Article III

Circadian Variations in Clock Gene Expression of Human Bone Marrow CD34$^+$ Cells

Oleg Tsinkalovsky,[*] Rune Smaaland,[†] Benedikte Rosenlund,[‡] Robert B. Sothern,[§]
Asle Hirt,[#] Solrun Steine,[*] Azadeh Badiee,[‡] Jenny Foss Abrahamsen,[†]
Hans Geir Eiken,[‡] and Ole Didrik Laerum[*,1]

[*]Stem Cell Research Group, the Gade Institute, Department of Pathology, [†]Department of Oncology, [‡]Center for Medical Genetics and Molecular Medicine, and [#]Department of Cardiology, Haukeland University Hospital, Bergen, Norway, [§]The Rhythmometry Laboratory, College of Biological Sciences, Biological Sciences Center, University of Minnesota, St. Paul

Abstract Time-dependent clock gene expression variations have recently been observed in mouse hematopoietic cells, but the activity of these genes in human bone marrow (BM) has so far not been investigated. Since such data can be of considerable clinical interest for monitoring the dynamics in stem/progenitor cells, the authors have studied mRNA expression of the clock genes h*Per1*, h*Per2*, h*Cry1*, h*Cry2*, h*Bmal1*, h*Rev-erb* α, and h*Clock* in human hematopoietic CD34-positive (CD34$^+$) cells. CD34$^+$ cells were isolated from the BM samples obtained from 10 healthy men at 6 times over 24 h. In addition, clock gene mRNA expression was analyzed in the whole BM in 3 subjects. Rhythms in serum cortisol, growth hormone, testosterone, and leukocyte counts documented that subjects exhibited standardized circadian patterns. All 7 clock genes were expressed both in CD34$^+$ cells and the whole BM, with some differences in magnitude between the 2 cell populations. A clear circadian rhythm was shown for h*Per1*, h*Per2*, and h*Cry2* expression in CD34$^+$ cells and for h*Per1* in the whole BM, with maxima from early morning to midday. Similar to mouse hematopoietic cells, h*Bmal1* was not oscillating rhythmically. The study demonstrates that clock gene expression in human BM stem/progenitor cells may be developmentally regulated, with strong or weaker circadian profiles as compared to those reported in other mature tissues.

Key words hematopoiesis, clock genes, stem cells, human bone marrow, circadian rhythm

The mammalian circadian system is composed of many individual tissue-specific clocks, which are important for maintenance of homeostasis in the body (Hastings et al., 2003). They react to external cues by generating physiological and behavioral responses, which are coordinated by the master circadian pacemaker in the SCN of the brain (Hastings et al., 2003). Recently, major advances in the understanding of the molecular and cellular basis of circadian clock components and mechanisms have been made due to the identification of so-called clock genes, which may modify transcription in nearly 10% of the genome (Panda et al., 2002). They code for positive (e.g., *Clock*, *Bmal1*) and negative (e.g., *Per 1-3*, *Cry 1-2*, *Rev-erb* α)

1. To whom all correspondence should be addressed: Ole Didrik Laerum, the Gade Institute, Department of Pathology, Haukeland University Hospital, N-5021 Bergen, Norway; e-mail: ole.larum@gades.uib.no.

JOURNAL OF BIOLOGICAL RHYTHMS
DOI: 10.1177/0748730406299078
© 200X Sage Publications

regulators modifying gene transcription. As a result, a feedback control mechanism is constituted, leading to oscillations of different cell functions in a circadian manner (Hastings et al., 2003). The most extensive characterization of clock gene expression has been done in the SCN and hypothalamic area of the brain. However, variations in clock gene expression have also been observed in peripheral tissues, including the oral mucosa, skin, liver, heart, kidney, lung, pancreas, skeletal muscle, bone marrow (BM), peripheral blood mononuclear and polymorphonuclear cells, and cultured fibroblasts (Balsalobre et al., 1998; Bjarnason et al., 2001; Boivin et al., 2003; Damiola et al., 2000; Kennaway et al., 2003; Kusanagi et al., 2004; Muhlbauer et al., 2004; Nagoshi et al., 2004; Oishi et al., 2003, 1998b; Tsinkalovsky et al., 2005; Welsh et al., 2004; Yamamoto et al., 2004). Only a few of these studies have been done on human cells (Bjarnason et al., 2001; Boivin et al., 2003; Kusanagi et al., 2004; Mormon et al., 2002; Teboul et al., 2005).

It is well established that hematopoiesis, in general, undergoes strong circadian variations, both in humans and rodents (Abrahamsen et al., 1997, 1998; Laerum, 1995; Smaaland et al., 1992, 2002). Thus, significant variations in cell cycling, as well as colony-forming ability, of the BM have been observed (Abrahamsen et al., 1997; Smaaland et al., 1992), which are related to corresponding variations of leukocyte numbers in peripheral blood (Smaaland et al., 1995). Recent findings suggest that time-dependent regulatory mechanisms may be operating locally. BM cells continued to proliferate rhythmically in mice with ablated SCN (Filipski et al., 2004). In addition, the response of BM cells to granulo-monocytic colony-stimulating factor displayed a sustained circadian rhythm over 4 days in culture (Bourin et al., 2002). BM engraftability is also subject to significant circadian variations (D'Hondt et al., 2004).

Recently, it was shown that clock genes are expressed in mouse whole BM (Chen et al., 2000; Granda et al., 2004; Tsinkalovsky et al., 2005) and isolated hematopoietic side population (SP) stem cells (Tsinkalovsky et al., 2005). Some of these genes oscillated rhythmically with patterns different between the whole BM and stem or progenitor cell populations (Chen et al., 2000; Tsinkalovsky et al., 2006). However, clock gene activity in human hematopoietic stem/progenitor cells has so far not been studied.

Based on this background, we have performed a comparative study of the clock genes h*Per1*, h*Per2*, h*Cry1*, h*Cry2*, h*Bmal1*, h*Rev-erb* α, and h*Clock* in BM CD34-positive ($CD34^+$) stem/progenitor cells. The aim was in particular to establish whether clock genes are expressed in this population of hematopoietic cells and can generate circadian rhythms. The population of $CD34^+$ cells comprises 1% to 3% of BM mononuclear cells and represents stem and progenitor cells capable of producing a progeny of all types of hematopoiesis. Although a combination of markers other than CD34 is reported to represent more primitive stem cell fractions, the whole population of $CD34^+$ cells was chosen as a subset used widely for clinical transplantations since these cells are isolated from BM by magnetic cell separation used routinely in clinical applications (Handgretinger et al., 1998). Our findings indicate a rhythmic systemic control of clock gene transcription in stem/progenitor cells, which may have consequences for the progeny of mature blood cells.

