Circadian-independent cell mitosis in immortalized fibroblasts

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Two prominent timekeeping systems, the cell cycle, which controls cell division, and the circadian system, which controls 24-h rhythms of physiology and behavior, are found in nearly all living organisms. A distinct feature of circadian rhythms is that they are temperature-compensated such that the period of the rhythm remains constant (~24 h) at different ambient temperatures. Even though the speed of cell division, or growth rate, is highly temperature-dependent, the cell-mitosis rhythm is temperature-compensated. Twenty-four-hour fluctuations in cell division have also been observed in numerous species, suggesting that the circadian system is regulating the timing of cell division. We tested whether the cell-cycle rhythm was coupled to the circadian system in immortalized rat-1 fibroblasts by monitoring cell-cycle gene promoter-driven luciferase activity. We found that there was no consistent phase relationship between the circadian and cell cycles, and that the cell-cycle rhythm was not temperature-compensated in rat-1 fibroblasts. These data suggest that the circadian system does not regulate the cell-mitosis rhythm in rat-1 fibroblasts. These findings are inconsistent with numerous studies that suggest that cell mitosis is regulated by the circadian system in mammalian tissues in vivo. To account for this discrepancy, we propose two possibilities: (*i*) There is no direct coupling between the circadian rhythm and cell cycle but the timing of cell mitosis is synchronized with the rhythmic host environment, or (*ii*) coupling between the circadian rhythm and cell cycle exists in normal cells but it is disconnected in immortalized cells.

cell cycle | reporter gene | temperature compensation | Cyclin B1 | cancer

Organization of physiology and behavior into specific time domains is a fundamental property of nearly all living organisms. Anticipation of periodic changes in the environment presumably increased survival and reinforced the development of endogenous circadian oscillators (1). Because cell division is critical to the survival of unicellular organisms and the integrity of DNA is susceptible to UV irradiation, the progression of the cell cycle was probably also strongly affected by daily changes in the environment. Indeed, multiple studies have measured diurnal fluctuations in cell division such that mitosis occurs at a specific time of day in numerous species ranging from unicellular organisms (2) to humans (3–5). These data suggest that the circadian and cell cycles may be coupled in vivo.

Circadian rhythms are self-sustained oscillations in physiology and behavior with endogenous periods of ≈24 h that can be synchronized, or entrained, to environmental cues such as the light/dark cycle or temperature (6). In mammals, the master circadian clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Genes that are important for circadian timekeeping are expressed not only in the SCN but also in many peripheral tissues, including fibroblasts (7–11). Immortalized embryonic fibroblasts exhibit circadian rhythms of gene expression (12) and, using single-cell imaging, it has been shown that individual fibroblasts contain circadian clocks similar to SCN neurons (13, 14). Consequently, immortalized fibroblasts have become a widely used tool for studying circadian rhythms.

The molecular mechanism of endogenous rhythm generation in circadian clocks is modeled as interlocking positive and negative transcriptional and translational feedback loops of clock gene expression (15–17). The transcription of two *Period* homologs (*Per1*, *2*) and two *Cryptochrome* genes (*Cry 1*, *2*) is activated by the binding of BMAL1/CLOCK or BMAL1/NPAS2 heterodimers to E boxes in the regulatory regions of the *Per* and *Cry* genes. As PER and CRY proteins accumulate, they form complexes and directly bind to BMAL1-CLOCK/NPAS2 heterodimers, thereby inhibiting their own transcription.

Similar to the molecular clockwork of circadian rhythms, transcriptional and posttranslational feedback loops drive transitions between, and passage through, phases of the cell cycle. Progression through the growth phases G1 and G2, S phase (DNA synthesis), and M phase (mitosis) directs the growth of a cell, the replication of its DNA, and the packaging and transmission of its chromosomes into each of two daughter cells (18). Complexes containing Cyclin-dependent kinases (Cdks) and Cyclins are synthesized, activated, and degraded at specific time points to ensure that the cell is prepared for the subsequent phase of the cell cycle. During G2, CYCLIN B1 (CCNB1) associates with Cdc2 and activation of the CCNB1-Cdc2 complex stimulates entry into mitosis (19). During late mitosis, CCNB1 is ubiquitinated and degraded, allowing exit from M phase.

