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Circadian expression of clock genes in purified hematopoietic stem cells is developmentally regulated in mouse bone marrow

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Objective. **Clock genes are known to mediate circadian rhythms in the central nervous system and peripheral organs. Although they are expressed in mouse hematopoietic progenitor and stem cells, it is unknown if they are related to circadian rhythms in these cells. We therefore investigated the 24-hour patterns in the activity of several clock genes in the bone marrow (BM) side population (SP) primitive stem cells, and compared these 24-hour patterns to clock gene variations in the whole BM and liver.**

Methods. **Cells were obtained from 84 B6D2F$_1$ mice in three replicate experiments on the second day after release into constant darkness from a standardizing light-dark schedule. mRNA expression of clock genes was measured with quantitative reverse transcriptase polymerase chain reaction.**

Results. **m*Per2* displayed circadian rhythms in SP cells, whole BM, and liver cells. m*Per1* and m*Rev-erbα* showed a circadian rhythm in whole BM and liver, but not SP cells. m*Bmal1* was not expressed rhythmically in SP cells, nor in the whole BM, contrary to rhythms observed in the liver.**

Conclusions. **With the exception of m*Per2,* most clock genes studied in primitive hematopoietic SP stem cells were not oscillating in a fully organized circadian manner, which is similar to immature cells in rapidly proliferating organs, such as the testis and thymus. These findings indicate that circadian clock gene expression variations in BM are developmentally regulated.** © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

The circadian system is an integral part of mammalian physiology and regulates most biological functions along a 24-hour time scale [1]. Circadian rhythms are generated by a molecular clock with specific clock genes whose proteins serve as self-regulating positive (CLOCK, BMAL1) or negative (PER1, PER2, PER3, CRY1, CRY2, REV-ERBα) elements. Clock proteins rhythmically modulate transcription of so-called "clock-controlled genes," which represent nearly 10% of the genome [2,3]. Clock genes are shown to be active not only in the suprachiasmatic nucleus (SCN) in the hypothalamus, the host of the central clock [1], but in many peripheral tissues, such as liver, heart, kidney, lung, pancreas, skeletal muscle, oral mucosa, skin, bone marrow (BM), and human peripheral blood mononuclear and polymorphonuclear cells [4–12]. The SCN is considered as a phase coordinator of persistently rhythmic peripheral clocks, rather than as a pacemaker driving peripheral oscillators [13]. Clocks in peripheral organs can express

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tissue-specific differences in period and phase [13], and are affected by changes of local conditions [6,8,14]. Recently, it was demonstrated that clock genes could be expressed in a constant, rather than rhythmic, manner in organs composed primarily of differentiating cells, like testis and thymus [15,16]. These findings led to a hypothesis that expression of clock genes is developmentally regulated [15], and that these clock genes do not operate in a fully organized circadian manner in immature cells [16].

Circadian rhythms have long been known for many hematopoietic functions in rodents or humans [17]. Significant 24-hour changes have been shown at several levels of hematopoiesis, including cell-cycle phase distribution in BM [18], the proliferative response of granulomonocytic precursors to growth factors [19], and mouse BM engraftability [20]. Recent findings support the hypothesis that BM contains self-sustained oscillators. BM cells continued to proliferate rhythmically in mice with ablated SCN [21], and the responsiveness of BM cells to granulomonocytic colony-stimulating factor displayed a sustained circadian rhythm over 4 days of culture [22]. Expression of key clock genes in mouse BM was recently demonstrated [23–25].

m*Per1* and m*Per2* were shown to oscillate over a 24-hour period in the BM in Balb/C mice [23,24], while m*Per2,* m*Bmal1* and m*Clock* were rhythmic in $B6D2F_1$ mice [24]. It is interesting to note that patterns of m*Per2* expression in BM were different in the two mouse strains. In addition, the peak of m*Bmal1* expression in $B6D2F_1$ mouse BM was different from those in other tissues [24], which could indicate that environmental factors in BM have a strong influence on time-dependent regulatory mechanisms.

Hematopoiesis in the BM is regulated locally by complex networks involving a multitude of growth factors, cytokines, chemokines, and other peptides [26,27]. This complicated regulation of the molecular clock may theoretically lead to unique quantitative properties of the circadian oscillators in BM.

BM is a rapidly proliferating tissue hosting many different cell types, mature and in various stages of proliferation and differentiation. The circadian patterns of clock gene variations appear to be lineage- and stage-dependent [23]. However, little is known on the molecular clock in different hematopoietic subpopulations. Recently, the presence of key clock genes was reported in purified mouse hematopoietic side population (SP) stem cells, with some differences compared with whole BM [25]. However, until now, circadian variations of clock gene expression in BM primitive stem cells have not been investigated.

Based on the hypothesis that clock genes could operate differently in immature and differentiating cells [16], we studied changes in clock gene expression throughout 24 hours in primitive hematopoietic stem cells, and compared them with patterns in similar clock genes simultaneously measured in the whole BM and liver in the mouse.

Materials and methods

Animals and experimental design

Eighty-four male $B6D2F_1$ mice, 5 to 6 weeks old (Charles Rivers, L'Arbresle, France) were synchronized for 3 weeks to standard lighting conditions consisting of 12 hours of light alternating with 12 hours of darkness (LD 12:12) with lights on from 06:00 to 18:00 hours. After this time, rectal temperatures (Digital Thermometer 49A; YSI, Dayton, OH, USA) were obtained around the clock from 28 mice in order to confirm synchronization to the LD schedule. Animals were then exposed to constant darkness (DD) for 24 hours in order to avoid any initial masking effect of the prior LD schedule on circadian rhythmic patterns during DD. Three studies using 28 mice each were performed, each involving 4 mice that were sacrificed at one of seven 4-hour circadian time points (CT) with reference to the time of previous light onset during LD (e.g., 06:00 hours = 00 CT). The seventh sampling point was a replicate of the first one and was obtained 24 hours later. Mice were sacrificed by CO_2 narcosis, and femurs, tibiae, and liver were removed. Also at sacrifice, heart blood was aspirated from the mice, plasma was extracted with diethyl ether, and corticosterone and melatonin were determined by radioimmunoassay as described earlier [28,29]. For corticosterone analysis, the residue was dissolved in assay buffer before incubation with 1,2,6,7-3H corticosterone (Amersham, Orsay, France) and rabbit anticorticosterone antibody (Valbiotech, Paris). For melatonin determination, antimelatonin antiserum and tracer were prepared according to [30], and diethyl ether was used for the extraction procedure. Sensitivity of the method was routinely 5 pg melatonin per mL plasma.

