Cancer Inhibition through Circadian Reprogramming of Tumor Transcriptome with Meal Timing

Xiao-Mei Li1,3, Franck Delaunay4, Sandrine Dulong1,3, Bruno Claustra6, Sinisa Zampera6, Yoshiro Fuji1,3, Michèle Teboul4, Jacques Beau1,3, and Francis Lévi1,2,3

Abstract

Circadian disruption accelerates cancer progression, whereas circadian reinforcement could halt it. Mice with P03 pancreatic adenocarcinoma (n = 77) were synchronized and fed ad libitum (AL) or with meal timing (MT) from Zeitgeber time (ZT) 2 to ZT6 with normal or fat diet. Tumor gene expression profiling was determined with DNA microarrays at endogenous circadian time (CT) 4 and CT16. Circadian mRNA expression patterns were determined for clock genes Rev-erba, Per2, and Bmal1, cellular stress genes Hspa8 and Cirbp, and cyclin A2 gene Ccn2a in liver and tumor. The 24-hour patterns in telemetered rest-activity and body temperature and plasma corticosterone and insulin-like growth factor-I (IGF-I) were assessed. We showed that MT inhibited cancer growth by ~40% as compared with AL (P = 0.011) irrespective of calorie intake. Clock gene transcription remained arrhythmic in tumors irrespective of feeding schedule or diet. Yet, MT upregulated or downregulated the expression of 423 tumor genes, according to CT. Moreover, 36 genes involved in cellular stress, cell cycle, and metabolism were upregulated at one CT and downregulated 12 h apart. MT induced >10-fold circadian expression of Hspa8, Cirbp, and Ccn2a in tumors. Corticosterone or IGF-I patterns played no role in tumor growth inhibition. In contrast, MT consistently doubled the circadian amplitude of body temperature. Peak and trough respectively corresponded to peak expressions of Hspa8 and Cirbp in tumors. The reinforcement of the host circadian timing system with MT induced 24-hour rhythmic expression of critical genes in clock-deficient tumors, which translated into cancer growth inhibition. Targeting circadian clocks represents a novel potential challenge for cancer therapeutics. Cancer Res. 70(8): 3351-60. ©2010 AACR.

Introduction

The mammalian circadian timing system is composed of a hypothalamic pacemaker, the suprachiasmatic nuclei (SCN), which generates or controls behavioral, physiologic, and metabolic rhythms over the 24 hours. In turn, this circadian physiology, including rhythmic body temperature, locomotor activity, and endocrine release, coordinates molecular clocks in peripheral organs (1, 2). Fifteen specific clock genes are organized through at least three interwoven transcription/translation feedback loops. This molecular clock organizes cellular metabolism and proliferation along the 24 hours by controlling key genes for these functions (3, 4).

Recently, the IARC (WHO) concluded that “shift work that involves circadian disruption was probably carcinogetic to humans” (5). Indeed, shift work has been associated with an increased risk of developing breast, colon, endometrium, or prostate cancer, as well as non-Hodgkin lymphoma (6–9). In addition, cancer patients can display disrupted rest-activity or cortisol patterns. Such circadian disruption predicted for poorer survival as compared with patients with near-normal circadian patterns, independently of all known prognostic factors (10). In mice, the disruption of circadian clocks through mutation of clock gene Per2 or chronic jet lag was associated with accelerated cancer progression (11, 12) and even promotion (13). The molecular clock further controls transcription of core genes for cell cycle, apoptosis, and DNA repair (14–17). However, not all types of molecular circadian disruption may be responsible for accelerated carcinogenesis, as shown for Cry1−/−Cry2−/− or Clock/Clock mutant mice (18–20). Taken together, the results suggest that some specific components in the host circadian timing system function as tumor suppressors.

The restriction of food availability for 4 to 6 hours during the light span in rats or mice shifts clock gene expression rhythms in liver or gastrointestinal tract, with little modifications, if any, in the SCN (21–23). Indeed, meal timing (MT) synchronized circadian molecular clocks in peripheral organs...
of mice despite prior SCN ablation (21) or jet lag–induced molecular clock disruption (12).

