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Clock gene expression in purified mouse hematopoietic stem cells

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***Objective*. Circadian genes have recently been characterized in many tissues, but not in hematopoietic stem cells. These cells are rare in the bone marrow (BM), which makes it difficult to collect enough cells for detailed molecular analysis in a short period of time without reduced RNA quality. The aim was to improve methodology and reliability of clock gene expression analysis in purified mouse hematopoietic stem cells.**

***Methods*. Stem cells were highly enriched by high-speed flow cytometric cell sorting of the side population (SP) cells from Hoechst 33342 (Hoechst)-stained mouse BM. Total RNA was isolated from sorted SP and whole BM cells and exposed to DNase treatment. The relative mRNA levels of major clock genes *mPer1, mPer2, mBmal1, mCry1, mClock,* and *mRev-erb α* were measured with real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) and normalized to *m36B4*, used as a reference gene. The clonogenity of sorted SP cells and whole BM; cells taken before and after sorting, were tested in colony-formation assay.**

***Results*. Clock gene activity in sorted SP cells showed pronounced relative differences compared with whole BM for *mPer1* and *mCry1*. The high-speed sorting procedure did not influence clock gene expression or cell clonogenity, even when this was performed with a delay period up to 24 hours.**

***Conclusions*. We demonstrated expression of six clock genes in mouse hematopoietic stem cells. A combination of high-speed flow cytometric sorting and Q-RT-PCR was shown to be useful and reliable for analysis of clock gene activity in small stem cell fractions.** © 2005 International Society for Experimental Hematology. Published by Elsevier Inc.

Adaptation of organisms to their periodically varying environment is mediated through the entrainment to circadian rhythms. A molecular and cellular basis of these rhythms in mammals has recently been provided through identification of clock genes and their products [1]. The clock mechanism involves transcription-translation feedback loops composed of positive (*mClock* and *mBmal1*) and negative (*mPer1, mPer2, mCry1, mCry2,* and *mRev-erb α*) elements [2]. Most detailed description of clock gene expression has been done for the suprachiasmatic nucleus cells—the main circadian pacemaker in mammals, located in ventral hypothalamus of the brain [2]. Recent studies have also shown that peripheral tissues such as liver, heart, kidney, lung, pancreas, skeletal muscles, oral mucosa, skin and bone marrow (BM), and mononuclear leukocytes express clock genes giving rise to circadian rhythms with a different phasing from that observed in suprachiasmatic nucleus ([3–8], reviewed in [9]). Even cell tissue culture, such as rat fibroblasts [7] or mouse smooth muscles [10], are capable of generating circadian rhythmicity of clock gene expression when entrained by humoral signals.

Although it is well established that hematopoiesis in general undergoes strong circadian variations in mammals [11–14], clock gene regulation of stem cells has not yet been analyzed. So far only *mPer1* and *mPer2* were analyzed in mouse BM [15], while clock genes in primitive stem cells have not been investigated. Hematopoietic stem cells are objects of considerable interest for research and clinical purposes [16,17], especially in the last years, when their high "plasticity" has been elucidated [17,18]. New data about clock gene expression in primitive stem cells could bring further insights into molecular mechanism controlling the

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circadian rhythms in hematopoiesis and stem cell biology in general.

The present lack of data on circadian gene expression in stem cells could partly be explained by methodological difficulties. Hematopoietic stem cells comprise a very small fraction (0.1–0.001%) of whole BM, and the long time necessary for enrichment of such rare cells in sufficient quantities for further analysis could increase the risk of RNA degradation. RNA quantity is critical for simultaneous analysis of all major clock genes in the evaluation of clock genes interactions. Moreover, when many samples have to be collected within 24 to 27 hours, a delay in cell flow cytometry analysis and sorting could also increase the risk of RNA degradation. Therefore, the aim of our study was to evaluate methodology for analysis of clock gene expression in primitive hematopoietic stem cells.

We employed a single-step Hoechst-33342 (Hoechst)-staining method for stem cell isolation described recently [19]. In Hoechst-stained BM a small fraction of the cells defined as side population (SP), efflux Hoechst dye, and express low Hoechst fluorescence in blue and red regions of the spectrum [19]. The process is dependent on the expression of the *Bcrp1* gene [20] and can be blocked by verapamil. SP fraction was shown to be highly enriched for long-term hematopoietic stem cells [19,21]. We performed high-speed flow cytometric sorting of SP cells followed by total RNA extraction and DNase treatment. This allowed us to get RNA of high quality and specifically analyze for the first time expression of the clock genes *mPer1, mPer2*, *mBmal1, mCry1*, *mClock,* and *mRev-erb* α*,* in mouse hematopoietic stem cells by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR).

