with normal peripheral tissues. Representative *in vivo* images of mice performed at ZT2 and ZT14. Each mouse bears HT1080-*Bmal1*-Luc xenograft on the right flank and HT1080-*Per2*-Luc – on the left flank. Both *Bmal1* and *Per2* display prominent oscillation in their expression with the corresponding peaks and troughs that are characteristic of normal tissues, with *Bmal1* peaking at ZT2 and *Per2* peaking at ZT14 within an individual animal.

FIGURE 5.

HT1080 xenografts are synchronized in phase by food availability independent from the SCN. Representative *in vivo* images of mice fed either *ad libitum* (A) or during lights-on time only (restricted feeding schedule, (B) performed at ZT2 and ZT14. Each mouse bears HT1080-*Bmal1*-Luc xenograft on the right flank and HT1080-*Per2*-Luc – on the left flank. *Bmal1* and *Per2* expression in HT1080 cells are synchronized with normal tissues in mice fed *ad libitum*. Regular pattern of oscillations is disturbed in mice on restricted feeding schedule. (C) Quantitative analysis of *Bmal1* and *Per2* gene expression in excised tumors (top panel) and liver (low panel) collected at ZT2 and ZT14. AL, *ad lib*; RF, restricted feeding. Luciferase signal in tumor lysates is normalized by protein concentration. Values represent mean ($n = 6$) $\pm$ SEM. In *ad libitum* fed mice *Bmal1* expression is higher and *Per2* expression is lower at ZT2 compared to ZT14 (* $p = 0.06$ and ** $p < 0.0001$ respectively, Student's *t*-test). Both *Bmal1* and *Per2* expression are reversed in phase in xenografts of mice on restricted feeding schedule with *Bmal1* expression getting lower and *Per2* expression getting higher at ZT2 compared to ZT14 (#, ## $p = 0.001$, Student's *t*-test). These changes match phase shifts induced by restricted feeding in the liver (bottom panel). * $p = 0.02$; ** $p = 0.002$; # $p = 0.0004$; ## $p = 0.004$; Student's *t*-test.