Cross-talks between Circadian Timing System and Cell Division Cycle Determine Cancer Biology and Therapeutics

F. Lévi,*†‡ E. Filipski,*† I. Iurisci,*† X.M. Li,*† and P. Innominato*†‡

*INSERM, U776 Rythmes biologiques et cancers, Hôpital Paul Brousse, Villejuif, F-94807, France; †Université Paris-Sud, UMR-S0776, Orsay, F-91405, France; ‡Assistance Publique-Hôpitaux de Paris, Chronotherapy Unit, Department of Oncology, Hôpital Paul Brousse, Villejuif, F-94807, France

The circadian clock orchestrates cellular functions over 24 hours, including cell divisions, a process that results from the cell cycle. The circadian clock and cell cycle interact at the level of genes, proteins, and biochemical signals. The disruption or the reinforcement of the host circadian timing system, respectively, accelerates or slows down cancer growth through modifications of host and tumor circadian clocks. Thus, cancer cells not only display mutations of cell cycle genes but also exhibit severe defects in clock gene expression levels or 24-hour patterns, which can in turn favor abnormal proliferation. Most of the experimental research actively ongoing in this field has been driven by the original demonstration that cancer patients with poor circadian rhythms had poor quality of life and poor survival outcome independently of known prognostic factors. Further basic research on the gender dependencies in circadian properties is now warranted, because a large clinical trial has revealed that gender can largely affect the survival outcome of cancer patients on chronotherapeutic delivery. Mathematical models further show that the therapeutic index of chemotherapeutic drugs can be optimized through distinct delivery profiles, depending on the initial host/tumor status and variability in circadian entrainment and/or cell cycle length. Clinical trials and systems-biology approaches in cancer chronotherapeutics raise novel issues to be addressed experimentally in the field of biological clocks. The challenge ahead is to therapeutically harness the circadian timing system to concurrently improve quality of life and down-regulate malignant growth.

INTRODUCTION

Dividing cells undergo a sequence of molecular and biochemical events that gate and monitor the traverse of the cell division cycle through four successive phases called Gap 1 (G_1), S (for DNA synthesis), G_2, and M (for mitosis). A complex gene and protein machinery regulates, gates, and times the transitions from one phase of this cycle to the next (Sanchez and Dynlacht 2005; Santamaría et al. 2007; Sclafani and Holzen 2007). Interconnected with the cell cycle are also the DNA repair/apoptosis/necrosis systems that limit genomic instability and prevent genetic mutations to accumulate and eventually result in malignant transformation (Shiloh 2003; Golstein and Kroemer 2006; Bartek and Lucas 2007). Indeed, the deregulation of the cell division cycle represents a main feature of malignant cells that can stem from cell cycle gene mutations as well as from hypoxia or altered available energy (Hanahan and Weinberg 2000; Keith and Simon 2007). Cell cycle phases also convey important therapeutic information regarding the cytotoxic potential of anticancer drugs (De Vita et al. 2007). For instance, S-phase cells are usually most susceptible to antimetabolites such as 5-fluorouracil, whereas antimitotic agents such as vinorelbine or taxanes exert greatest cytotoxicity on M-phase cells. Conversely, no cell cycle phase specificity seems to characterize the susceptibility for alkylating agents such as platinum complexes. Therefore, the timing of treatment delivery relative to cell cycle stages or events has guided the development of several chemotherapy protocols.

On another hand, 24-hour changes in cell divisions have long been known in healthy mammalian tissues from rodents or humans (Scheving 1959). The consequences of these rhythms for the determination of timing treatment delivery have also led to the development of cancer chronotherapeutics in order to minimize damage to healthy cells and to optimize malignant cell kill (Lévi 2001; Mormont and Lévi 2003).

CIRCADIAN GATING OF CELL DIVISION

Rhythmic DNA synthesis and mitotic activity have thus been demonstrated in most components of the hematopoietic and immune system, in all the segments of the gastro-intestinal tract, liver, skin, and cornea of rodents (Burns et al. 1976; Lakatua et al. 1983; Scheving et al. 1992). These rhythms have also been uncovered in human bone marrow, skin, and oral and rectal mucosae (Smaaland et al. 1991, 2002; Bjarnason et al. 2001; Bjarnason and Jordan 2002). The above 24-hour changes were first documented in laboratory rodents on light/dark (LD) synchronization and in humans on normal diurnal routine. Numerous experimental studies have shown that cell division rhythms were endogenous because they persisted in constant darkness (DD). Photoperiodic entrainment was further demonstrated through inverting the 24-hour pattern within 7–21 days of inversion of the LD 12:12 regimen. The robustness of the DNA synthesis and mitoses rhythms were further confirmed through their persistence despite ablation of adrenals, medulla, or pituitary (Scheving et al. 1992). Nevertheless, the 24-hour changes in cell divisions displayed altered rhythm characteristics in these animals. This observation suggested that these organs were not involved in the generation of the