Even though most biological reactions occur with a temperature coefficient (Q_{10}) of ~2 or 3, such that with every 10 °C increase in temperature the reaction rate approximately doubles or triples, the circadian system has developed temperature-compensated clocks to ensure that the length of the period remains relatively constant over a range of physiological temperatures. In mammals, the master pacemaker in the SCN, numerous peripheral tissues, and immortalized fibroblast cell lines are temperature-compensated in vitro (20–25). In contrast to temperature-compensated circadian clocks, the cell-growth rate is dependent on temperature (2, 23, 26). If the temperature-compensated circadian rhythm controls the cell cycle, as suggested by the numerous studies showing that cell division occurs at specific times of day, why is the duration of the cell-division cycle temperature-dependent? Studies in *Gonyaulax* (27), *Euglena* (26, 28), Chinese hamster lung fibroblasts (29), and *Chlamydomonas* (30) have demonstrated that it is the rhythm of cell mitosis rather than the cell growth rate that is temperature-compensated. The fact that the period of the cell-mitosis rhythm is relatively constant across a physiological range of temperatures provides evidence that the circadian system is gating progression through the cell cycle.

In the current study, we developed a system for monitoring the cell-cycle rhythm in real time by assessing luciferase activity that

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is driven by the *CCNB1* promoter in immortalized fibroblasts. Immortalized rat-1 fibroblasts exhibit a notable circadian feature: They have temperature-compensated circadian rhythms (22). We tested the hypothesis that the circadian and cell cycles are coupled in rat-1 fibroblasts by assessing temperature compensation of the cell-cycle gene expression rhythm.

Results

Real-Time Monitoring of the Cell-Cycle Gene Expression Rhythm in Synchronized Rat-1 Fibroblasts. To study the relationship between the circadian and cell cycles, we first established a real-time reporter method to monitor the cell cycle. We chose rat-1 fibroblasts for our experiments because circadian rhythms in this immortalized cell line were characterized previously (12, 22, 31). To avoid cross-talk from the circadian system, we searched for cell-cycle regulation genes that do not have circadian regulatory motifs in their promoter regions. Among several candidate genes, *Cyclin B1* (*CCNB1*) oscillates robustly and peaks at the G2/M phase of the cell cycle (32, 33). Although the human *CCNB1* promoter contains an E box, an element that is important for circadian rhythmicity (15, 34, 35), it likely does not have circadian function because *CCNB1* transcription is not activated by CLOCK/BMAL1 heterodimers (36). Therefore, we chose the human *CCNB1* promoter for our studies. To assess the timing of mitosis, our experiments required that we image luminescence so that we could visualize a cell splitting into daughter cells. We used green luciferase (PTGRm), identified in the luminescent Brazilian click beetle *Pyrearinus termitilluminans*, as our reporter because it is 21 times brighter than the widely used firefly luciferase (pGL3-basic) (37).

We next generated rat-1 fibroblasts which stably express the DNA reporter construct in which the human *CCNB1* promoter drives the expression of destabilized green luciferase (*CCNB1-dGluc*) and developed the optimal conditions for real-time analysis (*SI Text* and Fig. S1). Studies of circadian rhythmicity in rat-1 fibroblasts have demonstrated that intercellular coupling is weak and individual cells oscillate out of synchrony with each other (12–14). Numerous procedures can synchronize the phase of circadian gene promoters in cultures of rat-1 fibroblasts, including serum shock (12, 14, 22), treatment with forskolin (22, 38, 39) or dexamethasone (14, 40), and simply changing the media (13, 22). We tested whether these methods would synchronize the phase of the *CCNB1-dGluc* rhythm (*SI Text* and Fig. S2). We found that changing the media was most efficacious. Because we wanted to measure the cell-cycle gene expression rhythm for several days, it was necessary to plate cells at a low density (0.5%) so that we could visualize cell mitosis. However, at this low density, cells often arrest in G0. We found that replacing the growth media with recording media containing luciferin (41) 2 days after subculture stimulated progression through the cell cycle, as evidenced by an exponential increase in the number of cells compared with cells that remained in the original subculture growth media (Fig. S3). After the media change, the baseline of *CCNB1-dGluc* luminescence increased drastically as cells proliferated (Fig. 1*A*). Nonetheless, an oscillation with a 13-h period, representing the synchronized cell-cycle gene expression rhythm, was clearly resolved in the baseline-subtracted trace of *CCNB1-dGluc* luminescence (Fig. 1*B*).

If media change synchronizes the cell-cycle gene expression rhythm, then the rhythm of *CCNB1-dGluc* in subcultures prepared at the same time but that received a media change at different times should oscillate out of phase. We prepared subcultures, waited 2 days, and replaced the growth media with recording media in one set of dishes and then in another set of dishes 6 h later. The first peak of *CCNB1-dGluc* expression occurred 10–12 h after the media change such that there was an ≈6-h difference in phase for dishes that received the media change 6 h apart (Fig. 1*C*), suggesting that the *CCNB1-dGluc* rhythm synchronized to the time of the media change.

