# Time-dependent clock gene expression in mouse and human stem and progenitor cells

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Dissertation for the degree Philosophiae Doctor (PhD)

at the University of Bergen, Norway

2007

ISBN 978-82-308-0341-7
Bergen, Norway 2007

Printed by Allkopi Ph: +47 55 54 49 40

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*To my parents*

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## ABBREVIATIONS

ABCG2 - ATP-binding cassette transporter G2;

BM - bone marrow;

CK – Casein kinase;

Hoechst - Hoechst 33342;

Q-RT-PCR - quantitative real-time polymerase cell reaction;

$LIN^-$ - lineage negative;

SCN – the suprachiasmatic nucleus;

SP - side population

# PREFACE AND ACKNOWLEDGMENTS

The research project presented in this thesis was performed in the Section of Pathology of the Gade institute at the University of Bergen during the years 2000 - 2006. However, my first visit to this Department occurred more than 14 years ago. At that time I had graduated from the Medical University in Krasnodar and finished three years of postgraduate courses at the Institute of Immunology in Moscow. After I defended my thesis for the Candidate of Medical Sciences degree, I returned to my home city and started an academic career in the Department of Pathophysiology at the Medical University. Although I had been enjoying teaching, I wished to become more involved in research. A good opportunity occurred very soon. In 1992, I was invited to give a talk at the Congress of Immunorehabilitation in Dagomys, Russia, which was organized by professor Revaz Sepiashvili. I appreciate that he introduced me to professor Ole Didrik Laerum, who was a keynote speaker at the Congress. We discussed some research possibilities with professor Laerum and he suggested that I visit his laboratory in Bergen. At that time, professor Laerum, as the Rector of the University of Bergen, and Academician Rem Petrov, Vice-President of the Russian Academy of Sciences, were establishing a collaboration between their institutions. On the recommendation of Rem Petrov, which I appreciate very much, I was invited to Bergen for a three-month stay. The visit was successful and resulted in a paper being published in 1994 (Tsinkalovsky and Laerum, 1994). This inspired us to continue the collaboration, although it took some time to arrange my next visit.

In 2000, I came to Bergen again to start a PhD in professor Laerum's lab, where we created a Stem Cell Research Group, which has been a part of the Norwegian Stem Cell network since 2004. I express my sincere thanks to Ole Didrik Laerum. Meeting him changed my life in many ways. He inspired me to start work in the stem cell field, to learn flow cytometric techniques and to move to Norway. His passion in chronobiology has had a great influence on my research for several years. His supervision, optimism, and constant support were absolutely critical for this project. Lastly, I greatly appreciate his kind senior friendship.

My PhD was organized under an umbrella of the Centre for International Health. I thank professor Bernt Lindtjørn, the Director of the Centre, and Jon Dahl, the former Head of the administration, for their support. I also address my sincere gratitude to Solfrid Hornell for her kind secretarial help and invaluable advice. My special thanks go to Arvid Kleppe, Senior Executive Officer at the Department for Foreign Students at the University of Bergen, for continued support and helpful advice.

While working at the Gades Institute I met many nice people, both researchers and medical doctors, and I appreciate very much the friendly environment and nice working conditions. I especially wish to sincerely thank Gyrild Saldias, who provided excellent secretarial assistance, moral support and gentle kindness. I also keep warm memories of the previous secretary at the Gade Institute and a good friend, Aud Isaksen, who I met during my first visit to Bergen. We all regret her death in 2001.

I have appreciated very much the inspiring discussions and cooperation with my research colleagues and friends, Dana Costea, Jian Wang, Heike Immervoll and Per Øystein Sakariassen. Their good companionship has given continued moral support through many challenging research tasks. I am also grateful to several professors at the University of Bergen, including Anders Fjose, Rolf Bjerkvig, Frits Thorsen, Anders Molven and Hans Geir Eiken, for stimulating collaboration.

I thank Dr. Francis Lévi and Dr. Elisabeth Filipski from the Laboratory of Cancer Chronotherapeutics, Hospital Paul Brousse, Villejuif, France, for organizing my stay and experiments in Paris in 2002.

I especially owe a debt of gratitude to Benedikte Rosenlund and Robert Sothern, my unique research co-workers, whose contribution to this project was critical. Their professional skills and friendship have maintained my inspiration to continue the project and provided valuable moral support. Benedikte Rosenlund, working at the Centre for Medical Genetics and Molecular Medicine at Haukeland University Hospital, patiently and diligently taught me molecular biological methods, and personally contributed significantly to the analysis of mouse and human samples. Robert Sothern, an internationally recognized expert in chronobiology at the University of Minnesota in St. Paul, MN, provided invaluable help with statistical analysis of our data, creating figures and preparing manuscripts. I appreciate our stimulating discussion of the data and their interpretations. His great knowledge and experience in the field of chronobiology, as well as his writing skill, have considerably improved the articles. I am also grateful for his critical suggestions related to my thesis and the stylistic corrections of this text.

Much of my work was related to the flow cytometer sorter, MoFlo, so I would like to thank those who helped maintain this complex and delicate instrument. Gjert Bakkevold from Medical Research Centre the University of Bergen provided excellent managerial assistance in important financial issues related to the instrument. The experts, Gunnar Bøe at the Medical Technical Department of Haukeland Hospital, and Phil Whittle and Steven Lemoenic

from Dako, provided continuous and efficient technical support of the MoFlo, and greatly expanded my “technical” experience in flow cytometry.

I am especially grateful to Jon Sjursæther, the Head of the Department of Transport and Laundry at Haukeland Hospital, for his financial and moral support. During 4 years he has kindly provided me with a part-time job, which was especially critical during the first two years of my stay in Bergen. I have also learned from him about Norway, its culture and traditions.

I would like to thank my mother, Irina, a medical doctor, and my father, Rostislav, and uncle, Igor, (both were professors of medicine) for advising me on selecting medicine as my specialty and who continue to serve as an inspiration as I pursue research.

Finally, I am sincerely grateful to my wife, Raya, for her exceptional patience for my chronobiologically-shifted schedule during these years in research, and for her understanding and support of my work.

The project was supported by the Norwegian State Educational Loan Fund through the Quota Programme; the Stem Cell Research Program, the Norwegian Research Council; and the Aurora Program.

