

# Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation

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Circadian rhythms of cell and organismal physiology are controlled by an autoregulatory transcription-translation feedback loop that regulates the expression of rhythmic genes in a tissue-specific manner. Recent studies have suggested that components of the circadian pacemaker, such as the *Clock* and *Per2* gene products, regulate a wide variety of processes, including obesity, sensitization to cocaine, cancer susceptibility, and morbidity to chemotherapeutic agents. To identify a more complete cohort of genes that are transcriptionally regulated by CLOCK and/or circadian rhythms, we used a DNA array interrogating the mouse protein-encoding transcriptome to measure gene expression in liver and skeletal muscle from WT and *Clock* mutant mice. In WT tissue, we found that a large percentage of expressed genes were transcription factors that were rhythmic in either muscle or liver, but not in both, suggesting that tissue-specific output of the pacemaker is regulated in part by a transcriptional cascade. In comparing tissues from WT and *Clock* mutant mice, we found that the *Clock* mutation affects the expression of many genes that are rhythmic in WT tissue, but also profoundly affects many nonrhythmic genes. In both liver and skeletal muscle, a significant number of CLOCK-regulated genes were associated with the cell cycle and cell proliferation. To determine whether the observed patterns in cell-cycle gene expression in *Clock* mutants resulted in functional dysregulation, we compared proliferation rates of fibroblasts derived from WT or *Clock* mutant embryos and found that the *Clock* mutation significantly inhibits cell growth and proliferation.

cell cycle | circadian rhythms | Clock mutation | gene expression | protein-encoding transcriptome

Many organisms have  $\approx 24$ -h rhythms in metabolism, physiology, and behavior that are driven by cell autonomous circadian pacemakers (1). These circadian rhythms allow organisms to coordinate a myriad of physiological processes with the changing environment. In mammals, the circadian pacemaker is composed of interlocked transcription-translation feedback loops: the primary loop is composed of the basic helix-loop-helix transcription factors CLOCK and BMAL1, which drive transcription of the *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) genes (1, 2). PER and CRY proteins form the negative limb of the feedback loop by inhibiting their own CLOCK: BMAL1-induced transcription; turnover of PER and CRY allows the cycle to begin anew. The interlocked loop consists of REV-ERB- $\alpha$  and ROR $\alpha$ , which repress and activate the *Bmal1* gene, thereby modulating its function (3, 4). Mutation or deletion of *Clock* (5), *Bmal1* (6), *Per1/2* genes (7, 8), or *Cry1/2* (9, 10) genes results in behavioral arrhythmicity and disruption of the autoregulatory loop, whereas disruption of components of the secondary loop results in short period-length phenotypes (3, 4).

The molecular components of the circadian clock are present in the majority of neurons in the suprachiasmatic nucleus (SCN), a bilateral body in the anterior hypothalamus (1, 2, 11). Circadian transcription and neuronal activity in the SCN persist in the

prolonged absence of environmental input, and SCN-lesioned animals exhibit behavioral locomotor arrhythmicity. Therefore, these nuclei act coordinately as the primary timekeeper in mammals. It was long thought that SCN neurons were the only cells in the body with an intrinsic clock, but more recent studies have shown that neurons in different brain regions, glia, and diverse peripheral tissues such as liver, kidney, and lung express functional molecular clocks (12–16). These peripheral tissue clocks can sustain rhythmicity even in the absence of the SCN, although the phases of the different tissues are coordinated by signals originating from the SCN (17).

Microarray studies performed on brain, liver, and heart tissue have shown that up to  $\approx 10\%$  of the transcriptome is under circadian regulation (18–21). Interestingly, studies comparing two or more tissues suggested that, although the genetic components of the pacemaker are similar across tissues, the genes exhibiting rhythmic expression are tissue-specific: Panda *et al.* (19) found only 28 genes, of  $>7,000$  known genes, that were rhythmically expressed in both SCN and liver, while Storch *et al.* (20) identified a similarly low percentage of genes that were rhythmic in both heart and liver. These experiments raise the question of how circadian oscillators, expressed ubiquitously throughout the body, are able to regulate the expression of diverse genes in a tissue-dependent manner. It has been proposed that the majority of circadian output is achieved via a transcriptional cascade, with the CLOCK–BMAL1 complex directly inducing the expression of other transcription factors that are subsequently responsible for the majority of rhythmic, tissue-specific expression (22). This hypothesis has been supported by the observation that many transcription factors are rhythmic and are direct targets of the CLOCK–BMAL1 complex (23, 24). However, those studies were performed with microarrays that interrogate only approximately one-fourth of the protein-encoding mouse transcriptome, and therefore did not fully represent the genome.

