Effects of Light and Food Schedules on Liver and Tumor Molecular Clocks in Mice

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Background: Disrupted circadian coordination accelerates malignant growth, but the molecular mechanism is unclear.

Methods: Healthy or Glasgow osteosarcoma–bearing mice (n = 162) were synchronized with light and darkness over 2–3 weeks, submitted to an 8-hour advance of light every 2 days (chronic jet lag) to disrupt circadian coordination, or submitted to chronic jet lag and meal timing to prevent molecular clock alteration. The expression of molecular clock genes and of the cell cycle genes c-Myc and p53 in liver and tumor was determined with quantitative reverse transcription–polymerase chain reaction at six circadian times over a 24-hour period of light and darkness and analyzed with analysis of variance and cosinor. Tumor weight was measured daily over the course of the experiment. All statistical tests were two-sided. Results: In synchronized mice, mean mRNA levels of clock genes Rev-erbα, Per2, and Bmal1 varied by 206-, four-, and 26-fold, respectively, over the 24 hours in healthy mouse liver; by 36-, 35-, and 32-fold in the livers of tumor-bearing mice; and by 9.4-, 5.5-, and sixfold in tumor tissue (P = .046 to < .001). In mice subjected to chronic jet lag, the periodic changes were dampened and the clock gene rhythms were temporally shifted in liver and ablated in tumor, and tumor growth was accelerated. Meal timing reversed the chronic jet lag–induced alterations in Rev-erbα and Per2 expression in liver and of all three clock genes in tumor and slowed tumor growth. Tumor growth differed as a function of light and feeding schedules (P = .04). No obvious rhythm was detected for p53 or c-Myc in liver or tumor tissues of synchronized mice. In healthy mice subjected to chronic jet lag, the mean level of p53 expression was cut in half (P = .002), and a 12-fold circadian variation in c-Myc mRNA level (P = .03) was induced in the liver of healthy mice, whereas complex expression patterns were found in the liver and tumor of tumor-bearing mice. Conclusions: Altered light–dark or feeding schedules modified the expression of molecular clock genes and genes involved in carcinogenesis and tumor progression. [J Natl Cancer Inst 2005;97:507–17]
a recognizable circadian pattern in rest–activity cycles or body temperature. Moreover, the growth rate of a tumor transplanted into mice submitted to such chronic jet lag was accelerated compared with that in synchronized mice (11).

The complex machinery of the molecular clock has recently been shown (5,12,13) to exert a negative effect on the transcriptional activity of some key genes involved in cell cycle regulation, thereby suggesting that the circadian clock regulates cell proliferation. Indeed, circadian rhythms in cell cycle phase distribution have been extensively reported in both healthy and malignant mammalian tissues (14–17). Two recent studies have further identified the cell cycle genes c-Myc, p53, and Weel as clock-controlled genes (5,12). These genes have multiple cell cycle–related functions, with c-Myc and Weel controlling cell cycle progression from G1 to S and from G2 to M, respectively. In addition, c-Myc can exert pro-apoptotic effects through p53-dependent or -independent pathways (18–20).

Many other cell cycle–related genes display 24-hour rhythms in mRNA and/or protein expression in healthy tissues from rodents and/or humans. These genes include cdk2; cyclins A, B1, D, E, or mdm2, which control cell cycle checkpoints; or gadd45α, bcl2, and bax, which regulate apoptosis (5,12,15,21,22).

The molecular circadian clock exerts a rhythmic negative regulation on cell cycle progression, which possibly accounts for a tumor suppressor–like effect of the circadian clock in the context of γ radiation exposure (12). Indeed, mice with a constitutive mutation of clock gene Per2 not only lose circadian rhythmicity in locomotor activity when kept in constant darkness but are more susceptible to develop γ-radiation–induced cancers than wild-type mice (12).

Similarly, chronic jet lag, a condition that accelerates growth of transplanted tumors, also ablates the rhythms in mRNA expression of clock genes Per2 and Rev-erba both in the liver and in the tumor of mice with Glasgow osteosarcoma (11). By contrast, meal timing, a condition that resets circadian clocks in peripheral tissues (23,24), slows down tumor growth in mice (25).

Taken together, the results of the studies mentioned above support the finding that the circadian system exerts a negative regulation on malignant processes, be it through circadian physiology and/or molecular clocks (1,22). Here, we investigated whether the disrupting effect of chronic jet lag on the molecular circadian clock would, in turn, affect the mRNA expression of c-Myc and p53 and ultimately increase tumor cell proliferation. Two experiments were carried out to assess the effects of chronic jet lag on the 24-hour patterns in the expression of these clock genes and clock-controlled genes in the liver of mice with ablated suprachiasmatic nuclei (27), a condition that also disrupts circadian rhythms of activity and temperature (27).