Bergen, 03.11.2006
Oleg Tsinkalovsky

# GENERAL INTRODUCTION

Time is a fundamental part of all biological processes. During the whole process of evolution, living cells and organisms had to adapt to cyclic variations in the environment. In particular, the light and temperature conditions varied with day and night and with the seasons. The cellular functions and behavior in organisms were regulated according to their daily needs and provision of optimal conditions for survival.

Time functions in cells are of two kinds. One is the cyclic variation, where the same events come back with regular intervals. Thereby, cellular adaptation occurs in a cyclic manner. The second way is longitudinal time regulation. From conception on, the development of organs occur sequentially and strictly coordinated in time until a mature fetus is ready for delivery. The periods of gestation, infant, growth and maturity, as well as aging, are strictly regulated in all higher organisms. Total life span is also rather constant within each species, although there are differences of many fold between short-lived and long-lived species. Thus, all the different longitudinal time periods in the life within a species are strictly coordinated to each other and are in concordance with total life span.

Time regulation in single cells occurs at shorter intervals, both with cyclic and non-cyclic variations. The oscillations may be down to a few seconds or even parts of seconds, such as nerve pulses. Heartbeat in higher organisms usually occurs as a rhythm of about one second or less, respiratory rhythm is slower, while the rhythm of the blood pressure is according to day and night. As a result, many types of time regulation and adaptation are occurring simultaneously in the same organism. Until a few years ago, the study of such time keeping was largely confined to the observation of phenomena from the outside and with mainly a descriptive approach. This has been replaced by a deeper understanding of the underlying biology and regulatory mechanisms at the genetic level (for recent review see Koukkari and Sothern, 2006b).

This thesis deals with the hematopoietic system in mice and in men, a highly adaptable tissue with a high cell turnover and many different functions that cover all parts of the body. It is therefore no wonder that time keeping is an important part of the regulatory circuits. The main emphasis of this thesis is on the elucidation of local clock functions in cells of different stage of maturation, and discussion on how the molecular clock in bone marrow (BM) is unique compared to other tissues, as well as different between species.

## Hematopoiesis in general

During the last 40 years, extensive research has given a complex picture of the hematopoietic system. A total of 1.5-2.5 kg of hematopoietic tissue resides in the spongious part of bony tissue in the body and functions as a unity. Thus, the red BM has about the size of the liver, or more. A magnitude of nearly $10^{11}$ mature neutrophilic granulocytes are mobilized daily from the BM to peripheral blood. Turnover time for the whole BM is of a magnitude of two weeks, whereby most of the cells in the marrow are replaced at least twice every month. The half-life of granulocytes in peripheral blood is only about 7 hours in man, after which they leave the blood stream by penetrating the vascular wall and enter into peripheral tissues. Here they exert their final functions of phagocytosis and degradation of foreign material for a couple of days until they die. This rapid turnover of the cells is totally dependent on a correspondingly high rate of cell proliferation in the marrow (reviewed in Benestad and Laerum, 1989). The granulocyte numbers in peripheral blood are not constant. They vary within the day and in relevance with the physiological state of the organism. Thus, exercise or food administration rapidly mobilizes granulocytes into circulation. Pathological conditions induce changes that are even more pronounced. For instance, an acute infection can increase the number of peripheral blood granulocytes within a few days by a magnitude of up to 10. In addition, the BM functions as a reservoir for storing mature cells which can be released according to the actual needs.

Several types of blood cells are formed from specific precursor cells. These originate from progenitor cells, which are committed to one of the different cell lines in the marrow: granulopoiesis, erythropoiesis, megakaryopoiesis and lymphopoiesis. Progenitor cells are immature cells with high proliferative capacity, and most of them are actively cycling, producing new progeny. The development of the precursor cells occurs through a stepwise maturation, where each cell division is accompanied by a corresponding transcription of the genetic program for this particular cell type, ending with mature cells capable of exerting specialized functions. At this end stage, most of the mature cells have lost their ability to proliferate, such as erythrocytes and granulocytes, as well as megakaryocytes, and to some extent also monocytes and lymphocytes. Rapid cell division ends with loss of proliferative capacity. Hematopoiesis, therefore, consists of a rapid proliferation within strict limits. Progenitor cells are derived from stem cells, which in addition to unlimited cell proliferation

are able to produce new cells that can be committed for any of the cell lines in the hematopoietic tissue. This will be dealt with in the next section.

The hematopoietic system develops from the yolk sac. Later, the hematopoietic activity in the embryo moves to the liver, while after birth the BM takes over (Keller et al., 1999). BM stromal tissue has a significant influence on hematopoiesis. Stromal cells are important for anchoring of the hematopoietic cells, as well as for their nutrition and regulation by providing a microenvironment and producing growth factors and cytokines (Tavassoli and Friedenstein, 1983; Wolf, 1999). Stromal cells may support hematopoiesis in vitro, both as primary cells and as cell lines (Loeuillet et al., 2001).

The high plasticity of the hematopoietic system requires a dynamic type of organization and multiple interactions (Roeder and Loeffler, 2002). A multitude of regulatory factors are involved in both hematopoietic cell production and differentiation including cytokines, growth factors, chemokines, polypeptides, and oligopeptides (Rameshwar et al., 1997; Sachs, 1992; Thomas et al., 2004; Wolf, 1979). Several hormones and other cellular factors are involved in the expression of various specific genes and their products (Li et al., 2003), including the key cell cycle control genes. In addition, the BM has a high density of efferent nerves (Calvo and Forteza-Vila, 1969), indicating that the nervous system may also be involved.

Hence, hematopoiesis is a complex process regulated by many different factors. Its high proliferative activity combined with plasticity enables rapid adjustment to any alteration in the needs of the body. Due to the high ratio of cell production and release, hematopoietic tissue is completely dependent on continuous input of newly formed cells from the pool of stem cells.

## Properties and functions of stem cells

By definition, a stem cell is a cell with two main properties: it is capable of an unlimited number of divisions, and it can give rise to all cell types within a tissue (Weissman, 2000). Usually stem cells are in a resting state with low proliferative activity, but can, when activated, have a progeny of more than a million cells within a week. Once a stem cell enters differentiation into one or several specific cell types, it has been considered as primarily an irreversible process. To what extent this is really so, is at present a matter of debate. Possibly some cells with differentiated properties may still be able to exert stem cell functions.

There is a hierarchy of three levels in stemness: (1) totipotent stem cells, which can give rise to a new individual, including the placenta; (2) pluripotent stem cells, which can make at least two different tissues; (3) multipotent stem cells, which can develop into all types of cells within a tissue.