BM cell preparation and staining

BM cells were obtained by crushing both femurs and tibiae in a mortar using a pestle in cold Hank's balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) and 1 mM HEPES (all purchased from Gibco, Invitrogen Corporation, Carlsbad, CA, USA)− HBSS+. Cells from four mice were pooled, filtered through 70-μm nylon cell strainers BD Falcon (Becton Dickinson Labware Europe, Le Pont-de-Claix, France) and washed once. Cell pellets were incubated in 10 mL lysing solution (a gift by DakoCytomation, Denmark A/S) for 10 minutes in the dark. After centrifugation, cells were resuspended in 30 mL cold HBSS+ and counted. On average, $5 \pm 2 \times 10^7$ BM cells were recovered per mouse. Two-hundred microliters cell suspension ($2.0 \pm 0.3 \times 10^6$ BM cells) were spun down (300*g*, 5 minutes at + 4°C) in 2 mL cryotubes (TPP AG, Trasadingen, Switzerland), supernatants were discarded, and pellets snap frozen (30 seconds) in liquid nitrogen and stored at −80°C until use. The rest of the cell suspension was kept at 4°C until staining, which was performed when the samples from all time points had been collected. BM cells (10^6 cells/mL) were stained in Dulbecco's modified Eagle medium (DMEM) containing 2% FCS and 1 mM HEPES (all from Gibco, Invitrogen Corporation) with 5 μg/mL Hoechst 33342–Hoechst (Molecular Probes, Eugene, OR, USA) for 90 minutes at 37°C as described by Goodell et al. [31], spun down and resuspended in cold HBSS+ at 5–7 $\times 10^7$/mL. Cells were kept on ice until the sorting procedure. Propidium iodide (PI, 2 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to the samples before flow sorting to facilitate dead cell discrimination. Cell suspensions were filtered through 50 μm Filcons filter device (Dako, Denmark A/S) prior to flow cytometric analysis.

Flow cytometry and high-speed cell sorting

BM cell suspensions were analyzed and sorted on the MoFlo cell sorter (Dako, Fort Collins, CO, USA), equipped with Coherent Enterprise 621 argon-ion laser tuned to 488 nm (1 W) and 365 nm, ultraviolet (60 mW). Hoechst dye and PI were excited at 365 nm. Hoechst fluorescence was measured by two detectors through 450/20 BP (Hoechst "blue") and 675 EFLP (Hoechst "red") optical filters (Omega Optical, Brattleboro, VT, USA), and PI fluorescence through 675 EFLP. A 610 DMSP was used to separate emission wavelengths. The sorting gates for SP were established as described elsewhere [25]. Cells were sorted in "purify 1" mode and collected in 2 mL cryotubes (TPP AG, Trasadingen, Switzerland), containing cold HBSS with 10% FCS, that were placed on ice. Sorted cells (on average 1–5 $\times$ 10^4) were spun down, supernatants were discarded, pellets snap frozen in liquid nitrogen and stored at -80°C until use. Aliquots from some samples at the end of the sort were removed and reanalyzed for control of the sort purity that was $>97\%$, and viability by trypan blue staining that was $>97\%$. The recovery was $>70\%$.

RNA extraction, reverse transcription, primer and probe design

Total cellular RNA was extracted with the use of the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA), and was reverse transcribed (RT) using TaqMan RT reagents (Applied Biosystems, Foster City, CA) as described earlier [25]. Mouse BM total RNA (Clontech, Palo Alto, CA, USA) was used as an exogenous control.

Primer Express software (Applied Biosystems) was used for primer and probe design for all of the TaqMan assays. Fluorogenic TaqMan probes, incorporating the reporter dye FAM on the 5′ end and the quencher dye TAMRA on the 3′ end, were used. The designed primers and probes for eight target genes (m*Per1,* m*Per2,* m*Bmal1,* m*Cry1*, m*Clock,* m*Rev-erbα,* m*Wee1,* and m*Dbp*) were purchased from MedProbe (Oslo, Norway). Three housekeeping genes m*36B4*, *18S,* and m*GAPDH* were chosen as reference genes (endogenous control). *18S* and m*GAPDH* were bought commercially from Applied Biosystems with a reporter dye labeled VIC. Sequences of clock genes and m*36B4* are given in ref. [25]. Probes and primers of m*Wee1* and m*Dbp* were the following: m*Wee1*-Probe, AGA GCC GGA ATC AAT AAC TCG CGC TTT; m*Wee1*-For, CGC ACA CTC CCA AGA GTT TG; m*Wee1*-Rev, ATG AAT AGA GAA CTA CCC CGG AGT TT; m*Dbp*-Probe, TTC ATT GTT CTT GTA CCT CCG GCT CCA GT; m*Dbp*-For, GCC TGA GGA ACA GAA GGA TGA G; m*Dbp*-Rev, TCT TGC ATC TCT CGA CCT CTT G. As an extra control, m*Bmal1* was analyzed using an alternative primer sequence as described by Tamaru et al. [32].

Real-time quantitative RT polymerase chain reaction of BM samples

Real-time quantitative RT polymerase chain reaction (Q-RT-PCR) was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with 96 wells. A standard curve consisting of six points was included in each run. This was obtained by performing a twofold serial dilution of control RNA starting at 250 ng. A "no template control" was also included in each run. Each sample was analyzed in triplicate. All samples from a tested cell population (the whole BM and SP) were run on a single plate. Coefficient of variation calculated from control Q-RT-PCR was $0.40 \pm 0.09\%$ for *mPer1* ($n = 30$). O-RT-PCR was performed as earlier described [25].

The standard curves obtained from control cDNA were used to evaluate the quality and efficiency of each reaction, as well as in estimating the relative quantity of each sample. The standard curves exhibited slopes with a mean of -3.1 to -3.3, which indicated efficient Q-RT-PCRs.

Q-RT-PCR of liver samples

RNA extraction was performed as described for BM. All primers for Q-RT-PCR were obtained from Invitrogen Life Technologies (Cergy Pontoise, France). Sequences were the following: m*Per2*-For, GTG AAG CAG GTG AAG GCT AAT G; m*Per2*-Rev, AAG CTT GTA AGGGGT GGT GTA G; m*Rev-erbα*-For, TGG CCT CAG GCT TCC ACT ATG; m*Rev-erbα*-Rev, CCG TTG CTT CTC TCT CTT GGG; m*Bmal1*-For, TGT CAC AGG CAA GTT TTA CAG AC; m*Bmal1*-Rev, ACA GTG GGA TGA GTC CTC TTT G; m*36B4*-For, ATG TGC AGC TGA TAA AGA CTG G; m*36B4*-Rev, AGG CCT TGA CCT TTT CAG TAA G. One-step Q-RT-PCR was performed with a LightCycler instrument (Roche Diagnostics, Meylan, France) in a total volume of 20 μL containing 1.0 μL (500 ng) total RNA, 2.4 μL (6 mM) $MgCl_2$, 2 μL (0.5 μM) of each primer, 4.0 μL LightCycler Q-RT-PCR Reaction Mix SYBR Green I, 0.4 μL LightCycler Q-RT-PCR Enzyme Mix and 10.2 μL distilled water. RT was performed at 55°C for 10 minutes. Denaturation and amplification conditions were 95°C for 30 seconds followed by 40 cycles of Q-RT-PCR. Each cycle included immediate denaturation at 95°C, 10 seconds of primers annealing at 60°C and 13 seconds of extension/synthesis at 72°C.

At the end of the extension step, fluorescence of each sample was measured at 84°C (85°C for m*Bmal1*) to eliminate the background fluorescence due to primer dimers. The specificity of the Q-RT-PCR products was assessed by the melting curve analysis, and by agarose gel electrophoresis. As an additional control, liver mRNA was tested with Q-RT-PCR using exactly the same method as for SP cells and BM, which produced similar results.