cell division rhythms, but rather in the circadian coordination and phase setting of dividing cells. Even the suprachiasmatic nuclei (SCN) do not seem to be indispensable for rhythmic divisions to occur in synchrony in mouse intestine or corneal epithelium (Scheving et al. 1983), as well as in mouse bone marrow (Filipski et al. 2004a). In a study involving 52 mice with histologically proven SCN ablation and 34 sham-operated controls kept under LD 12:12 synchronization, the rest-activity cycle was suppressed in all of the mice with SCN ablation whereas the plasma corticosterone rhythm persisted yet with a nonsinusoidal pattern, a damped amplitude, and a phase-advance by a few hours (Filipski et al. 2004a). The bone marrow proliferation rhythms appeared to be least affected by SCN ablation. Thus, the count in bone-marrow-nucleated cells as well as the proportions of cells in G_1, S, or G_2/M displayed highly statistically significant rhythms, with similar amplitudes and phases in mice with SCN ablation or sham operation (Fig. 1). In addition, bone marrow hematopoiesis was accelerated in the mice with ablated SCN, as shown by a nearly 25% increase in the 24-hour mean of bone-marrow-nucleated cell count and a doubling in the 24-hour mean of circulating neutrophil count (Filipski et al. 2004a). These findings indicated the ability of peripheral circadian oscillators, and in particular those that regulate cellular proliferation, to remain synchronized in the absence of the established central SCN pacemaker. In a separate study, mouse bone marrow cells were sampled and processed for liquid culture for 96 hours. Samples from the bone marrow liquid culture were obtained every 3–4 hours for up to 4 days and exposed to granulocyte macrophage–colony-stimulating factor (GM-CSF) on agarose culture, a method used to determine the number of bone marrow GM progenitors. The study revealed proliferative circadian rhythms in cultured bone marrow cells that persisted for 3–4 days (Bourin et al. 2002). Interestingly, the circadian maximum recurred daily near CT3, which is the time of peak ex vivo proliferative response of mouse bone marrow cells to GM-CSF exposure (Perpoint et al. 1995). The ability of peripheral oscillators in proliferating tissues to maintain circadian rhythms in the absence of a central pacemaker has been further demonstrated for gene expression in cultured dividing fibroblasts (Nagoshi et al. 2004) and in some tumors, yet with rapid dampening (Balsalobre et al. 1998; Delaunay and Laudet 2002). Usually, the circadian rhythms in nondividing cultured cells tend to fade away unless a stimulation (glucocorticoid or serum shock) or an environmental 24-hour cycle (temperature and light) is introduced after a few days in culture, suggesting the need for a regular resetting of free-running peripheral oscillators in order for their coordination to be maintained (Balsalobre et al. 1998; Balsalobre 2000; Brown et al. 2002). This indeed may also be the case for cultured proliferating tissues. However, the above results support the assumption that LD and/or other periodic signals can be conveyed to proliferative tissues, among other peripheral oscillators, via structures other than the SCN, and at least partly synchronize the molecular or cellular rhythms.

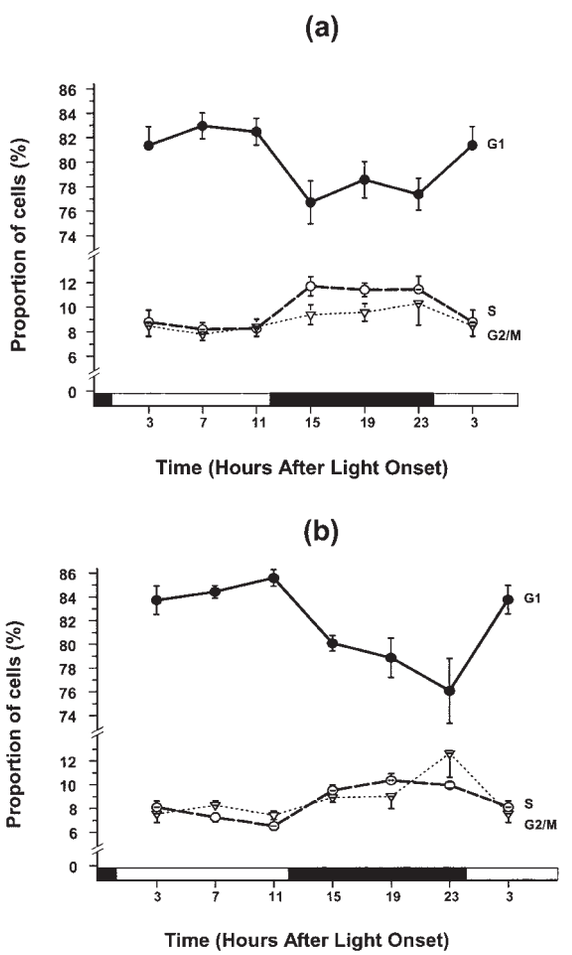

Figure 1. Circadian regulation of bone marrow proliferation rhythms in ♂ B6D2F1 mice kept in LD 12:12. Twenty-four hour changes in the proportions of whole bone marrow cells in G_1, S, or G_2-M phases of the cell cycle (mean ±S.E.M.), with light onset as time reference. (*a*) Circadian pattern in healthy mice; (*b*) persistent rhythms with similar patterns despite SCN ablation 4 weeks earlier. (Modified from Filipski et al. 2004a.)

DOWN-REGULATION OF TUMOR GROWTH BY THE CIRCADIAN TIMING SYSTEM

The very first results supporting the concept that the circadian timing system could down-regulate cancer progression stem from clinical investigations, where 24-hour rhythms are determined in patients and their patterns are correlated with clinical endpoints. These results have called for further confirmatory clinical investigations and experimental demonstrations of the relevance of the circadian timing system for controlling tumor progression.

CLINICAL STUDIES

The rest-activity rhythm is a well-established physiological output of the circadian timing system that can be easily and noninvasively recorded for several days with a wrist-worn monitor. A 3-day time series in rest-activity provide a window on the circadian timing system and can discriminate cancer patients with near-normal rhythms

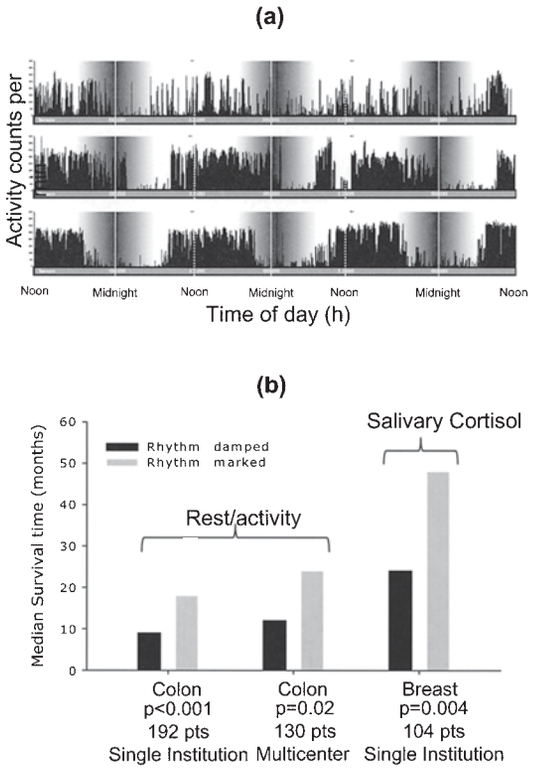

Figure 2. Relevance of circadian physiology for the survival outcome of cancer patients. Circadian physiology can be estimated with rest-activity monitoring for 3 days or more and/or by diurnal patterns in plasma or salivary cortisol concentration. Relations are shown between maintained or disrupted circadian physiology and survival outcome in patients with metastatic colorectal cancer or metastatic breast cancer. (*a*) Different rest-activity circadian patterns in three patients with metastatic colorectal cancer; (*b*) prediction of better survival with marked rhythm in rest-activity or salivary cortisol in cancer patients. (Adapted from Mormont et al. 2000; Sephton et al. 2000; P. Innominato et al., in prep.)