The next level in this hierarchy is a progenitor cell, which is already committed to become the ancestor of one cell type. Until a few years ago, "stem cell" was the denomination of these two functions, and very little was known about its morphology. With modern biological methods, the stem cell surface antigens and other biological properties have been characterized. Thereby, they can be specifically sorted out and analyzed morphologically. In consequence, this has led to experiments where the progeny of one single stem cell can be studied (Krause et al., 2001; Osawa et al., 1996). The development of flow cytometry and sorting methods has greatly contributed to the understanding of stem cell functions and other characteristics (see below).

With new methods for studying the clonogenic potential of cells in vitro as well as in vivo, the occurrence of tissue-specific stem cells has been studied all over the body. Almost an explosion in the numbers of articles on this issue has resulted, and in addition, the therapeutic potential of stem cells for reconstituting damaged tissues has been explored (Gammaitoni et al., 2003). However, the limited potential for growth and renewal *in vitro* has so far been an obstacle to extensive clinical use. The use of supporting tissues, specific growth and chemotactic factors, as well as other factors in the microenvironment transferred to *in vitro* may be promising tools for overcoming this limitation (Moore et al., 1997; Zhong et al., 2004). Telomerase activity may be critical for this self-renewal, although the limitations of the *in vitro* environment have so far not been solved (Morrison et al., 1996).

Although the concept that single immature cells could give rise to all types of hematopoietic cells was developed in the 1950s, more systematic attempts of isolating and characterizing of stem cells first came with the development of flow cytometry and flow sorting starting in the 1970s (van Bekkum et al., 1985; Visser and de Vries, 1994; Weissman, 2002). Later, it was recognized that both in the unperturbed and irradiated mouse, sequential recruitment of stem cells in the marrow was responsible for the maintenance of steady state hematopoiesis (Drize et al., 2001). Then, the subclasses of progenitors in BM (e.g., responsible for lymphopoiesis) were further characterized (Kondo et al., 1997).

By use of combinations of different methods, pluripotent stem cells have during the last 5-10 years been purified from the brain (Uchida et al., 2000), the skin (Toma et al., 2001), the lung (Kim et al., 2005) and some other tissues (Clarke, 2005; Qu-Petersen et al., 2002; Yu

et al., 2006). Furthermore, specific neoplastic stem cells with pluripotent properties can be isolated from human tumors (Al-Hajj and Clarke, 2004; Collins et al., 2005; Kim et al., 2005; Singh et al., 2004). An important part of this scenario is the identification of acute myeloid leukemia as a stem cell disease, wherein a subpopulation of the neoplastic cells retain stem cell functions (Lecuyer and Hoang, 2004).

In recent years, particular attention has been paid to the possibility that hematopoietic stem cells, being essentially mesodermal in origin, can be harbored by other tissues (Lecuyer and Hoang, 2004). Thus, hematopoietic stem cells have been isolated both from brain and skeletal muscle (Jackson et al., 1999). Consequently, numerous attempts have been made to inject hematopoietic stem cells into other tissues both in the steady state and after injury and in the course of regeneration. Hematopoietic stem cells seem to be able to convert to other tissues, such as skeletal muscle in cell culture (Abedi et al., 2004), as well as develop *in vivo* into myocytes of skeletal or heart muscle (Muguruma et al., 2003), or hepatocytes (Lagasse et al., 2000). However, to what extent this phenomenon can be explained by a real plasticity at the single cell level, the cell capability for "re-programming" and/or "transdifferentiation", or it has occurred due to the process of fusion with local differentiated cells, is not yet settled (Camargo et al., 2003; Krause, 2002; O'Malley and Scott, 2004; Wagers and Weissman, 2004). Although more studies have to be done to describe the mechanisms of this phenomenon (Wagers and Weissman, 2004), stem cell plasticity in general seems to provide an important tool for inducing local repair in damaged tissues of different origins (Poulsom et al., 2002).

One important reason for the large body of data on hematopoietic stem cells is their accessibility from various sources. In contrast to most other tissues in humans, they can easily be harvested from peripheral blood, from fetal tissues and umbilical cord blood, as well as by sequence sampling of the marrow cavity in the skeleton (Huang et al., 1998). These cells can be serially transplanted in immunosuppressed rodents and be easily traced for comparison between short term and long term repopulating cells of the perturbed host (Lanzkron et al., 1999; Spangrude et al., 1995). The cells can also be used for clinical purposes in man. Human and rodent stem cells express characteristic surface antigens (Spangrude et al., 1988), in particular CD34 (Guo et al., 2003) (although the earliest progenitor cells in a resting state may be negative for this marker), c-kit (Kawashima et al., 1996), sca-1 (Spangrude and Scollay, 1990), but not CD38 (Poznansky et al., 2001) (**Figure 1**). The combination of these markers is successfully used for flow cytometric cell sorting (Spangrude et al., 1988). In addition, stem cells have a low number of mitochondria (confirmed by low rhodamine 123

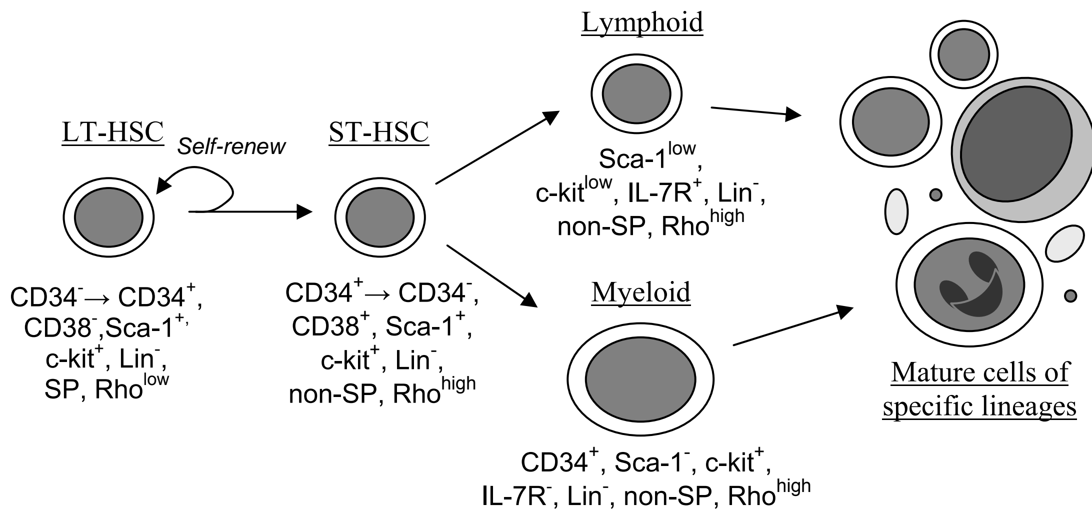


**Figure 1. Lineage negative (Lin$^-$) long- and short-term repopulating hematopoietic stem cells (LT-HSC and ST-HSC, respectively) and precursors can be separated by expression of surface markers and staining with Hoechst and Rhodomine (Rho).** Most primitive HSC in Hoechst-stained bone marrow are contained in the side population (SP, see also Figure 2), and are characterized by low Rho fluorescent.