Studies of mice harboring the *Clock* mutation have suggested a role for CLOCK in a wide variety of physiological processes. Recent

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Abbreviations: CT, circadian time; DD, constant darkness; LD, light/dark; MEF, mouse embryonic fibroblast; MMC $\beta$ , multiple measures correct  $\beta$ ; SCN, suprachiasmatic nucleus.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE3751).

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studies have implicated circadian genes in functions as diverse as obesity and glucose tolerance (25, 26), reproduction (27), tumor susceptibility (28), and response to chemotherapeutic agents (29). One of the most interesting roles identified for peripheral circadian rhythms is in regulation of the cell cycle: Matsuo *et al.* (30) found that, after partial hepatectomy, livers from mice lacking both *Cry* genes regenerated more slowly than livers from WT mice. In the same study, the expression of several genes key to cell-cycle progression, including *Wee1* and *Cdc2*, was found to be under transcriptional regulation by the CLOCK–BMAL1 complex. Other groups have identified circadian regulation of transcription of *cyclin D1* and *c-myc* and protein levels of BCL2 and BAX, all key factors in cell-cycle regulation (28, 31). Furthermore, it has been shown that individual cells continue to rhythmically express clock proteins during cell division, and that the timing of cell division is gated by the circadian clock (14). In addition to regulating circadian gene transcription, the *Clock* mutation has been shown to affect many nonrhythmic genes, suggesting that core circadian components, including CLOCK, play important roles in noncircadian functions (19).

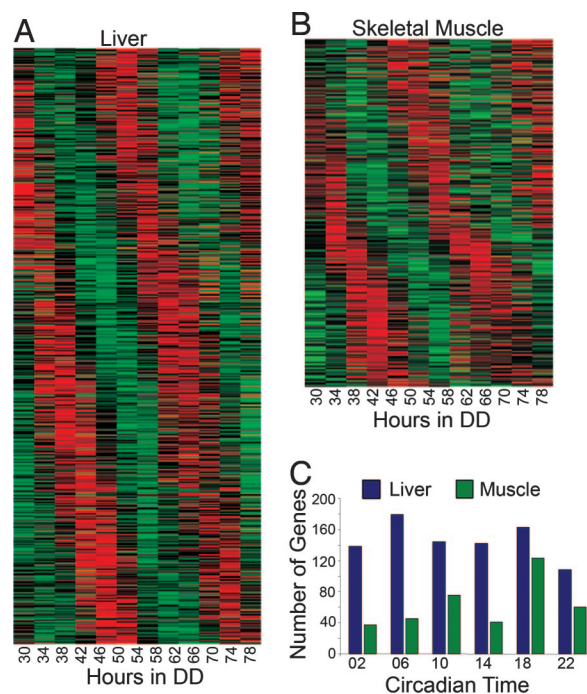
To address the role that the circadian clock plays in whole-genome gene regulation and in cell growth and proliferation, we analyzed RNA expression from WT liver and skeletal muscle tissue by using a microarray chip (GNFIM) designed to interrogate 36,182 nonredundant transcripts derived from protein-encoding genes (32). We also examined the same tissues from *Clock* mutant mice to investigate both circadian and noncircadian roles for CLOCK. We then used targets generated from analysis of microarray data to identify pathways disrupted in *Clock* mutants, including cell growth and proliferation, and tested whether changes in gene expression resulted in functional abnormalities of cell-cycle progression in *Clock* mutant fibroblasts.