Materials and methods

Animals, cell preparation, and staining

Twenty-three C57 black male mice were synchronized to standard lighting conditions (12-hour light/12-hour dark cycle) for 3 weeks with a standard diet and tap water provided ad libitum. Mice were sacrificed in the morning between 9:00 and 9:30 by neck dislocation, and BM cells were obtained by crushing 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)—$HBSS^+$. Cells from all 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 dissolved in 10 mL Lysing solution (a gift by DakoCytomation, Denmark A/S) for 10 minutes in the dark. After centrifugation, cells were resuspended in 31 mL cold $HBSS^+$ and counted. On average, 7.5×10^7 BM cells were recovered per mouse. Part of the cell suspension (10^8 cells) was used for the experiments on the whole BM, and remaining cells were stained with Hoechst (Molecular Probes, Eugene, OR, USA).

Experiments on the whole BM samples. Approximately 10^8 cells were used for analysis of gene expression and colony-forming assay before and after sorting of whole BM samples. Cells (2.5×10^6) were distributed into five 2-mL cryotubes (TPP AG, Trasadingen, Switzerland) (5×10^5 per tube), and spun down (300*g*, 5 minutes at +4°C). Supernatants were discarded, pellets snap-frozen (30 seconds) in liquid nitrogen and placed at −80°C until use. An aliquot of the cells (2×10^5) was used for colony-forming assay. The rest of the whole BM suspension was kept on ice until the sorting procedure.

Experiments on SP cell samples. Thirty milliliters of cell suspension were divided into three equal parts. One part was immediately stained with fluorescent dye Hoechst. Two other parts were kept at +4°C for 12 hours and 24 hours, respectively, until staining. Before staining, cells were spun down and resuspended in prewarmed Dulbecco's modified Eagle's medium with 2% FCS and 1 mM HEPES (all purchased from Gibco) at 10^6 cells/mL. BM cells were stained with 5 μg/mL Hoechst for 90 minutes at 37°C as described elsewhere [19], spun down and resuspended in cold $HBSS^+$ at 5–7 $\times 10^7$/mL. A parallel aliquot was stained, as described, in the presence of 50 μM verapamil (Sigma-Aldrich, St. Louis, MO, USA). For surface stem cell markers Sca-1 and c-Kit analysis, an aliquot of 5×10^5 Hoechst-stained cells was washed, resuspended in 80 μL cold $HBSS^+$ and incubated for 30 minutes on ice with 10 μL fluorescein isothiocyanate (FITC)-conjugated Sca-1 and 10 μL R-phycoerythrin (RPE)-conjugated c-Kit antibodies (both from PharMingen, San Diego, CA, USA) followed by washing in $HBSS^+$. Cells were kept on ice until flow cytometric analysis and sorting. Propidium iodide (PI, 2 μg/mL; Sigma-Aldrich) was added to Hoechst-stained samples before flow cytometric analysis to facilitate dead cell discrimination. Cell suspension was filtered through a 50-μm Filcons filter device (DakoCytomation, Denmark A/S) prior to flow cytometry analysis.

Flow cytometry and high-speed cell sorting

Cells were analyzed and sorted on the MoFlo cell sorter (DakoCytomation, Denmark A/S; former Cytomation, USA), equipped with Coherent Enterprise 621 dual-laser tuned to 488 nm (1 W) and 365 nm, UV (60 mW). Cells were sorted in "purify 1" mode with high speed up to 30,000 events (cells) per second, and collected in 5-mL polypropylene round-bottom Falcon tubes (Becton Dickinson Labware Europe, Le Pont-de-Claix, France), containing cold HBSS with 10% FCS, that were placed on ice.

Experiments on the whole BM samples. Doublets were discriminated using a forward light scatter vs pulse width after 488-nm excitation. Created region was gated into forward light scatter vs side light scatter bivariate, and then all displayed nucleated BM cells were sorted. Sorted cells were counted and 25×10^5 cells were distributed into five 2-mL cryotubes (TPP AG, Trasadingen, Switzerland), 5×10^5 per tube, and spun down (300*g*, 5 minutes at +4°C). Supernatants were then discarded; pellets snap-frozen (30 seconds) in liquid nitrogen and placed at −80°C until use; 2×10^5 cells were used for colony-forming assay.