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uptake) (Uchida et al., 1996), and they are able to pump out the vital dye Hoechst 33342 (Hoechst), constituting a so-called side population (SP) cells, enabling their specific flow cytometric cell sorting (Goodell et al., 1996) (also see later).

Extensive attempts have been made to characterize the gene expression pattern responsible for “stemness”. Thus, for example, several home box genes have been identified as important for the development of primitive hematopoietic stem cells (Akashi et al., 2003; Terskikh et al., 2003; Zhou et al., 2001). However, surprisingly, a single gene expression studied by quantitative real-time polymerase cell reaction (Q-RT-PCR), as well as different types of microarrays, have not yielded a consistent pattern (Lemischka, 2001). Thus, there were considerable differences between embryonic and adult stem cells, and also between the different sources, including human and mouse stem cells (Lu et al., 2002). Therefore, there has been an urgent need for finding a more consistent pattern of key gene expression and not only a wide variety of stem cell-related gene expression, which may be more or less global. It also remains to be shown to what extent there is a dynamic variation in this gene expression, both with time and with the functional state of these cells.

## Flow cytometry and high-speed cell sorting: application in hematopoiesis

Flow cytometry means quantitative and qualitative measurements of single cells in suspension that pass through a light beam with high velocity. The measurements both include the light scattering that reflects the size and optical properties of the cells, and the absorption of light and emission of fluorescence from cell components that have been stained with a fluorescent dye (Shapiro, 2003b). Thereby, multiple cell parameters can be measured simultaneously, and by a combination of photodetectors and computer technology, these parameters can be analyzed in relation to each other. The flow of cell suspension can be broken up into electrically charged droplets after leaving a nozzle and having passed the light source. Cells containing specific signals can then be sorted out by passing a static electrical field (Shapiro, 2003a).

The first commercial flow cytometers became available in the early 1970s. These were both built as analyzers and as cell sorters by use of electrostatic deflection of charged droplets. However, a limiting factor was their rate of measuring or sorting, which usually was less than 1000 cells (events) per second. Consequently, it took a rather long time to obtain a high number of cells, which, for example, could be used for isolating RNA and studying gene expression. Especially when sorting out rare subpopulations, in many cases it was impossible to obtain a sufficient amount of cells for molecular biological analysis. A great step forward was the introduction of commercially available high-speed cell sorters in the early 1990s. By combining high pressure and high velocity of cell flow with several coupled photodetectors, the rate of sorting and analysis could be increased by a factor of 10-20 (van den Engh, 2000). Hence, using modern instruments, it is possible to sort routinely with speeds of 20-30,000 cells (events) per second (Tsinkalovsky et al., 2005), keeping a high purity of sorted cell populations (**Figure 2**). It should be noted, however, that sorting speed depends on several parameters, such as cell source, type, and quality of the sample. Thus, flow sorting has moved from being an end-stage analytical tool to the combination of multiparameter analysis and based on this, isolating and further processing the cells for other types of methods, including cell culture studies. The sorting procedure can be accelerated by using immunomagnetic beads as a pre-enrichment step for cell isolation. For instance, these beads can be used for removing cells expressing lineage-specific markers, by so-called “lineage negative” ($Lin^-$) selection. Another useful combination of these techniques is the application of immunomagnetic beads for selecting a specific cell population (for instance $CD34^+$ cells)

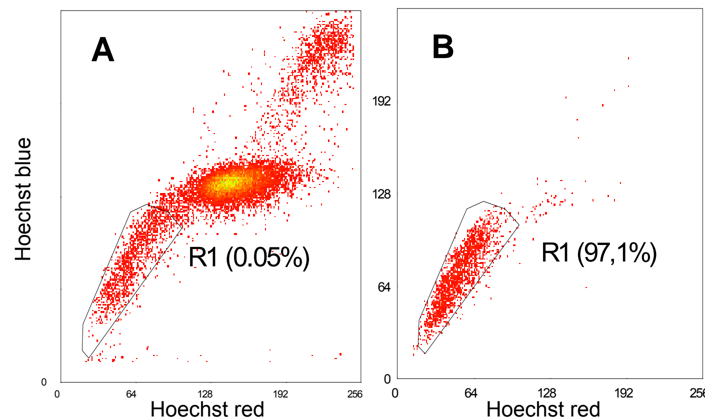


**Figure 2. The flow cytometric analysis of cells displayed in region 1 (R1, A) after sorting (B) shows a high purity of chosen population.** Side population (SP) cells from Hoechst-stained mouse bone marrow were isolated in this experiment.

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with further analysis of isolated cell fraction purity by flow cytometry.

Rather early, flow cytometry was used in hematopoiesis as a method of choice for analyzing the heterogeneous BM populations (Visser, 1992). Later, it was developed to become an everyday tool for studying both normal and pathological marrow (Knapp, 1992; Smaaland et al., 1992).

An extensive survey of different applications of flow cytometry in hematology would be outside the scope of this introduction. However, it should be emphasized that flow cytometry has served as the main tool for isolation of primitive hematopoietic stem cells (Spangrude et al., 1988; Spangrude and Scollay, 1990; Visser, 1992). The employment of flow cytometry allows enrichment for quite primitive hematopoietic stem cell subsets using a combination of specific surface markers mentioned before (see previous section). Thus, sorting of $Lin^-$ cells positive for c-kit, sca-1 and Thy-1 low (mice), or $CD38^-$ subset of $CD34^+$ cells (human) will represent stem cells with long-term reconstituting ability tested in lethally irradiated host mice.