Tumor Implantation

Mice were anesthetized with a single intraperitoneal injection of 0.5-mL solution of 10 g of 2,2,2-tribromoethanol (Fluka, Saint-Quentin-Fallavier, France) in 10 mL of 2-methyl-2-butanol (Fluka) diluted 1:39 in 0.9% NaCl. Ten days after the beginning of chronic jet lag, eventually combined with meal timing, all the mice in experiment 2 received a subcutaneous implantation of a 3-mm³ fragment of Glasgow osteosarcoma in both flanks (10). The growth of this transplanted tumor has previously been shown to be regulated by the hypothalamic clock (10). Tumor weight was measured daily, as previously described (10).

Tissue Sampling

Ten days after the beginning of chronic jet lag (experiment 1) or 15 days after tumor inoculation (experiment 2), subgroups of mice from each group were killed at one of six different circadian times separated by 4 hours. The sampling circadian times were relative to the time of light onset, CT0. All mice were exposed to constant darkness during the 2 days preceding sampling to prevent any masking effect of light on circadian rhythms (28). There were 10 control and 10 jet-lagged mice per time point in experiments 1 and two or three mice per time point and per group in experiment 2. Approximately 30 mg of liver (experiment 1) or...
liver and tumor (experiment 2) was surgically removed from each mouse, frozen immediately in liquid nitrogen, and stored later at −80 °C until RNA extraction.

**Quantitative Reverse Transcription–Polymerase Chain Reaction**

Circadian expression patterns were determined for the clock genes Per2, Cry1, Bmal1, and Rev-erbα and the cell cycle–controlled genes c-Myc and p53 in the liver of healthy mice in experiment 1. In experiment 2, circadian expression was measured for Per2, Bmal1, Reverba, c-Myc, and p53 in liver and in tumor samples.

Total RNA was extracted from the frozen tissue specimens using GenElute Mammalian total RNA kit (Sigma-Aldrich Chimie, St. Quentin-Fallavier, France) for experiment 1 or RNeasy Mini Kit (Qiagen S.A., Courtabeuf, France) for experiment 2, according to the manufacturers’ instructions. Extracted RNA concentration and purity were assessed by optical density at 260 and 280 nm. RNA integrity was assessed by the visualization of 18S and 28S RNA subunits following 1 M formaldehyde–1% agarose gel electrophoresis and ethidium bromide staining (0.01 μg/mL) (29). The RNA was stored at −80 °C until use.

All oligonucleotide primers for PCR were obtained from Invitrogen Life Technologies (Cergy Pontoise, France). All primer pairs were designed with at least one primer comprising two successive intron–exon splicing sequences of the appropriate gene pairs to avoid amplification of contaminating genomic DNA. Respective forward and reverse sequences of primers were as follows: 5′-ATG TGC AGC TGA TAA AGA CTG G-3′ and 5′-AGG CCT TGA CCT TTT CAG TAA G-3′ (ARP/36B4); 5′-GGT GGT GTA G-3′ and 5′-AAG CCT GTA AGG GTT GGT GTA G-3′ (Per2); 5′-TGG CCT CAG GCT TCC ACT ATG-3′ and 5′-CCG TTG CTT CTC TCT CCT GGG-3′ (Rev-erbα); 5′-TGT CAC AGG CAA GTT TTA CAG AC-3′ and 5′-ACA GTG GGA TGA TGC CTC TTT G-3′ (Bmal1); 5′-GAA GAG CCT GCC TTT GAT ACA G-3′ and 5′-CAA GGG ATC TGA ACA CAG ATG G-3′ (Cry1); 5′-CTC AAA AAA CTT ACC AGG GC-3′ and 5′-CAC CAC GCT GTG CCG AAA AGT CTG-3′ (p53); 5′-AGT GCA TTG ACC CTC AGT CCT TCC A-3′ and 5′-CAG CTC CTT CCT CTT CGT ACG TCT AAT GAC GTT-3′ (c-Myc).