Experiments on the SP cell samples. The 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" and PI) optical filters (Omega Optical, Brattleboro, VT, USA). A 610 DMSP was used to separate the emission wavelengths. FITC and RPE emissions were observed through 530/40 and 580/30 filters, respectively, after 488-nm excitation.

Doublets were discriminated using a forward light scatter vs pulse width. Sort regions were detected on a Hoechst red vs Hoechst blue emission bivariate (both on a linear scale). The sorting gates for SP were established as described elsewhere [19]. Briefly, after excluding the doublets, a live gate was defined on Hoechst red vs blue dot plot with the exclusion of dead cells (as positive for PI, far right on Hoechst "red" axis) and debris (no Hoechst staining). After collecting 6–7 $\times$ 10^4 cells in a live gate, SP was clearly defined and a new gate was established on this population (Fig. 1A). SP cells represented 0.05% to 0.07% of Hoechst-stained BM. Sorted SP cells were counted and 5–7.5 $\times$ 10^4 cells were distributed into five 2-mL cryotubes (TPP AG, Trasadingen, Switzerland), 1–1.5 $\times$ 10^4 per tube, spun down (300*g*, 5 minutes at +4°C), supernatants were discarded, pellets snap-frozen in liquid nitrogen and placed at −80°C until use. A part of each sorted sample (10^4 cells) was used for colony-forming assay. Aliquots from some samples at the end of the sort were removed and reanalyzed for control of the sort purity that was >97% (Fig. 1C), and viability by trypan blue staining that was >97%. Recovery was >70%.

Gates for analysis of antibody staining were set using isotype controls so that <1% of cells were positive for isotype antibody staining, and SP cell region was gated on c-Kit vs Sca-1 bivariate (Fig. 1D).

Colony-forming assay

Whole BM and SP cells were cultured in pretested 35-mm culture dishes in complete methylcellulose medium MethoCult 3434 (both from Stem Cell Technologies, Vancouver, Canada) containing 3 U/mL erythropoetin, 10 ng/mL interleukin (IL)-3, 10 ng/mL IL-6, and 50 ng/mL stem cell factor. Clonogenic progenitors were counted on day 12 of culture according to standard criteria on the basis of an identifiable colony of more than 50 cells (http://www.stemcell.com/technical/28405_methocult%20M.pdf). Plating density for whole BM was 2 $\times$ 10^4 cells/mL (2.2 $\times$ 10^4 cells per dish). As a colony assay for mouse SP cells is not standardized we tried different concentrations (20–1000 cells/mL) in preliminary experiments and selected 100 cells/mL (110 cells per dish) that gave the best ratio colony/plated cells. One experienced person read all colonies, and single lots of reagents were used for all experiments to reduce day-to-day variability in colony cell numbers.

RNA extraction

Total cellular RNA was extracted with the use of the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to manufacture's instructions. All samples were subjected to DNase treatment followed by phenol-chloroform extractions (Promega, Madison, WI, USA) to avoid DNA contamination that would result in inaccurate quantification [22].The extracted RNA was dissolved in 10 µL of RNA storage solution (Ambion, Houston, TX, USA). Each sample (1 µL) was run on the Agilent 2100 Bioanalyzer Nano LabChip for quality control and quantification of total RNA as described by the manufacturer. A ladder (Ambion, Houston, TX, USA) including six RNA fragments was run on each chip for quantitation of the samples. It also served as a built-in measure of assay quality control. The total RNA was stored at −80°C until use. On average 3.2 to 4.1 µg total RNA were obtained from BM cell samples and 50 to 240 ng from sorted SP cell samples (Fig. 2). Mouse BM total RNA (Clontech, Palo Alto, CA, USA) was used as exogenous control.

Reverse transcription

Total RNA was reverse transcribed using TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA). Final concentration of the reaction mix was 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 2 mM dNTP mixture (500 µM of each dNTP), 2.5 µM random hexamers, 0.4 U/µL RNase inhibitor, 1.25 U/µL Multiscribe reverse transcriptase, 1 µL of RNA (in different concentrations), and RNase-free water to a total volume of 50 µL.

The incubation was as follow: 25°C for 10 minutes (primer incubation), 48°C for 30 minutes (reverse transcription) and 95°C for 5 minutes (inactivation of reverse transcriptase). All complementary DNA samples were stored at −20°C.