Previous studies have shown that *CCNB1* mRNA and protein are rhythmically expressed such that expression is low in G1, rises gradually in S phase, and peaks at the G2/M transition (42–45). We next investigated whether the timing of cell mitosis occurred at

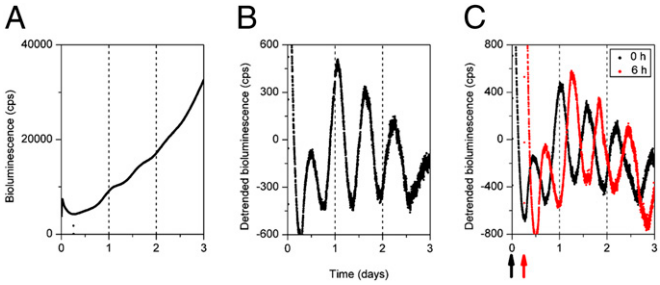

Fig. 1. Rhythmic *CCNB1-dGluc* expression in rat-1 fibroblasts is synchronized by changing the media. Rat-1 fibroblasts (0.5% confluency) stably expressing *CCNB1-dGluc* were subcultured in growth media and maintained in a CO_2 incubator. Two days later, the growth media were replaced with recording media and luminescence was recorded. (*A*) Raw *CCNB1-dGluc* bioluminescence (counts/s) was measured for 3 days. (*B*) Detrended *CCNB1-dGluc* bioluminescence (counts/s) was obtained by subtracting the 14-h moving average from the raw data. A representative example is shown in *A* and *B* (from a total of eight dishes). (*C*) Two days after subculture, the growth media were replaced with recording media in one set of dishes at 0 h (black trace; $n = 4$) and 6 h later in another set of dishes (red trace; $n = 4$). Detrended (subtracted 14-h moving average) *CCNB1-dGluc* bioluminescence is shown. Arrows indicate the time of media change (luminescence recording start time).

a specific phase of the 13-h rhythm of *CCNB1-dGluc* expression that we measured in rat-1 fibroblasts. Using a low-luminescence imaging system, we imaged *CCNB1-dGluc* expression in rat-1 fibroblasts for several days. From these images, the rhythm of *CCNB1-dGluc* expression and the timing of cell division (one cell visibly splitting into two cells) were determined (Fig. 2*A*). Consistent with previous reports showing that *CCNB1* expression peaks at the G2/M transition, cell division always occurred at the peak of *CCNB1-dGluc* expression in that cell (Fig. 2*A*). In addition, the cell always split at the peak of *CCNB1-dGluc* expression and never skipped a cycle of the *CCNB1-dGluc* rhythm. Quantification of bioluminescence and the timing of cell mitosis showed that the periods of the *CCNB1-dGluc* rhythm and of cell mitosis were ≈13 h (Fig. 2*B*). Following cell division, the phase and period of *CCNB1-dGluc* expression in daughter cells were equivalent to that of the parent cells such that daughter cells exhibited ≈13-h periods of rhythmic *CCNB1-dGluc* expression and cell division (Fig. 2*C*). Importantly, the period of rhythmic *CCNB1-dGluc* expression did not change throughout the recording even though cell density changed during this time (0.5–20% confluency). Taken together, our findings suggest that the timing of cell division can be monitored in real time by imaging rhythmic *CCNB1-dGluc* expression in individual cells or by measuring total bioluminescence emitted from rat-1 fibroblast cultures synchronized by media change.

Real-Time Monitoring of Circadian Rhythms from Dividing Cells. Most previous studies analyzing circadian rhythms in rat-1 fibroblasts were performed at 100% cell confluency. To examine the relationship between the circadian and cell cycles, we first needed to characterize the circadian rhythm in dividing rat-1 fibroblasts because we assessed the cell-cycle rhythm in dividing cells. We created a stable line of rat-1 fibroblasts in which the mouse *Bmal1* promoter (46) drives the expression of *dGluc* (*Bmal1-dGluc*) (*SI Text*). Although the baseline of *Bmal1-dGluc* expression continually increased during the recording (due to the increasing cell number), a stable circadian oscillation of *Bmal1-dGLuc* expression was observed (Fig. 3 *A* and *B*). To determine whether media change could synchronize the circadian rhythm in dividing rat-1 fibroblasts, we prepared subcultures, waited 2 days, and then replaced the growth media with recording media in one set of dishes and then in another set of dishes 6 h later. There was an ≈6-h difference in the phase of *Bmal1-dGluc* expression for dishes that received the media change 6 h apart (Fig. 3*C*), suggesting that the *Bmal1-dGluc* rhythm syn-