from those with clearly abnormal 24-hour patterns (Fig. 2a). Two rhythm parameters, autocorrelation coefficient r24 and dichotomy index I < O, respectively, estimate the regularity of the pattern over 24 hours and the relative amount of activity In-bed versus Out-of-bed (Mormont et al. 2000). In a single institution study, rest-activity rhythm as assessed with both parameters was found to be an independent predictor of survival for 192 patients with metastatic colorectal cancer, 62% of whom had received prior chemotherapy before circadian physiology assessment (Mormont et al. 2000). In a second multicenter international prospective study, rest-activity rhythm was confirmed as an independent predictor of survival in 130 patients with metastatic colorectal cancer who had not received any previous chemotherapy (P. Innominato et al., in prep.). In a third single institution study, salivary cortisol rhythm was estimated through repeated daily autosampling by 104 patients with metastatic breast cancer (Sephton et al. 2000). Large interpatient differences in daily patterns of serum or salivary cortisol were confirmed, with nearly flat patterns in some patients and clearly rhythmic patterns in others (Touitou et al. 1995). The salivary cortisol rhythm also independently predicted for survival in these patients with metastatic breast cancer (Sephton et al. 2000). Taken together, these three studies reveal that the risk of an earlier death from metastatic colorectal or breast cancer is significantly lower and the median survival is nearly twice as high in the patients with near-normal circadian physiology outputs as compared to those with damped or abnormal rhythms (Fig. 2b).

Experimental Demonstrations

In a first series of studies, mice kept in LD 12:12 were submitted to SCN ablation or sham operation and then received bilateral subcutaneous implants of a fragment of Glasgow osteosarcoma (GOS) or pancreatic adenocarcinoma (P03) and were monitored for tumor growth and survival. Following inoculation of healthy recipient mice, the doubling time is rapid for GOS (2–3 days) and slower for P03 (4–5 days). In the current study, the tumor grew significantly faster in the mice with bilateral SCN destruction (a posteriori documented) as compared to those that were sham-operated. These differences further translated into highly statistically significant differences in survival (Fig. 3a) (Filipski et al. 2002). In a second series of experiments, mice were submitted to chronic jet lag (CJL). The CJL regimen consisted in 8-hour advances of light onset of LD 12:12 every 2 days. It was selected as being the most disturbing one for the rest-activity rhythm. GOS was inoculated in mice exposed to CJL for 10 days (a condition that was maintained thereafter) or in mice kept in LD 12:12. Mice on CJL had complete suppression or severe dampening of their rest-activity and body temperature rhythms, whereas the serum corticosterone rhythm became bimodal (Filipski et al. 2004b). The tumor grew significantly faster in the mice on CJL as compared with those on LD 12:12, resulting in statistically significant differences in survival (Fig. 3b) (Filipski et al. 2004b). In a further experiment, the hypothesis that feeding synchronization could counterbalance the deleterious effect of CJL on malignant growth was tested. Mice to receive GOS were either kept in LD 12:12 or exposed to CJL alone or exposed to CJL with food availability regularly alternating between availability for 12 hours and no food for 12 hours. Feeding synchronization moderately slowed down malignant growth as compared to CJL (Filipski et al. 2005). In additional experiments, food availability was limited to 4 hours ("meal timing") for several weeks before GOS or P03 inoculation and thereafter. This procedure allowed the mice on meal timing to regain weight following initial loss, so that their body weights were similar to those of mice on LD 12:12 upon tumor inoculation. Meal timing, a procedure known to entrain peripheral clocks, proved to be an effective method to slow down tumor growth (Wu et al. 2004).

The results then emphasize that both an anatomical structure such as the SCN and lifestyle-related factors such as the LD exposure cycle and feeding schedules can impact on tumor growth rate. This effect can be mediated through host circadian physiology, central circadian coordination, and/or molecular clocks in healthy and/or malignant tissues.