Primer and probe design

Primer Express software (Applied Biosystems) was used for primer and probe design for all of the TaqMan assays (Table 1). 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 were purchased from MedProbe (Oslo, Norway). Six assays were prepared for target genes: *mPer1, mPer2, mBmal1, mCry1, mClock,* and *mRev-erb* α. A persumed

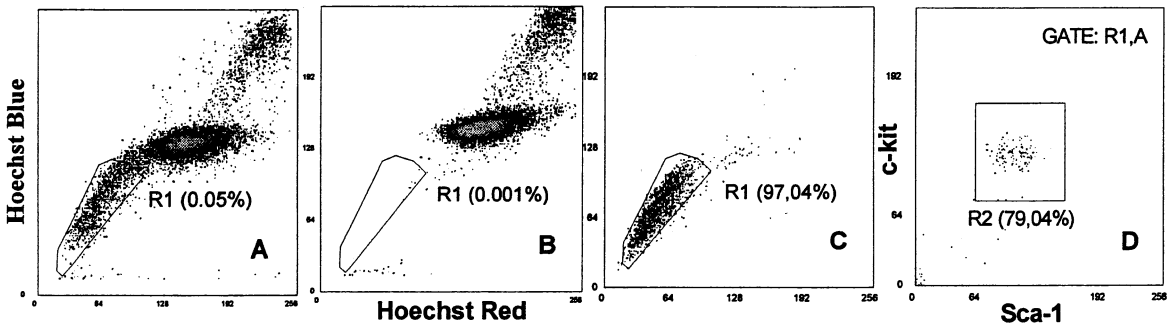

Figure 1. Flow cytometric analysis of side population (SP) cells in Hoechst-stained bone marrow (BM). R1 (Region 1): SP region of whole BM stained with Hoechst without (**A,C**) or in the presence (**B**) of 50 µM verapamil. A percent of cells in correspondent region is given in the brackets. (**C**) Reanalysis of sorted SP (purity = 97.04%). (**D**) Expression of c-Kit and Sca-1 surface antigens in SP cells (R1 gated from A); R2 (Region 2) comprises SP cells positive for both markers. Debris and dead cells were excluded as described in Materials and Methods section.

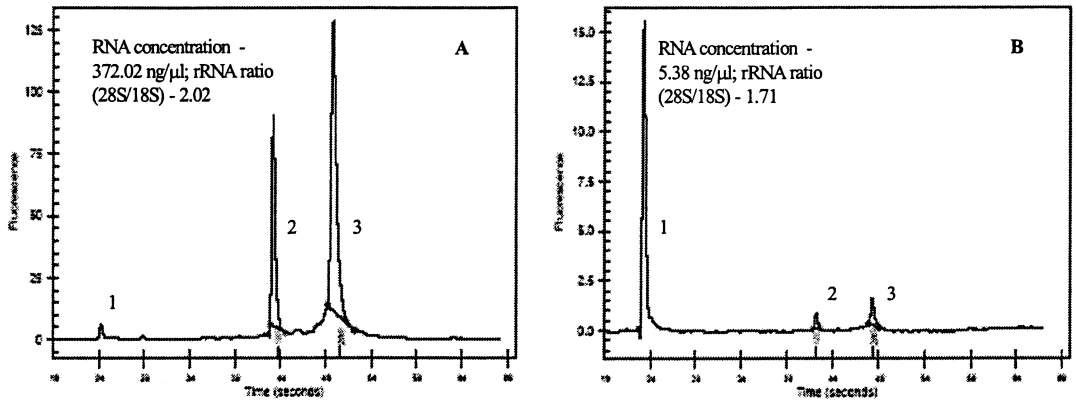

Figure 2. Total RNA of sorted whole bone marrow (BM) (**A**) and side population (SP) cells (**B**) analyzed on Nano LabChip on Agilent 2100 Bioanalyser. 1: the ladder, 2: 18S, 3: 28S rRNA; no signs of gDNA were detected.

noncircadian gene, *m36B4,* was used as reference gene (endogenous control).

Q-RT-PCR

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.

Polymerase chain reaction was performed in a 25-µL reaction volume consisting of 12.5 µL of a 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM (0.25 µL) forward primer, 300 nM (0.25 µL) reverse primer, and 200 nM (1 µL) TaqMan probe for the majority of genes. For *mRev-erb* α 500 nM TaqMan probe was used. The thermal cycling parameters were as follow: two presteps at 50°C for 2 minutes (UNG incubation) and 95°C for 10 minutes (AmpliTaq Gold activation) followed by 40 cycles at 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension).