One-step RT-PCR was performed with a Light Cycler instrument (Roche Diagnostics, Meylan, France) in a total volume of 20 μL containing 500 ng of total RNA, 7 mM MgCl2, 0.75 μM each primer, Light Cycler RT-PCR Reaction Mix SYBR Green I, and Light Cycler RT-PCR Enzyme Mix. All reagents were purchased from Roche. The protocol consisted of four stages: reverse transcription of template RNA, denaturation of the cDNA–RNA hybrid, amplification of cDNA, and melting curve analysis for product identification. Reverse transcription was performed at 55 °C for 10 minutes. The denaturation and amplification conditions were 95 °C for 30 seconds followed by 40 cycles of PCR. Each cycle of PCR included immediate denaturation at 95 °C, 10 seconds of primer annealing at 60 °C (59 °C for c-Myc), and 13 seconds of extension/synthesis at 72 °C. At the end of the extension step, the fluorescence of each sample was measured at 84 °C (85 °C for Bmal1) to eliminate the background fluorescence generated by primer dimers. After 40 cycles of amplification, a cDNA melting curve was obtained by heating the samples at 20 °C/s to 95 °C, cooling them at 20 °C/s to 65 °C, and slowly heating them again at 0.1 °C/s to 95 °C, whereas fluorescence data were collected at 0.1 °C intervals. The intraassay and interassay variation coefficients were less than 1% and less than 6%, respectively. The efficiency of each primer pair to amplify was validated by construction of serial dilution curves and was comparable among all the genes studied, being approximately 90% (data not shown). The specificity of the PCR products was assessed by melting curve analysis and by agarose gel electrophoresis to check for the presence of nontarget products and to confirm that the size of the product corresponded to that of the expected amplicon.

**PCR Data Analysis**

The relative levels of each mRNA of the genes of interest were normalized to that of 36B4 by the following formula: relative mRNA level = 2^([Cp[target gene] - Cp[reference gene]], where Cp (crossing point) is the cycle number at which the fluorescence signal reaches the threshold of detection. The crossing point was determined using Lightcycler software version 3.5 either using the second derivative maximum method (experiment 1) or using the fit points method (experiment 2). In the fit points method, the noise band was set above the background fluorescence in each run, and the threshold for analysis was adjusted according to the fluorescence level of the known positive standard sample added to each run and related to a previously constructed standard curve using 10-fold serial dilutions. As a result, all six time points were analyzed in duplicate for each gene and each group in a single run. The fit points method was considered as being possibly more precise than the second derivative maximum method and was used in experiment 2 because of the limited number of samples per time point.

**Statistical Analysis**

Seven-day time series of body temperature (°C) and activity data (arbitrary units) of each mouse were analyzed by spectral analysis (Fourier transform analysis) using Mathcad 6.0 (Integral Software, Paris, France), complemented with cosinor analysis for periods of 24, 12, or 8 hours (10,30,31) (data not shown).

For each gene, mean expression was calculated as a function of sampling time, tissue, and experimental condition. Intergroup differences were compared using two-way analysis of variance (ANOVA) for experiment 1. Three-way ANOVA was performed on gene expression data to estimate the respective roles of sampling time, tissue (liver versus tumor), and experimental condition (standard conditions versus chronic jet lag versus combined chronic jet lag and meal timing) and to identify possible interactions in experiment 2.

The statistical significance of sinusoidal rhythmicity was further documented by cosinor analysis (30,31), which characterizes a rhythm by parameters of the fitted cosine function best approximating all data. Periods τ = 24 hours and τ = 12 hours were considered a priori. The rhythm characteristics estimated by this linear least squares method include the mesor (rhythm-adjusted mean), the double amplitude (difference between minimum and maximum of fitted cosine function), and the acrophase (time of maximum in fitted cosine function, with light onset as phase reference). Time measurements are indicated as hours (minutes). A rhythm was detected if the null hypothesis was rejected with P<.05.
RESULTS

Effects of Chronic Jet Lag on Gene Expression Patterns in Liver of Healthy Mice

Marked 24-hour variations were found for the mRNA expression of the clock genes Cry1, Rev-erb, Per2, and Bmal1 in the liver of mice synchronized to standard lighting conditions (12 hours of light and 12 hours of darkness for 2–3 weeks) (Fig. 1, left panel). The relative peak-to-trough differences were 10-, 206-, four-, and 26-fold, respectively. All 24-hour changes were statistically validated with both ANOVA and cosinor. Cosinor analysis further documented sinusoidal circadian rhythms for the expression of all four genes, with acrophases occurring in the early light span for Cry1 and Rev-erb, shortly after the light–dark transition for Per2, and near the end of the dark span for Bmal1 (Table 1).