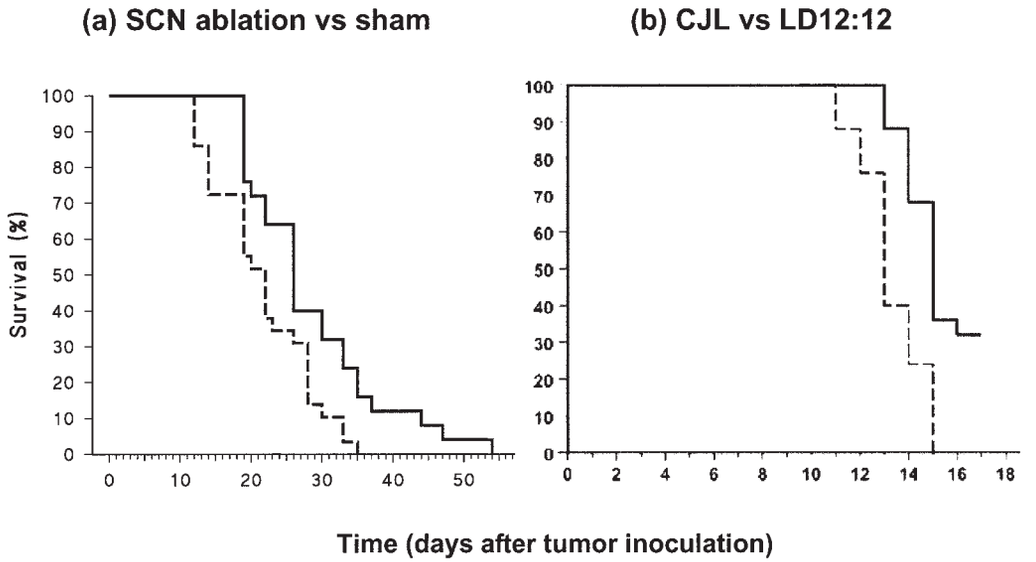

Figure 3. Relevance of the circadian timing system for experimental tumor progression and survival in ♂ B6D2F1 mice. (*a*) Survival curves of mice inoculated with GOS or pancreatic adenocarcinoma P03 4 weeks after ablation of SCN (*dashed line*) or sham-operated (*solid line*) and kept in LD 12:12 (log rank test adjusted for tumor type, $p = 0.006$) (Modified from Filipski et al. 2002.). (*b*) Survival curves of mice inoculated with GOS 10 days after beginning exposure to chronic jet lag (*dahed line*) or maintained on LD 12:12 (*solid line*) (log rank test, $p < 0.001$). (Modified from Filipski et al. 2004b.)

INTERACTIONS BETWEEN MOLECULAR CLOCK AND CELLULAR PROLIFERATION

At least three molecular mechanisms link molecular circadian clock with the cycling of cell division. The molecular clock controls *Wee1* transcription through an E-box-mediated mechanism. *Wee1* negatively controls the activity of CDK1/cyclin B1 that regulates the G_2/M transition (Matsuo et al. 2003). In addition, the BMAL1: CLOCK heterodimers activate *Per2* and *Rev-erbα* transcription and repress c-Myc transcription through E-box-mediated reactions in the c-*myc* gene P1 promoter (Fu and Lee 2003). *Per2* can also suppress c-*myc* expression indirectly by stimulating *Bmal1* transcription.

Both *Per1* and *Per2* as well as possibly other clock genes also control DNA repair through interactions with ATM and *mdm2* and maintain genome stability (Hunt and Sassone-Corsi 2007). However, circadian clock gene expression in bone marrow display damped or ablated patterns for *Bmal1* and persistent *Per1* and *Per2* rhythms (Granda et al. 2005; Tsinkalovsky et al. 2006).

CJL not only profoundly alters circadian physiology as previously mentioned, but also severely disrupts the molecular clock in the SCN and in liver. Thus, the rhythm in PER1 protein expression was suppressed in the SCN of mice on CJL, and so were the 24-hour rhythms in the mRNA expression of clock genes *Rev-erbα*, *Cry1*, and *Bmal1* in liver. However, *Per2* mRNA retains a circadian rhythm yet with a nearly 10-hour phase-advance as compared with mice on LD 12:12 (Filipski et al. 2005). Interestingly, CJL also resulted in the repression of *p53* and the derepression of c-*myc* mRNA expression in liver. Furthermore, the expression pattern of c-*myc* became prominently rhythmic in the liver of mice on CJL (Fig. 4a) (Filipski et al. 2005). *p53* is a tumor suppressor gene whose activation can induce cell cycle arrest, DNA repair or apoptosis, or senescence, depending on the differential activation of *p53* target genes (Vogelstein et al. 2000; Liu and Chen 2006). Recent data further reveal that the tumor suppression activities of *p53* could result from the control this transcription factor exerts on energy metabolism, including glycolysis and mitochondrial respiration (Bensaad and Vousden 2007). Conversely, c-*myc* is an oncogene that has been linked with many human cancers. This transcription factor in the basic helix-loop-helix zipper family regulates up to 15% of all cellular genes, promotes entry into the cell cycle, and transition from G_1 to S. c-*myc* also impinges on global chromatin structure both directly and indirectly via both the regulation of histone acetyltransferase (HAT) GCN5, which in turn globally acetylates chromosomal histones, and histone methylation (Knoepfler 2007).

The role of the circadian clock in mediating the effect of CJL on p53 and c-Myc transcription patterns is further supported by very similar findings in the liver of mice with a constitutive *Per2* mutation (Fig. 4b) (Fu and Lee 2003) . Taken together, the results suggest that genetic or functional disruption of the molecular circadian clock results in genomic instability and accelerated cellular proliferation, two conditions that favor carcinogenesis (Hanahan and Weinberg 2000). Indeed, exposure of mice with a constitutive *Per2* mutation to γ-radiation resulted in shortened survival and increased incidence of tumors as compared with normal mice with a similar genetic background (Fu et al. 2002). No such effect was found in mice with the *Cry1* and *Cry2* double mutation (Gauger and Sancar 2005). However, the in vivo part of this study was performed in double-mutant mice kept in LD 12:12, an environmental condition that dampens, yet does not disrupt, 24-hour physiology, whereas DD exposure does

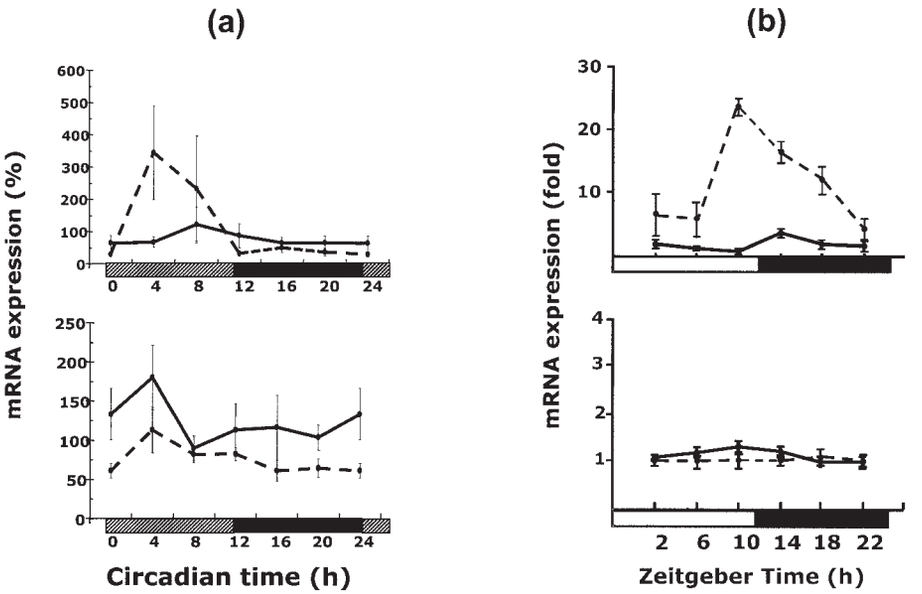

Figure 4. Downstream effects of molecular clock disruption on the mRNA expression patterns of c-Myc (*upper panels*) and p53 (*lower panels*) in mouse liver. (*a*) Chronic jet lag (*dashed lines*) versus LD 12:12 synchronization (*solid lines*). (Modified from Filipski et al. 2005.) (*b*) Constitutive *Per2* mutation (*dashed lines*) versus wild type (*solid lines*). (Modified from Fu et al. 2002.)