Another flow cytometric approach for hematopoietic stem cells isolation is based on the analysis of the functional properties of cells. One of the most useful methods is detection of the efflux of the DNA-binding vital dye Hoechst. This method, which was originally developed by Goodell et al. (1996) for isolation of mouse BM hematopoietic stem cells, yielded a distinct SP that displayed very low blue/red fluorescent emission intensities. This property depends on active efflux that is determined by the specific expression of the ATP-binding cassette transporter G2 (ABCG2) in these particular cells (Zhou et al., 2001). The SP was shown to be highly enriched for primitive stem cells with long-term reconstitution ability

(Goodell et al., 1996) and was suggested as a marker for quiescent hematopoietic stem cells with anti-apoptotic properties (Arai et al., 2004). Additional studies have demonstrated that SP cell purification can be used as a general method for enrichment of stem cells from normal hematopoietic tissues of mammalian species, including humans (Bhattacharya et al., 2003; Goodell et al., 1997). Interestingly, SP cells are also present and exhibit stem cell properties in several nonhematopoietic tissues (reviewed in Challen and Little, 2006). Hence, the SP phenotype is regarded as a specific marker for stem cells from various sources (Bunting, 2002; Zhou et al., 2001). SP cells have also been detected in some malignant tissues, where they may represent cancer stem cells (Haraguchi et al., 2006; Hirschmann-Jax et al., 2004; Kondo et al., 2004; Patrawala et al., 2005).

In conclusion, flow cytometry (also with the application of immunomagnetic beads) is an important tool for isolating and characterizing hematopoietic stem and progenitor cells with high purity and specificity.

## Chronobiology in general, circadian variations in hematopoiesis, and statistical evaluation of rhythms

Chronobiology is in the widest sense the knowledge of how the organisms adapts to the physical conditions in the environment. Most functions in the body show systematic 24-h (circadian) variations (Koukkari and Sothern, 2006b).

The central pacemaker for circadian and seasonal variations in the body is situated in the suprachiasmatic nucleus (SCN), which in the human brain is situated above the chiasmatic crossing of the optical nerves and has a diameter of a few millimeters (Bartness et al., 2001). The SCN is the principle generator of circadian rhythms and is a part of an entrainment system that synchronizes a human being or an animal with its environment. Through complex nerve tracts, the SCN is connected to the pineal gland, where melatonin secretion is an important effector. In addition, the autonomous nervous system is responsible for peripheral tissue connections. Thus, the SCN has been found to be a part of the sympathetic outflow from the brain to different organs and tissues in the body, including the adrenal gland, and seems to be related to the modulation of neuroendocrine systems connected to the hypothalamus (Bartness et al., 2001). The SCN is also involved in the parasympathetic nervous system innervations of organs as the thyroid, the liver, the pancreas and the submandibular glands.

Specific photoreceptors in the eye connect the SCN through the optical tract to the environment. The ganglion cells of the inner retina contain the pigment melanopsin, which

forms dendritic plexuses in a network that allows these cells to capture photostimuli across broad spatial domains (Ruby et al., 2002). The surrounding light is an important entrainment factor for the adaptation of the body to the environment and connects many important functions in the body to environmental cues. Alterations in the light conditions induce circadian phase shifting. The length of the day also modulates the light responses and mediates important hormonal reactions. Thus, important body functions, such as temperature, respiration, blood pressure, and heart beat, vary with day and night and with the succession of seasons (Bartness et al., 2001). The same applies to the secretion of many hormones, of which each has a characteristic pattern. For example, cortisol varies strongly with the time of the day, reaching a maximum in the early morning and a minimum during the night.

The cell kinetics of different tissues also undergo strong circadian variations, and in particular rapidly proliferating tissues such as the BM, the gut mucosa, other surface epithelia and the epidermis (Smaaland et al., 1995a). Knowledge about such rhythms has been important for the understanding of cytotoxic effects on various tissues, and in particular, side effects of cytostatic therapy to normal tissues (Laerum et al., 1995).

Is has long been known that the different parts of hematopoiesis undergo strong circadian and seasonal variations. This applies to the numbers of stem/progenitor cells and to the proliferation of precursor cells of the different cell lines in the marrow sampled from human volunteers (Smaaland et al., 2002). In addition, peripheral blood cells undergo similar variations, and for example, circulating granulocyte numbers can vary with several orders of magnitude that is largely related to variation in serum cortisol (Abrahamsen et al., 1993; Laerum, 1995). It is also known that immune functions, including the tendency to allergic reactions, undergo considerable circadian variations (Ratajczak et al., 1993; Smaaland et al., 2002). Both the clonogenicity of human CFU-GM *in vitro* and the total cell proliferation ability in the marrow are varying in parallel with a maximum during the day, roughly coinciding with the body temperature rhythm (Smaaland et al., 1992). Even the engraftment of stem cells in animal hosts has shown circadian variations, indicating that at least in mice, the time of injecting the cells may be critical for the result (D'Hondt et al., 2004). Interestingly, after transfer of murine BM progenitor cells to *in vitro*, the circadian rhythm may persist for several days (Bourin et al., 2002). It is therefore a good reason to claim that hematopoiesis largely occurs in rhythms, reflecting the renewal of hematopoietic cells as a labile pulsating organ (Laerum, 1995). Surprisingly, rhythmic variations could not be found in malignant lymphoma cells involving cervical lymph nodes (Smaaland et al., 1995b), indicating that malignant growth may be less dependent on environmental cues.

The understanding of circadian rhythms in hematopoiesis has been important for guiding cancer chemotherapy in order to enable higher doses to be given at time points of low proliferative activity. Thereby, toxic side effects due to high doses of cell cycle specific cytostatical drugs may be circumvented, giving the opportunity for administering higher doses to patients with malignant tumors unrelated to hematopoietic cells (Eriguchi et al., 2003; Levi, 2006).A critical part of identification of circadian rhythms is the use of an appropriate statistical analysis for identification of sequential patterns of variations (Koukkari and Sothern, 2006a). In the area of medicine, one of the most commonly used computerized methods is the *cosinor* analysis. Originally introduced by the pioneer Franz Halberg in 1972, it has become an important statistical tool for biological studies of rhythms (Koukkari and Sothern, 2006a). The cosinor technique involves fitting a curve to a time series of data of a predefined period (24 h is often used to approximate the circadian rhythm) by least squares linear regression. The main parameters derived from the fitted cosine include (1) a *p-value* from a zero-amplitude test, (2) the *double amplitude* 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 a defined reference point (e.g., local midnight or the previous time of light-onset during light-darkness standardization) **(Figure 3)**. Time series are also often analyzed for a time-effect by a one-way analysis of variance (ANOVA).