The circadian expression patterns of all four clock genes of mice exposed to conditions mimicking chronic jet lag (10 days of serial 8-hour advances of light–dark cycles every 2 days) were markedly altered compared with those in synchronized mice (Fig. 1, right panel versus left panel). The mean mRNA expression levels of Cry1, Per2, and Bmal1 were nearly halved, and that of mean Rev-erb expression was more than threefold lower than that of synchronized mice. The Cry1 and the Rev-erb rhythms were severely flattened. The Per2 rhythm persisted, yet it was dampened, and its peak was shifted back 8 hours (from CT12 to CT4). The Bmal1 pattern became bimodal, with peaks at CT0 and at CT12. Cosinor analysis indicated that the Cry1 rhythm was ablated (P = .23), yet the minor changes in Rev-erb expression over the 24-hour period were fitted with a 12-hour periodic component (P = .03) (Table 1). Per2 expression displayed circadian rhythm despite chronic jet lag, yet with markedly altered characteristics: the mesor was reduced by 40% (0.081 [95% CI = 0.05 to 0.12] compared with 0.13 [95% CI = 0.09 to 0.18] in synchronized mice), and its acrophase advanced by nearly 10 hours (3.20 [95% CI = 0.10 to 7.20] compared with 14.20 [95% CI = 10.20 to 18.20] in synchronized mice) (Table 1). Finally, the Bmal1 rhythm also displayed a statistically significant dominant 12-hour periodic component (P < .001) (Table 1).

In synchronized mice, the expression pattern of c-Myc was highest at CT8 with low values from CT16 to CT0, whereas p53 peaked at CT4, with low values from CT8 to CT20 (Fig. 1, left panel). The 24-hour changes were close to statistical significance for the expression of c-Myc but not for that of p53 (P from ANOVA = .055 and .13, respectively); no sinusoidal circadian or 12-hour rhythm was observed with cosinor analysis for either gene (Table 1).

In mice that experienced chronic jet lag, the 24-hour mean expression of p53 was approximately half that of synchronized mice. c-Myc expression increased by nearly 50%, and its rhythmicity was amplified 12-fold over the 24-hour period compared with that in synchronized mice (Fig. 1). Indeed, c-Myc expression in the jet-lagged mice followed a sinusoidal circadian rhythm (cosinor P = .03), the double amplitude was 235% of the mesor, and acrophase was located near midnight (Table 1).

Based upon two-way ANOVA of the gene expression data, statistically significant differences were found as a function of circadian time for Cry1 (two-sided P = .046), Rev-erbα (P = .001), and Bmal1 (P < .001), but not c-Myc (P = .055). The analysis validated differences in mean level of expression between synchronized mice and mice undergoing chronic jet lag for Rev-erbα (P = .026), Per2 (P = .035), Bmal1 (P = .01), and p53 (P = .002). Furthermore, a statistically significant interaction was found between circadian time and light–dark schedule for Rev-erbα (P = .002), Per2 (P = .008), and Bmal1 (P < .001), but not for c-Myc (P = .12).

Effects of Chronic Jet Lag and Meal Timing in Tumor-Bearing Mice

Meal timing was used here as a means to entrain the circadian clocks otherwise disrupted by chronic jet lag. Meal-fed mice were fed from CT12, the onset of activity, to CT24, the onset of rest.

The body weight of the ad libitum–fed synchronized mice or those that underwent chronic jet lag conditions increased similarly. The meal-fed mice lost weight during the initial 4 days of meal timing, and then their weight increased. The ad libitum–fed mice gained weight slowly during this time. On the day of tumor inoculation, mean body weight (±SD) in both groups of the ad libitum–fed mice was 27.7 (±0.3), and that of the meal-fed mice that had undergone chronic jet lag was 27.1 (±0.5) (P from ANOVA = .46).

Tumors grew more quickly in the mice undergoing chronic jet lag than in synchronized mice (Fig. 2), whereas restricted feeding moderately counterbalanced the effect of jet lag; the comparison of all three curves with ANOVA indicated statistically significant differences (P = .04). On day 12, prior to tissue sampling, mean tumor weight (95% confidence interval [CI]) was 1317 mg (95% CI = 1067 to 1567) in synchronized mice (n = 13), 1997 mg (95% CI = 1458 to 2536) in chronic jet-lagged mice fed ad libitum (n = 14), and 1567 mg (95% CI = 1032 to 2101) in chronic jet-lagged, meal-fed mice (n = 15).

Regardless of light–dark synchronization and meal timing, Rev-erbα, Per2, Bmal1, and c-Myc mRNA expression in tumor-bearing mice varied over the 24-hour period in liver and in tumor, as indicated by a statistically significant effect of sampling time with three-way ANOVA for each of the genes (P < .001, P = .005, P = .048, and P = .009, respectively). By contrast, no temporal change in p53 expression was statistically validated (P = .96). The 24-hour patterns of Rev-erbα and Bmal1 expression in liver and tumor differed statistically significantly as a function of light–dark and feeding schedules (Rev-erbα P = .002, Bmal1 P = .008), as indicated by analyzing sampling time by tissue interaction terms with three-way ANOVA. This analysis also showed that the 24-hour patterns of Per2 and Bmal1 expression were statistically significantly altered in mice that underwent chronic jet lag or the combination of chronic jet lag and meal timing (Per2 P = .019 and Bmal1 P = .001).