Here, we first seek whether progression of a pancreatic cancer model can be halted with MT during the light span. We then investigate circadian clock gene expression in mouse liver and tumor and perform the first circadian assessment of tumor transcriptome as a function of feeding schedule. We searched for some form of circadian induction mechanism with MT that would match that previously reported for soli-ciclib (24). This leads to the identification of a critical role of body temperature rhythm in the 24-h entrainment of tumor genes involved in malignant progression.

Materials and Methods

Animals and synchronization. Two experiments (Exp I and Exp II) involved male B6D2F1 mice ages 6 wk (JANVIER), kept in an alternation of 12 h of light (L) and 12 h of darkness (D) from 7:00 a.m. to 7:00 p.m., with food ad libitum (AL) during the first week and water AL throughout the entire experiment. Mice were randomly allocated to one of three or four groups in Exp I and Exp II, respectively. These groups differed according to feeding schedule (AL or MT) and diet [normal (N) or fat (F)]. Mice on MT had access to food from Zeitgeber time (ZT) 2 to ZT6 (ZT0 corresponds to light onset and ZT12 to dark onset). MT was initiated 1 wk after arrival of mice at our facility. Both normal and fat diets contained similar amounts of casein (21.4% and 23%), sugar (51.7% and 52%), cellulose (4.9% versus 5%), and vitamins and minerals (5.1% and 5.0%). However, the lipid and calorie contents of the normal diet were 5% and 3,200 kcal/kg, respectively, with 12.2% moisture (04-10, SAFE), whereas corresponding values were 15% and 4,300 kcal/kg for the fat diet (D 279, INRA). All manipulations during the dark span were done under dim red light (7 lux). Both experiments were conducted in accordance with the guidelines approved for animal experimental procedures by the French Ethical Committee, decree 87-848.

Study design. All the mice received a s.c. implantation of a 3-mm³ fragment of P03 pancreatic adenocarcinoma in both flanks 5 or 3 wk after MT onset in Exp I and Exp II, respectively. Tumor weight was measured daily at ZT2 as previously described (25). Body weight was measured daily at ZT2 and ZT16 for all groups.

In Exp I, the effects of MT and diet on tumor progression were related to the circadian characteristics of rest-activity and body temperature. Fifteen mice (five per group) were randomly allocated to AL-N, MT-N, or MT-F.

In Exp II, 62 mice (15–17 per group) were randomized to AL-N, AL-F, MT-N, or MT-F.Twenty-one days after tumor inoculation, all the mice were exposed to constant darkness for the 2 d preceding sampling to avoid masking effects of L (26). In such case, time was expressed as circadian time (CT), with CT0 corresponding to subjective L onset and CT12 corresponding to subjective D onset. Blood, liver, and tumor were obtained at CT0, CT4, CT9, CT12, or CT16 in two to four mice per CT. Blood was obtained in EDTA, then plasma was extracted by centrifugation at 3,000 × g for 25 min at 4°C and stored at −80°C until corticosterone or insulin-like growth factor-I (IGF-I) determinations. Liver and tumor fragments (300 mg) were immediately frozen in liquid nitrogen, then stored at −80°C until RNA extraction. DNA microarrays were done on the tumors sampled at CT4 or CT16. Circadian changes in mRNA expression of selected genes according to microarrays were further determined with quantitative reverse transcription-PCR (RT-PCR) in tumor and liver.

Quantitative RT-PCR. Total RNA from liver and tumor was purified using the method of Chomczynski (27) and stored at −80°C until use. Five micrograms of total RNA were treated with DNase I (Invitrogen) and converted into cDNA using High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s recommendations. RT-PCR was done to determine the mRNA expression of clock genes Rev-erba, Per2, and Bmal1, cellular stress genes Hspa8 and Cirbp, cyclin A2 gene Cen2a, and housekeeping reference gene 36B4, using QuantiTect SYBR Green PCR kit (Qiagen S.A.) and Light Cycler 2.0 (Roche Diagnostics) under conditions of amplification as previously described (24). Respective forward and reverse sequences of specific primers were designed (three clock genes and 36B4 gene; ref. 24): Hspa8, 5′-CGTACCTCGGAAAGACGGTA-3′ and 5′-TCCTTTCACTCGACCTCTC-3′; Cirbp, 5′-GCAGATCTCC-GAAGTGCTG-3′ and 5′-CAAGCTGTCTCAACTCTGAT-3′; Cen2a, 5′-AAAGCTCGAGGTTGGTC-3′ and 5′-CCAGGGCATCTCACACCTCT-3′. All primers were obtained from Invitrogen Life Technologies. The relative expressions of the target genes were normalized to 36B4 (24).