MATERIALS AND METHODS

Subjects

Ten healthy male students or graduates, ages 21 to 28 years, were studied: 5 over a 4-week period from December 1999 to January 2000 and another 5 from December 2003 to January 2004. All volunteers provided written, informed consent after learning the nature and possible consequences of the study, which was approved by the Regional Medical Ethics Committee. All procedures were performed in accordance with the international ethical guidelines for biomedical research involving human subjects (Council for International Organizations of Medical Sciences [CIOMS], 2002). None of the subjects had been doing any night work during the previous month, and all of them followed a regular diurnal activity schedule, rising between 0700 and 0900 h and going to bed between 2300 and 0030 h for at least a week before the experiments. Meals were taken according to Norwegian standards with breakfast in the morning, lunch between 11:30 a.m. and 12 p.m., warm meal between 4 and 5 p.m., and a light supper between 7 and 8 p.m. The sleeping time was approximately the same as in daily life for each subject. Individual interviews reported no extreme chronotypes.

BM sampling

BM was obtained 6 times over 24 h by sequentially puncturing the sternum and the posterior iliac crests on a rotating schedule at 0800, 1230, 1700, 2130, 0200, and 0700 h. The sequence of sampling from the 3

different anatomical sites was randomized to avoid systematic errors. In addition, the experiment was started at 0800 h for one half and at 1230 h for the other half of the volunteers for the same purpose. The puncture site was infiltrated with 8 mL of 10 mg/mL lidocain (Astra, Sweden) for local anesthesia, and 5 mg/mL chlorhexidin with iodine was used for disinfection. No other premedication was given. Then, 8 mL of BM was collected in a 20-mL syringe, washed with 100 IE/mL heparin (Leo Pharma AS, Oslo, Norway), and immediately transferred to a 50-mL tube (TPP AG, Trasadingen, Switzerland) with 13 mL phosphate-buffered saline (PBS) containing 2 mM EDTA (Sigma-Aldrich, St. Louis, MO) and carefully mixed.

Serum

Venous blood was collected from the subjects at the same times as the BM samplings. The blood was obtained as the initial procedure to avoid artificially increased levels of cortisol resulting from the puncture procedure itself. A small portion was taken for the white cell counts (subjects 6-10 only). Then, 8 mL venous blood was drawn into two 5-mL Vacutainer tubes (Becton Dickinson Labware Europe, Le Pont-de-Claix, France) and centrifuged 5 min at 300 *g*. The top transparent layer of serum was transferred to 2-mL cryotubes (TPP AG) and placed at –80 °C until analysis. Serum cortisol, growth hormone, and testosterone concentrations were measured at the Hormone Laboratory of Haukeland University Hospital to ensure that subjects exhibited a standard diurnal pattern in relation to their sleep/activity schedule.

Cell Preparation

Of the cell suspension, 1 mL ($7 \pm 2 \times 10^6$ BM cells) was spun down (250 *g*, 5 min at 4 °C), and cell pellets were incubated in 10 mL lysing solution (Dako, Denmark A/S) for 10 min in the dark. After centrifugation, cells were resuspended in 500 µL PBS containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 2 mM EDTA—PBS+, filtered, counted, and spun down (300 *g*, 5 min at 4 °C) in 2-mL cryotubes (TPP AG). Supernatants were discarded and pellets snap frozen in liquid nitrogen and placed at –80 °C until analysis.

The remainder of the BM sample was carefully layered over 2.5 mL Lymphoprep (Nycomed, Oslo, Norway) in four 15-mL conical tubes (TPP AG) (5-mL cell suspension per tube) and centrifuged 25 min at 800 *g* at 20 °C in a swinging-bucket rotor (without brake). Interphase cells (i.e., low-density mononuclear cells) were carefully collected with a Pasteur pipette, filtered through 70-µm nylon BD Falcon cell strainers (Becton Dickinson Labware Europe), pulled in a new 15-mL tube, washed twice by adding PBS+, and centrifuged 10 min at 250 *g*. Supernatants were removed completely and cells resuspended in 330 µL PBS+ and counted. Then, 30 µL was stored at 4 °C until flow cytometric analysis, and the remainder of the cell suspension was submitted to magnetic labeling and separation.

Isolation of $CD34^+$ Cells

The hematopoietic stem cells were magnetically separated from low-density mononuclear cells using the Direct CD34 Progenitor Cell Isolation Kit and MS^+ Column (both from Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells in 300 µL PBS+ were magnetically labeled with 100 µL MACS CD34 Micro Beads by incubation for 30 min at 4 °C in the presence of 100 µL FcR Blocking Reagent. After washing, cells were resuspended in 500 µL PBS+, filtered through 50-µm Falcon filter devices (Becton Dickinson Labware Europe), and passed through a MS^+ positive selection column in the magnetic field of a MACS separator (Miltenyi Biotec). Retained cells were eluted, and the separation step was repeated with a new column. Cells in 1 mL of PBS+ were counted, 80 µL was taken for flow cytometric analysis, the rest was spun down (300 *g*, 5 min at 4 °C) in 2-mL cryotubes (TPP AG), supernatants were discarded, and pellets were snap frozen in liquid nitrogen and then stored at –80 °C until use.