In the livers of tumor-bearing synchronized mice, mRNA levels of Rev-erbα, Per2, and Bmal1 peaked during the light span; peak-to-trough differences were 36-, 35-, and 32-fold, respectively (Fig. 3, left panel). Sinusoidal 24-hour rhythms in Rev-erbα and Per2 mRNA expression were further validated by cosinor analysis (Table 2). For comparison, the respective peak-to-trough differences in Rev-erbα, Per2, and Bmal1 gene expression were 9.4-, 5.5-, and sixfold in tumor (Fig. 3, right panel);
Fig. 1. Circadian expression of clock genes Cry1, Rev-erbα, Per2, Bmal1, c-Myc, and p53 in the livers of healthy mice synchronized by standard lighting conditions (left panel) or undergoing chronic jet lag (right panel). The relative mRNA expression of each gene of interest was normalized to that of 36B4. Each point represents the mean of 10 mice. The mice in both groups were exposed to constant darkness during the 2 days preceding sampling. The sampling circadian times (CTs) are relative to the time of light onset, CT0. Hatched rectangles indicate subjective light span and black rectangles indicate dark span. To better visualize the rhythm during the 24-hour period, data obtained at CT0 were entered at CT24 and connected with a broken line to data at CT20.
Table 1. Circadian rhythms of gene expression in the liver of healthy, synchronized mice or those undergoing chronic jet lag*

| Group            | Gene  | t (h) | P    | Mesor (95% CI) | Double amplitude (95% CI), % of mesor | Acrophase (95% CI), hour
|------------------|-------|-------|------|----------------|---------------------------------------|----------------------
| Synchronized     | Cry1  | 24    | .019 | 0.0217 (0.01 to 0.0323) | 203 (27 to 369) | 410 (0.10 to 8.10)
|                  | Rev-erbα | 24 | .0016 | 0.2774 (0.1092 to 0.4456) | 326 (108 to 541) | 520 (2.30 to 8.90)
|                  | Per2  | 24    | .018 | 0.1338 (0.0912 to 0.1764) | 131 (18 to 239) | 144 (16.20 to 18.80)
|                  | Bmal1 | 24    | <.001 | 0.1390 (0.0919 to 0.1699) | 204 (92 to 306) | 2340 (21.40 to 25.90)
|                  | c-Myc | 24    | .39  | 0.0200 (0.0136 to 0.0264) | 02 (92 to 306) | 9.60
|                  | p53   | 24    | .43  | 0.2532 (0.1992 to 0.3072) | 40 | 2.80
| Chronic jet lag  | Cry1  | 24    | .23  | 0.0092 (0.0036 to 0.0056) | 1 | 5.00
|                  | Rev-erbα | 24 | .82  | 0.0881 (0.0453 to 0.1309) | 43 | 20.50
|                  | Per2  | 24    | .012 | 0.0842 (0.0476 to 0.1286) | 176 (15 to 340) | 65 (4.00 to 9.10) and 185 (16.00 to 21.10)
|                  | Bmal1 | 24    | .16  | 0.0474 (0.0550 to 0.0938) | 127 (22 to 222) | 35 (10.00 to 72.00)
|                  | c-Myc | 24    | .032 | 0.0307 (0.0119 to 0.0495) | 170 (92 to 247) | 115 (1.00 to 12.50) and 235 (23.00 to 50.00)
|                  | p53   | 24    | .09  | 0.1594 (0.1332 to 0.1856) | 235 (13 to 456) | 58 (1.00 to 10.00)

*The relative mRNA level of each gene of interest was normalized to that of 36B4. All circadian parameters are given as means and 95% confidence intervals (CI) using cosinor analysis; P values are two-sided. Mesor = rhythm-adjusted mean. Double amplitude = difference between minimum and maximum of the fitted cosine function. Acrophase = time of maximum in fitted cosine function, with light onset as phase reference. A rhythm was detected if the null hypothesis was rejected with P<.05.

only Per2 exhibited a sinusoidal circadian rhythm (Table 2). Small variations were found in c-Myc and p53 expression over the 24-hour time period in liver and in tumor, and no sinusoidal waveform was detected (Fig. 3, Table 2).