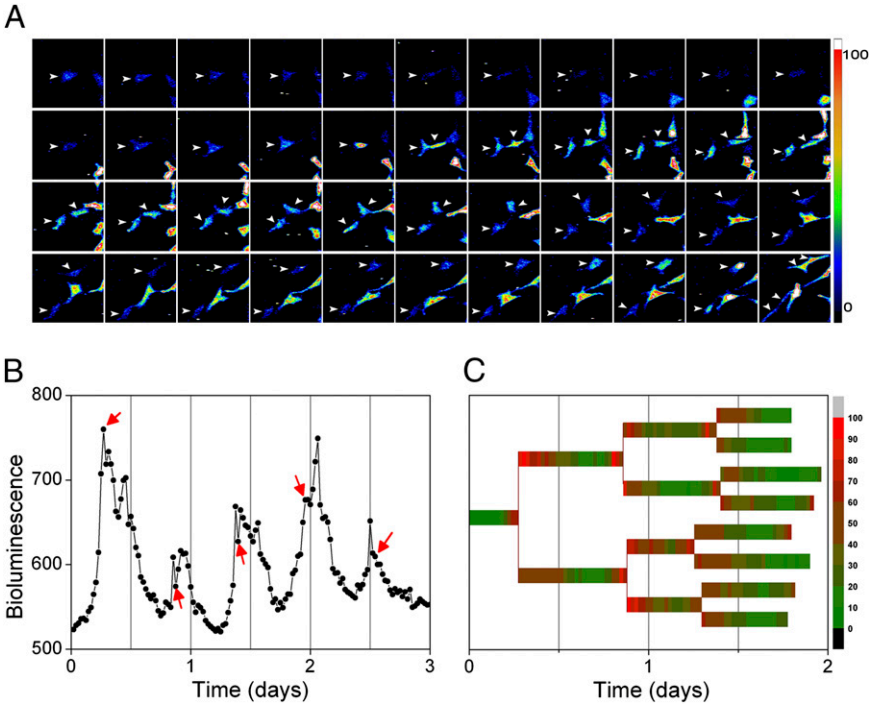

Fig. 2. Cell division occurs at the peak of the *CCNB1-dGluc* rhythm in rat-1 fibroblasts. Images (29-min exposures) of *CCNB1-dGluc* bioluminescence from rat-1 fibroblasts after a media change were collected every 30 min. Eight individual cells from two independent dishes were analyzed. (*A*) The relative intensity of *CCNB1-dGluc* expression (22 h are shown) is indicated by the color gradient, where white is high expression and black is no expression. Arrowheads indicate cells that originated from the same parent cell. (*B*) The optical density of *CCNB1-dGluc* bioluminescence in a single cell is plotted as a function of time. The times when the cell divided (one cell visibly splitting into two cells) are indicated by red arrows. (*C*) The phase and period of *CCNB1-dGluc* expression are similar in parent and daughter cells. Relative intensity of *CCNB1-dGluc* expression as a function of time is indicated by the color gradient, where red is high expression and green is low expression. Branch points represent the time when the parent cell split into two daughter cells.

chronized to the time of the media change but not to the time of subculture preparation. Therefore, similar to the *CCNB1-dGluc* rhythm, media change synchronized the circadian rhythm in the culture even though the cells were dividing.

The Cell-Cycle Rhythm Is Independent of the Circadian Rhythm in Rat-1 Fibroblasts. Previous studies have shown that cell mitosis occurs at a specific time of day in animal tissues in vivo (2–4, 47–49), suggesting that the timing of cell mitosis is gated by the circadian cycle. Therefore, we hypothesized that there would be a consistent phase relationship between the circadian and cell-cycle rhythms in cultured cells. We compared the phase and period of the *CCNB1-dGluc* and *Bmal1-dGluc* expression rhythms under the same conditions and recorded at the same time. Surprisingly, we found that there was no consistent phase relationship between the cell-cycle and circadian rhythms (Fig. 4 and Fig. S4). These data suggest that cell mitosis is not controlled by the circadian rhythm in rat-1 fibroblasts.