## Results and Discussion

### Rhythmic Gene Expression in Liver and Skeletal Muscle from WT Mice.

To identify circadian-regulated transcripts, WT mice were entrained to a light/dark (LD) 12:12 cycle and then released into constant darkness (DD). Starting at 30 h in DD, corresponding to circadian time (CT) 18 (CT18), liver and skeletal muscle samples were collected from mice every 4 h for 48 h. For each time point, RNA was extracted from each tissue and pooled into two separate samples (each representing five mice), providing two independent biological replicates per time point. The samples were then hybridized to duplicate sets of custom-made genome arrays composed of 36,182 probe sets designed against the mouse protein-encoding transcriptome. Duplicate intensity values for two full circadian cycles (2 days) for each probe set were evaluated for circadian rhythmicity by COSOPT, as described (19). If significance of fit to a cosine wave was determined, the COSOPT algorithm provided a multiple measures correct  $\beta$  (MMC $\beta$ ) value indicating goodness of fit to a cosine wave form over a  $\approx$ 24-h period. MMC $\beta$  values for genes with a known circadian expression pattern were then used to set the MMC $\beta$  significance cutoff, in this case 0.2; any probe sets with MMC $\beta \leq 0.2$  were considered rhythmic [see supporting information (SI) Fig. 5 and Table 1]. To assess the validity of this cutoff further, four genes (*Timp3*, *Herpud1*, *Dscr1*, and *Gilz*) that were not previously known to be rhythmic but that had an MMC $\beta < 0.3$  in both tissues were chosen for follow-up analysis. WT tissues were collected every 4 h for 24 h, and mRNA expression levels were measured by real-time PCR. In all cases where RT-PCR was performed on separate tissue samples, microarray results were confirmed, suggesting that the combination of separate replicates for each genotype and time point with the GCRMA algorithm produces replicable results (33).

COSOPT analysis identified 854 probe sets in liver and 383 probe sets in muscle that had an MMC $\beta \leq 0.2$  (Fig. 1 *A* and *B* and SI Tables 1 and 2). These data were then subjected to an intensity cutoff value of 500 as the optimal discriminant between expressed



**Fig. 1.** Rhythmic expressed genes in WT liver and skeletal muscle. (*A* and *B*) Liver and gastrocnemius muscle tissues were collected from WT mice over a 48-h period (30–74 h in DD), and mRNA expression was determined by using a custom Affymetrix whole mouse genome microarray. Expression data were subjected to COSOPT cosine analysis to identify transcripts that were expressed with an  $\approx$ 24-h period. Rhythmic (MMC $\beta < 0.2$ ) genes were plotted by peak phase for liver (*A*, 854 genes) and skeletal muscle (*B*, 383 genes). (*C*) These genes were then separated into phase clusters derived from COSOPT analysis. Rhythmic liver genes exhibited large-phase clusters at CT06 and CT18, whereas the largest skeletal muscle-phase cluster occurred at CT18.

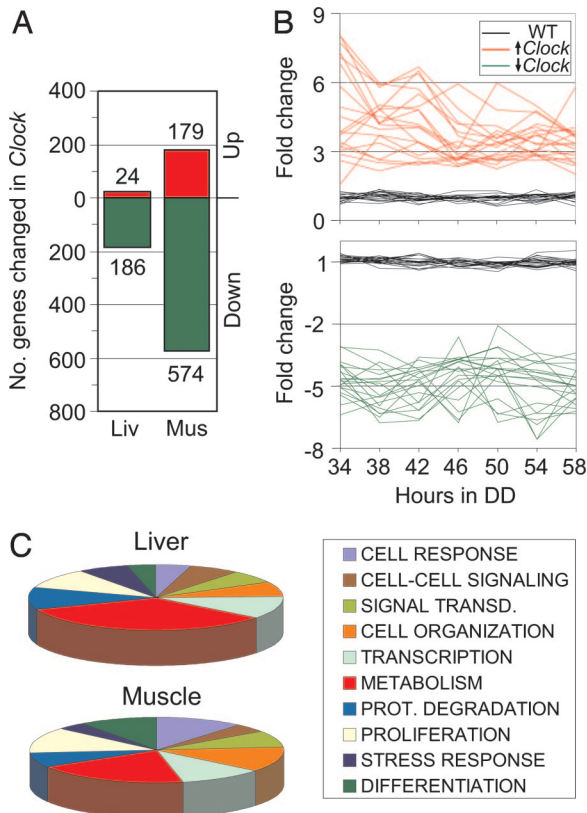
and nonexpressed genes. This analysis resulted in the identification of 716 rhythmic genes of 6,818 genes expressed in the liver (10.5%) and 267 rhythmic genes of 7,824 genes expressed in skeletal muscle (3.4%). The liver and skeletal muscle values are consistent with past reports (19, 20) regarding the percentage of rhythmically expressed genes in these tissues. As in previous studies, our results indicate that only a small number of genes are rhythmically expressed in more than one tissue, even when no intensity value cutoff is imposed. Only 57 genes had an MMC $\beta$  value  $\leq 0.2$  in both tissues sampled, many of which have previously been shown to be circadian (SI Table 3). These genes include core pacemaker components (*Per2* and *Cry2*), their regulators (*Nr1d1 = Rev-erba* and *Stra13 = Dec1*), and their immediate downstream targets (*Dbp* and *Tef*).