DNA microarrays. Total tumor RNA was obtained from three AL-N and three MT-N mice at CT4 and at CT16. It was retrotranscribed, converted to cRNA, and hybridized to Mouse Genome 430A 2.0 GeneChips (Affymetrix) according to the manufacturer’s recommendations. Processing of the raw data and identification of the differentially expressed genes as well as the enriched functional classes between the two feeding regimens were done using the ChipInspector and Bibliosophe softwares (Genomatix; see Supplementary Data).

Rest-activity and body temperature. A telemetric sensor (PhysioTel, TA10TA-F20, Data Sciences, Inc.) was implanted i.p. under general anesthesia in all the mice in Exp I, 3 wk after MT onset (25). Rest-activity and body temperature were recorded every 10 min for 1 wk before and 4 wk after tumor inoculation.

Corticosterone and IGF-I determinations. Corticosterone and IGF-I concentrations were determined by RIA (28). Briefly, for corticosterone, 5 μL of plasma were extracted with 3 μL of ethyl ether; the residue was dissolved in 100 μL of assay buffer before incubation with 1,2,6,7-[3H]corticosterone (Amersham) and rabbit anti-corticosterone antibody (Valbionech). For IGF-I assay, a rat IGF-I RIA kit (DSL-2900, Diagnostic Systems Laboratories) was used. IGF-I was extracted from plasma with acid-ethanol according to the manufacturer’s recommendations. The cross-reactivity of mouse IGF-I is 75%.

Statistical analyses. Mean and SEM were computed for each variable and/or time point. One-way or multiple-way ANOVA with Scheffé post hoc test was done to validate intergroup differences. For comparisons of longitudinal tumor weight data, repeated-measures ANOVA was used. For gene
expression data, single cosinor analysis provided mesor (rhythm-adjusted mean), double amplitude (difference between minimum and maximum of fitted cosine function), and acrophase (time of maximum in best-fitting cosine function, with light onset as phase reference), with their respective 95% confidence limits. The significance of the difference according to feeding regimes was calculated by using t-test approximation of the standard F-statistics (29).

Locomotor activity and body temperature records of each mouse were split into five time series of 7 d each, including baseline (1 wk before tumor inoculation). The dominant period with the largest amplitude was determined in each time series with power spectrum analysis (Complex Fast Fourier Transform) using Mathematica (Wolfram Research, Inc.). Population rhythm parameters were computed for periods of 24, 12, and 8 h for each group by using standard population cosinor procedures. Obtained parameters were compared with Hotelling’s $T^2$ test (29) and summed up to reconstruct the circadian signal. All statistical analyses were carried out with dedicated tools developed under SPSS (v16, SPSS, Inc.).

**Results**

**Body weight change.** Feeding schedule had similar effects on body weight in Exp I and Exp II. Overall, mice on MT lost 9.9 ± 0.8% of their body weight while on MT-N, as compared with 9.4 ± 0.7% in mice on MT-F within the 3 days following MT onset. Body weight then gradually recovered to values similar to those in AL-N mice. Conversely, body weight increased gradually in mice on AL-N, and it did more prominently so in the mice on AL-F. Mean body weight on the day preceding tumor inoculation was highest in the mice on AL-F ($P = 0.014$, ANOVA; Supplementary Fig. S1A). Scheffe’s post hoc test further revealed statistically significant difference between AL-F and MT-N only ($P = 0.02$). Similar results were found 21 days after tumor inoculation, yet no difference was statistically validated ($P = 0.4$; Supplementary Fig. S1B).

**Tumor progression.** Tumors started to become measurable 1 week after inoculation in both experiments. In Exp I, tumor growth rate was similar in both MT groups without any diet-related difference. However, tumor grew faster in the AL mice. Twenty-eight days after tumor inoculation, mean tumor weight was nearly twice as large on AL-N as compared with MT-N or MT-F (Fig. 1A).