The Period of Rhythmic *CCNB1-dGluc* Expression Is Not Temperature-Compensated in Rat-1 Fibroblasts. To further investigate the relationship between the circadian and cell cycles, we measured the timing of cell-mitosis and *CCNB1-dGluc* expression rhythms in rat-1 fibroblasts cultured at different temperatures (Fig. 5). In contrast to our previous report showing that the period of the circadian rhythm measured with the *Per1-luc* reporter in rat-1 fibroblasts was temperature-compensated (Q_{10} = 0.85–0.88) (22), rhythmic expression of *CCNB1-dGluc* was not temperature-compensated (Q_{10} = 4.3; Fig. 5*C*). Imaging of individual rat-1 fibroblasts showed that the period of *CCNB1-dGluc* expression was longer at 33 °C than at 37 °C (Fig. 5*B*). Cell division was not temperature-compensated, because it occurred at every peak of *CCNB1-dGluc* expression at both 33 °C and 37 °C. These data suggest that the circadian system is not gating the timing of cell division in immortalized rat-1 fibroblasts.

Because our previous experiments analyzing temperature compensation of the circadian rhythm in rat-1 fibroblasts were performed under different conditions from those that we have used to measure temperature compensation of the cell-cycle rhythm (e.g., cell density, synchronization method, culture me-

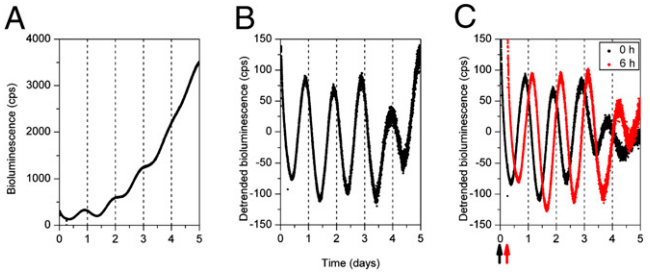

Fig. 3. Rhythmic *Bmal1-dGluc* expression in rat-1 fibroblasts is synchronized by changing the media. Rat-1 fibroblasts (0.5% confluency) stably expressing *Bmal1-dGluc* were subcultured in growth media and maintained in a CO_2 incubator. Two days later, the growth media were replaced with recording media and luminescence was recorded. (*A*) Raw *Bmal1-dGluc* bioluminescence (counts/s) was measured for 5 days. (*B*) Detrended *Bmal1-dGluc* bioluminescence (counts/s) was obtained by subtracting the 24-h moving average from the raw data. A representative example is shown in *A* and *B* (from a total of seven dishes). (*C*) Two days after subculture, the growth media were replaced with recording media in one set of dishes at 0 h (black trace; n = 3) and 6 h later in another set of dishes (red trace; n = 4). Detrended (subtracted 24-h moving average) *Bmal1-dGluc* bioluminescence is shown. Arrows indicate the time of media change (luminescence recording start time).

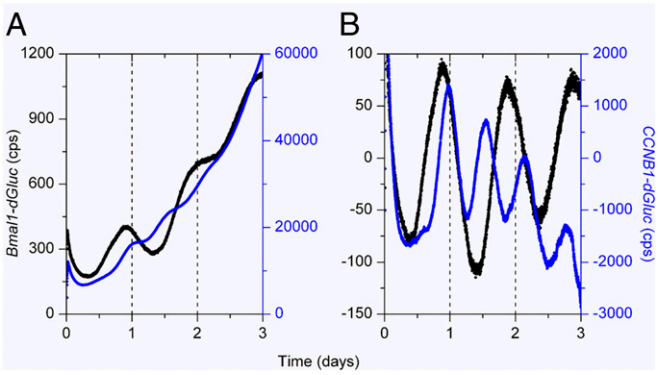

Fig. 4. Circadian and cell-cycle gene expression rhythms in rat-1 fibroblasts are independent from each other. Rat-1 fibroblasts stably transfected with *Bmal1-dGluc* or *CCNB1-dGluc* (0.5% confluency) were subcultured in growth media and maintained in a CO_2 incubator. Two days later, the growth media were replaced with recording media and luminescence was recorded. Raw (*A*) and detrended (*B*) data are shown. To compare the phase of the rhythms to each other, the *Bmal1-dGluc* (black trace) and *CCNB1-dGluc* (blue trace) were recorded at the same time under the same conditions and both were detrended by subtracting the 24-h moving average. The first peak of the *CCNB1-dGluc* rhythm was masked by subtracting the 24-h moving average. An independent experiment showed nearly identical results.