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

To find out the intraexperimental coefficient of variation (CV) 10 equal samples of 100 ng mouse BM total RNA (Clontech, Palo

Table 1. Primers and probes for tested genes (TaqMan assay)

Gene symbol	Accession number	Primer probe[a]	Sequence 5′ → 3′
mPer1	011065	Forward	TGAGAGCAGCAAGAGTACAAACTCA
		Reverse	CTCGCACTCAGGAGGCTGTAG
		Probe	AGAGCCCATCCCCACCCAGCAG
mPer2	011066	Forward	GTCCACCTCCCTGCAGACAA
		Reverse	TCATTAGCCTTCACCTGCTTCAC
		Probe	CTGGCAACCCTGAAGTATGCCCTGC
mBmal1	007489	Forward	AGAGGTGCCACCAACCCATA
		Reverse	TGAGAATTAGGTGTTTCAGTTCATCAT
		Probe	CACAGAAGCAAACTACAAGCCAACATTTCTATCAG
mCry1	007771	Forward	CTCGGGTGAGGAGGTTTTCTT
		Reverse	GACTTCCTCTACCGAGAGCTTCAA
		Probe	AAGCCGGGCTCATAACCGACACCT
mClock	007715	Forward	CAAAATGTCACGAGCACTTAATGC
		Reverse	ATATCCACTGCTGGCCTTTGG
		Probe	TCAGGAATCTATAGTAACACGATTTGCCTTTTCCATATT
mRev-erb α	145434	Forward	GATAGCTCCCCTTCTTCTACATCATC
		Reverse	TTCCATGGCCACTTGTAGACTTC
		Probe	TCATCCTCTTCATCCTCCTCCTCCTTCTATAACG
m36B4	007475	Forward	GGCACCATTGAAATTCTGAGTGA
		Reverse	ATGTTCAGCATGTTCAGCAGTGT
		Probe	TGGCTCCCACCTTGTCTCCAGTCTTTATC

[a]Probes for all target genes and for m36B4 have the reporter dye FAM incorporated on the 5′ end and the quencher dye TAMRA on the 3′ end. All primers span one intron.

Alto, CA, USA) were analyzed on the same 96-well plate twice for a target gene (*mPer1*) and a reference gene (*m36B4*) in triplets.

Calculations and statistics

The standard curve method was used for calculations of gene expression, as previously described (User Bulletin #2 ABI PRISM 7700 Sequence Detection System, http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). This was done to standardize the data of the clock genes run on different plates. Each sample was normalized by dividing the quantity of the target gene by the quantity of *m36B4* (the quantity for each sample was estimated from the corresponding standard curve) to correct for variation of input RNA between the samples. 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%. GraphPad Prism, version 3 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis and the creation of graphs. Mann-Whitney nonparametric *U*-test was performed to calculate whether the difference between values was statistically significant.

Results

Isolation of SP cells and extraction of total RNA

In our experiments cells located in SP region (Fig. 1A) comprised 0.05% to 0.07% of mouse Hoechst-stained BM and were sensible to verapamil (Fig. 1B). Analysis of early hematopoietic progenitor antigens Sca-1 and c-Kit expression on SP cells showed that 75% to 85% of cells were positive for both antigens (Fig. 1D). SP cells were highly clonogenic and multipotent in colony assay in complete methylcellulose medium MethoCult 3434 (Table 2). The average ratio colony per plated cells for total colony number in SP was 1/3.2. This means the SP cells were 76.5 times enriched for clonogenicity in comparison with the whole BM, where this index was 1/244.9 (Table 2).

We employed flow cytometric cell sorting, performed at a speed of approximately 30,000 events (cells) per second for the enrichment of SP cell fraction. This enabled us, in a short period of time, to get fractions of viable primitive stem cells of high purity (>97 %, Fig. 1C) and yielded sufficient quantities of total RNA for further gene expression analysis. High-speed sorting did not induce RNA degradation in SP cells as all samples were of high quality confirmed by Nano LabChip tests run on Agilent 2100 Bioanalyzer (Fig. 2B).

Clock gene activity in SP and whole BM cells

All clock genes were expressed both in SP and whole BM cells, with some similarities and differences for particular genes (Fig. 3). Thus, in both cell populations *mPer2, mBmal1,* and *mClock* showed similar patterns of relative gene expression. mRNA levels of *mPer1* were found to be different in precursor and progenitor cells: in SP cells *mPer1* mRNA levels were much higher (approximately three times) than any of the other genes, while in the whole BM it was similar to *mPer2, mBmal1*, and *mClock.* Furthermore, *mCry1* showed the highest expression in whole BM while in SP cells its expression was similar to the other genes, with the exception of *mPer1* (Fig. 3).