(Nagashima et al. 2005). Cultured fibroblasts from the *Cry* double mutants also did not display an altered response to radiation exposure (Gauger and Sancar 2005). However, recent data show that *Cry*s do not seem to be required for normal circadian clock function in mouse fibroblasts (Fan et al. 2007). Thus, the hypothesis that core circadian clock disruption favors malignant growth irrespective of its mechanisms remains plausible.

The hypothetical molecular circadian clock (Lévi and Schibler 2007) can be estimated to be functional through the relative phase relations among three core clock genes whose transcription is regulated by one another: *Rev-erbα* down-regulates *Bmal1*, *Bmal1* up-regulates both *Rev-erbα* and *Per2*, and *Per2* down-regulates both *Rev-erbα* and its own transcription. This simplified model is built through the adjustment of a 24-hour cosine function to the mRNA expression data of each of the three genes in tissues sampled from mice kept in DD for 24–60 hours. Figure 5 depicts the circadian clock of GOS under various experimental conditions, in comparison with that in healthy liver. The acrophases (maxima in fitted 24-hour cosine function) of the mRNA rhythms occur at $CT5^{20}$ for *Rev-erbα*, $CT14^{20}$ for *Per2*, and $CT23^{40}$ for *Bmal1* in healthy mouse liver (Fig. 5a). In GOS, the clock gene transcription rhythms are ablated and not statistically significant (Fig. 5b).

Exposure of GOS-bearing mice to CJL results in further apparent alteration of the simplified tumor clock (Fig. 5c). However, feeding synchronization (FS 12:12) induces a statistically significant rhythm in the transcription of all three clock genes despite CJL. Yet, the mRNA rhythms of these three clock genes occur in coincidence near CT6, which suggests that the molecular clock remains partly abnormal (Fig. 5d). This is also the case for the liver of these tumor-bearing mice, which display near-normal rhythms in mRNA expression of *Rev-erbα* and *Per2*, yet without any significant *Bmal1* rhythm (Filipski et al. 2005).

Some drugs, such as the cyclin-dependent kinase inhibitor (CDKI) seliciclib, arrest cycling cells in both G_1-S and G_2-M stages by preventing assembly of CDK and cyclins through competing with ATP for the binding to the catalytic site of CDKs. Seliciclib was used as a pharmacologic tool to study the cross-talk between the circadian clock and cell division cycle in GOS-bearing mice. Seliciclib treatment did produce rhythmic clock gene expression patterns in advanced malignant tumors with otherwise disrupted or uncoordinated molecular clocks (Iurisci et al. 2006). More specifically, seliciclib induced a near-normal molecular clock in the tumors of mice dosed at zeitgeber time 3 (ZT3), i.e., in the early light span (Fig. 5e). No such effect was found in the tumors of mice receiving seliciclib at ZT19, i.e., in the second half of the dark span (Fig. 5f). The pharmacologic induction of the tumor clock is associated with the best antitumor effect, with both tumor clock induction and antitumor effects being greatest following treatment at ZT3 (Iurisci et al. 2006).

Circadian clock induction improves control of G_2/M gating through an enhancement of *Wee1* transcription, a gene that is unidirectionally controlled by CLOCK:BMAL1. In the case of seliciclib, the induction of the molecular clock involves inhibition of CK1δ/ε, a key determinant for circadian clock function. Thus, the CK1δ/ε mutation or alteration produces disorders of rest-activity or sleep-wakefulness rhythm both in rodents and in humans (Hastings et al. 2003; Xu et al. 2005). The inhibition of CK1δ/ε by seliciclib results in increased *Bmal1* transcription, an effect that likely results from decreased PER2 degradation and nuclear translocation.

Because highly coordinated sequential transcription is a major mechanism of circadian rhythms, programmed