Flow Cytometric Analysis

The purity of the samples enriched for $CD34^+$ cells by MACS was evaluated by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) equipped with an Innova 90C laser tuned to 488 nm. $CD34^+$ cell samples were compared to the whole BM samples taken before MACS separation (Figure 1). Prior to flow cytometry, cells were stained with phycoerythrin-conjugated anti-CD34 and fluorescein isothiocyanate-conjugated anti-CD45 antibodies (both from Miltenyi Biotec) for 10 min at 4 °C in the dark, followed by washing with PBS+. Then, 1 µg/mL 7-aminoactinomycin D (Sigma-Aldrich) was added to the samples

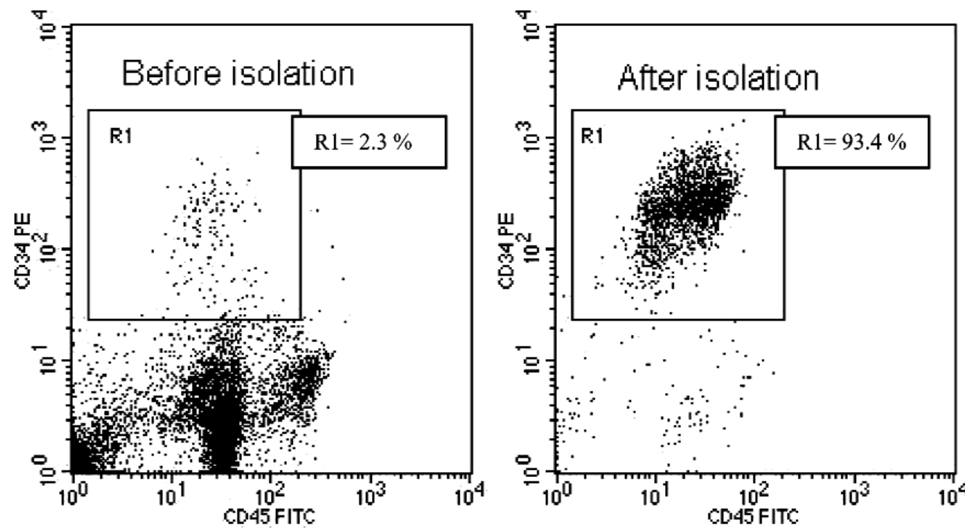

Figure 1. Flow cytometric analysis of bone marrow CD34+ cells (R1, region 1) before (left) and after (right) magnetic bead isolation. Cells stained with phycoerythrin-conjugated anti-CD34 and fluorescein isothiocyanate-conjugated anti-CD45 antibodies; 7-aminoactinomycin D was added to discriminate dead cells that were excluded from analysis.

15 min before flow sorting to facilitate dead cell discrimination. Fluorescein isothiocyanate, phycoerythrin, and 7-aminoactinomycin D emissions were observed through 530/30, 585/42, and 670LP filters, respectively, after 488-nm excitation. Gates for analysis of antibody staining were set using isotype controls, so that less than 1% of cells were positive for isotype antibody staining. The cell suspensions were filtered through a 50-µm Falcon filter device (Dako, Denmark A/S) prior to flow cytometric analysis.

The living CD34+ cells exhibited low-density CD45 expression and low side-scatter properties (Figure 1) and thus had the characteristics as described by others (Sutherland et al., 1996). The purity of MACS-isolated CD34+ cells ranged from 70.3% to 95.6% (median: 82.3%).

RNA Extraction

Total cellular RNA was extracted with the use of the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD) according to the manufacture's instructions. All samples were subjected to DNase treatment by the phenol-chloroform method (Promega, Madison, WI). The extracted RNA was dissolved in 10 µL of RNA storage solution (Ambion, Houston, TX). Then, 1 µL of each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip for quality control, as well as concentration measurement of total RNA. The remaining RNA was stored at –80 °C until analysis. The total RNA in the samples was of high quality, and no signs of DNA contamination or RNase-induced degradation were detected. Human BM total RNA (Clontech, Palo Alto, CA) was used as exogenous control.

Reverse Transcription and Primer Design

Total RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) as previously described (Tsinkalovsky et al., 2005). The thermal cycling parameters were as follows: 25 °C for 10 min (primer incubation), 48 °C for 30 min (reverse transcription), and 95 °C for 5 min (inactivation of reverse transcriptase). All complementary DNA (cDNA) samples were stored at –20 °C.

Seven assays were prepared for the target genes h*Per1*, h*Per2*, h*Cry1*, h*Cry2*, h*Bmal1*, h*Rev-erb* α, and h*Clock*. The primers for these genes were designed by Primer Express software (Applied Biosystems) and purchased from MedProbe (Oslo, Norway). The primer sequences were the following: h*Per1*-For, ACA CAC TTC AGA ACC AGG ATA CCT T; h*Per1*-Rev, TGC TCC GAA ATG TAG ACG ATT C; h*Per2*-For, GTC CAC CTC CCT GCA GAC AA; h*Per2*-Rev, CTG GTA ATA CTC TTC ATT GGC TTT CA; h*Cry1*-For, GCC AGG CGG AGA AAC TGA A; h*Cry1*-Rev, AAA ATT TGC CAC CCA AGC TTT; h*Cry2*-For, CCT ACC TGC GCT TTG GTT GT; h*Cry2*-Rev, TGC TGT TCC GCT TCA CCT TT; h*Rev-erb* α-For, CCA TGA ACC TGG CCA ACA A; h*Rev-erb* α-Rev, GTG CTG GGT GGG TGA AGT CT; h*Bmal1*-For, GAA ATC ATG GAA ATC CAC AGG ATA A; h*Bmal1*-Rev, GAG GCG TAC TCG TGA TGT TCA AT; h*Clock*-For, CTA CAC CTC AGT TCA TCA AGG AAA TG; h*Clock*-Rev, ATA TCC ACT GCT GGC CTT TGG. Four housekeeping genes—*18S*, h*P0*, hβ-*actin*, and *GAPDH*—were chosen as reference genes (endogenous control). All 4 were purchased commercially from Applied Biosystems labeled with VIC reporter dye.

Table 1. Circadian Variations in Whole-Blood Variables in Healthy Men during Bone Marrow Sampling[a] Results for Time-Effect by Analysis of Variance (ANOVA) and Single Cosinor[b]

*Variable (*n *Subjects)*	*ANOVA (*p *Value)*	*Cosinor (*p *Value)*	*Double Amplitude (%)*	*Acrophase (95% Limits)*
Cortisol (10)	< 0.001	< 0.001	129	0938 h (0836, 1036 h)
Growth hormone (10)	< 0.001	< 0.001	346	0131 h (0016, 0244 h)
Testosterone (10)	< 0.001	< 0.001	45	0827 h (0720, 0936 h)
White blood cells (5)	0.005	0.004	39	2341 h (2112, 0152 h)
Neutrophils (5)	0.003	< 0.001	44	2204 h (1956, 0004 h)
Lymphocytes (5)	< 0.001	< 0.001	41	0154 h (0020, 0320 h)

a. Blood sampled every 4 to 5 h for 24 h (6 values/subject). Data from each subject normalized to percentage of mean and combined for analysis. Cosinor analysis = fit of a 24-h cosine to all data by least squares linear regression (single cosinor technique). p value determined from zero-amplitude test; double amplitude = difference between peak and trough of fitted cosine; acrophase = time of peak in fitted cosine (units = hh:mm from local midnight = 0000 h); 95% limits of acrophase listed if fit of cosine significant at $p \leq 0.05$.
b. Individual data can be provided upon request to the corresponding author.