Mice that underwent chronic jet lag had dampened 24-hour changes in liver and in tumor Rev-erbα expression, and the sinusoidal waveform was suppressed, as shown by the lack of any 24-hour rhythm with cosinor (Table 2). Chronic jet lag also clearly altered the circadian patterns in Per2 and Bmal1 mRNA expression in both tissues. In the livers of jet-lagged, tumor-bearing mice, the 24-hour rhythms of Per2 and Bmal1 expression displayed peaks nearly 12 hours out of phase with those in synchronized mice (Fig. 4). Chronic jet lag further ablated the rhythmic changes in tumor Bmal1 expression (Fig. 4, Table 2).

Meal timing restored near-normal rhythmic sinusoidal 24-hour patterns of both Rev-erbα and Per2 expression but ablated that of Bmal1 in liver of tumor-bearing mice (Fig. 4, left panel and Table 2). However, meal timing imposed large 24-hour sinusoidal rhythms in the expression of all three clock genes in tumor tissue (Fig. 4, right panel and Table 2). Chronic jet lag increased the magnitude of the 24-hour changes in p53 and c-Myc expression in both the liver and tumor of the tumor-bearing mice, although a sinusoidal rhythm was seen for p53 in liver only (Fig. 4, Table 2). Meal timing reinforced the c-Myc 24-hour pattern in liver but dampened it in tumor (Fig. 4).

### Discussion

Our results demonstrate that chronic jet lag severely altered the circadian rhythms in the expression of clock genes in liver and tumor of mice bearing Glasgow osteosarcoma tumors. We also observed modifications in the expression patterns of the downstream genes c-Myc and p53 and accelerated tumor growth compared with that in control synchronized mice. Meal timing (i.e., from CT12, the onset of activity, to CT24, the onset of rest) prevented the alterations produced by chronic jet lag in the expression patterns of Per2 and Rev-erbα in liver and tumor and Bmal1 in tumor and slowed tumor growth.

We previously reported (11) that an 8-hour advance of light onset every 2 days markedly alters the circadian patterns of rest-activity and body temperature, two main circadian outputs that are directly controlled by the suprachiasmatic nuclei, the central pacemaker in the hypothalamus. In this study, similar results were obtained in both healthy mice and mice bearing tumors (data not shown).

The transcription of clock genes Cry1, Rev-erbα, Per2, and Bmal1 varied largely as a function of circadian time in the liver of synchronized healthy mice, with peak times and amplitudes similar to those in previous reports (1,3,6,12,32). Over a 24-hour period, the mean maximum expression of Cry1 was nearly sixfold lower than mean maximum expression of Per2 or Bmal1 and 25-fold lower than that of Rev-erbα. For this reason, only the last three clock genes were measured in mice with Glasgow osteosarcoma. For Rev-erbα, Per2, and Bmal1, mRNA expression in the liver of tumor-bearing mice displayed circadian changes that were similar to those of healthy mice. The 24-hour patterns in mean Rev-erbα or Per2 expression as determined here with quantitative RT–PCR were in agreement with those previously reported using

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**Fig. 2.** Tumor growth in mice synchronized by standard lighting conditions (dashed line, n = 13), undergoing chronic jet lag (solid line, n = 14), or the combination of meal timing and chronic jet lag (dotted line, n = 15). Mean tumor weight and 95% confidence intervals at each time point over the 12-day span following inoculation of Glasgow osteosarcoma are shown.
Fig. 3. Circadian expression of clock genes Rev-erbα, Per2, Bmal1, c-Myc, and p53 in the livers (left panel) or the tumors (right panel) of tumor-bearing mice synchronized to standard lighting conditions. The relative mRNA expression of each gene of interest was normalized to that of 36B4. Gene expression was calculated as a percentage of the 24-hour mean value in each group. Each point represents the mean of two to three mice. The mice in both groups were exposed to constant darkness during the 2 days preceding sampling. The sampling circadian times (CTs) are relative to the time of light onset, CT0. To better visualize the rhythm during the 24-hour period, data obtained for CT3 are repeated 24 hours later and connected with a broken line to data at CT23. Hatched rectangles indicate subjective light span and black rectangles indicate dark span.
Table 2. Circadian rhythms of gene expression in tumor-bearing mice synchronized, undergoing chronic jet lag, or undergoing chronic jet lag and meal timing*  