Using information derived from COSOPT analysis, we determined the phase distribution of rhythmic genes (Fig. 1*C*). In liver, we found the largest clusters of rhythmic genes at CT06 and CT18, 6 h after the onset of subjective day and subjective night, respectively. These data are in agreement with previous studies on the phase distribution of gene expression in the liver and likely represent physiological demands on metabolism and catabolism (19). In contrast, in muscle we identified a single large-phase cluster at CT18. As CT18 represents the midpoint of the active phase,  $\approx$ 6 h after the onset of activity, this peak may be activity-induced. It has previously been shown that resistance exercise can directly affect expression levels of key clock components and downstream targets in muscle (34); our data suggest that locomotor activity may act to phase-coordinate expression of rhythmic genes in skeletal muscle.

We used GeneSpring software to sort highly significant rhythmically expressed genes (MMC $\beta < 0.1$ ) into functional categories and hand-annotated the list for accuracy. In both tissues, genes







**Fig. 3.** Effect of the *Clock* mutation on gene expression. Gene expression in liver and gastrocnemius muscle from *Clock* mice was compared with gene expression in WT mice. (A) Bar graphs represent the number of genes whose average expression over a 24-h period showed significant, >2-fold change in expression in *Clock* tissue. In both liver and muscle, the majority of CLOCK-controlled genes were down-regulated compared with WT expression. (B) Each graph shows 20 examples of genes whose average expression was up-regulated (Upper) or down-regulated (Lower) in *Clock* muscle. (C) Pie charts depict the most highly represented functional categories of genes changed in *Clock* mouse liver or muscle.

fold change of  $\geq 2.0$  or  $\leq 0.5$  were considered to be differently expressed between WT and *Clock* mutants (SI Tables 6 and 7). Using no intensity cut-off, we found that  $\approx 200$  genes in the liver and 750 genes in the muscle differed in expression level between WT and *Clock* mice (Fig. 3A and B). In both liver and skeletal muscle, the *Clock* mutation resulted in the down-regulation of gene expression (86% and 76% of significantly changed genes in liver and muscle, respectively) more frequently than up-regulation. In sum, these results suggest that many genes regulated by CLOCK are nonrhythmic, and, furthermore, that CLOCK is directly or indirectly involved in both transcriptional repression and activation in the periphery.

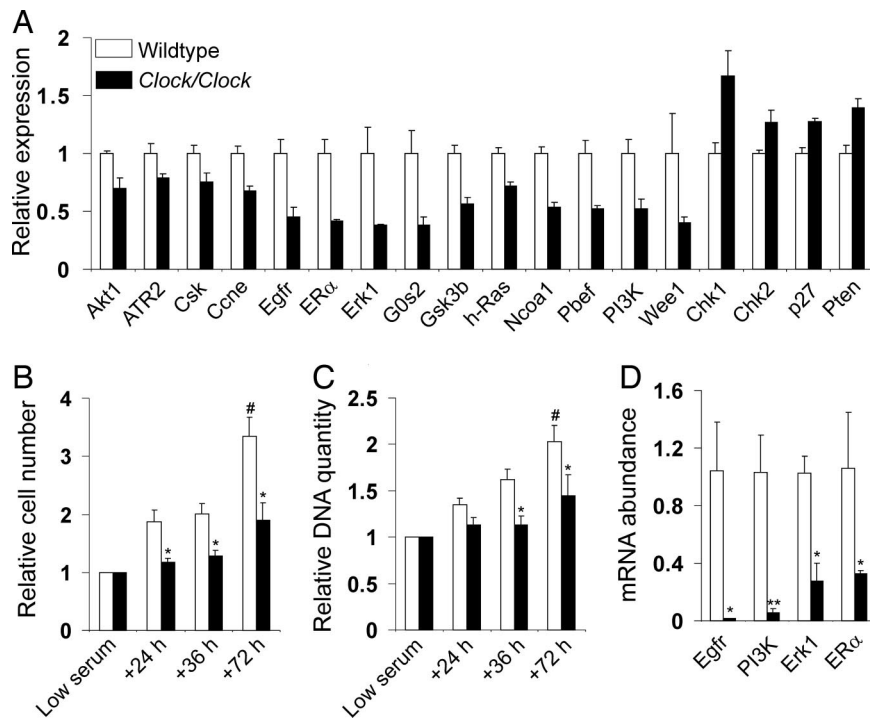
We used GeneSpring to categorize CLOCK-regulated genes by function and found that genes involved in cell growth and proliferation represented one of the largest functional categories in both liver and skeletal muscle (Fig. 3C). In the liver, 10% of CLOCK-regulated genes were involved in cell growth and proliferation, and in muscle 13% of CLOCK-regulated genes fell into the growth and proliferation category. Included in the cell proliferation-associated genes whose expression was affected by the *Clock* mutation were *p21* (*Cdkn1a*), a key inhibitor of cell-cycle progression that is up-regulated in peripheral *Clock* mutant tissue, *ki-67*, a marker of cell proliferation, and *hspca* (SI Table 8). Coupled with the above observations, these results suggest that the circadian oscillator and the CLOCK protein may play regulatory roles in cell-cycle control.