Intraexperimental CV for different runs of Q-RT-PCR

The intraexperimental CVs calculated from control Q-RT-PCR were determined to be for *mPer1* (n = 30): 0.43% and 0.26%, respectively, for plate 1 and 2, and for *m36B4* (n = 30): 0.43% and 0.45%. The distributions of *mPer1* relative expression (%) of 10 samples on 2 plates with SEM for each plate are shown in Figure 4.

Possible influence of cell sorting on mRNA quantification

To find out whether the high-speed sorting itself could influence on gene expression we performed QRT-PCR on total RNA from whole BM cell samples taken before and after sorting with a high speed at rate up to 30,000 events per second. Sorting did not induce RNA degradation: RNA from samples taken before and after sorting were similar and of good quality confirmed by Nano LabChip tests run on Agilent 2100 Bioanalyzer (data not shown). No statistically significant differences were found for expression of any of the analyzed genes in sorted cells compared to cells that were not sorted (Fig. 5A). Similar result was shown by colony-forming assay: sorted cells cultured in complete methylcellulose medium MethoCult 3434 were able to

Table 2. Colony forming assays of side population (SP) and whole bone marrow (BM) cells

Sample (plated cells/mL)	N	Colony-forming units[a]					
		GM	GEMM	BFU	Total	Colony/Cell-plated	Enrichment
SP (100)	7	23.46 ± 9.1	8.50 ± 2.7	3.63 ± 2.1	35.60 ± 9.9	1/3.2	×76.5
BM before sorting (20×10^3)	5	42.33 ± 3.1	18.00 ± 5.2	21.33 ± 2.5	81.67 ± 7.8	1/244.9	
BM after sorting (20×10^3)	5	45.67 ± 2.3	18.67 ± 3.1	13.67 ± 5.9	78.00 ± 5.2	1/256.4	

Whole BM and SP cells were cultured in complete methylcellulose medium MethoCult 3434 (Stem Cell Technologies, Vancouver, Canada) containing 3 U/mL erythropoetin, 10 ng/mL IL-3, 10 ng/mL IL-6, and 50 ng/mL stem cell factor. Clonogenic progenitors were counted on day 12 of culture based on standard criteria on the basis of an identifiable colony of more than 50 cells. n = number of experiments. The classes of mouse hematopoietic progenitors detected: GM = granulocyte-macrophage; GEMM = granulocyte, erythroid, macrophage, megakaryocyte; BFU = burst-forming unit-erythroid.

[a]Values are mean ± SD.

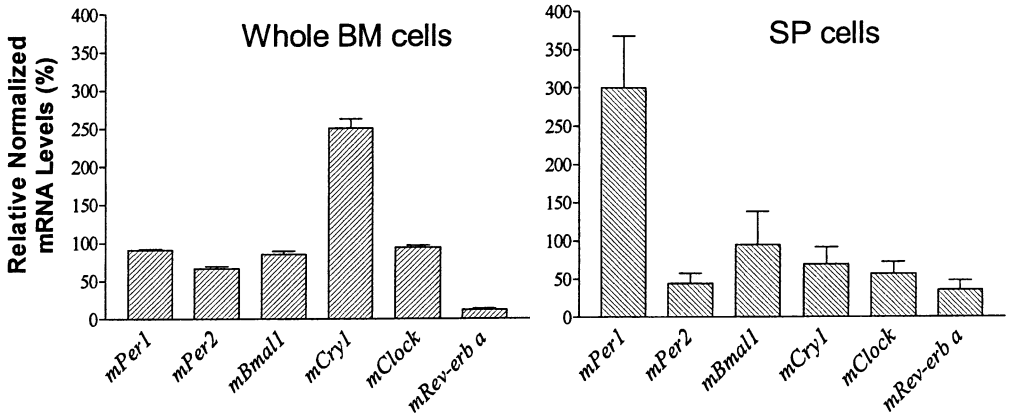

Figure 3. Comparison of relative mRNA levels of clock genes whole bone marrow (BM) (left) and side population (SP) (right) cells. Samples were normalized to m36B4 and values calculated as described in Materials and Methods section. Values are mean ± SEM of five samples.

give rise to the same number of colonies as cells plated before sorting (Table 2).

As the circadian studies with continuous cell sampling during 24 hours may induce a delay in staining and sorting of SP cells, we tested if such kind of experimental design could influence on clock gene expression. The delay in sorting did not influence on the RNA profile confirmed by Nano LabChip tests run on Agilent 2100 Bioanalyzer (data not shown). As shown in Figure 5B, mPer1 expression in SP cells sorted from whole BM stained with Hoechst 12 or 24 hours after preparation of cell suspension was similar to SP samples sorted from BM stained without delay. The same results were found for the other clock genes (data not shown).