Calculations and statistics

The standard curve method was used for calculations of gene expression, as previously described [33]. This was done to standardize the data of the clock genes run on different plates. To reduce the possible error related to the use for normalization of only one housekeeping gene as an endogenous control, the normalization strategy described previously [34] with the geometric mean of three endogenous control genes (based on the average quantity values from triplets) was used for SP and the whole BM samples. Each sample was normalized by dividing the quantity mean of the target gene by the quantity of the geometric mean (the quantity for each sample was estimated from the corresponding standard curve). The liver samples were normalized in the same way to the corresponding m*36B4* instead of the geometric mean. For each study, the average of the normalized values of all samples within each gene was calculated, and each value was then expressed as a percentage of relative expression in which the average of all normalized values was taken as 100%. The normalized values from all three studies were then combined for analyses for overall time effects.

Time series of body temperature (°C), serum corticosterone, plasma melatonin, and expression of each mRNA clock gene in liver and BM (whole and SP) were analyzed for circadian rhythm by the single cosinor method using the Chronolab software

package [35]. This method involves fitting a curve of a predefined period (in this case, 24.0 hours was used to approximate the circadian rhythm during the second day in the free-running DD condition) by least squares linear regression, as discussed elsewhere [36]. Parameters derived from the fitted cosine include 1) a p value from a zero-amplitude test, 2) the double amplitude (2A) indicating the predictable range of change in the time series from peak to trough of the curve, and 3) the acrophase or peak of the fitted curve, in hours and minutes from the previous time of light-onset during LD standardization. Each time series was also analyzed for a time-effect by one-way analysis of variance (ANOVA).

Results

Rhythms of three body markers indicate a circadian coordination in mice during the second day in constant darkness

Mean core body temperature varied significantly as a function of sampling time (ANOVA: $p < 0.001$, Table 1), and a 24-hour rhythm was documented with cosinor analysis ($p < 0.001$, Table 1 and Fig. 1). The peak occurred near the middle of the previous dark period (17:40 hours), which corresponds to the period of maximal locomoter activity in mice. High amplitude and significant rhythms were observed in both corticosterone and melatonin, with peaks occurring at 12:40 and 10:50 hours, respectively (Fig. 1 and Table 1). The timing of peaks for these two hormones were slightly advanced from the usual peak time of corticosterone [37] and melatonin [38] in early dark in LD, indicating that these rhythms were beginning to free-run in DD, with a period differing from precisely 24.0 hours. Nevertheless, these results show that the mice still maintained circadian coordination during the second day in constant darkness, when BM and liver were sampled for gene expression analysis.

Differences in relative expression level of clock genes in SP and whole BM cells are most pronounced for mPer1 and consistent throughout 24 hours

The relative mRNA levels differed between SP cells and the whole BM at each CT in three replicate experiments (Suppl. Fig. 1). The most pronounced difference was found for m*Per1* expression. In SP cells, it was approximately 2 to 2.5 times as high as the other clock genes at all time points. Conversely, in the whole BM, m*Per1* showed low-to-moderate expression levels when compared with other clock genes, depending on circadian time. In SP cells, this relative distribution did not change much over the 24-hour period, while in contrast, there were some variations in the BM cells (Suppl. Fig. 1).

Expression of mPer1 throughout 24 hours is rhythmic in the whole BM, but not in SP cells

Expression of m*Per1* in SP cells displayed large temporal changes in each of three experiments (Fig. 2), but individual patterns were highly variable from one experiment to another. As a result, no overall 24-hour rhythm in m*Per1* expression in SP cells was validated by cosinor analysis ($p = 0.602$, 2A = 13%, Fig. 2 and Table 2). In contrast, a robust high-amplitude circadian rhythm for m*Per1* was found in the whole BM, where temporal variations of this gene were both large and consistent in all three studies, with an overall 2A of 90% ($p < 0.001$) (Fig. 2 and Table 2).

We observed two distinct peaks in each of the three individual studies for m*Per1* in SP cells ($p = 0.0355$, 0.065, and 0.007 for the best-fitting ultradian cosine in each study, with mean period = 16.1 hours), but with peaks failing to coincide across studies, resulting in a nonsignificant circadian pattern when data from all studies were combined (Fig. 2, left column).

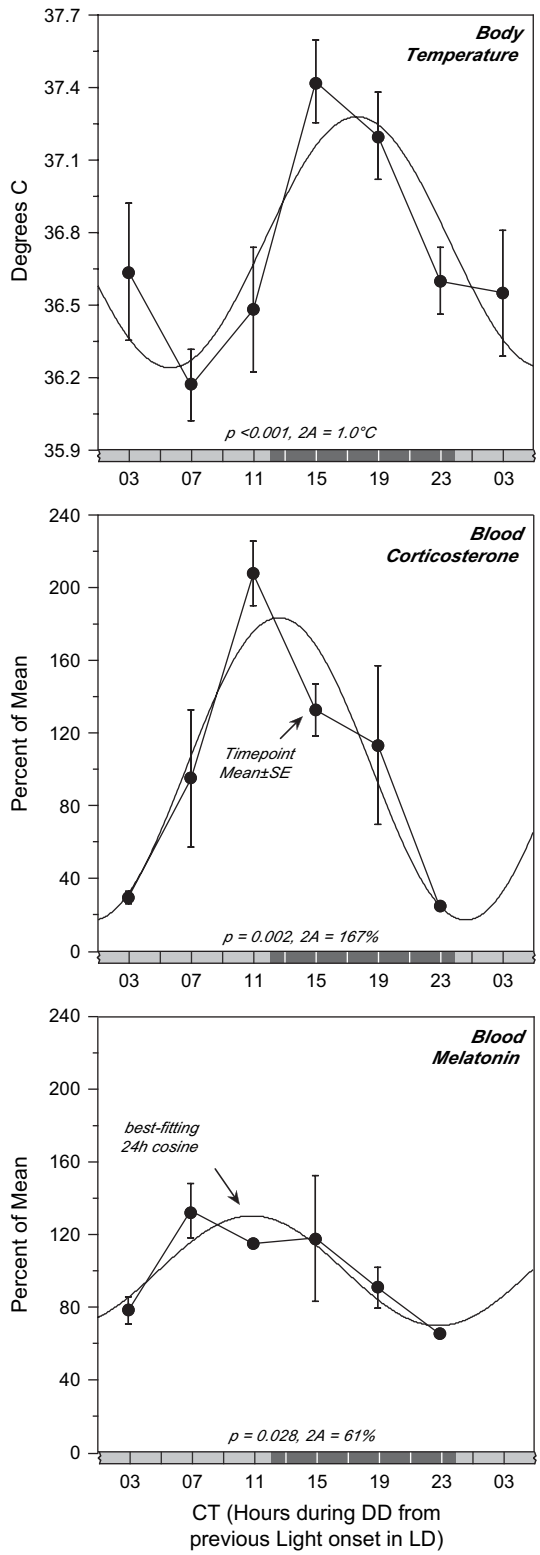
The 24-hour variations of mPer2, mRev-erbα, and mBmal1 expression are different in SP cells, whole BM, and liver