**CLOCK Regulation of Cell Growth and Proliferation.** Disrupting circadian rhythms has been shown to have an impact on cell cycle *in vivo*. In *Cry* double knockout mice, liver regeneration proceeds more slowly than normal, likely because of constitutive up-regulation of *Wee1*, a checkpoint kinase (30). *Per2* knockout mice show elevated levels of *c-myc* (a proliferative gene) and reduced *p53* (antiproliferative) expression concomitant with increased  $\gamma$ -irradiation-induced rates of cell proliferation and lymphoma (28). In contrast, overexpression of *Per1* sensitizes cells and inhibition of *Per1* abrogates the response to DNA damage-induced apoptosis (37). In *Clock* mutant mice, we found that cell-cycle inhibitory genes were generally up-regulated (e.g., *p21*, *p27*, *Chk1*, *Chk2*, and *Atr1*) or nonrhythmic (*Wee1*), whereas proproliferative genes such as *Jak2*, *ER $\alpha$* , *Pbif*, *Akt1*, *Cdk2*, *cyclins D3* and *E1*, and the TGF $\beta$  and EGF receptors were down-regulated (Fig. 4A and SI Table 8).

We hypothesized that the effect of the *Clock* mutation on cell-cycle gene expression might result in altered proliferation of *Clock* mutant cells. To test this, we measured DNA synthesis and cell growth in 12 WT and 10 *Clock/Clock* independently derived mouse embryonic fibroblast (MEF) lines. Cells were plated in 96-well plates at  $1\text{--}2 \times 10^4$  cells per well (three to five replicates per MEF line). To synchronize cell growth and circadian rhythms, the cells were subjected to 48 h of low serum (2% BCS) conditions, then reintroduced to 10% FBS. The amount of DNA and the number of cells were determined after 3 and 48 h in low-serum conditions and at 24, 36, and 72 h after introduction of high serum. As neither genotype showed a change in DNA quantity or cell number during low serum, the 3- and 48-h low-serum values for each genotype were averaged and used to normalize high-serum values by dividing each replicate's high-serum reading by its average low-serum reading. We observed that *Clock* MEFs exhibit reduced DNA synthesis and cell proliferation compared with WT MEFs (Fig. 4B and C). Seventy-two hours after introducing 10% FBS, DNA quantity and cell number had increased significantly in WT cells; in contrast, there was no significant increase in *Clock* MEF DNA quantity or cell number at any time point examined. For both DNA quantity and cell number, there were main effects of genotype, with WT values significantly elevated over *Clock* values, and time, with 72 h significantly different from all other time points (two-way ANOVA with repeated measures;  $P < 0.0001$ ). These effects of the *Clock* mutation depended on cell plating density and were seen at low, but not high, cell densities.