Real-Time™ Quantitative Reverse Transcription Polymerase Chain Reaction

For the quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with 96 wells was used. Samples collected at all time points in a single experiment (for both BM and $CD34^+$ cells) were run on 2 plates per gene due to the high number of samples being tested and the fact that each sample was analyzed in triplicate. A standard curve consisting of 9 points was included in each run. This was obtained by performing a 2-fold serial dilution of human bone marrow total RNA (Clontech) starting at 500 ng. A “no template control” was also included in each run.

Q-RT-PCR was performed in a 25-µL reaction volume consisting of 12.5 µL of a 2× TaqMan Universal PCR Master Mix (Applied Biosystems) or a 2× SYBR Green Universal PCR Master Mix (MedProbe) for the endogenous control genes and the target genes, respectively; 300 nM (0.25 µL) forward primer; and 300 nM (0.25 µL) reverse primer. For *18S*, h*P0*, hβ-*actin*, and *GAPDH*, 1.25 µL of a 20× mix of primers and probe was used. Then, 15 µL and 6 µL of the cDNA template were used for the endogenous control genes and the target genes, respectively. Water was then added to a total volume of 25 µL. A 500-fold dilution of cDNA was performed for *18S* due to its high expression. The thermal cycling parameters were as follow: 2 presteps at 50 °C for 2 min (UNG incubation) and 95 °C for 10 min (AmpliTag Gold activation), followed by 40 cycles at 95 °C for 15 sec (denaturation) and 60 °C for 1 min (annealing/extension).

The standard curves obtained from control cDNA were used to evaluate the quality and efficiencies of each Q-RT-PCR. Most of the standard curves exhibited slopes with a mean of –3.1 to +3.3.

Calculations and Statistics

To reduce variations from running the samples on 2 different plates during the Q-RT-PCR, we used the standard curve method for calculating gene expression and the strategy described previously for normalization of the values (Tsinkalovsky et al., 2006). Briefly, the normalized value for each sample was calculated by dividing the target gene by the geometric mean of 4 endogenous control genes (based on the average quantity values from triplets). A percentage of relative gene expression was calculated for each sample in which the average of all normalized values was taken as 100%. We reexpressed each series as a percentage of its own mean to reduce differences in overall levels, which for some clock genes were considerable. For example, the 24-h means ranged from 0.15 to 0.20 (33%) for h*Per1* but from 0.19 to 1.04 (447%) for h*Bmal1*.

After normalizing data for each variable (serum hormones, white blood cell count, and each mRNA clock gene) to percentage of the individual mean to reduce intersubject variability in levels, we combined all data at each time point to have 10 replicate values for statistical testing. Grouped data for each variable were analyzed for time-effect across the 6 time points by 1-way analysis of variance (ANOVA) and for diurnal rhythm by the single cosinor method (fitted period = 24.0 h) using the Chronolab software package, as described previously (Tsinkalovsky et al., 2006).

RESULTS

Rhythms of Serum Hormones Levels and White Blood Cell Counts Show a Circadian Coordination in Subjects during Sampling

The level of each hormone (cortisol, growth hormone, testosterone), as well as total white blood cell