In SP cells, a significant circadian rhythm was demonstrated for m*Per2* (ANOVA: $p = 0.006$; cosinor, $p = 0.025$, Table 2, Fig. 3). This gene was also rhythmic in the whole BM ($p = 0.021$) and liver ($p < 0.001$) (Fig. 3). Peak time of m*Per2* expression in SP cells occurred near the end of the subjective light (rest) period (10:40 hours), while in the whole BM and liver it was

Table 1. Circadian changes in three marker rhythms indicating persistence of the circadian body clock in mice during second day in constant darkness. Results for time-effect by analysis of variance and single cosinor

Variable	Units	ANOVA (p value)	Cosinor[a] (p value)	Double amplitude[b]	Acrophase[c] (95% confidence limits)
Body temperature	°C	<0.001	<0.001	1.04°C	17:40 (15:21, 19:24)
Blood corticosterone	% of mean	0.016	0.002	167%	12:40 (10:56, 14:20)
Blood melatonin	% of mean	0.146	0.028	61%	10:50 (08:00, 13:48)

Male B6D2F$_1$ mice synchronized to 12-hour light-to-dark cycle for 3 weeks and exposed to constant darkness for 24 hours prior to study. Data from each of three studies normalized to percent of mean and combined for analysis (n = 100 for temperature, n = 12 for hormones).
ANOVA = analysis of variance.
[a]Cosinor analysis = fit of a 24-hour cosine to all data by least-squares linear regression (single Cosinor technique). p value determined from zero amplitude test.
[b]Double amplitude = difference between peak and trough of fitted cosine.
[c]Acrophase = time of peak in fitted cosine (units = hours:minutes from usual light onset = 00:00 hours); 95% confidence limits (hours) of acrophase listed if fit of cosine significant at $p \leq 0.05$. Reference: usual light onset.



near the beginning of the subjective dark (active) period at 13:30 and 12:50 hours, respectively (Table 2). The double amplitude of m*Per2* variations was considerably lower in the SP cells (43%) and BM (28%) than in liver (215%) (Table 2).

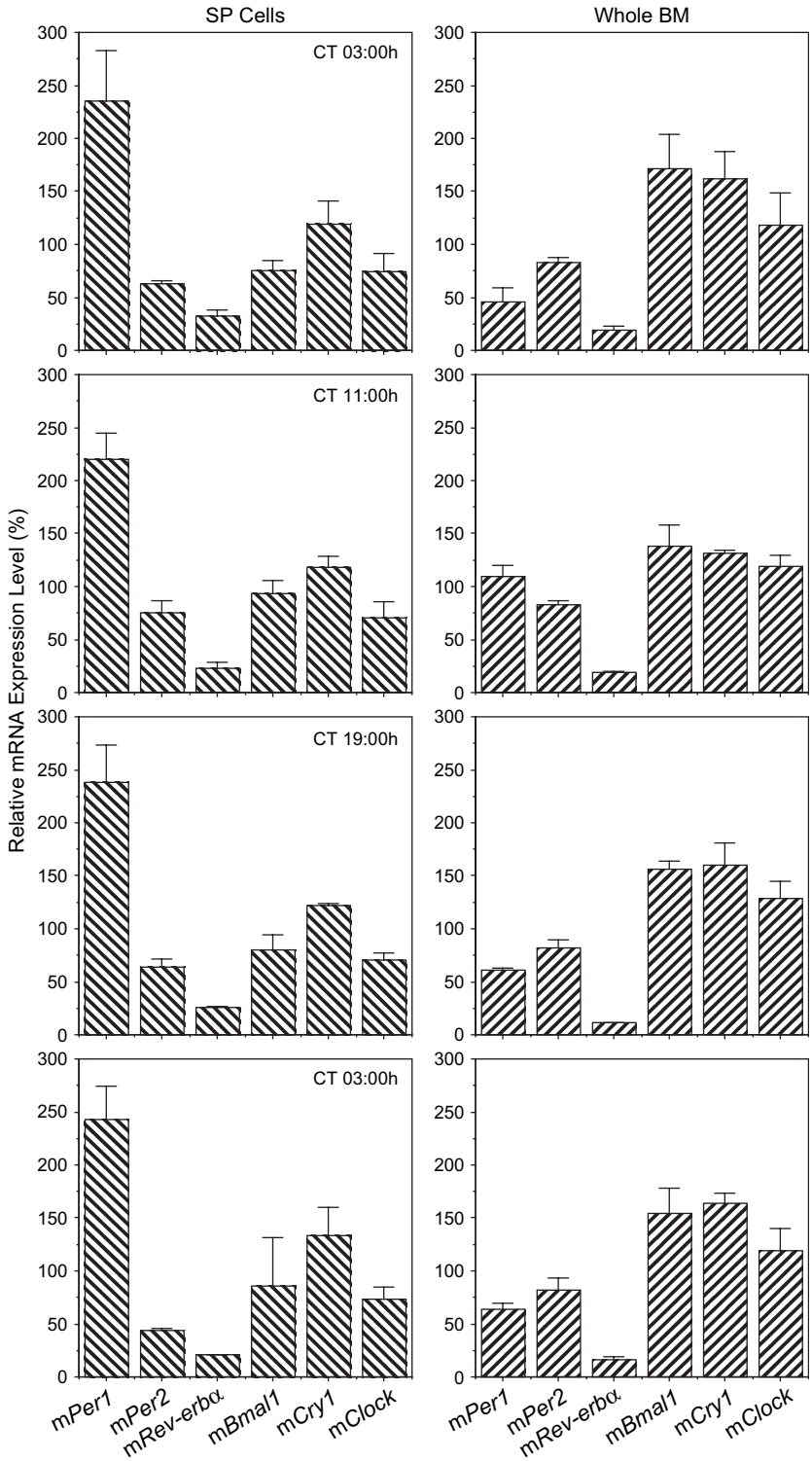
Variations of m*Rev-erbα* expression in SP cells were not rhythmic ($p = 0.206$, 2A = 52%). In contrast, a significant 24-hour rhythm of *mRev-erbα* was found in the whole BM ($p < 0.001$) and liver ($p = 0.024$) (Table 2, Fig. 3). Peaks of expression were detected in the subjective light (rest) period (Fig. 3 and Table 2). As in the case of m*Per2*, the double amplitude of m*Reverbα* was much lower in the BM than in liver (63% vs 226%, respectively, Table 2). The most striking difference found in 24-hour clock gene patterns in BM cell populations as compared to liver was m*Bmal1* expression variations. No rhythm was found for m*Bmal1* expression in either SP ($p = 0.522$, 2A = 25%) or the whole BM cells ($p = 0.605$, 2A = 14%) (Fig. 3, Table 2). In contrast, m*Bmal1* was expressed rhythmically in liver with a large amplitude (cosinor, $p = 0.016$, 2A = 169%), and a peak at 01:40 hours, near the onset of the subjective light (rest) period (Fig. 3).