## Clock genes and their relevance for circadian rhythms

For several years, it was an open question how the time functions could be regulated in tissues and in cells. Over years, researchers had observed that biological time was measured by cycles that range from milliseconds to years. Circadian rhythms, which measure time on a scale of approximately 24 h, are generated by one of the most ubiquitous and well-studied timing systems. Although this was found to be timed by a master clock in the brain that coordinated tissue-specific rhythms according to light input from the outside world, very little was known about the underlying mechanisms (Reppert and Weaver, 2002).

A genetic basis for circadian timing was demonstrated about 30 years ago. The existence of clock genes was first proven in *Drosophila melanogaster, Chlamydomonas reinhardi* and *Neurospora crassa* by the isolation of circadian mutants. Later, mutations in clock genes were also identified in mammals, such as mouse, hamster and man. The *Drosophila* period (*per*) gene was the first clock gene to be cloned in 1984, and in the following years, all the currently

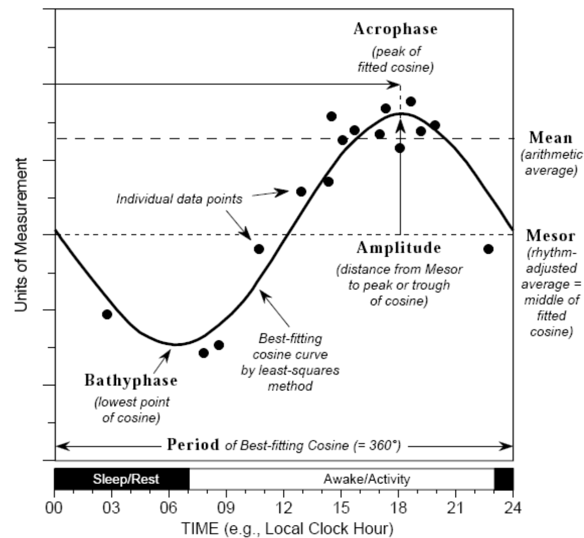


**Figure 3. Rhythmic characteristics derived from a fitted cosine** ***(created by R.B.Sothern).***

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known mammalian clock genes have been cloned. A clock gene is usually defined as one whose product is required for the generation and/or maintenance of the circadian clock.

The cloning of the clock genes had a profound effect on the understanding of time regulation in peripheral tissues and led to the identification of clock genes and their protein products in different tissues/organs. In particular, the understanding of circadian rhythmicity at the intracellular level was significantly improved, thereby giving a clearer picture of nuclear and cytoplasmic events related to 24-h oscillations. The general picture is that clock genes are ubiquitous in cells including in prokaryotes, in plants, and in lower and higher animals. During evolution, such time-keeping functions at the genetic level seemed to occur for the first time about 700 million years ago and then have gradually been developing and specializing in different species. In a broader sense, all living cells contain internal time keeping functions by which they can be in phase with the environment. In mammals, circadian oscillations of clock gene expressions have been observed not only in the SCN, but also in many peripheral tissues, such as liver, heart, kidney, lung, pancreas, skeletal muscle, oral mucosa, skin, BM, and human peripheral blood mononuclear and polymorphonuclear cells (Balsalobre et al., 2000; Bjarnason et al., 2001; Boivin et al., 2003; Bourin et al., 2002; Chen et al., 2000; Damiola et al., 2000; Muhlbauer et al., 2004; Oishi et al., 1998).

The question remained to what extent clock functions in peripheral tissues were independent or whether they were regulated by the central master clock in the SCN. Surprisingly, it has been shown that even in rat fibroblasts cultured *in vitro* for more than three decades,circadian gene expression can still be induced by using a serum shock

(Balsalobre et al., 1998). In general, the peripheral clocks seem to be phase-delayed as compared to the master clock by a magnitude of about 4 hours (Balsalobre et al., 1998). Thus, the model has been proposed that tissues have their own independent clock functions which are coordinated by the master clock. If the master clock is severely disturbed or non-functioning, these may take over as time-regulatory functions. The expression "master and slave clocks" therefore has been used for this dependence.

Traditionally, the clock gene functions and their regulation is described as a complex relationship between positive and negative feedback loops of genes and their products (reviewed recently by Ko and Takahashi (2006) (**Figure 4**). In the primary feedback loop, the positive elements include members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, CLOCK and BMAL1. These proteins form heterodimers that initiate transcription of target genes containing E-box CIS-regulatory enhancer sequences, including Period (in mice, *Per1*, *Per2* and *Per3*) and cryptochrome (*Cry1* and *Cry2*). PER:CRY heterodimers then translocate back to the nucleus and repress their own transcription by acting on the CLOCK:BMAL1 complex completing a negative feedback loop. Another regulatory loop is induced by CLOCK:BMAL1 heterodimers activating transcription of orphan nuclear receptors, *Rev-erbα* and *Rora*. REV-ERBA and RORa regulate the circadian oscillation of *Bmal1* subsequently competing for binding orphan receptor response elements (ROREs) present in *Bmal1* promoter. It has been shown that RORs activate transcription of *Bmal1*, whereas REV-ERBs repress the transcription process. The autoregulatory feedback loops take approximately 24 h to complete a cycle and constitute a circadian molecular clock. The 24-h molecular clock generation is modified by post-translational modifications, such as phosphorylation and ubiquitination. These processes significantly contribute to the precision of the mammalian clock by affecting the stability and nuclear translocation of core clock proteins. Casein kinase 1 epsilon and Casein kinase 1 delta (CK1ε and CK1δ) are critical factors that regulate the core circadian protein turnover in mammals. Mutations in CK1ε and CK1δ can have dramatic effects on circadian period.

It should be noted that this traditional concept of circadian clockwork in mammals was developed by analogy with the *Drosophila* system, and evidence in favor of this concept is still incomplete (Hastings and Herzog, 2004). Some anomalous observations that run counter to the predictions of transcription/translation oscillator model were summarized and discussed in a recent review (Lakin-Thomas, 2006). In a recent study it was shown that *Clock*, one of the core clock genes, was not required for rhythmicity (Debruyne et al., 2006). Although CLOCK-deficient mice had an altered response to light, they continued expressing robust

circadian rhythms. Hence, it is likely that the traditional model of molecular clock components interactions can be modified, at least for the mammalian system.