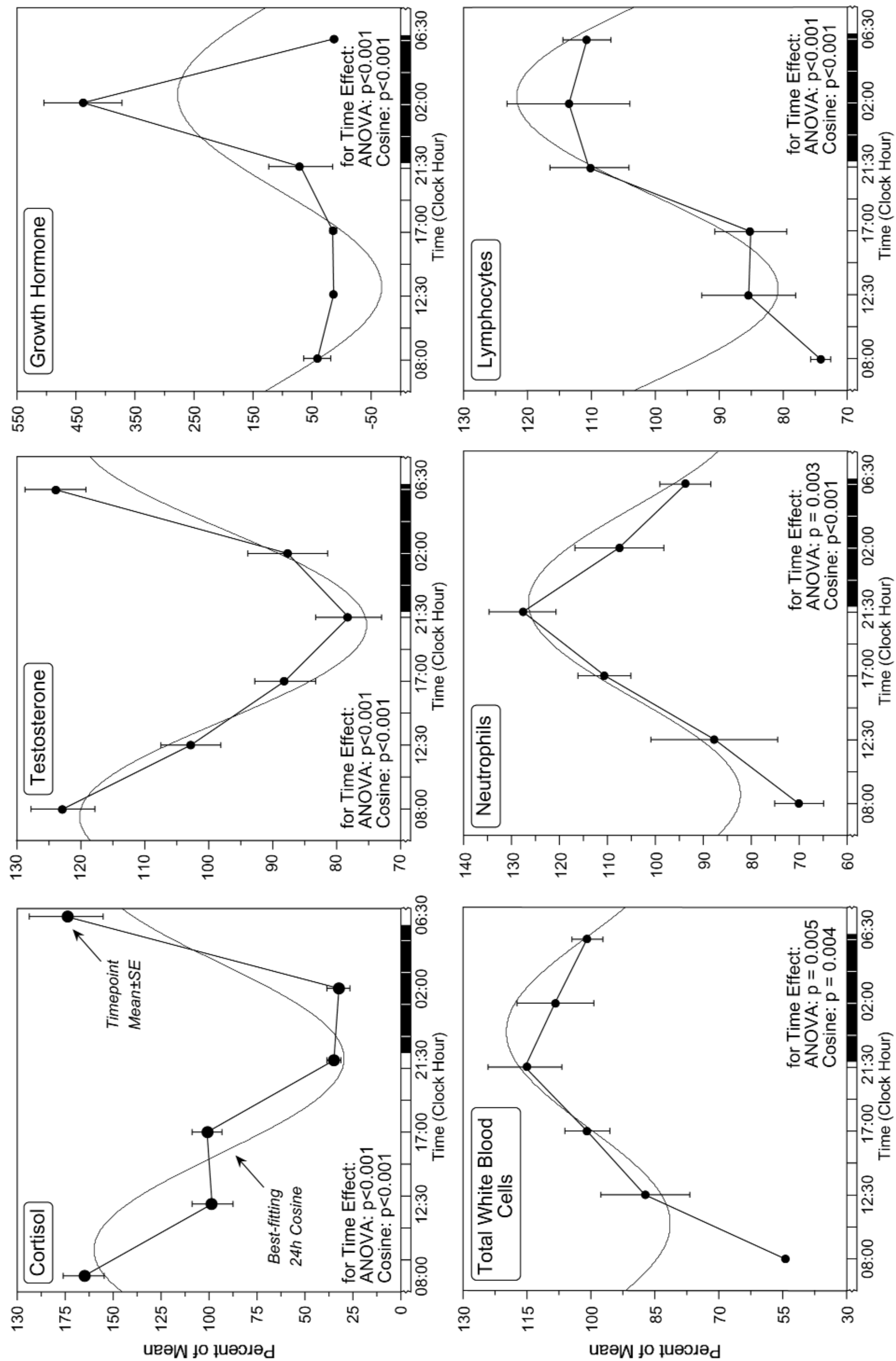

Figure 2. The 24-h variations for variables in blood of healthy men during bone marrow sampling. Blood sampled from 10 men for cortisol, testosterone, and growth hormone (top) and 5 men for total white blood cell, neutrophil, and lymphocyte counts (bottom) every 4 to 5 h for 24 h (3-6 samples/subject). Data for each variable expressed as percent of mean for each subject and combined overall for analysis for time-effect by analysis of variance (ANOVA) and by the least squares fit of a 24-h cosine (see *p* values). Time point means ± SE are shown with best-fitting 24-h cosine. Abscissa represents time; white and dark bars along *x*-axis indicate the duration of the activity and rest/sleep periods, respectively.

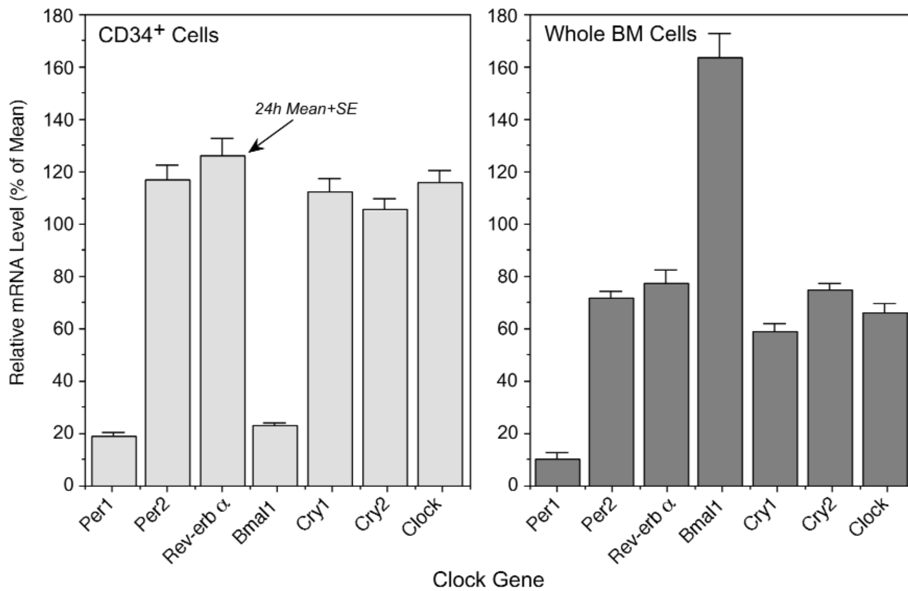

Figure 3. Comparison of relative expression of clock genes in human CD34+ (left) and whole bone marrow (BM, right) cells. Level of relative expression is the average quantity for each gene, based on the normalized quantity from all samples estimated from the standard curve. The normalized value for each sample was calculated by dividing the target gene by the geometric mean of 4 endogenous control genes and standardized to percentage of study mean. Values are mean ± SEM from 18 samples collected over 24 h from 3 healthy men. Whole BM and CD34+ population levels of gene expression could not be compared directly because they had different cell number and RNA concentrations and were analyzed in different quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) reactions (on the different plates with different standard curves).

(WBC), neutrophils, and lymphocytes, varied significantly as a function of sampling time by ANOVA, and a 24-h rhythm was documented for each variable by cosinor analysis (Table 1, Fig. 2). The peak of cortisol and testosterone occurred early in the morning, while growth hormone peaked close to midnight, which is similar to what has been reported by others (Krieger et al., 1971). The total white cell count, as well as separate neutrophil and lymphocyte counts, demonstrated the expected 24-h variation for all individuals. The timing of peaks for these cells occurred near the beginning of sleep/rest.

These results indicate that the subjects had diurnal coordination during the 24–h period of BM sampling, with timing of their marker rhythms similar to earlier published data (Smaaland et al., 1995).

Relative Clock mRNA Expression Is Similar in CD34+ Cells and Whole BM with the Exception for h*Bmal1*

In the 3 men monitored for clock gene–relative mRNA expression in total BM, CD34+ and whole BM cells showed similar levels of relative expression of clock genes within each cell type, with the exception for h*Bmal1*. In both cell types, h*Per1*, h*Per2*, h*Cry1*, h*Cry2*, h*Rev-erb* α, and h*Clock* were expressed at about the same levels within each cell population, while h*Per1* expression level was several-fold lower (Fig. 3). Of interest, expression of h*Bmal1* was as low as the h*Per1* in CD34+ cells, while it was the highest of all clock genes in the whole BM.

The 24-h Variations of h*Per1*, h*Per2*, and h*Cry2* Expression in CD34+ Cells Are Rhythmic

A significant 24-h rhythm was demonstrated for 3 of the 7 clock genes: h*Per1*, h*Per2*, and h*Cry2* (Table 2). Diurnal patterns of these genes were very similar, with the highest expression during the first part of the day (Fig. 4). The peaks of gene expression followed a sequence, with the peak of h*Per1* detected at 0839 h, followed by h*Per2* at 1052 h and h*Cry2* at 1146 h (Table 2). The double amplitude, as representative of the predictable diurnal range of change from lowest to highest values during the 24–h period, was significantly higher for h*Per1* (66%) than for h*Per2* (22%) and h*Cry2* (14%) (Table 2).