In order to exclude the possibility that the absence of m*Bmal1* rhythmicity in the BM populations was due to the primers/probe design, we repeated the analysis of m*Bmal1* expression in the whole BM using another primer/probe set [32]. No circadian rhythm was detected by this second method ($p = 0.172$, 2A = 28%). Thus, identical results were obtained with the two primer sets (Fig. 3, lower mid-panel and Suppl. Fig. 2).

mCry1, mClock, mWee1, and mDbp do not exhibit circadian rhythms in BM cell populations

Expression of m*Cry1* and m*Clock* did not vary significantly throughout 24 hours by either ANOVA or cosinor analysis in either SP cells or the whole BM (Table 2, Suppl. Fig. 3). Double amplitude for circadian variations in both genes was very small when compared with the amplitudes for clock genes mentioned above (Table 2). In addition to the analysis of key clock genes, we studied circadian variations of the expression of two clock-associated genes, m*Wee1* and m*Dbp*. Both genes were reported to be rhythmic in the SCN and liver and controlled by clock (CLOCK-BMAL1) proteins [5,39]. m*Wee1* and m*Dbp* were expressed in SP and the whole BM, but did not exhibit

Figure 1. Circadian rhythms in core body temperature (top), serum corticosterone (middle) and melatonin (bottom) in mice during second day in constant darkness. Abscissa represents the circadian time points (CT). Gray and black bars along X-axis indicate the duration of prior light and dark periods in LD, respectively. Data from each study (hormones normalized to percent of mean) were combined for analysis, n = 28 for temperature, n = 12 for hormones. CT means and standard error (SE) are depicted along the 24-hour time scale. The best fitting 24-hour single component cosine curve, *p* value and double amplitude (2A) from cosinor analysis are shown. DD = constant darkness.



Supplemental Figure 1. Comparison of relative mRNA expression level (%) of clock genes in side population (SP) cells (left column), and the whole bone marrow (BM) (right column) at four circadian time points (CT) during 24-hour period. Clock genes are indicated along the X-axis, from the left to the right: m*Per1*, m*Per2*, m*Rev-erbα,* m*Bmal1*, m*Cry1,* and m*Clock*. Level of relative expression is the mean and standard error (SE) of the values from three independent experiments, each with four pooled mice. Quantities of all samples were estimated from the corresponding standard curves and normalized to the geometric mean of three endogenous control genes.

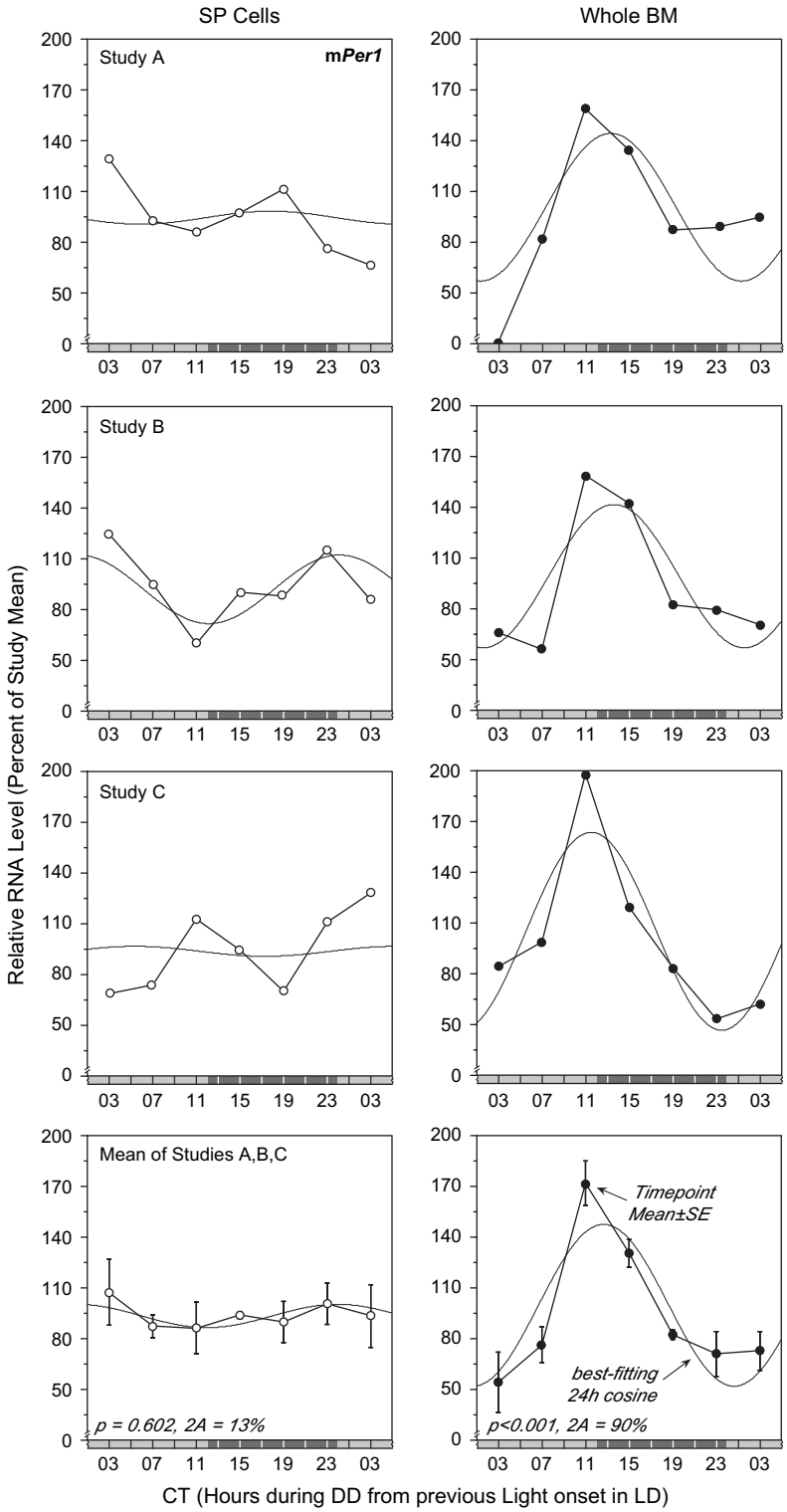


Figure 2. The timing of m*Per1* relative mRNA expression (%) in single studies (top) and pooled (bottom) in side population (SP) cells (left column) and the whole bone marrow (BM) (right column). Abscissa represents circadian time points (CT); gray and black bars along X-axis indicate the duration of prior light and dark spans in LD, respectively. Samples were normalized to the geometric mean of three endogenous control genes, then standardized to percent of study mean. Values represent three independent studies to show reproducibility with four-pooled mice each (upper three curves), and overall means ± standard error (SE) from the three experiments (bottom curve). The best fitting 24-hour single component cosine curve, *p* value and double amplitude (2A) from cosinor analysis are shown.