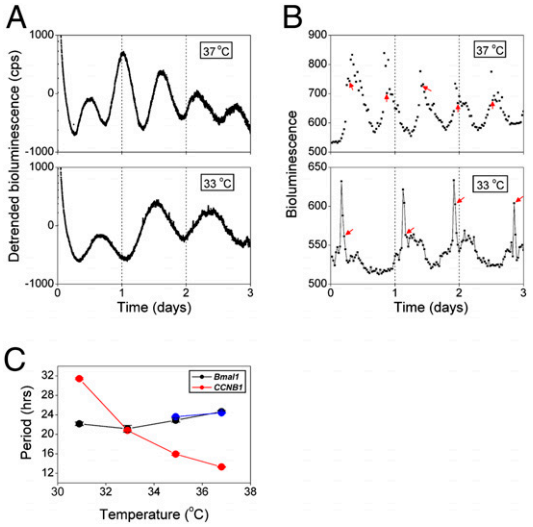

Fig. 5. The period of rhythmic *CCNB1-dGluc* expression is not temperature-compensated in rat-1 fibroblasts. Rat-1 fibroblasts, stably transfected with *CCNB1-dGluc* or *Bmal1-dGluc*, were subcultured (0.5% confluency) in growth media and maintained in a CO_2 incubator. Two days later, the growth media were replaced with recording media and cells were placed in a non-CO_2 incubator at 31 °C, 33 °C, 35 °C, or 37 °C. (*A*) Representative traces of detrended *CCNB1-dGluc* expression at 37 °C (14-h moving average) and 33 °C (24-h moving average) measured in LumiCycle software. (*B*) The optical density of *CCNB1-dGluc* bioluminescence in a single cell imaged at 37 °C or 33 °C. The timing of cell mitosis is indicated by red arrows. (*C*) The periods (hours) of the *CCNB1-dGluc* (red) and *Bmal1-dGluc* rhythms are plotted as a function of temperature (°C). Because the period of the *CCNB1-dGluc* rhythm varied with temperature, the following moving averages were subtracted from the raw data: 31 °C: 35 h; 33 °C: 24 h; 35 °C: 17 h; 37 °C: 14 h (n = 7 at each temperature). Raw *Bmal1-dGluc* bioluminescence was detrended by subtracting the 24-h moving average. We could only consistently detect rhythmic *Bmal1-dGluc* induced by a medium change at 35 °C and 37 °C (blue; n = 3 at each temperature). To measure the period of the circadian rhythm at low temperatures, we stimulated rat-1 fibroblasts expressing *Bmal1-dGluc* with dexamethasone for 3 h and then recorded bioluminescence at 31 °C, 33 °C, 35 °C, or 37 °C (n = 4 at each temperature). The periods of the dexamethasone-stimulated *Bmal1-dGluc* rhythms (black) were similar to the rhythms measured without dexamethasone stimulation (blue) at 35 °C and 37 °C. Data are presented as the mean ± SEM. Error bars are plotted but are smaller than the symbols. Note: At 37 °C, there was no significant difference (P = 0.61) between the periods of the *CCNB1-dGluc* rhythm after dexamethasone stimulation (13.03 ± 0.41 h) and after the media change (13.20 ± 0.08 h).

dia), we reevaluated temperature compensation of the *Bmal1-dGluc* rhythm in rat-1 fibroblasts using conditions identical to those used for analyzing temperature compensation of the cell-cycle rhythm. We were able to measure the rhythms of *Bmal1-dGluc* expression after a media change at high recording temperatures (35 °C and 37 °C; Fig. 5*C*, blue circles), but we did not consistently detect a robust rhythm after media change at low temperatures (31 °C and 33 °C). Because we could not always reliably measure the period of the circadian rhythm after media change at low temperatures, we instead stimulated the rat-1 fibroblasts with dexamethasone for 3 h before the recording. At high temperatures, the phase of the dexamethasone-stimulated circadian rhythm was slightly advanced compared with the phase of *Bmal1-dGluc* after the media change, but the period of the circadian rhythm was similar in both conditions (Fig. 5*C*). In addition, the period of the *Bmal1-dGluc* rhythm at low cell density was slightly overcompensated and the Q_{10} (0.82) was similar to the Q_{10} (0.85–0.88) of the circadian rhythm in high-cell-density rat-1 fibroblast cultures (22).

We occasionally observed rhythmic *Bmal1-dGluc* expression after the media change without dexamethasone stimulation at 31 °C. Phase analysis of the *CCNB1-dGluc* and *Bmal1-dGluc* rhythms after media change at 31 °C showed that there was no consistent phase relationship between the circadian and cell-cycle rhythms at 31 °C (Fig. S5).

Because the period of the *CCNB1-dGluc* rhythm is highly sensitive to temperature and the core body temperature of rats oscillates with an amplitude of about 1 °C, we tested whether the cell-cycle rhythm in rat-1 fibroblasts could be entrained to a 24-h temperature cycle (*SI Text*). Although the results were variable, we found that the *CCNB1-dGluc* rhythm in rat-1 fibroblasts could be entrained to a 24-h temperature cycle with an amplitude of 1 °C (*SI Text* and Fig. S6). These data suggest that it may be possible for the cell-cycle rhythm to be entrained to 24-h fluctuations in temperature in a rhythmic host environment.