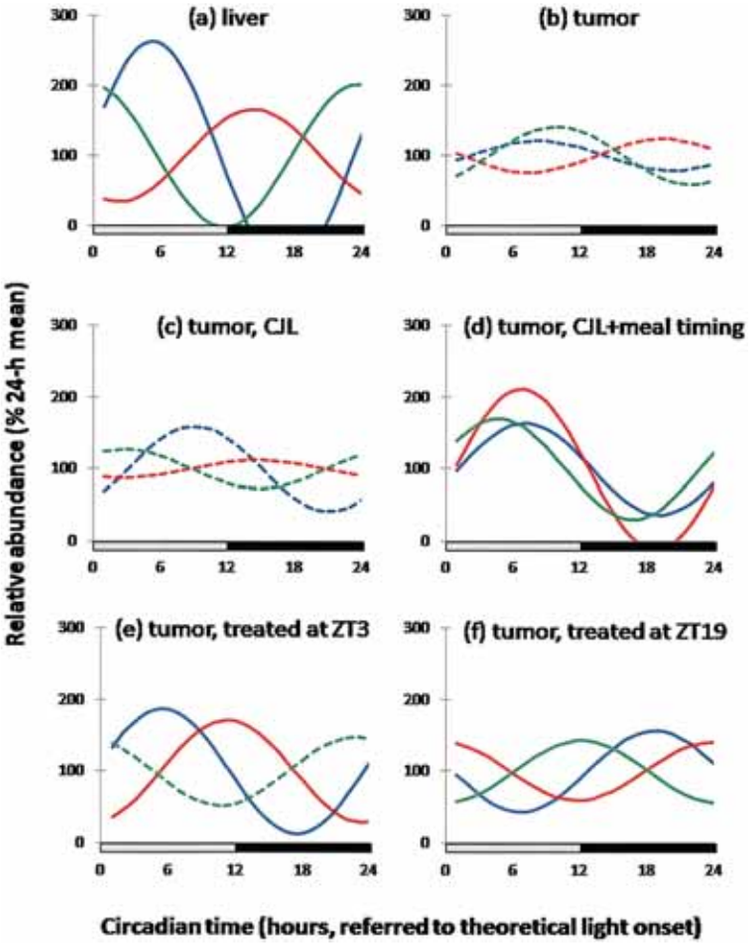

Figure 5. Molecular clock disruption in experimental tumor (GOS) at an advanced stage of growth. Effects of light, food, and drug on the circadian patterns in mRNA expression of *Rev-erbα* (*blue line*), *Per2* (*red line*), and *Bmal1* (*green line*). All studies in ♂ B6D2F1 mice. (*Solid line*) Statistically significant cosine fit for a 24-hour period; (*dashed line*) best-fitting yet nonsignificant 24-hour cosine function. (Modified from Filipski et al. [2005] and Iurisci et al. [2006].) (*a*) Liver clock, taken as a reference of a healthy functional peripheral clock in mice synchronized with LD 12:12. (*b*) Tumor clock (GOS) in mice synchronized with LD 12:12. (*c*) Tumor clock in mice on chronic jet lag for 10 days before and during the course of malignant growth. Chronic jet lag consisted of 8-hour advances of light onset of LD 12:12 every 2 days. (*d*) Tumor clock in mice on chronic jet lag (same as above) and meal timing, consisting of fixed daily food availability for a 12-hour span. (*e*) Tumor clock in mice synchronized with LD 12:12 following treatment with the cell cycle inhibitor seliciclib dosed at ZT3, i.e., the time that achieved best antitumor efficacy. (*f*) Tumor clock in mice synchronized with LD 12:12 following treatment with the cell cycle inhibitor seliciclib at ZT19, i.e., the time that achieved poorest antitumor efficacy.

food availability or daily seliciclib acts as a strong resetter of tumor cells that have lost synchrony in functional clocks, through transient inhibition of CK1δ/ε or other pathways within permissive time windows.

Recent studies in our group reveal that clock gene transcription is indeed rhythmic at an early stage of GOS growth, whereas the rhythms in *Rev-erbα*, *Per2*, and *Bmal1* mRNAs become ablated at a late stage of tumor growth. Concurrently, the circadian control of cell cycle phase distribution disappears and the rate of apoptotic cells decreases markedly (X.M. Li et al., unpubl.). Consistent with these data is the high and nonrhythmic expression of antiapoptotic protein BCL-2 in another advanced mouse tumor (mammary adenocarcinoma MA13/C) with presumably an altered molecular clock (Granda et al. 2005). Conversely, antiapoptotic BCL-2 and proapoptotic BAX, respectively, vary threefold and fivefold over 24 hours in mouse bone marrow. The circadian peak occurs at early light for BCL-2 and near mid dark for BAX, revealing the fine tuning of apoptosis circadian control in rapidly dividing healthy tissues (Granda et al. 2005).

The molecular clock seems to follow similar dynamics in other mouse tumor models. In early-stage mouse sarcoma180, clock gene mRNAs were rhythmic, with peaks occurring near the LD transition for *Per1* and *Per2* and at late dark/early light for *Bmal1* (Koyanagi et al. 2003). In a mouse breast cancer model at a more advanced stage of growth (early to late), *Per1* and *Per2* mRNAs lacked any circadian periodicity, whereas *Bmal1* mRNA remained rhythmic, yet with a 13-fold reduction in circadian amplitude (You et al. 2005). An elegant study performed in rats with diethylnitrosamine-induced hepatocarcinoma, a very slow growing tumor, further shows that the entrainment properties of *Per1* in cancerous liver tissue differ from

those of the healthy liver tissue it originates from (Davidson et al. 2006).

Taken together, the results from these and other studies indicate that clock genes are expressed in most malignant tumors that are currently used as experimental cancer models. The tumor molecular clock appears to switch from near normal to disrupted along the course of tumor growth. Such clock disruption is associated with altered transcription patterns of cell cycle genes so that proliferation and genomic instability are favored, apoptosis is down-regulated, and malignant progression is accelerated.

The increased proliferation and decreased apoptosis that are associated with clock disruption in tumors can be reverted through overexpressing *Per1* or *Per2* in malignant cells (Gery et al. 2006; Hua et al. 2006, 2007). Thus, overexpression of *Per1*-sensitized human cancer cells to low-dose radiation that produced DNA-damage-induced apoptosis, whereas inhibition of *Per1* cells blunted apoptosis in these tumor cells (Gery et al. 2006). Radiation triggered an p53-independent increase in c-Myc expression and a decrease in p21 expression in the tumor cells that were overexpressing *Per1*, but not in those with blunted *Per1*. The mechanisms of *Per1* in the elicitation of the apoptotic response to radiation involve interactions with Chk2 and activation of the ATM checkpoint pathway (Gery et al. 2006). Timeless, another clock protein, also has an important role in the ATR pathway, another arm of the checkpoint network (Unsal-Kaçmaz et al. 2005). In addition, *Per1* overexpression decreases the levels of Wee1, cyclin B1, and CDK1 (Gery et al. 2006). The CDK1-cyclin B1 complex gates the G_2/M transition and it is negatively regulated by Wee1 (Matsuo et al. 2003). Similarly, overexpression of *Per2* reduced cellular proliferation and increased apoptosis in mouse Lewis lung carcinoma (LLC) and mammary carcinoma (EMT6) cell lines (Hua et al. 2006). *Per2* overexpression down-regulated c-Myc, Bcl-X(L), and Bcl-2 and up-regulated p53 and Bax, thus promoting apoptosis in both cancer cell lines. However, no such effect was found for NIH-3T3 cells (Hua et al. 2006). Furthermore, intratumoral *Per2* gene delivery significantly slowed down the growth of LLC transplanted in mice through inhibition of proliferating cell nuclear antigen (PCNA) expression and apoptosis induction (Hua et al. 2007).