Interestingly, we found a significant 24-h rhythm for *hPer1* also in the whole BM samples analyzed only in 3 subjects (ANOVA: $p = 0.004$; cosinor analysis: $p = 0.03$; Fig. 4, upper left panel). The peak of *hPer1* expression in the whole BM (0853 h) coincided with that in CD34+ cells (0840 h), and the double amplitude was also large (81% vs. 66%). Additional conclusions regarding any time effects for the other clock genes (that did not show diurnal rhythm) in whole BM cells could not be made at this time due to the small group size of only 3 subjects (results not shown).

Table 2. Circadian Variations in mRNA Expression of Clock Genes in CD34$^+$ Bone Marrow Cells in 10 Healthy Men during Standardized Light-Dark Conditions[a]: Results for Time-Effect by Analysis of Variance (ANOVA) and Single Cosinor

Gene	*ANOVA (p Value)*	*Cosinor (p Value)*	*Double Amplitude (%)*	*Acrophase (95% Limits)*
h*Per1*	< 0.001	< 0.001	66	0839 h (0652, 1024 h)
h*Per2*	0.015	0.027	22	1052 h (0732, 1352 h)
h*Cry2*	0.080	0.050	14	1146 h (0800, 1500 h)
h*Cry1*	0.422	0.331	12	1018 h
h*Rev-erb* α	0.544	0.269	10	0622 h
h*Bmal1*	0.742	0.338	17	2152 h
h*Clock*	0.173	0.890	5	1411 h

a. Bone marrow sampled every 4 to 5 h for 24 h (6 values/subject). Data from each subject normalized to percentage of mean and combined for analysis. Cosinor analysis = fit of a 24-h cosine to all data by least squares linear regression (single cosinor technique). *p* value determined from zero-amplitude test; double amplitude = difference between peak and trough of fitted cosine; acrophase = time of peak in fitted cosine (units = hh:mm from local midnight = 0000 h); 95% limits of acrophase listed if fit of cosine significant at $p \leq 0.05$.

h*Cry1*, h*Bmal1*, h*Rev-erb* α, and h*Clock* Do Not Exhibit Rhythms during the 24–h Period

Although the time point means for h*Cry1*, h*Bmal1*, and h*Rev-erb* α (but not h*Clock*) appeared to be rhythmic, with higher values in the morning for h*Cry1* and h*Rev-erb* β and in the evening for h*Bmal1*, the analysis of these clock genes for time effect did not reach statistical significance by either ANOVA or cosinor analysis (Table 2, Fig. 4). A multiple-component cosine model (i.e., 24 h and 12 h) made only a slight statistical improvement in the overall result for the genes that were rhythmic but did not reveal improvements in rhythm detection for the other clock genes compared to a 24-h single component cosine (results not shown).

DISCUSSION

Rhythmic expression of the core circadian gene *Per1* has been identified in both central and peripheral tissues and is regarded as a key marker of the circadian molecular clock. We show here for the first time that key clock genes are expressed in human hematopoietic BM, and 3 of them—namely, h*Per1*, h*Per2*, and h*Cry2*—exhibit a significant 24-h rhythm in the CD34$^+$ stem/progenitor cell fraction, as well as h*Per1* in whole BM.

h*Per1* showed the highest amplitude compared to other rhythmic genes and had a peak in the morning at approximately the same time as serum cortisol. In contrast, we have recently shown that circadian variations of m*Per1* in mouse primitive SP stem cells were weak and/or undeveloped (Tsinkalovsky et al., 2006). Differences were also observed in relative levels of *Per1* expression in the studied BM populations in mice and humans. In the current study, we found that h*Per1* showed the lowest level of relative expression compared to other clock genes both in CD34$^+$ and whole BM cells. In mouse hematopoietic SP stem cells, however, the relative level of m*Per1* was much higher (approximately 3 times) than any of the other genes (Tsinkalovsky et al., 2006), while in the whole BM, it was similar to others. Whether this reflects differences in species or in the stem cell populations (mouse primitive SP stem cells vs. human CD34$^+$ cells) is not clear. The CD34$^+$ cell fraction in human BM not only contains primitive stem cells but also different progenitors. However, we chose this population for our study because human CD34$^+$ cells isolated by magnetic cell separation are used routinely in clinical transplantations (Handgretinger et al., 1998). Getting further enrichment in primitive stem cells (e.g., isolation of lineage-negative CD34$^+$CD38$^-$ subpopulations) would also dramatically reduce cell numbers. Since obtaining BM is a painful procedure and may result in bleeding afterward, we decided to keep the number of samplings and the sample volume to a minimum.

We show here that h*Bmal1* was not significantly rhythmic over 24 h in CD34$^+$ cells, even though the highest mean values were located in the evening and at night and were lower during the day. h*Bmal1* has been shown to oscillate significantly in human oral mucosa and skin, with peaks of expression in the evening in both tissues (Bjarnason et al., 2001). Antiphase oscillation of *Bmal1* and *Per* genes has also been demonstrated in many tissues of several different species (Oishi et al., 1998a). However, our results are consistent with previous data obtained in mouse hematopoietic tissue: no significant circadian rhythms