Table 2. Circadian changes in mRNA expression of clock and clock-controlled genes in bone marrow cells and liver in mice during second day in constant darkness

		Results for time-effect by analysis of variance and single cosinor			
Gene	Site	ANOVA (p value)	Cosinor[a] (p value)	Double amplitude[b] (%)	Acrophase[c] (95% confidence limits)
m*Per1*	SP	0.921	0.602	13	24:00
	BM	<0.001	<0.001	90	12:40 (11:16, 14:12)
m*Per2*	SP	0.006	0.025	43	10:40 (08:00, 13:36)
	BM	0.190	0.021	28	13:30 (10:40, 16:44)
	Liver	<0.001	<0.001	215	12:50 (11:12, 14:24)
m*Rev-erbα*	SP	0.130	0.206	52	06:20
	BM	0.011	<0.001	63	09:10 (07:36, 10:44)
	Liver	0.098	0.024	226	07:30 (04:36, 10:28)
m*Bmal1*	SP	0.730	0.522	25	17:10
	BM	0.845	0.605	14	18:50
	Liver	<0.001	0.016	169	01:40 (22:56, 04:24)
m*Cry1*	SP	0.740	0.560	14	01:20
	BM	0.824	0.350	17	18:10
m*Clock*	SP	0.740	0.791	11	01:00
	BM	0.769	0.175	25	14:10
m*Wee1*	SP	0.747	0.441	10	16:40
	BM	0.975	0.720	5	05:40
m*Dbp*	SP	0.110	0.257	46	20:00
	BM	0.167	0.119	22	12:20

Male B6D2F$_1$ mice synchronized to 12-hour light-to-dark cycle for 3 weeks and exposed to constant for 24 hours prior to study. Original data represent calculated mean mRNA expression from three studies involving four mice at each of seven time points (28 mice/study). Data from each study normalized to percent of mean and combined for analysis (n = 21 values).

ANOVA = analysis of variance; BM = whole bone marrow; SP = side population bone marrow cells.

[a]Cosinor analysis = fit of a 24-hour cosine to all data by least-squares linear regression (single cosinor technique). p Value determined from zero amplitude test.

[b]Double amplitude = difference between peak and trough of fitted cosine.

[c]Acrophase = time of peak in fitted cosine (units = hours:minutes from usual light onset = 00:00 hours); 95% confidence limits of acrophase listed if fit of cosine significant at $p \leq 0.05$. Reference: usual light onset.

a significant 24-hour rhythm (Suppl. Fig. 3, Table 2). Some temporal nonsignificant changes in m*Dbp* activity in individual studies were more pronounced in SP cells compared with whole BM (data not shown).

Discussion

Here we report for the first time that, with the exception of m*Per2,* the majority of key clock genes in mouse primitive hematopoietic stem cells are not expressed rhythmically throughout 24-hour periods in the absence of LD synchronizing conditions. The pattern of molecular clocks in the stem cells is different from that in the whole BM, where circadian rhythms were also validated for m*Per1* and m*Rev*-erbα during constant conditions (DD) of the study. The variations of clock gene expression in the BM thus appear to be tissue-specific, and are different from other peripheral organs, such as liver, with regard to phase, amplitude, and rhythmicity. Our findings confirm the concept that, in addition to peripheral tissue/organ-specific, cell-specific synchronization mechanisms of molecular clocks do exist [13]. It was recently demonstrated that in testis, and to some extent in thymus, the expression of clock genes is constant over 24 hours [15,16]. Based on those results, the authors hypothesized that clock gene expression is developmentally regulated [15], and that the circadian clock does not operate in immature cells that are undergoing differentiation [16]. This concept appears to be supported by our findings of nonrhythmic expression in most of the key clock genes in mouse marrow SP cells. SP was found to be highly enriched for primitive stem cells with long-term reconstitution ability [31], and is comprised mostly of quiescent hematopoietic stem cells [40]. We show here that the molecular clock in mouse BM displays a circadian organization, which is different between precursors and stem cells. Thus, m*Per1* and m*Rev-erbα* were

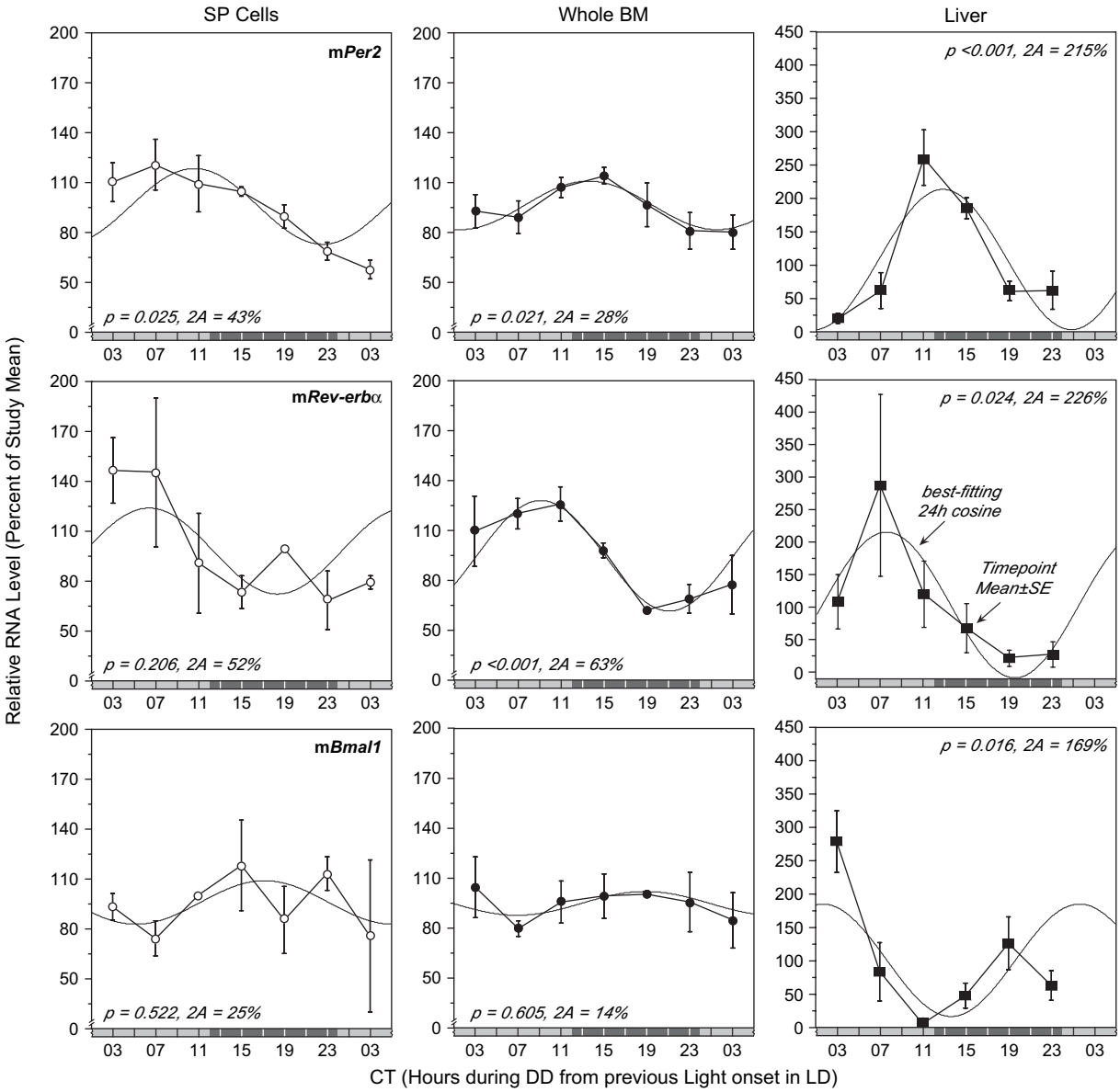
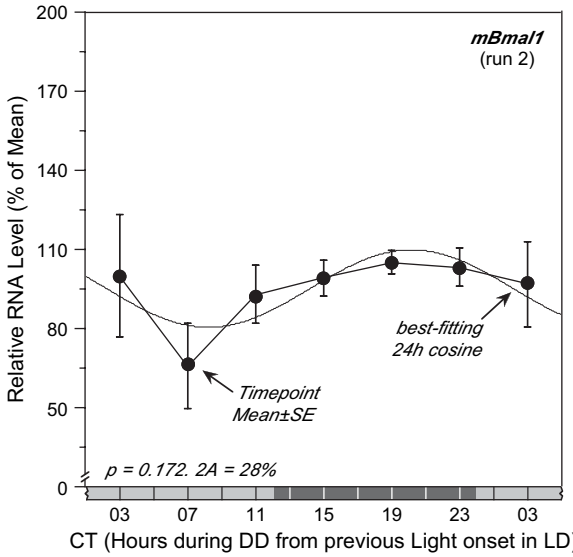