The deficiency in cell proliferation and lack of DNA synthesis suggests that *Clock* cells fail to respond to mitogenic signals and/or experience a block at the  $G_1/S$  transition. S-phase entry is dually regulated by the Src-Shc-Ras-Raf-MEK pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (40, 41), both of which are activated by growth factors, including EGF and estrogen, present in the fetal serum used in cell culture media. Therefore, we measured the expression of components involved in stimulating the  $G_1/S$  transition, including epidermal growth factor receptor (*Egfr*), estradiol receptor  $\alpha$  (*ER $\alpha$* ), and the kinases ERK1 and PI3K, and found that all four proproliferative genes were significantly down-regulated in *Clock* MEFs, in agreement with the microarray results described above ( $P < 0.05$  for all; *t* test) (Fig. 4D). Thus, *Clock* cells appear to combine overexpression of factors that inhibit cellular proliferation, such as *p27* and *p21*, with a reduced ability to respond to mitogenic signals.

Despite the failure of *Clock* MEFs to proliferate *in vitro*, *Clock* mutant mice show no apparent developmental abnormalities (5). One possibility, as suggested by DeBruyne *et al.* (42), is that other genes, such as *Npas2*, can compensate for the lack of functional CLOCK. It would be of interest to examine whether the *Npas2* knockout mice also exhibit deficits in MEF proliferation (43). Another possibility is that *Clock* mutant mice may not have been carefully examined for developmental defects, and that these mice may have subtle deficits. Adult *Clock* mutants have been shown to be more susceptible than WT mice to side effects of the chemo-



**Fig. 4.** DNA synthesis and cell proliferation is diminished in *Clock* primary cells. (A) The average expression intensities from 24-h microarray data are shown for many genes that are involved in cell proliferation and exhibit significantly ( $P < 0.05$ ; see SI Table 8) changed expression in *Clock* liver. (B and C) MEFs from WT and *Clock* embryos were cultured in 2% BCS for 48 h and then switched to media containing 10% FBS. The number of live cells (B) and quantity of DNA (C) were measured during low-serum and high-serum (+24 h, +36 h, +72 h) conditions. At least three wells per culture were measured at each time point, and all data were normalized to the average low-serum values for each culture. Asterisks represent the significant difference observed for both the relative cell number and relative DNA quantity of WT MEF cells versus *Clock* MEF cells at +36 h and +72 h, and the relative cell number of WT MEFs versus *Clock* MEFs at +24 h (\*, effect of genotype; two-way ANOVA). In addition, WT MEFs exhibited significantly higher relative cell number and DNA quantitation at +72h, compared with other time points (#, effect of time; two-way ANOVA). Error bars represent standard deviation from the means of normalized data from 12 independent WT and 10 independent *Clock* MEF cultures. (D) mRNA expression from MEFs grown in 10% FBS was determined by RT-PCR. Expression of the proliferative genes *Erk1*, *ERα*, *Egfr*, and *PI3K* was significantly reduced in *Clock* MEFs (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; Student's *t* test).

therapeutic drug cyclophosphamide, exhibiting reduced B cell survival and recovery after treatment (29). Although the genes responsible for reduced B cell proliferation after chemotherapy in *Clock* mutants were not identified, our present results suggest that CLOCK-mediated up-regulation of antiproliferative signals, particularly those sensitive to DNA damage that would be activated by cyclophosphamide (44), combined with a reduced response to proliferative signals, could underlie the observed sensitivity. As in the liver regeneration model in *Cry* knockout mice, deficits in cell cycle and development may become apparent only upon perturbation of the system (30). It is also possible that during development maternal factors override the inhibitory effects of the *Clock* mutation on growth in fetal cells; after birth, proliferation occurs at a much lower level, and therefore defects may not be apparent unless the system is challenged. Given that studies in humans have shown an association between circadian disruption induced by shift work and the development of breast cancer (45), and that several clock gene mutations have been identified in humans (46, 47), the association between circadian rhythms and development, cell proliferation, and physiological responses to environmental insults presents a fertile field for future research.

## Materials and Methods

**Animals.** A total of 190 WT male C57BL/6J mice age 7–10 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and entrained to a L/D 12:12 cycle for 2 weeks. Mice were then placed in light-tight boxes on a L/D 12:12 cycle for 4 weeks, then released into DD. Starting 30 h after entry into DD (CT18), tissues from 5 (skeletal muscle, gastrocnemius) or 10 (liver) WT mice were

collected every 4 h for 48 h, for a total of 12 time points. At time points 34–58 h in DD, tissues from age-matched male C57BL/6J *Clock* homozygous mutant mice that had been treated with the same light entrainment protocol as the WT mice were collected. Tissues were collected from 5 *Clock* mutants at each time point except for 34 and 46 h after the onset of DD, when tissues from 10 *Clock* mice were collected and run as independent replicates. Mice were euthanized by cervical dislocation, and the liver was removed, divided into several 2-mm cubed sections, and snap-frozen on dry ice. The gastrocnemius muscle was removed from each hind leg and frozen in liquid nitrogen.