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<th>Tissue</th>
<th>Gene</th>
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<td>Chronic jet lag + meal timing</td>
<td>Liver</td>
<td>Rev-erbα</td>
<td>.008</td>
<td>263 (70 to 456)</td>
<td>700 (300 to 1000)</td>
</tr>
<tr>
<td></td>
<td>Per2</td>
<td>.009</td>
<td>188 (48 to 329)</td>
<td>1300 (1000 to 1600)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bmal1</td>
<td>.88</td>
<td>22</td>
<td></td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>c-Myc</td>
<td>.03</td>
<td>182 (16 to 348)</td>
<td>600 (230 to 1110)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p53</td>
<td>.72</td>
<td>41</td>
<td></td>
<td>1210</td>
</tr>
<tr>
<td>Tumor</td>
<td>Liver</td>
<td>Rev-erbα</td>
<td>.07</td>
<td>127</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>Per2</td>
<td>.01</td>
<td>223 (40 to 406)</td>
<td>600 (330 to 1010)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bmal1</td>
<td>.01</td>
<td>140 (28 to 251)</td>
<td>400 (190 to 800)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-Myc</td>
<td>.35</td>
<td>93</td>
<td></td>
<td>790</td>
</tr>
<tr>
<td></td>
<td>p53</td>
<td>.77</td>
<td>76</td>
<td></td>
<td>1110</td>
</tr>
</tbody>
</table>

*The relative mRNA level of each gene of interest was normalized to that of 36B4. Due to the method used, all circadian parameters are given as means and 95% confidence intervals (CI). Mesor = rhythm-adjusted mean. Double amplitude = difference between minimum and maximum of the fitted cosine function. Acrophase = time of maximum in fitted cosine function, with light onset as phase reference. A rhythm was detected if the null hypothesis was rejected with $P<.05$.

RNase protection assay and only four time-points (11). However, this study showed that, in tumor-bearing mice, the waveform of Bmal1 mRNA expression in liver was modified so that the sinusoidal rhythm observed in healthy mice was not detected. Nevertheless, the overall characteristics of the liver molecular clock were only slightly affected by the presence of a tumor since the transcriptional activity of the positive feedback loop of the clock, here assessed with Rev-erbα and Bmal1 (32), peaked in the first half of the light span, and the transcriptional activity of the negative feedback loop peaked near the transition from light to darkness, as is the case in healthy mice.

Chronic jet lag markedly reduced the average level of clock gene expression and severely altered their rhythms in healthy mouse liver. Interindividual variability in clock gene expression was reduced in the mice that underwent chronic jet lag compared with synchronized mice, suggesting that the ablation of circadian rhythmicity could not result from increased interindividual variability in circadian phases. Instead, it is likely that the rhythms were indeed ablated in each mouse.

Chronic jet lag further revealed differences in the molecular clock response to environmental changes between healthy mice and tumor-bearing ones. Chronic jet lag induced bimodality in the Rev-erbα and Bmal1 expression patterns of the liver clock in healthy mice. In tumor-bearing mice, by contrast, chronic jet lag suppressed the circadian rhythm in Rev-erbα, a result similar to that of a previous report using RNase protection assay (11). Chronic jet lag also shifted the Bmal1 circadian rhythm back by nearly 14 hours compared with that of synchronized mice. The relationship between Per2 and Bmal1 peak expression over the circadian time scale constitutes the core part of a functional molecular clock (33). Chronic jet lag altered this relationship in the liver of healthy mice but maintained it in the liver of tumor-bearing mice, although with an advance of nearly 8 hours. Thus, the tumor itself could have a role in the entrainment of the liver clock, in the absence of fit host circadian coordination.

Meal timing entrains circadian rhythms in the expression of clock genes and clock-controlled genes in liver and in other peripheral oscillators (23, 24), even in the absence of the hypothalamic pacemaker (27). We found that meal timing prevented the circadian patterns of Rev-erbα and Per2 mRNA expression from being disrupted by chronic jet lag, yet Bmal1 rhythm was suppressed. This finding suggests that only some molecular components of the liver circadian clock can be entrained by meal timing.