A HYPOTHETICAL MODEL OF THE INTERACTIONS BETWEEN CIRCADIAN TIMING SYSTEM AND TUMOR PROLIFERATION

A synthetic view of the interactions between the circadian timing system and the cell division cycle is depicted in Figure 6, with a major emphasis on the cell cycle in cancer cells. The panel on the left part of the figure illustrates the fact that circadian physiology represents the most obvious rhythms that reflect circadian timing system function. The rhythms in rest-activity, core body temperature, feeding behavior, and hormones are generated or controlled by the SCN in the hypothalamus through diffusible signals that include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), prokineticin-2, and cardiotrophin-like cytokine (Kramer et al. 2001; Cheng et al. 2002; Kraves and Weitz 2006).

The SCN can control the molecular clocks in peripheral tissues through sympathetic and parasympathetic pathways (Kalsbeek et al. 2006). These clocks are also redundantly regulated and coordinated through circadian physiology (Balsalobre et al. 2000; Brown et al. 2002; Kornmann et al. 2007). In turn, constitutive mutations in the molecular clock can alter both SCN functions and circadian physiology (Hastings et al. 2003). Molecular clocks in healthy cells control the cell division cycle through *Clock:Bmal1* regulation of several key cell cycle genes (Fu and Lee 2003; Matsuo et al. 2003). Circadian physiology can also and redundantly regulate cell cycle traverse (Dickmeis et al. 2007).

As indicated in the lower part of Figure 6, cancer cells are characterized by a deregulated cell cycle through numerous mutations that critically affect the transition

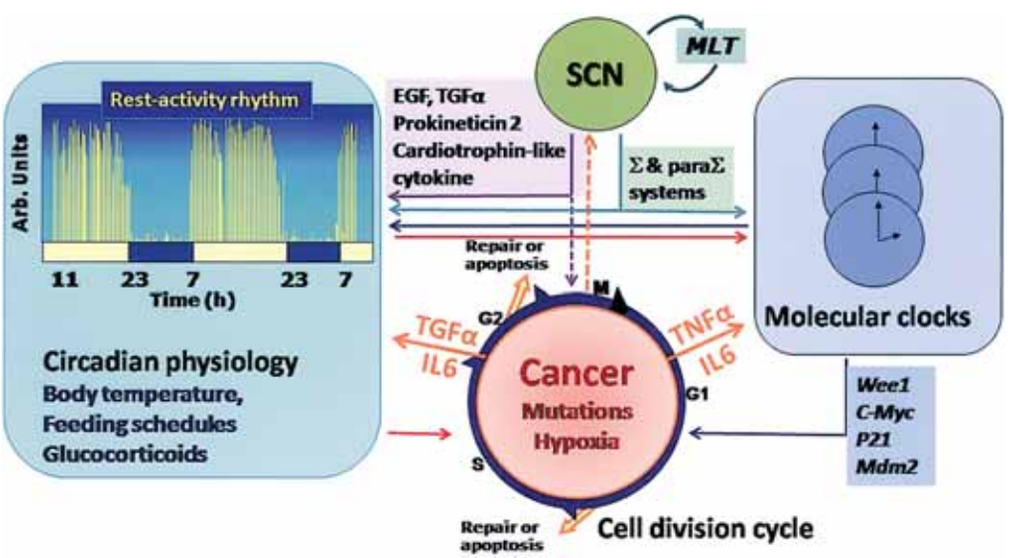

Figure 6. Schematic representation of the interactions among the circadian timing system, the cell division cycle, and cancer.

from G_1 to S in most tumors (Hanahan and Weinberg 2000). Hypoxia is also a classical feature that modifies both cellular proliferation (Keith and Simon 2007) and the circadian clock (Chilov et al. 2001; Tu and McKnight 2006). Furthermore, rhythmic metabolism can also drive rhythmic cell divisions (Chen et al. 2007).

Both gene mutations and metabolism alterations in cancer cells could thus account for defective circadian clock control of malignant cell proliferation. Additionally, malignant tumors can directly or indirectly produce cytokines, such as TGF-α, interleukin-6 (IL-6), or tumor necrosis factor-α (TNF-α) (Aggarwal et al. 2006; Rajput et al. 2007). These cytokines can interfere at various levels of the circadian timing system and result in circadian disruption or alteration (Kramer et al. 2001; Motzkus et al. 2002; Kraves and Weitz 2006; Cavadini et al. 2007). Indeed, severe blunting of the rest-activity rhythm was strongly related to increased serum levels of TGF-α, IL-6, and TNF-α in patients with metastatic colorectal cancer. Significant correlations were found between serum levels of TGF-α and IL-6, circadian patterns in wrist activity, and serum cortisol and tumor-related symptoms in 80 patients with metastatic colorectal cancer. These data support clinically relevant links between tumor cytokines and the circadian timing system (Rich et al. 2005; Rich 2007).

IMPLICATIONS FOR CANCER CHRONOTHERAPEUTICS

The circadian timing system controls cellular proliferation as well as drug metabolism over 24 hours through molecular clocks in each cell, circadian physiology, and the SCN, the hypothalamic pacemaker that coordinates circadian rhythms (Lévi and Schibler 2007). As a result, both the toxicity and efficacy of more than 30 anticancer agents vary by more than 50% as a function of dosing time in experimental models (Mormont and Lévi 2003). The circadian timing system also down-regulates malignant growth in several experimental models and in several clinical situations, as previously discussed.

Programmable-in-time infusion pumps and rhythmic physiology monitoring devices have made possible the application of chronotherapeutics to more than 2000 cancer patients without hospitalization. This treatment method consists in the chronomodulated administration of anticancer agents with appropriate selection of peak times of drug delivery within the 24-hour timescale. This strategy first revealed the antitumor efficacy of oxaliplatin, which is now a main drug used against colorectal cancer. In this disease, international clinical trials have shown a fivefold improvement in patient tolerability and near doubling of antitumor activity through chronomodulated administration, in comparison to constant-rate delivery of the same drug combination, 5-fluorouracil-leucovorin and oxaliplatin (Lévi et al. 1997; Lévi 2001). Recent clinical trials have further shown the relevance of the peak time of the chronomodulated delivery of these cancer medications along the 24 hours for achieving best tolerability: The incidence of severe adverse events varied up to fivefold as a function of the choice of when during the 24 hours the peak dose of the medications was timed. Gender was an important determinant of drug schedule tolerability in this trial (Lévi et al. 2007).