In Exp II, similar differences in tumor growth curves were found between the AL-N groups on one hand and both the MT-N and MT-F groups on the other hand (Fig. 1B). Pooled analysis of both Exp confirmed that MT significantly slowed down tumor growth as compared with AL, without any significant influence of diet (Fig. 1C).

**Clock gene expression patterns in liver and tumor.** In the liver of AL-N mice with pancreatic adenocarcinoma, mean mRNA expression of clock genes Rev-erha, Per2, and Bmal1 oscillated with 26-, 10-, and 10-fold respective magnitudes over the 24 hours. Peaks occurred at CT4 for Rev-erha, at CT12 for Per2, and at CT20 for Bmal1 (Fig. 2A1). MT-N advanced peak times by 12 hours for Rev-erha and by 8 hours for Per2 and Bmal1 (2A2). Change in diet composition exerted no apparent effect (Fig. 2A3 and A4). According to single cosinor comparison tests, MT significantly shifted the circadian acrophases of all three clock genes in liver ($P < 0.002$). MT further increased the circadian amplitude of Rev-erha ($P = 0.009$) and Per2 ($P = 0.00005$), yet it reduced that of Bmal1 ($P = 0.0005$) in this organ.
In the tumor of mice fed with normal diet, no circadian rhythm was observed for Rev-erbα, Per2, or Bmal1 whatever the feeding schedule (P ≥ 0.1; Fig. 2B1 and B2), whereas a low amplitude rhythm was validated with cosinor for Rev-erbα and Bmal1 in AL-F mice (P < 0.04) and for Rev-erbα in MT-F mice (P = 0.0007; Fig. 2B3 and B4).

**Time-qualified response of tumor transcriptome to MT.**

MT significantly modified the expression patterns of 423 genes in tumor according to CT. Pairwise comparisons between AL-N and MT-N indicated that most of the transcriptional changes were detected at CT16 as compared with CT4, with a majority of the genes being upregulated (Fig. 3A).

Functional categorization revealed a large proportion of the differentially expressed transcripts involved in cellular stress, cell cycle, and metabolism domains (Fig. 3B; Supplementary Tables S5–S8). A gene ontology (GO)-based statistical analysis following a biological process–based filtering of the gene lists showed that most overrepresented GO terms were related to response to unfolded proteins or stress, cell cycle, and immune system (Supplementary Tables S1–S4). The significant enrichment for these three biological processes indicated that specific key determinants of tumor growth were regulated by MT.

MT even upregulated mRNA expression at one CT and downregulated it 12 h apart for 36 genes, most of which

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**Figure 2.** Effects of MT on the mRNA expression patterns of clock genes in mice bearing advanced pancreatic adenocarcinoma. The circadian clock is described by the adjustment of a cosinor curve with a 24-h period for Rev-erbα (blue), Per2 (red), and Bmal1 (green). Solid line, P < 0.05; dotted line, P > 0.05, by cosinor analysis. Respective acrophases (computed time of maximum) and amplitudes (half the computed extent of variation) with their 95% confidence limits (C.L.) are shown for each clock gene by horizontal and vertical bars, respectively. A, liver clock genes from mice on AL-N (A1), MT-N (A2), AL-F (A3), or MT-F (A4). Note that left ordinate correlates to Rev-erbα expression and right ordinate to Per2 and Bmal1. Cosinor analyses revealed sinusoidal circadian rhythms for the liver of each clock gene expression in both feeding schedules (P ≤ 0.01). Three-way ANOVA validated interactions between feeding schedule and CT for each clock gene (P < 0.001). Neither a significant role of diet (Rev-erbα, P = 0.128; Per2, P = 0.451; Bmal1, P = 0.991) nor an interaction between diet and feeding schedule (P ≥ 0.48) was found. B, tumor clock genes from mice on AL-N (B1), MT-N (B2), AL-F (B3), or MT-F (B4). Three-way ANOVA: synchronizing effect of MT for Rev-erbα only (feeding schedule and CT interaction, P = 0.049). No significant role of diet, feeding schedule, and CT for each clock gene was validated (P ≥ 0.20). The relative mRNA expression of each interest gene was normalized to the level of 36B4 gene. Horizontal axis, hatched and black rectangles indicate subjective light and dark spans of photoperiodic cycle, respectively.
control cellular stress, cell cycle, and cell organization (Fig. 3C). For instance, Klf6, a zinc finger transcription factor and tumor suppressor gene that also arrests cells at G1-S transition (30), was upregulated at CT16, when Sfi1, which halts G2-M transition (31), was downregulated. Twelve hours later, Klf6 was downregulated and Sfi1 was upregulated. Expressions of most stress and unfolded protein response genes, including heat shock proteins (Hsp), were upregulated at CT4 and downregulated at CT16. Conversely, expressions of Cirbp and genes that regulate cell cycle or cell organization were downregulated at CT4 and upregulated at CT16. Moreover, expressions of the positive regulators of pancreatic differentiation, Sox9 and Insm1, were downregulated at CT16 and upregulated at CT4 (Fig. 3C). MT further resulted in the overexpression of Bhlhb8, another pancreatic differentiation regulator, at CT4 (Supplementary Table S6). Only two genes were either upregulated (Nov) or downregulated (Alas2) at both time points. Thus, MT resulted in an extensive and time-dependent transcriptional reprogramming within the tumors. Microarray data are available at EBI ArrayExpress.