The effects of light and food schedule on gene expression were further investigated in the tumor. Tumor Per2 and Bmal1 transcription remained rhythmic in light-dark synchronized mice. The time lag between expression peaks in tumor differed from that in liver, however, and the pattern of Rev-erbα expression in tumor, was dampened compared with that in liver. This finding suggested that the tumor clock was altered despite photoperiodic synchronization. The observation that chronic jet lag clearly ablated sinusoidal circadian rhythms in clock gene expression in the tumor raises the possibility that additional molecular clock
Fig. 4. Data for 24-hour changes in the mRNA expression of Reverbα, Per2, Bmal1, c-Myc, and p53 in the livers (left panel) or the tumors (right panel) of tumor-bearing mice undergoing chronic jet lag (solid line) or the combination of meal timing and chronic jet lag (dashed line). The relative mRNA expression of each gene of interest was normalized to that of 36B4. Gene expression was calculated as a percentage of the 24-hour mean value in each group. Each point represents the mean of two to three mice. The mice in both groups were exposed to constant darkness during the 2 days preceding sampling. The sampling circadian times (CT) are relative to subjective time of light onset, CT0. To better visualize the rhythm during the 24-hour period, data obtained at CT3 are repeated 24 hours later and connected with a broken line to data at CT23. Hatched rectangles indicate subjective light span and black rectangles indicate dark span.
deregulation within the tumor itself leads to subsequent tumor progression, in agreement with prior observations (10,11). By contrast, the combination of meal timing and chronic jet lag induced nearly synchronous rhythmic 24-hour changes in Rev-erbα, Bmal1, and Per2 expression in the tumor. Because ad libitum– and meal-fed mice had similar weights when tumors were injected, we believe that the tumor growth differences in these two groups were not related to food restriction but rather depended upon the expression patterns of the molecular clock and cell cycle genes. The restored tumor molecular clock exerted a modest yet effective negative control of tumor progression, despite the atypical phase relations within the clock’s core mechanisms. In agreement with this, the restriction of food availability to 4–6 hours during the light span was able to largely slow the growth of Glasgow osteosarcoma (25). Thus, selective inhibition of certain components of the tumor molecular clock would speed up malignant growth through accelerated cell cycle progression, stimulated angiogenesis, or reduced apoptosis, whereas partial restoration or reinforcement of the tumor molecular clock would slow down malignant growth through opposite effects on these mechanisms.

Cell cycle phase distribution and apoptotic pathways are under circadian regulation in healthy, rapidly proliferating tissues, such as bone marrow (16,34–36). In tumors, G2/M gating and tumor angiogenesis are well coordinated by the circadian system, whereas the G1/S transition or apoptotic pathways display temporal changes in different tumor types (17,21,37,38). In the liver of healthy mice, we found that the severe clock dysfunction produced by chronic jet lag increased the overall level of c-Myc expression and amplified its rhythm and nearly halved the overall level of p53 expression. Our results, obtained with functional clock disruption, parallel those reported in mice with germlinal Per2 null mutation (12). Per2/−/− mice displayed disrupted circadian clock function and displayed an increased susceptibility to develop cancers following exposure to γ-radiation (12). Thus, chronic jet lag exposure could also favor carcinogenesis through increased cellular proliferation and subsequent genomic instability induced by carcinogenic exposure. Indeed, a single 8-hour advance in the light–dark cycle also decreased apoptosis in the mammary tissues of mice exposed to γ-radiation (39). Both promotion and progression of liver tumors resulted from prolonged exposure of rats to constant light (7,40), a lighting condition that disrupts the circadian system of rats (41). In another rat model, accelerated tumor progression was related to enhanced tumor fatty acid uptake and metabolism, supporting a role for food intake in malignant progression (40).

In tumor-bearing mice, the molecular clock of the tumor clearly interacted in a complex manner with the host’s response to chronic jet lag and to the combination of chronic jet lag and meal timing, as indicated by observed changes in liver c-Myc and p53 transcriptional activities under these conditions. Interestingly, the combination of meal timing and chronic jet lag dampened the 24-hour variations in c-Myc and p53 expression in the tumor, suggesting that negative regulation of proliferation was stronger in the “restored” yet altered tumor circadian clock.

The results from this study do not rule out the possibility that light and food schedules modify transcriptional activity of only a few genes in the molecular clock and the cell cycle without further downstream consequences. This possibility is unlikely because light and food schedules have been repeatedly shown to alter tumor progression (10,25). In addition, potential downstream consequences of altered transcription of clock genes and cell cycle genes other than tumor growth suppression were not investigated in this study. Indeed, light and food schedules could also modify the dynamic patterns in transcription of other clock and cell cycle genes and may also affect translation, conformation, and/or heterodimerization of clock and cell cycle proteins. Systematic exploration of the crosstalk between the circadian clock and the cell cycle in cancer processes will be necessary to provide new therapeutic opportunities to prevent or treat cancers.

Several epidemiologic studies emphasize the clinical relevance of our findings because people in repeatedly altered environmental cycles through prolonged shift work or iterative transmeridian flights had a statistically significantly increased risk of breast or colorectal cancer compared with that of people who are exposed to regular 24-hour cycles (42–47).

In conclusion, we showed here that the molecular clocks in liver and in tumor were severely altered by the disruption of regular photoperiodic synchronization, a condition that also accelerated tumor growth. The deleterious effects of jet lag on the molecular clock in liver tissue were partly counteracted by meal timing, and more so in the tumor itself, the growth of which was slowed. The effects were partly mediated by the relationship between the molecular clock and the expression patterns of c-Myc and p53.

REFERENCES


Notes