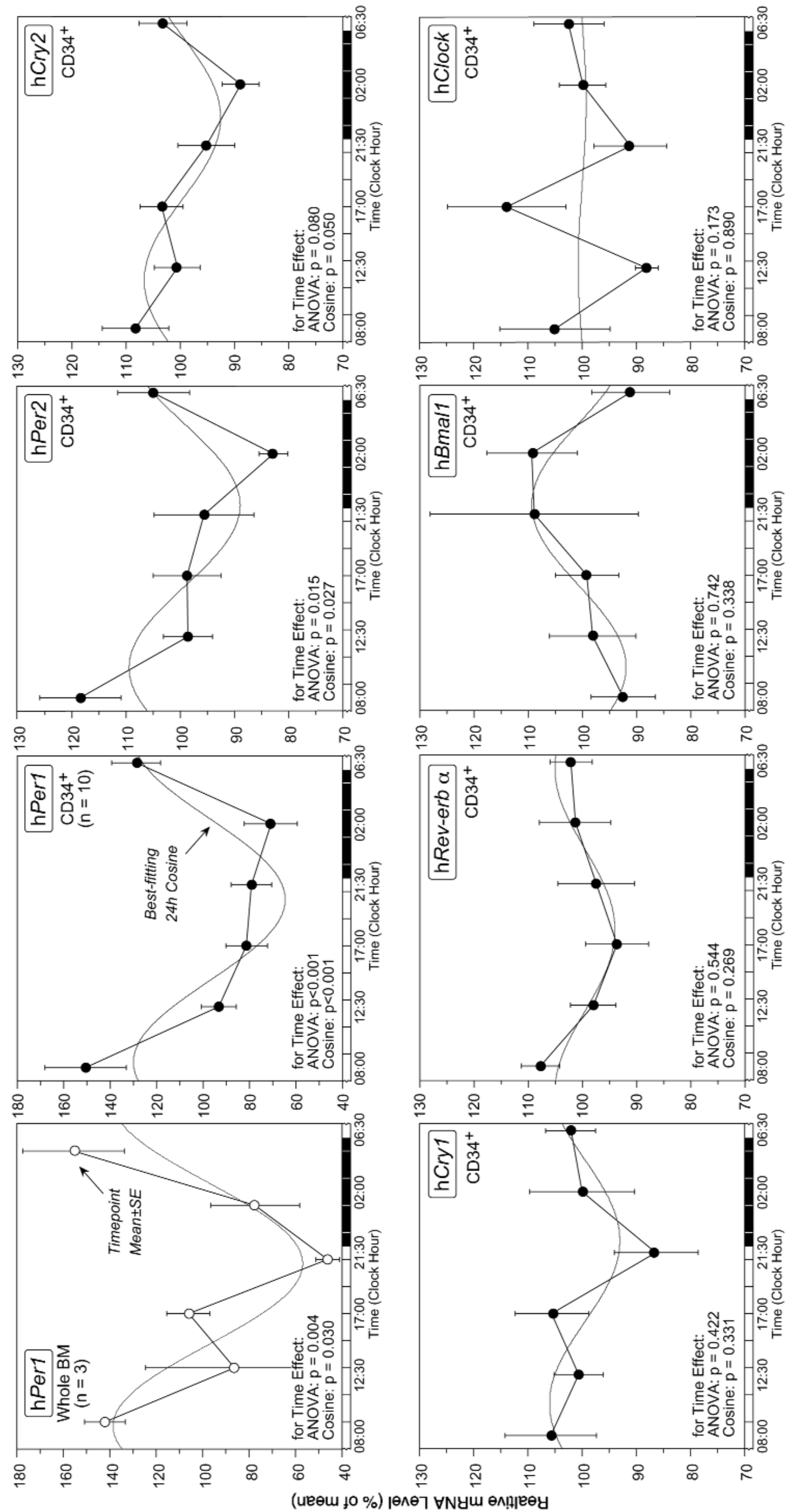

Figure 4. The 24-h variations in relative mRNA expression (see text for calculations) for h*Per1*, h*Per2*, and h*Cry2* (top) and h*Cry1*, h*Rev-erb* α, h*Bmal1*, and h*Clock* (bottom) in bone marrow (BM) CD34⁺ cells sampled every 4 to 5 h over 24 h in 10 healthy men (h*Per1* also sampled in total BM cells in 3 men—upper left panel). Data for each variable expressed as percentage of the mean for each subject and combined overall for analysis for time effect by analysis of variance (ANOVA) and by the least squares fit of a 24-h cosine (see *p* values). Time point means ± SE are shown with best-fitting 24-h cosine. The *y*-axis for h*Per1* was expanded for remaining clock genes for clarity. Abscissa represents time; white and dark bars along the *x*-axis indicate the duration of the activity and rest/sleep periods, respectively.

were found for m*Bmal1* expression in the whole BM or SP stem cells, contrary to the liver analyzed in the same study (Tsinkalovsky et al., 2006). This is also in agreement with reports from other laboratories showing that clock genes may have totally different phase relationships in circadian output protein among different tissues, including peripheral blood (Teboul et al., 2005). Our results confirm that BM could have a specific organization of the molecular clock, reflecting specific control of the timing of hematopoiesis, and different from other organs/tissues.

Correlation of the timing of clock gene expression with the timing of specific cell cycle stages suggests that the circadian clock might be involved in the ordering of cell cycle events, as has been shown for mouse liver (Matsuo et al., 2003). Similarly, in human oral mucosa, the major peak of h*Per1* expression coincides with the G1-phase, whereas the peak in h*Bmal1* may coincide with the peak in M-phase (Bjarnason et al., 2001). Earlier, it was found that both the fraction of cells in DNA synthesis and the clonability of myelogenic progenitor cells in human BM exhibit similarly timed circadian variations (Abrahamsen et al., 1998). These roughly follow the circadian variations of core body temperature and have a maximum in the day, or 4 to 6 hours later than we found for h*Per1* in the stem/progenitor cells. Since the corresponding synthesis of PER1 protein necessarily will be completed several hours later, this is not incompatible with a covariation. Thus, hematopoietic activity in general may occur in circadian waves, as we have indicated earlier (Smaaland et al., 2002).

Per genes have been reported to play an important role in cell cycle events and growth (Matsuo et al., 2003) and in a wide range of physiological effects, including cancer development. *Per* and other clock components have also been implicated in normal metabolic regulation, and their disruption has been associated with an increasing number of biological consequences, including disease. To what extent our findings have clinical implications at the present is not clear. However, elucidation of regulatory transcriptional mechanisms occurring with rhythmic patterns that are physiologically relevant opens the opportunity to manipulate both proliferation and differentiation of stem and progenitor cells for clinical purposes.

In conclusion, we found that 3 of 7 key clock genes expressed in human BM CD34$^+$ progenitor/stem cells—h*Per1*, h*Per2*, and h*Cry2*—exhibit a significant circadian rhythm. h*Bmal1* did not oscillate significantly in these cells, which confirms our previous findings in mouse hematopoietic BM cells that some clock genes in primitive hematopoetic stem cells do not oscillate rhythmically and/or may be developmentally regulated, as has been noted in other immature or differentiating tissues. This could indicate that circadian rhythmicity in *Bmal1* is undeveloped in rapidly proliferating hematopoietic tissues. Nevertheless, our study demonstrates that clock gene expression in human BM stem/progenitor (CD34$^+$) cells can be circadian rhythmic, but with some differences from those reported in other species and/or mature tissues.

ACKNOWLEDGMENTS

We thank Raymond Lygre for technical assistance with flow cytometry analysis and the volunteers for giving their bone marrow. This work was supported by the stem cell program of the Research Council of Norway, Aurora program, and the Norwegian Cancer Society.