Figure 3. Timing of relative mRNA expression (%) of m*Per2* (top), m*Rev-erbα* (middle) and m*Bmal1* (bottom) in side population (SP) cells (left column), the whole bone marrow (BM) (middle column), or liver (right column). Abscissa represents circadian time points (CT), gray and black bars along X-axis indicate the duration of prior light and dark periods in LD, respectively. Samples were normalized to the geometric mean of three endogenous control genes, then standardized to percent of study mean. Overall CT means and standard error (SE) from three independent experiments, each with four pooled mice, are depicted along the 24-hour time scale. The best fitting 24-hour single component cosine curve, *p* value, and double amplitude (2A) from cosinor analysis are shown.

rhythmic in the whole BM, but not in SP cells. The macroscopic peaks of m*Per2,* which showed 24-hour rhythmic expression in both cell populations, appeared to differ by up to 8 hours, with the highest time point means at 07 CT in SP cells and at 15 CT in BM, but acrophases (10:40 vs 13:30 hours) and their 95% confidence limits were not significantly different ($p = 0.145$) (Table 2). The relative level of clock gene expression in SP cells was also entirely different as compared with the whole BM. m*Per1* dominated in SP cells at each of seven circadian test times, with average expression approximately 2 to 2.5 times higher relative to other clock genes. In the whole BM, m*Per1* expression level was mostly lower as compared with the other clock genes, depending on circadian time. A similar discrepancy was demonstrated in C57Bl6 mice kept on standard LD 12:12 lighting conditions [25]. A low level of m*Per1* expression in BM was also reported by others [24]. It is interesting to note that in mouse testis, the expression level of m*Per1,* which was constant over 24 hours, was higher than the peak levels of this gene in kidney and muscles [15], where it was rhythmic.

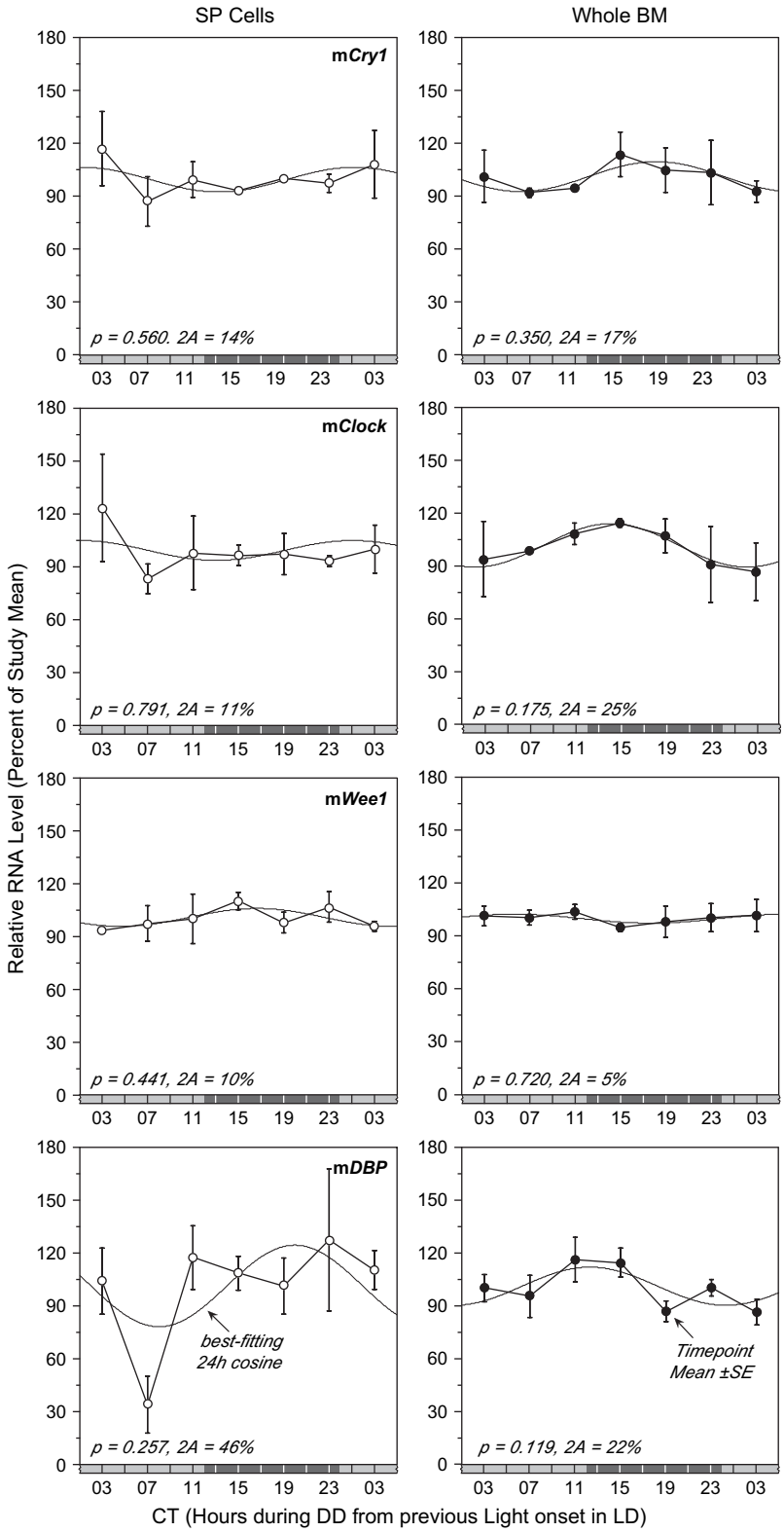


Supplemental Figure 2. Timing of m*Bmal 1* relative mRNA expression (%) in bone marrow (BM) analyzed with primers used by Tamaru et al [32]. Abscissa represents circadian time points (CT); gray and black bars along X-axis indicate the duration of subjective light and dark phases, respectively. Samples were normalized to the geometric mean of three endogenous control genes. Overall CT means and SE from three independent experiments, each with four pooled mice are depicted along the 24-hour time scale. The best fitting 24-hour single component cosine curve, *p* value and double amplitude (2A) from cosinor analysis are shown.