In mammals, many of the known clock genes have recently been experimentally disrupted. The genetic manipulations have resulted in variable phenotypes, whereby a disturbance of the circadian rhythmicity is obtained. For example, m*Per3*-deficient mice exhibit about ½ hour shorter periods than control animals. Total arrhythmicity has been observed in m*Bmal1*-deficient mice (for review see Balsalobre, 2002). A study of circadian rhythmicity in triple mutant mice lacking m*Per1*, m*Cry* and either m*Clock* or m*Bmal* revealed that certain combinations of m*Per* and m*Cry* are necessary to drive the circadian clockwork. The presence of a single gene alone (e.g., m*Per1* or *mCry1*) does not seem to be sufficient to induce rhythmic expression of downstream genes, but in this case, light-dark conditions may drive the rhythmic oscillator of some output genes (Oster et al., 2003).

The proteins coded by clock genes and their protein heteromers are transcription factors expressed as a circadian cycle. In the widest sense of the word, a clock gene therefore denotes a transcription factor responsible for time regulation of other gene(s) as a more or less cascade effect (Balsalobre, 2002). These findings are largely based on the studies of neurons in the central nervous system and, in particular, the SCN. Thus, the clock genes were shown to constitute an important effector function related to input nervous signals induced by light effects of the retina.

The use of systematic gene expression studies has given important information about output components of the clock system. Peripheral clocks seem to regulate the temporal variations in diverse biological processes by modulating so called "clock-controlled genes". In a comparative analysis of circadian gene expression in mouse liver and heart representing 12,488 genes, it was found that 8-10% of the genes were expressed rhythmically (Storch et al., 2002). It is important to note that only a minor overlap of oscillating genes between tissues was found in this study.

It is probable that most of clock-controlled genes are not directly regulated by clock genes, but rather by transcription factors that accumulate or are controlled in a circadian manner (Balsalobre, 2002). An interesting example of such a directly regulated gene is *Dbp*, which oscillates in many tissues including the liver. *Dbp* encodes a transcription factor that controls the circadian expression of several digestive enzymes in the liver (Lavery et al., 1999). It was suggested that the *Dbp* expression is directly controlled by

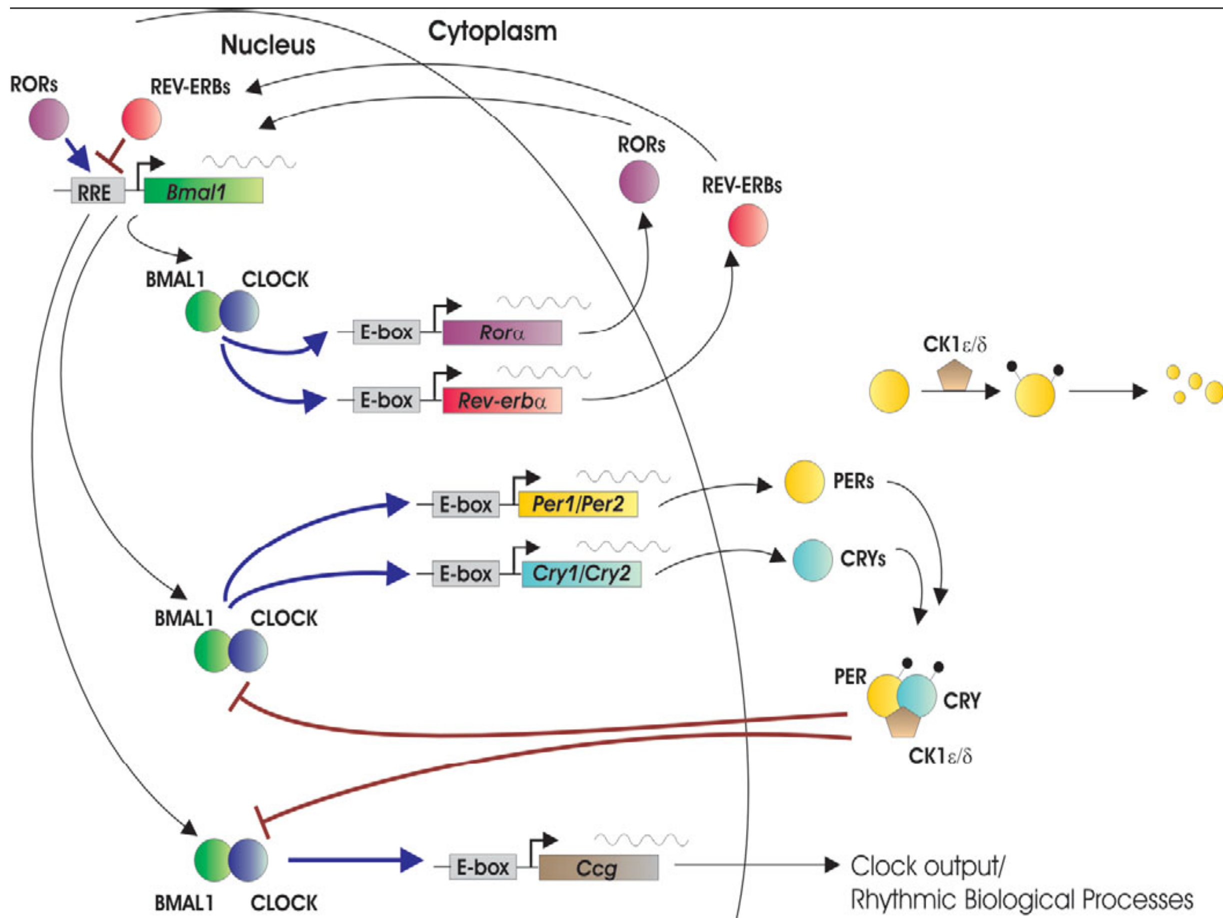


**Figure 4. Transcription/translation oscillator model of the molecular clockwork.** A complex of CLOCK and BMAL1 proteins activates transcription of *Per1/Per2, Cry1/Cry2, Rev-Erbα* and *Rorα* genes. A complex of PERs and CRYs proteins inhibits the positive effects of CLOCK:BMAL1. REV-ERBα and RORα proteins modify transcription of *Bmal1*. Casein kinases (CK) are regulatory factors for PER and CRY proteins. CLOCK:BMAL1 can also regulate expression of clock control genes (*Ccg*).

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CLOCK:BMAL1 heterodimers through an E-box (Gachon et al., 2004) **(Figure 4)**. It is therefore likely that one common core oscillator in peripheral tissue may regulate many tissue-specific clock-controlled genes by directly modulating a relatively small number of tissue-specific circadian transcription factors that would then regulate multiple targets (Balsalobre, 2002).