Figure 3. Circadian reprogramming of pancreatic adenocarcinoma transcriptome with MT. Results from DNA microarray in tumor. A, bar graph representing the number of genes upregulated (red) or downregulated (green) with MT as compared with ad libitum according to CT. B, pie charts depicting the most highly represented functional categories of genes with significantly modified expression on MT at CT4 or at CT16 as compared with ad libitum. C, heat map showing opposite transcriptional changes at CT4 and CT16 in mice on MT as compared with AL.
Circadian changes in mRNA expression of selected genes in tumor and liver. Quantitative RT-PCR confirmed microarray assessment of downregulation and/or upregulation with MT. Because largest upregulations and downregulations were found for Hspa8, Cirbp, and Ccna2 in the microarray study, the circadian patterns of these three genes in mice on normal food were further determined with quantitative RT-PCR.

In tumor, no 24-h pattern was validated for the mRNA expression of Hspa8, Cirbp, and Ccna2 in mice on AL-N (cosinor, P = 0.08, P = 0.90, and P = 0.15, respectively). Conversely, MT-N induced a >10-fold 24-h variation in the transcriptional activity of these three genes, with peak expression occurring at CT0 for Hspa8 and Ccna2 and at CT16 for Cirbp (Fig. 4A–C).

Hspa8 expression more than tripled along the 24 hours in the liver of mice on AL-N, with a maximum at CT9 and a trough at CT16 (P = 0.008). MT-N advanced peak time of liver Hspa8 by 5 hours (P < 0.001; data not shown). Conversely, Cirbp was arrhythmic in the liver of mice on AL-N. MT-N induced a circadian rhythm in Cirbp expression, with a peak near CT16 (P = 0.008; data not shown). No circadian variation was documented in liver for Ccna2 whatever the feeding schedule (P > 0.3).

Circadian biomarkers. Mice on AL-N displayed a reproducible rest-activity rhythm, with high activity during darkness, on the week preceding tumor inoculation as well as on the following weeks. Conversely, mice on MT-N or MT-F displayed bimodal rest-activity rhythm, with high activity both in the early light and dark spans, respectively. The rest-activity rhythm of the mice in each group gradually dampened and became fragmented along the course of tumor growth (Fig. 5A). Conversely, body temperature values were highest during darkness in mice on AL-N and during light in mice on either MT-N or MT-F. The body temperature rhythmic patterns remained similar along the course of tumor growth in the three groups (Fig. 5B).

Spectral analysis of baseline time series of rest-activity revealed that the dominant period was 24 h in AL-N mice and 12 h in MT-N or MT-F mice with an 8-h component. The reconstructed circadian signal confirmed prominent differences in the rest-activity 24-h pattern as a function of feeding schedule and diet (Fig. 5A).