Discussion

We show that the clock genes *mPer1, mPer2, mBmal1, mCry1, mClock,* and *mRev-erb* α are active in mouse hematopoietic stem cells. To be able to study gene expression we have used a combination of high-speed flow cytometric cell sorting and Q-RT-PCR.

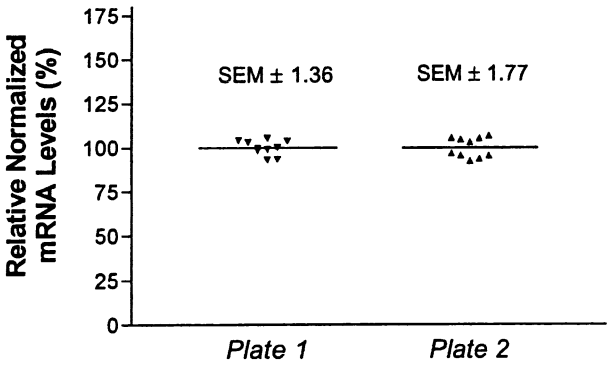

Figure 4. Relative mRNA levels (%) of *mPer1* from 10 equal samples with 100 ng mouse bone marrow total RNA analyzed twice on two different plates. Samples were normalized to *m36B4* and values calculated as described in Materials and Methods section. For those calculations the target and the reference gene values were taken from different plates. SEM of values is shown for each of the two plates.

It is well established that hematopoiesis in general undergoes strong circadian variations both in human beings and rodents (for review see [23]). Yield of positive selected human $CD34^+$ progenitor stem cells showed significant circadian variation [24]. Colony-forming ability of human BM, an indicator of progenitor cells, showed the same circadian variation as cell cycling [12]. Distinct different lineage-dependent circadian patterns of mouse BM progenitors have been reported [25]. A possible mechanism responsible for the different degrees of mouse marrow damage sustained from a fixed dose of a cytotoxic agent at different times of the day have been suggested to the circadian organization of cell cycle events [25]. Most recently, significant circadian variations of mouse BM engraftability were demonstrated [26]. Mouse BM granulo-monocytic precursors were able to form rhythmically colonies in vitro over the first 4 days of culture [27] that could be evidence of the presence of a circadian time-keeping system within these cells. Thus, circadian rhythmicity might be a profound part of regulatory mechanisms in hematopoiesis.

mPer1 and *mPer2* have been studied in mouse BM, where they were found to be expressed and oscillated robustly over a 24-hour period [15]. The patterns of *mPer* gene expression found in BM were different from the ones shown in other tissues. This may indicate that time-dependent regulatory mechanisms in marrow are operating locally. BM represents a heterogeneous population of stem cells and various hematopoietic progenitors and precursors whose proliferation and differentiation are both controlled by generally acting and unique factors. Until now, primitive hematopoietic stem cells have never been studied for the clock gene expression. This can be partly explained by methodological difficulties: stem cells comprise a quite small population in the whole BM (<0.1%), which makes it difficult to enrich in a short period of time a pure fraction of viable cells for further