REFERENCES

Abrahamsen J, Smaaland R, Sothern R, and Laerum O (1997) Circadian cell cycle variations of erythro- and myelopoiesis in humans. Eur J Haematol 58:333-345.

Abrahamsen J, Smaaland R, Sothern R, and Laerum O (1998) Variation in cell yield and proliferative activity of positive selected human CD34+ bone marrow cells along the circadian time scale. Eur J Haematol 60:7-15.

Balsalobre A, Damiola F, and Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93:929-937.

Bjarnason GA, Jordan RCK, Wood PA, Li Q, Lincoln DW, Sothern RB, Hrushesky WJM, and Ben-David Y (2001) Circadian expression of clock genes in human oral mucosa and skin: Association with specific cell-cycle phases. Am J Pathol 158:1793-1801.

Boivin DB, James FO, Wu A, Cho-Park PF, Xiong H, and Sun ZS (2003) Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood 102:4143-4145.

Bourin P, Ledain A, Beau J, Mille D, and Levi F (2002) In-vitro circadian rhythm of murine bone marrow progenitor production. Chronobiol Int 19:57-67.

Chen Y-G, Mantalaris A, Bourne P, Keng P, and Wu JHD (2000) Expression of mPer1 and mPer2, two mammalian clock genes, in murine bone marrow. Biochem Biophys Res Commun 276:724-728.

Council for International Organizations of Medical Sciences (CIOMS) (2002). *International Ethical Guidelines for Biomedical Research Involving Human Subjects*. Geneva: Council for International Organizations of Medical Sciences.

D'Hondt L, McAuliffe C, Damon J, Reilly J, Carlson J, Dooner M, Colvin G, Lambert J, Hsieh C, Habibian H,

et al. (2004) Circadian variations of bone marrow engraftability. J Cell Physiol 200:63-70.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, and Schibler U (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14:2950-2961.

Filipski E, Delaunay F, King VM, Wu M-W, Claustrat B, Grechez-Cassiau A, Guettier C, Hastings MH, and Francis L (2004) Effects of chronic jet lag on tumor progression in mice. Cancer Res 64:7879-7885.

Granda TG, Liu X-H, Smaaland R, Cermakian N, Filipski E, Sassone-Corsi P, and Levi F (2004) Circadian regulation of cell cycle and apoptosis proteins in mouse bone marrow and tumor. FASEB J 19:304-306.

Handgretinger R, Lang P, Schumm M, Taylor G, Neu S, Koscielnak E, Niethammer D, and Klingebiel T (1998) Isolation and transplantation of autologous peripheral CD34+ progenitor cells highly purified by magnetic-activated cell sorting. Bone Marrow Transplant 21: 987-993.

Hastings M, Reddy A, and Maywood E (2003) A clockwork web: Circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci 4:649-661.

Kennaway DJ, Varcoe TJ, and Mau VJ (2003) Rhythmic expression of clock and clock-controlled genes in the rat oviduct. Mol Hum Reprod 9:503-507.

Krieger D, Allen W, Rizzo F, and Krieger H (1971) Characterization of the normal temporal pattern of plasma corticosteroid levels. J Clin Endocrinol Metab 32:266-284.

Kusanagi H, Mishima K, Satoh K, Echizenya M, Katoh T, and Shimizu T (2004) Similar profiles in human period1 gene expression in peripheral mononuclear and polymorphonuclear cells. Neurosci Lett 365:124-127.

Laerum OD (1995) Hematopoiesis occurs in rhythms. Exp Hematol 23:1145-1147.

Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, and Okamura H (2003) Control mechanism of the circadian clock for timing of cell division in vivo. Science 302: 255-259.

Mormon MC, Bogdan A, Cormont S, Touitou Y, and Levi F (2002) Cortisol diurnal variation in blood and saliva of patients with metastatic colorectal cancer: Relevance for clinical outcome. Anticancer Res 22:1243-1249.

Muhlbauer E, Wolgast S, Finckh U, Peschke D, and Peschke E (2004) Indication of circadian oscillations in the rat pancreas. FEBS Lett 564:91-96.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, and Schibler U (2004) Circadian gene expression in individual fibroblasts: Cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell 119:693-705.

Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G-I, Ohkura N, Azama T, Mesaki M, Yukimasa S, et al. (2003) Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem 278:41519-41527.

Oishi K, Sakamoto K, Okada T, Nagase T, and Ishida N (1998a) Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. Biochem Biophys Res Commun 253:199-203.

Oishi K, Sakamoto K, Okada T, Nagase T, and Ishida N (1998b) Humoral signals mediate the circadian expression of rat period homologue (rPer2) mRNA in peripheral tissues. Neurosci Lett 256:117-119.

Panda S, Antoch M, Miller B, Su A, Schook A, Straume M, Schultz P, Kay S, Takahashi J, and Hogenesch J (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109:307-320.

Smaaland R, Laerum O, Sothern R, Sletvold O, Bjerknes R, and Lote K (1992). Colony-forming unit-granulocyte-macrophage and DNA synthesis of human bone marrow are circadian stage-dependent and show covariation. Blood 79:2281-2287.

Smaaland R, Sothern R, Laerum O, and Abrahamsen J (2002) Rhythms in human bone marrow and blood cells. Chronobiol Int 19:101-127.

Smaaland R, Sothern R, Lote K, Sandberg S, Aakvaag A, and Laerum O (1995) Circadian phase relationships between peripheral blood variables and bone marrow proliferative activity in clinical health. In Vivo 9:379-389.

Sutherland D, Anderson L, Keeney M, Nayar R, and Chin-Yee I (1996) The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 5:213-226.

Teboul M, Barrat-Petit MA, Li XM, Claustrat B, Formento JL, Delaunay F, Levi F, and Milano G (2005) Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. J Mol Med 83: 693-699.

Tsinkalovsky O, Filipski E, Rosenlund B, Sothern RB, Eiken HG, Wu MW, Claustrat B, Bayer J, Levi F, and Laerum OD (2006) Circadian expression of clock genes in purified hematopoietic stem cells is developmentally regulated in mouse bone marrow. Exp Hematol 34:1248-1260.

Tsinkalovsky O, Rosenlund B, Laerum OD, and Eiken HG (2005) Clock gene expression in purified mouse hematopoietic stem cells. Exp Hematol 33:100-107.

Welsh DK, Yoo S-H, Liu AC, Takahashi JS, and Kay SA (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14: 2289-2295.

Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, and Takumi T (2004) Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Mol Biol 5:18.