The baseline body temperature rhythm was reconstructed using statistically validated dominant 24-h period and 12-h harmonic for all three groups and 8-h harmonic for both MT groups. Such signals differed markedly as a function of feeding schedule, with minimal influence of diet composition (Fig. 5B). Thus, baseline body temperature increased from a nadir at ZT8 to a higher maximum at ZT15 in AL-N mice; a second maximum was found at ZT2. Conversely, body temperature increased from a nadir at ZT21 to a maximum at ZT3 in both MT groups. The peak-to-trough difference in the reconstructed circadian signal was 3.9°C on MT-N and 2.7°C on MT-F as compared with 1.7°C on AL-N (Hotelling’s t test, P < 0.001).

Corticosterone and IGF-I rhythms. Mean plasma corticosterone concentration was significantly increased with MT (P = 0.017) and reduced with fat diet (P = 0.013) according to three-way ANOVA. This analysis further revealed an interaction term between feeding schedule, diet, and CT (P = 0.047). Mean concentration of corticosterone increased 10-fold from a nadir at CT0 to a maximum at CT12 on AL-N. MT-N shifted this rhythm by 12 hours. The 24-h pattern in plasma corticosterone was flattened in mice on fat diet whatever the feeding schedule (Supplementary Fig. S3A).

Mean plasma IGF-I concentration varied according to CT (P = 0.013), without any significant effect of feeding schedule.
(P = 0.103) or diet (P = 0.558) or any interaction, according to three-way ANOVA. Plasma IGF-I concentration was highest at CT0 and reached a nadir at CT16 in AL-N mice. This pattern dampened in mice on MT-N, AL-F, or MT-F (Supplementary Fig. S3B).

**Discussion**

Meal timing during the light span, when mice usually rest, significantly slowed down the growth rate of a transplantable pancreatic adenocarcinoma in two separate experiments compared with ad libitum feeding. This result confirmed our earlier report in the same mouse strain for the growth rate of Glasgow osteosarcoma (25). Tumor growth inhibition through limited access to food was initially thought to be the consequence of calorie restriction (32, 33). In the current investigation, mice on MT-N initially lost nearly 10% of their body weight, yet they had completely recovered within 2 weeks. Body weights subsequently remained similar in mice on AL-N, MT-N, or MT-F. High-fat diet increased body weight in all groups.

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**Figure 5.** Amplification and phase shift of circadian rhythms in locomotor activity and core body temperature with MT. Results are shown for mice on AL-N (left), MT-N (middle), or MT-F (right). Double-plotted representation of locomotor activity (A, top row) and body temperature (B, top row). Horizontal axis, the open and dark rectangles respectively represent the alternation of 12 h of light and 12 h of darkness for 2 consecutive days; food availability is depicted with hatched rectangular boxes. Arrow, day of tumor inoculation. Reconstructed circadian signal of locomotor activity (A, bottom row) and body temperature (B, bottom row) based on statistically validated 24 h, 12 h ± 8 h harmonics identified with spectral and cosinor analyses of 7-d time series before tumor inoculation.
weight in AL mice only, yet this was not associated with any acceleration of tumor growth. Therefore, the current study supports a major role of feeding times rather than diet composition or calorie intake in the control of tumor growth. Diet composition did not significantly modify tumor progression in mice on either feeding schedule, despite altering the 24-h patterns of plasma corticosterone and IGF-I. Thus, neither corticosterone nor IGF-I rhythms seemed to play any relevant role in the circadian control of tumor progression.

The circadian expression patterns of clock genes Rev-erba, Per2, and Bmal1 remained unaltered in the liver of mice fed AL, despite the mice bearing growing s.c. P03 pancreatic adenocarcinoma (34). High-fat diet did not modify peak times, mean levels, or amplitudes of any of these three clock genes. MT advanced by 8 to 12 hours the sequence of the expression patterns of the three clock genes, consistent with other reports in healthy rodents (21, 22). MT further significantly increased the circadian amplitude of Rev-erba by 40% and that of Per2 by 93%. This observation at tissue level could result from an increased coordination of circadian clocks by MT in individual liver cells.

No circadian rhythm was observed for the three clock genes in P03 pancreatic adenocarcinoma whatever the feeding condition. Consistently, the microarray study showed that no clock gene was identified in the data sets influenced by MT. Thus, the molecular circadian clock in this pancreatic cancer model remained severely disrupted despite MT. The limited effects of MT on the tumor molecular clock contrasted with the consistent inhibition of tumor growth in MT mice. MT thus bypassed the defective tumor clock and entrained clock-controlled pathways possibly through rhythmic availability of metabolic cues. Indeed, a significant proportion of the clock-controlled transcriptional program in peripheral tissues was entrained by systemic cues rather than by the local molecular clock system (35). In the current study, the ability of MT to entrain tumor cellular stress response and cell cycle genes was emphasized in the microarray study and confirmed with quantitative RT-PCR.