Discussion

DNA synthesis, mitosis, and the expression of cell-cycle proteins in mammalian tissues (e.g., skin, bone marrow, gut, tongue, and oral mucosa) occur with a 24-h cycle in vivo (3–5, 49–55) and circadian periodicity of cell mitosis persists in rodents in constant darkness (2, 5). Daily rhythms of cell-cycle protein expression (*Cyclins E*, *A*, and *B1*) were also observed in human oral epithelium sampled by biopsy at 4-h intervals for 24 h from healthy human subjects (3). In addition, Bjarnason and colleagues found that circadian genes were rhythmically expressed in human oral epithelium, suggesting that the timing of mitosis in this tissue could be controlled by the circadian clock (54). Taken together, the numerous studies showing that the timing of mitosis occurs at a specific time of day in mammals in a light/dark cycle and with circadian periodicities in constant darkness suggest that the circadian system is gating the progression of the cell cycle.

Based on the extensive evidence in the literature that cell mitosis occurs on a 24-h cycle, we expected that the circadian and cell cycles would be coupled in immortalized rat-1 fibroblasts. Surprisingly, though, we found that the period of the *CCNB1-dGluc* expression rhythm in rat-1 fibroblasts was not 24 h. In addition, there was no consistent phase relationship between the *CCNB1-dGluc* and *Bmal1-dGluc* rhythms, suggesting that the circadian and cell cycles are not coupled in rat-1 fibroblasts. Our findings appear to be in conflict with a previous study that con-

cluded that the circadian clock gates cell-division timing in immortalized NIH 3T3 fibroblasts (14). In that study, Nagoshi et al. stimulated NIH 3T3 cells with dexamethasone to synchronize the circadian rhythm in the cells and then visualized cell division. Although Nagoshi et al. concluded that dexamethasone did not directly synchronize the cell-cycle rhythm in NIH 3T3 cells, we found that dexamethasone did synchronize the *CCNB1-dGluc* rhythm in rat-1 fibroblasts. Because the efficiency of cell-cycle synchronization varies in different cell lines, the discrepancy between our results and those of Nagoshi et al. may be attributed to the use of different cell lines.

We also analyzed temperature compensation of the cell-cycle rhythm. If the circadian and cell cycles are coupled in rat-1 fibroblasts, then the period of the cell-mitosis rhythm, which is measured by analyzing the *CCNB1-dGluc* rhythm (mitosis always occurs at the peak of the *CCNB1-dGluc* rhythm), should be temperature-compensated. In low-density cultures, the circadian rhythm had a temperature-compensated period of about 24 h. In contrast, we found that the cell-cycle gene expression rhythm was not temperature-compensated in immortalized, dividing rat-1 fibroblasts.

Our data suggest that circadian regulation of cell mitosis is absent in rat-1 fibroblasts. This finding is inconsistent with the numerous published reports that suggest that cell mitosis is regulated by the circadian system in mammalian tissues in vivo. There are at least two possible explanations for this discrepancy: (*i*) There is no direct intracellular coupling between the circadian rhythm and cell cycle, but in vivo circadian factors synchronize the timing of cell mitosis or (*ii*) coupling between the circadian rhythm and the cell cycle exists in normal cells but it is disconnected in immortalized cells.

Direct or indirect interactions between the circadian and cell cycles could account for the observation that cell mitosis occurs at a specific time of day in mammals in vivo. Circadian regulation of the cell cycle could occur through direct intracellular molecular interactions. Alternatively, the rhythmic in vivo environment generated by the SCN and peripheral oscillators could indirectly affect the progression of the cell cycle without the necessity for interactions between the molecular components of the circadian and cell-cycle machineries. For example, the period of the cell cycle could be regulated by the 24-h rhythm of body temperature or hormone levels controlled by the SCN. The phase and amplitude of rhythms of cell division are altered in SCN-lesioned mice, suggesting that the circadian system can regulate cell proliferation in vivo (56). A previous study also showed that the circadian rhythm of circadian gene mutant primary fibroblasts acquired the periodicity of the host rhythm when the fibroblasts were transplanted into wild-type mice (57). Although it is clear that the rhythmic in vivo environment has a strong effect on the circadian rhythmicity of transplanted primary fibroblasts, it is unknown whether the timing of cell mitosis can be synchronized to the host environment.