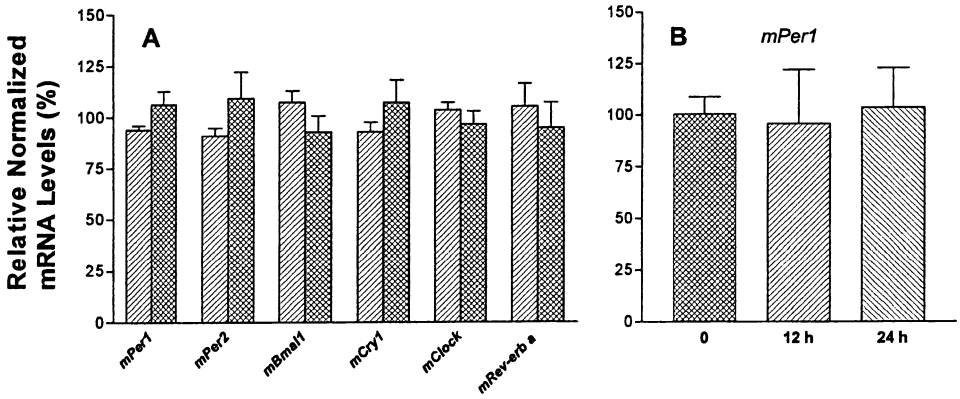

Figure 5. Stem cell sorting and mRNA quantification. (**A**) Relative mRNA levels (%) of *mPer1, mPer2, mBmal1, mCry1, mClock,* and *mRev-erb* α in the whole bone marrow (BM) cells before (left column) and after (right column) the flow cytometry cell sorting. (**B**) Relative mRNA levels (%) of *mPer1* in side population (SP) cells sorted from BM stained with Hoechst in three different intervals. Fresh BM cell suspension was stained with Hoechst immediately (left column), with 12 (center column) and 24 hour-delay (right column). Thereafter, SP cells were sorted by the flow cytometer. Samples were normalized to *m36B4* and calculated as described in Materials and Methods section. Values are mean ± SEM of five experiments.

detailed molecular analysis. A contamination by mature cell populations may mask the true gene expression profiles, as the expression patterns of many gene in subsets of mouse hematopoietic stem and progenitor cells were shown to be different [28,29]. Differences were also reported in circadian expression of *mPer1* and *mPer2*: gene expression patterns exhibited two peaks over a 24-hour period in unfractionated BM compared to one peak in myeloid-enriched (Gr-1 positive) cell fraction [15].

We have used a simple single-step Hoechst-staining method [19] that allows to get a small "side population" highly enriched for long-term progenitor cells. In our study SP cells from C57Bl mouse BM represented <0.07% of the cell population, and were sensible to verapamil. Cultured SP cells showed more than 75 times enrichment in clonogenity as compared to whole BM; high proportion of SP cells (approximately 80%) also expressed early hematopoietic progenitor antigens Sca-1 and c-Kit. This indicated that the population was highly enriched for hematopoietic stem cells and similar to results described by others [19].

High-speed flow cytometric cell sorting appears to be the only appropriate method for SP cell isolation. New generations of flow cytometric sorters give an advantage to work with higher speeds. This is crucial for sorting of small (<0.1%) cell populations, like subpopulations of BM stem cells, for further molecular analysis, as prolongation of sorting reduces cell viability and respectively increases a risk of RNA degradation induced by RNase contamination. The sorting of SP cells with speed up to 30,000 events (cells) per second allowed us in a reasonably short time period to get sufficient RNA quantities for clock gene expression analysis by the Q-RT-PCR technique. This technique has been widely used for quantification of RNA levels as well as characterizing gene expression patterns in different samples (for review see [22,30]), and was recently employed for studies in small subsets of hematopoietic cells [31]. Control real-time Q-RT-PCR experiments with standard BM RNA showed very low intraexperimental CV (<0.5%), which confirmed the accuracy of the method in our hands. As successful Q-RT-PCR is highly dependent on the quality of RNA [22], we exposed all RNA samples to DNase treatment to avoid DNA contamination that would cause inaccurate quantification. Results of Nano LabChip analysis performed on the Agilent 2100 Bioanalyzer demonstrated high quality of total RNA obtained from sorted SP cells, indicating that enrichment by high-speed flow cytometric cell sorting induces little or no RNA degradation. Similar results of clock gene expression and colony-forming ability of BM samples taken before and after sorting showed that high-speed sorting procedure did not influence on clock gene expression profile or cell clonogenicity. We believe this method can be employed for circadian variation studies as well as for chronobiological experiments when collecting and proceedings of the samples cannot be done simultaneously (delay period for 12 or even 24 hours).

We showed the clock gene activity in SP cells, and found pronounced relative differences compared to the whole BM for *mPer1* and *mCry1*. In stem cells *mPer1* showed the highest expression, while it was similar to *mPer2, mBmal1,* and *mClock* in the whole BM. In SP cells, *mCry1* was expressed at the same level as the others genes, except *mPer1*, while its expression was higher in the whole BM compared to the other genes. It should be taken into consideration that this was a comparison of the relative values of gene activities, not the absolute quantities. Because we run single assays for each gene expression analysis independent of each other, we cannot exclude the possibility that Q-RT-PCR efficiency could differ between assays. However, standard curves included in each run indicate that all assays in our study were efficient. Although small differences in

Q-RT-PCR efficiency could result in slight errors in quantity comparison, the comparison of relative values could be performed. Peculiarities of clock gene activity in primitive stem cell fraction may indicate that these cells have a local regulatory control. Future studies on circadian gene expression variations in different BM fractions are now possible using improved methodology.

Our results suggest that the described methodological approach combining flow cytometric high-speed sorting of SP cells with Q-RT-PCR is useful for clock gene expression analysis and circadian variations studies in small stem cell fractions.

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