The role of gender on cancer chronotherapeutics is consistent with recent results from a large randomized clinical trial involving 554 patients with metastatic colorectal cancer, where gender also predicted survival outcome on chronotherapy but not on conventional drug delivery (Giacchetti et al. 2006). In this trial, the survival of men with colorectal cancer was significantly improved with the chronotherapy schedule that was applied, whereas that of women was significantly reduced as compared with conventional delivery. The "gender x schedule" interaction remained highly statistically significant following multivariate analyses, suggesting that gender could have an essential role in the determination of optimal chronotherapeutic schedule (Fig. 7) (Giacchetti et al. 2006). A recent study has further revealed that gender largely determines the circadian transcriptome in human oral mucosa, a finding that further supports the hypothe-

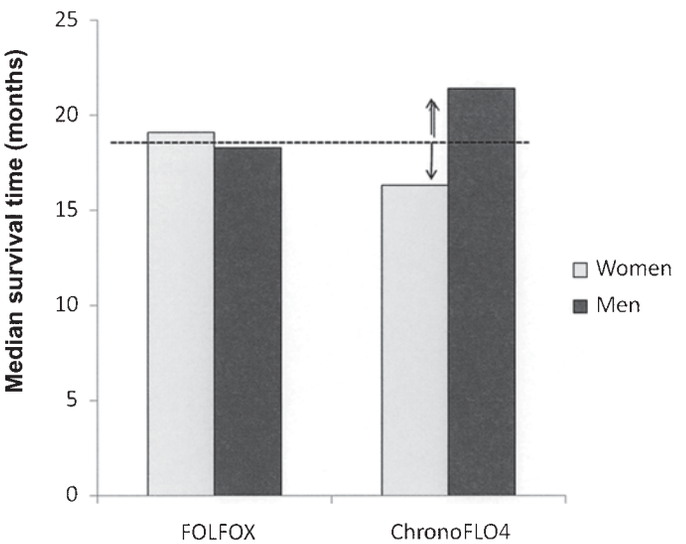

Figure 7. Relevance of gender for the survival outcome of patients with metastatic colorectal cancer receiving conventional delivery (FOLFOX) or chronomodulated infusion (ChronoFLO4). Median survival time of women and men on either delivery schedule in a randomized international trial in 554 patients (gender x schedule interaction test, $p < 0.0001$). (*Dotted line*) Median survival time for all patients on FOLFOX. (Modified from Giacchetti et al. 2006.)

sis of gender dependencies in optimal chronotherapeutics (Bjarnason et al. 2007).

Ongoing translational studies and technology developments are exploring new methods for tailoring cancer chronotherapeutics to the main rhythmic characteristics of the individual patient, an approach needed in view of the dynamic cross-talks between the circadian timing system and the cell cycle along the course of cancer processes. Thus, impaired expressions of the clock genes *Per1*, *Per2*, or *Per3* have been reported in human cancers originating from breast, lung, endometrium, pancreas, or colon, as well as in human myeloid leukemia (Chen et al. 2005; Gery et al. 2005, 2006, 2007a,b; Yeh et al. 2005; Shih et al. 2006; Krugluger et al. 2007). Promoter methylation defects may account for such impaired clock gene expression (Yeh et al. 2005). In human colorectal cancers, a close association has further been shown among decreased *Per1* expression, rapid cellular proliferation, and decreased expression of dihydropyrimidine dehydrogenase, the rate-limiting enzyme of 5-fluorouracil catabolism. In this study, an interaction with gender was also found (Krugluger et al. 2007). The yet limited human cancer data thus corroborate rather well the results from studies in mouse tumors regarding the occurrence of defective circadian clocks in malignant cells and its implications for tumor proliferation and susceptibility to therapeutic agents.

Recent approaches in theoretical models help identify and take into account the main factors that can impinge upon the success of cancer chronotherapeutics. Modeling based on experimental chronotherapeutic data is now confirming the role of an infusional circadian schedule of 5-fluorouracil and oxaliplatin upon toxicity and efficacy. The cell cycle automaton model reveals the key roles of variability in circadian entrainment and cell cycle length upon therapeutic activity of different sinusoidal delivery or constant-rate infusion schedules. This theoretical study pinpoints the need for novel biological assessments in the individual patient and his or her tumor in order to take full advantage of cancer chronotherapeutics (Altinok et al. 2007). A pharmacokinetic-pharmacodynamic model of chemotherapy with circadian periodic dimension investigates the effects of temporal drug-delivery dynamics on a population of tumor cells and its tolerance by a population of fast-renewing healthy cells. The model parameters are based on the experimental chronotherapeutics of mouse GOS with oxaliplatin, whose main toxicity target is the jejunal mucosa. Here also, the model shows the advantage of a periodic time-scheduled regimen, compared to the conventional continuous constant infusion of the same daily dose, when the biological time of peak infusion is correctly chosen. Furthermore, mathematical optimization methods of drug infusion flow, choosing tumor population minimization as the objective function and healthy tissue preservation as a constraint, reveal nonintuitive optimal dynamic schedules of drug delivery that depend on both host/tumor status and therapeutic objectives (Clairambault 2007).

CONCLUSIONS

The circadian timing system controls healthy cells and malignant proliferation. The genetic, epigenetic, and lifestyle factors that impinge on the several components of this system can interfere with the cross-talks between the circadian clock and the cell cycle. This can contribute to the large variability in outcome of patients harboring a similar cancer type. Therefore, targeting therapeutic delivery to the dynamics of the cross-talk among the circadian clock, the cell division cycle, and pharmacology pathways represents a new multidisciplinary challenge to concurrently improve quality of life and survival through personalized cancer chronotherapeutics.

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F. Lévi, E. Filipski, I. Iurisci, et al.

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