The circadian rhythm of temperature, which is controlled by the SCN, is a salient feature of the in vivo environment that could entrain the cell cycle. To determine whether a 24-h temperature cycle could entrain the cell cycle in rat-1 fibroblasts, we exposed cultures to a temperature cycle with an amplitude of 1 °C, similar to the oscillation in core body temperature in rats. We found that a 24-h temperature cycle could, in some cases, entrain the *CCNB1-dGluc* rhythm in rat-1 fibroblasts. Therefore, it is possible that the circadian temperature cycle could entrain the cell-cycle rhythm in vivo.

Alternatively, it is possible that an absence of mutual coupling between the circadian and cell cycles may be a characteristic of immortalized cells. To the best of our knowledge, no studies have measured both the circadian rhythm and the cell cycle in normal cells in vitro, but several studies have described potential molecular links between the circadian and cell cycles (58). If the circadian rhythm gates cell mitosis in vivo, then we expect that the phase of the circadian gene expression rhythm in each tissue will always have a fixed phase relationship with the timing of cell division in that tissue. Although it is known that the phases of the cell-cycle rhythms in various tissues are widely distributed similar to the wide distribution of the phases of circadian oscillations in different tissues, no systematic study has been conducted to measure both the circadian and cell-cycle gene expression rhythms in several tissues.

Mutual interactions between the circadian and cell cycles may be affected in carcinoma cells and tumors. Numerous studies have found that the expression of circadian genes is altered in cancerous tissues compared with normal tissues (59–65). We do not know whether these changes in circadian gene expression in tumor cells cause disconnection between the circadian and cell cycles. Furthermore, our findings in rat-1 fibroblasts may not extend to cancer cells, because immortalized cells and tumor cells have different characteristics. But if coupling between the circadian and cell cycles exists in normal cells and the disconnection of these timekeeping systems is a characteristic of cancer cells, then restoration of coupling could be a novel approach for controlling tumor progression. Identification of the mechanisms that result in disconnection of the circadian and cell cycles in cancer cells could be important for diagnosing and treating cancer.

Materials and Methods

Cell Culture and Recording Procedures. Subcultures containing 5,000 cells/35-mm dish were prepared and maintained in a CO_2 incubator (5% CO_2). Forty-eight hours later, growth media (Dulbecco's modified Eagle medium; DMEM; Gibco) containing 5% fetal bovine serum (FBS) were replaced with recording media (DMEM no phenol red; Sigma) with sodium bicarbonate (350 mg/L; Sigma), Hepes (10 mM; Sigma), 5% FBS (FPJ21922; HyClone), and luciferin (0.1 mM; Promega) and the luminescence signal was measured. The detailed methods for real-time measurement of luminescence have been described (41). Bioluminescence was measured using a photon-counting head (H6240; Hamamatsu) in a temperature-controlled environmental room (Environmental Growth Chambers). Temperature fluctuations (±0.02 °C) next to the culture dish were monitored by a temperature logger (HOBO H8 Pro; Onset). Bioluminescence imaging was performed using a water-cooled CCD camera (ORCAIIERW; Hamamatsu). The cultures were placed on an x-y-z stage in a dark box in an incubator (VWR) and the image was processed through direct coupling of a noninfinity corrected microscope objective (NPLM20×; Union Optical) to the camera with a 3-cm-long extension tube. Focus was adjusted by moving the motorized z stage controlled from outside the box. Twenty-nine-minute exposures were collected every 30 min.

Data Analysis. Total bioluminescence was analyzed using LumiCycle and ClockLab software (Actimetrics). The moving average was subtracted from the raw luminescence data using LumiCycle software. Because the period of the *CCNB1-dGluc* rhythm varied with temperature, the moving average value that was closest to the estimated period length was subtracted from the raw data (31 °C: 35 h; 33°C: 24 h; 35 °C: 17 h; 37 °C: 14 h; except data presented in Fig. 4). Because the period of the *Bmal1-dGluc* rhythm was always close to 24 h regardless of temperature, the 24-h moving average was subtracted from the raw *Bmal1-dGluc* rhythm. Baseline-subtracted data were then exported to ClockLab. The period was determined by fitting a regression line to the acrophase of at least three cycles of the luminescence rhythm and the phase was determined from the maximum bioluminescence of the first peak after synchronization.

The optical density from images of bioluminescence was analyzed using ImageJ software (National Institutes of Health). Individual cells were outlined by freehand drawing and the optical density inside the outline was measured for each image. The average signal intensity inside the selected area was measured. After a parent cell split into two daughter cells, one daughter cell was chosen at random and followed, so that time-series data could be collected from an individual cell. Because baseline *CCNB1-dGluc* bioluminescence did not increase, we did not perform baseline subtraction. Data are presented as the mean ± SEM.

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