The question remains as to why out of the two m*Per* genes tested in SP cells in our studies, only m*Per2* was circadian rhythmic. As recently suggested, it is possible that *Per* genes could be regulated differently, and that the m*Per1* locus is more susceptible to damping during constant lighting conditions than the m*Per2* locus [13]. In addition, primitive SP cells may be controlled by an ultradian (i.e., $<$20 hours) time structure, wherein oscillations occur at frequencies higher than circadian (20 to 28 hours) that may or may not be synchronizable to an LD schedule. This could indicate a developmental aspect to time-dependent control of clock genes in BM, with clock gene expression in SP cells characterized by more ultradian oscillations that are not yet under full control by the circadian clock. The significantly different clock gene expression profiles in SP cells compared with the whole BM cannot be explained by the influence of some environmental factors in the process of preparation and isolation of SP cells, since this has previously been investigated [25]. The SP profile was highly reproducible in all samples and the number of sorted SP cells was also very similar. Even though SP is a relatively homogeneous cell population from a morphological point of view, we cannot exclude individual variations in minor subpopulations that could partly modify observed curves. However, based on our data from whole BM population as compared with SP, we do not find this likely. It is tempting to speculate that time regulation in primitive stem cells with progenies evolving or maturing into several lineages at different rates and proliferation/maturation requirements must necessarily be more complex than in precursor cells, which are committed for one direction and with another global gene expression pattern [41–44]. Thus, accessibility for transcription of multilineage-affiliated genes is shown to be hierarchically controlled in early hematopoietic stem cells [45].

Upon analyzing m*Per1* and m*Per2* variations in the BM, we found single daily peaks in expression of both genes. The pattern of m*Per2* expression in BM in our study was similar to the one demonstrated recently in the BM of $B6D2F_1$ [24]. However, our results are somewhat in contrast to those reported by Chen et al. [23]. They found two daily synchronous peaks in m*Per1*and m*Per2* in the whole BM of Balb/C mice aged 3 to 4 weeks. Discrepancies between results might be explained by differences in normalization procedures of gene expression data, the method of rhythm validation, and/or experimental conditions. In the normalization procedure of the target genes, we used a recently described strategy [34] with the geometric mean of three endogenous control genes, which has been found as a more stable reference. In contrast, Chen et al. [23] used a single reference gene *18S*, whose expression was recently shown to vary in human BM subpopulations [46]. In addition, data presented by Chen et al. were not validated by cosinor analysis. Experimental conditions in the mentioned study were also different from ours: mice were from a different strain, 2 to 3 weeks younger and were not released into constant darkness before sampling. In our study, mice were kept in constant darkness for 24 hours before cell sampling, which allowed us to avoid any immediate masking effect of light on endogenous rhythms. The robust rhythms of core body temperature, and blood corticosterone and melatonin showed the persistence of the circadian clock in our mice on the second day in constant darkness, when cell samplings were performed.

In our study, the circadian organization in the BM was different from liver, where the circadian pattern of the clock genes mRNA expression was similar to that reported by several others, including one in $B6D2F_1$ mice [28]. Thus, the amplitudes of rhythmic m*Per2* and m*Rev-erbα* variations were several-fold less in the whole BM (and for m*Per2* in SP cells) as compared with liver. In both hematopoietic cell fractions, m*Cry1* was expressed at more or less constant levels throughout the day, while in liver this gene is transcribed rhythmically [47]. Furthermore, m*Bmal1*, a positive regulator of the molecular clock, was not expressed rhythmically in mouse primitive stem cells or whole BM, i.e., all stages of hematopoiesis, contrary to significant rhythms observed in liver. This was unexpected, because *Bmal1* is known to be expressed rhythmically in anti-phase to *Per1* and *Per2* in many of the peripheral tissues, such as the liver and oral mucosa, in different species [12,36]. We showed that our finding of a nonrhythmic m*Bmal1* expression profile was reproducible with two different primer



Supplemental Figure 3. The timing of relative mRNA expression (%) of (from the top to the bottom) m*Cry1*, m*Clock*, m*Wee1*, and m*Dbp* in side population (SP) cells (left column) and the whole bone marrow (BM) (right column). Abscissa represents circadian time points (CT); gray and black bars along X-axis indicate the duration of subjective light and dark phases, respectively. Samples were normalized to the geometric mean of three endogenous control genes. Overall CT means and standard error (SE) from three independent experiments, each with four pooled mice are depicted along the 24-hour time scale. The best fitting 24-hour single component cosine curve, *p* value, and double amplitude (2A) from cosinor analysis are shown.

sequences, as the analysis with primers/probe set used by Tamaru et al. [32] gave the same result. Expression of *Bmal1* is regulated by REV-ERBs and RORs proteins, which activate and inhibit *Bmal1* transcription, respectively [48,49]. Expression patterns of RORs, shown to be quite different in peripheral tissues [50], suggest *Bmal1* regulation may also be tissue-specific.

We cannot exclude that a circadian rhythm in m*Bmal1* could be masked due to a limited number of observations. However, recent studies demonstrated that the *Bmal1* rhythm could be unstable in some tissues. An unusual phase relationship of h*Per2* and h*Bmal1*, with peaks at approximately the same time, was recently demonstrated in human peripheral blood mononuclear cells [51]. An atypical rhythm in m*Bmal1* expression with a peak located only 2 hours after that in m*Per2* has also been observed in BM cells from $B6D2F_1$ mice that were not released into constant darkness before total BM sampling [24]. Comparison of the results in our study and those in the literature might suggest that turning lights off could alter the circadian expression pattern of m*Bmal1* in the BM clock without changing it in liver.

Differences in BM and liver intracellular clockworks were also demonstrated in two genes related to the output pathway of the circadian system. m*Wee1* displays a robust circadian rhythm in normal mouse liver and has been suggested to be directly regulated by the clock genes [39]. In our study, m*Wee1* was expressed at more or less constant levels through the day in both of the BM populations, with very low variations in all three experiments. No rhythm was observed for the mRNA expression of m*Dbp*—another clock-controlled gene that is expressed rhythmically in SCN and several peripheral organs, such as liver [52]. Our findings suggest that BM has a specific organization of the molecular clock, making it different from other organs investigated so far. The difference in timing and content of clock gene regulation in peripheral tissues demonstrated recently [2] suggests a specialized role of molecular clocks in each tissue corresponding to the control of the timing of different functions [53]. The high-density oligonucleotide array analysis of temporal gene expression in BM may reveal circadianly regulated output genes implicated in hematopoiesis.

In conclusion, as compared with whole BM, expression of several key clock genes in mouse primitive stem cells displays only weak or undeveloped circadian rhythmicity. This strongly suggests a developmental organization in BM circadian oscillators.

Acknowledgments

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