We showed a >10-fold rhythmic induction of Hspa8, Cirbp, and Ccna2 transcription in tumors by MT. Physiologic temperature fluctuations drive rhythmic DNA binding of heat shock factor-1, which in turn regulates HSP transcription (36). We then hypothesized that the endogenous circadian rhythm in core body temperature could play a critical role in the reprogramming of tumor transcriptome. Indeed, irrespective of diet, MT consistently modified the circadian patterns in rest-activity and body temperature, two main outputs of the hypothalamic pacemaker. More specifically, MT not only advanced the phase of host body temperature rhythm by ~12 hours but also nearly doubled its circadian amplitude. In mice bearing Glasgow osteosarcoma, MT during darkness doubled the circadian amplitude in body temperature and reduced tumor growth by ~30% as compared with ad libitum feeding. MT during light nearly tripled the circadian amplitude of body temperature and reduced tumor growth by 62% (25). This finding further supports a critical role of the circadian amplitude in host core body temperature for cancer control. In the tumors of the mice on MT, Hspa8 peaked near body temperature maximum, whereas Cirbp, which encodes for a cold inducible protein, peaked near body temperature trough. These results are consistent with the divergent temperature-dependent transcription of Hspa8 and Cirbp (36, 37).

Hspa8 transcriptional activity reportedly facilitates G1-S transition in cycling cells (38). This accords well with the coincident peak expressions of Hspa8 and Ccna2 in the tumors of mice on MT. On the contrary, Cirbp transcription results in the gating of G1-S transition (39). Thus, MT likely imposed alternative activation and gating of G1-S transition in tumors, which occurred 12 hours apart. Consistently, the microarray study revealed a major enrichment of stress and cell cycle genes, which were upregulated or downregulated with MT according to circadian time. Many types of mammalian cells failed to undergo the G2-M transition after cooling from 37°C to 16–20°C. One-hour exposure to severe hypothermia (4–10°C) further induced a high degree of mitotic synchrony, which indicates that cell cycle checkpoint could be triggered in response to cold shock (40). However, the temperature values that produced such cell cycle effects were largely below the nadir of body temperature in our study. Our results

Figure 6. Hypothetical scheme integrating MT effects on the circadian organization of healthy and cancer tissues. The circadian timing system is coordinated by the SCN, an endogenous pacemaker, whose periodicity is calibrated to precisely 24 h by the alternation of 12 h of light and 12 h of darkness (top). The SCN generate the circadian rhythm in core body temperature. Physiologic time cues emanating from the SCN coordinate functional circadian clocks and downstream clock-controlled pathways in healthy peripheral tissue (bottom left). MT then resets the clocks in these tissues and shifts its circadian organization. Conversely, cancers lack functional clocks at tissue level (bottom right). Amplification of body temperature with MT then induces rhythmic reprogramming of tumor transcriptome via cell stress genes, including Hspa8 and Cirbp. Such reprogramming mainly affects cell cycle, cell organization, and metabolism genes and halts tumor progression (bottom right).
support an essential role for the amplification of the circadian rhythm in body temperature rather than instantaneous temperature values for the reprogramming of critical genes involved in cancer progression. About one third of patients with metastatic cancer displayed circadian disruption, which has been associated with poor survival (10, 41). The results in the current study emphasize that timing of meals or parental nutrition could both revert circadian disruption and prolong survival in patients. Such novel circadian-based supportive care deserves clinical testing.

In conclusion, we show that MT induces rhythmic expression of critical genes in tumors in spite of nonfunctional molecular clocks. We identify the endogenous circadian rhythm in core body temperature as a likely circadian physiologic signal for entrainment of cell cycle and metabolism genes in tumor through cellular stress genes (Fig. 6). As a result, MT partly inhibits cancer progression. We expect that these findings will foster the integration of circadian clocks into a novel paradigm of cancer prevention and therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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