For verification of microarray data by real-time PCR (TaqMan), 60 male WT mice, aged 12–15 weeks, and 27 age-matched *Clock* male mice were subjected to the same light entrainment protocol described above. Beginning 34 h after entry into DD (CT22), liver and muscle were collected from eight WT mice and four *Clock* mutant mice per time point every 4 h for 24 h.

**RNA Extraction and Probe Hybridization.** RNA was extracted from  $\approx 100$  mg of frozen tissue by using TRIzol (Invitrogen, Carlsbad, CA). Tissues destined for microarray analysis were further processed with a RNeasy miniprep kit (Qiagen, Chatsworth, CA), and pooled into two independent groups representing five mice each per time point. These groups were kept separate throughout the microarray process. Five micrograms of total RNA per group was used as a template to synthesize cDNA and biotinylated cRNA (Enzo kit; Affymetrix, Santa Clara, CA) using standard Affymetrix protocols. The microarray used was a custom design by Affymetrix and the Genomics Institute of the Novartis Research Foundation



(GNF) and is described in Su *et al.* (32). After hybridization, the arrays were washed with a custom GNF fluidics machine, scanned with an Affymetrix GCS3000 scanner, and analyzed with the GCRMA algorithm (33).

**Real-Time PCR.** TaqMan RT-PCR technology (Applied Biosystems, Foster City, CA) was used to validate microarray results and measure expression of genes in adult tissue and MEF cells. Probe and primer sets were designed with Primer Express software (Applied Biosystems) to span exon-exon junctions of target cDNA sequences (see SI Table 9 for sequence information). One hundred nanograms of each RNA sample was reverse-transcribed, and the resulting cDNA was amplified by using the TaqMan EZ RT-PCR kit (Applied Biosystems). For adult tissues, *Gapdh* was used as an internal reference control, and probe and primer concentrations were optimized so that the target and control reactions could be performed in the same tube. Expression of the target gene was determined by using the comparative Ct (cycle of threshold detection) method to normalize target expression relative to *Gapdh* expression. For MEF RNA samples, RNA concentration was verified by spectrophotometry, and target gene Ct values were normalized to average WT values, as *Gapdh* was significantly lowered in *Clock* MEF cells (data not shown).

**MEF Cell Culture.** Embryos were removed from mice on days 12–14 of pregnancy. The uterine and placental tissues were dissected away, and the embryos were washed in cold PBS. The head and peritoneal organs were removed and used to genotype embryos, and the remaining tissue was minced and agitated at 37°C for 30 min in PBS containing 1× trypsin/EDTA with 100 μg/ml DNaseI, followed by two more agitations with equal volumes of PBS

containing 1× trypsin/EDTA for 30 min each. Enzymatic dissociation of the cells was stopped with DMEM plus 10% FBS, and cells were centrifuged and resuspended in DMEM plus 10% FBS. Cells were plated on 10-cm dishes at a density of  $3 \times 10^6$  cells per dish. Passage 2 and 3 cells were plated in 96-well plates at  $1\text{--}2 \times 10^3$  cells per well in DMEM plus 2% BCS (low-serum condition). Twelve WT and 10 *Clock/Clock* MEF cell lines were used, and each line was represented by three to five replicates. Three and 48 h after plating, cells were collected to quantitate the number of live cells and amount of DNA. After 48 h, cells were placed in media with 10% FBS. The number of live cells and the amount of DNA were quantitated at 0, 24, 48, and 72 h after placing cells in 10% serum. The number of cells per well was measured with an MTT-conversion assay (Vybrant, Molecular Probes, Eugene, OR), and the amount of DNA per well was measured by using a fluorescence-based assay (CyQuant; Molecular Probes) per the manufacturer's instructions. To measure the expression of genes involved in the regulation of cell proliferation, WT and *Clock* MEFs were grown to confluency in 10% FBS. RNA from the cells was extracted by using TRIzol, diluted to 100 ng/μl, and relative abundance of gene expression was measured by RT-PCR, as described above.

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