The question can be raised to what extent local clock gene expression can be regulated by input signals other than from the central master clock. This also seems to be case. For example, it was shown that the DNA-binding activity of CLOCK:BMAL1 heterodimers were drastically influenced by the ratio between the amount of reduced and oxidized forms of nicotinamide adenine dinucleotide, NAD (Rutter et al., 2002). Since these ratios are affected

by cellular metabolism, restricted feeding may thus directly reset peripheral clocks through metabolic activity. Temperature and temperature changes effect on circadian clocks was shown in cyanobacteria, unicellular algae, plants, fungi, arthropods, and vertebrates (reviewed in Rensing and Ruoff, 2002).

Therefore, clock genes seem to be an integral part of local feedback regulation in cellular functions, both by constituting and modifying oscillations according to local and central needs.

## Development of chronobiological studies of hematopoiesis at the Gade Institute

An important basis for the present thesis has been more than 25 years of systematic research on circadian and circannual variations in hematopoietic tissues. Thus, Nils Petter Aardal defended his thesis on circadian patterns in the BM of mice, showing rather labile variations of very high magnitude (Aardal, 1983). Olav Sletvold later followed this up by studying how this circadian pattern varied with the age of the animal (Sletvold, 1988). Thus the aging process in mice was accompanied by both lowered amplitude and alterations of the phasing. Robert Sothern in his thesis showed how the application of chronobiology could reduce the toxic effect of drugs and chemotherapy, and improve anticancer treatment (Sothern, 1992). This was followed by studies on humans by Rune Smaaland. He showed that there were strong circadian variations in hematopoietic tissues, based on sequence sampling from male volunteers (Smaaland, 1994). In particular, cell cycle distribution varied with the circadian period, and as already mentioned, in parallel with clonability of myelopoietic progenitor cells. In the work by Smaaland. a statistically significant circadian rhythm was found in cell-cycle variations (e.g., DNA S-phase) for Non-Hodgkin's lymphomas (Smaaland et al., 1995b). However, at that time nothing was known about effector functions in the cells, including clock genes and timing at the cellular level. This prompted us to pursue a similar study on clock gene expression.

In addition to the studies on the total bone marrow population and their progenitor cells, it was of particular interest to know how the precursor cells varied, and if this was in accordance with the other circadian rhythmic patterns. This was addressed in a thesis by Jenny Foss Abrahamsen, combined with systematic methodological studies of the sampling technique (Abrahamsen, 1997).

Flow cytometric measurements have been an important tool in the studies included in all of these theses, combined with other methods. The Gade Institute started work on flow

cytometry in 1975 and was the first laboratory in Norway that employed the method on clinical material. This included sequence studies on leukemia treatment, as well as the development of a new method for quantitative and qualitative studies of phagocytosis (Aardal et al., 1979; Bassø, 1983; Bjerknes, 1984).

High-speed cell sorting was first started in Norway in 2000.

# PROBLEM FORMULATION AND AIMS OF THE STUDY

Both hematopoiesis in general and stem/progenitor cells in particular are subjects to a multitude of influences. It has been demonstrated that many hematopoietic functions in rodents or humans are regulated in a circadian manner. Clock genes have been shown to play an important role in different tissues but have only been scarcely studied in hematopoiesis. On this background, the following aims of the present study were formulated:

1. To create and standardize the protocol for gene expression analysis in hematopoietic cells, including primitive stem cells and following preparative high-speed cell sorting (Article I).

2. To study clock gene expression variations in mouse whole BM population and the fraction of primitive stem cells using the advantage of high-speed flow cytometric analysis and cell sorting (Article II).

3. To study clock gene expression variations in human BM stem/progenitor cells (Article III).

## LIST OF ARTICLES I – III

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

II. Tsinkalovsky, O., Filipski, E., Rosenlund, B., Sothern, R.B., Eiken, H.G., Wu, M.W., Claustrat, B., Bayer, J., Levi, F., and Laerum, O.D. (2006) Circadian expression of clock genes in purified hematopoietic stem cells is developmentally regulated in mouse bone marrow. *Exp Hematol* **34**:1248-1260.

III. Tsinkalovsky, O., Smaaland,R., Rosenlund, B., Sothern, R.B., Hirt, A., Steine, S., Badee, A., Abrahamsen, J.F., Eiken, H.G., and Laerum, O.D. (2007) Circadian variations in clock gene expression of human bone marrow CD34-positive cells (*J Biol Rhythms* **in press**).

# GENERAL DISCUSSION

## Methodological considerations

### *Stem/progenitor cell isolation by magnetic beads and flow cytometric sorting*

As the present lack of data on circadian gene expression in hematopoietic stem/progenitor cells could partly be explained by methodological difficulties we first aimed at improving and standardizing the protocol for gene expression analysis in these cells (**Figure 5**).

In the study on mouse BM, we were able to target rare stem cell fractions for analysis, as it was possible to collect initially more BM cells by increasing the number of experimental animals. For detection of mouse hematopoietic stem cells, we employed a single step Hoechst staining method (Goodell et al., 1996). This simple and cheap method has been used extensively for purification of mammalian HSCs, and the SP phenotype was clearly shown to be a specific marker of primitive stem cells with long-term reconstitution ability (Goodell et al., 1996; Goodell et al., 1997). It should be noted, however, that technical issues in cell preparation and staining (cell number, dye concentration, duration of staining) and in flow cytometric analysis (filter setup, laser alignment) are known to influence SP and could cause some discrepancies in data interpretation (Montanaro et al., 2004). In our lab using the basic protocol of Goodell et al. (1996) for staining of mouse BM cells, we obtained results very similar to others (Article I). In our study, SP cells represented $< 0.07\%$ of the cell population, and were senstive to ABCG2 transporter inhibition by verapamil, reserpine or a fumitremorgin C. Approximately 80% of SP cells also expressed the early hematopoietic progenitor antigens sca-1 and c-Kit (Article I). High-speed flow cytometric sorting was chosen as a method for SP cell isolation. For the first time we performed a colony-forming assay of sorted SP cells (**Figure 6**) and showed that these cells were more than 75 times enriched in clonogenicity as compared to whole BM (Article I). This confirmed that the SP isolated in our lab was highly enriched for hematopoietic stem cells.

As a target cell fraction in the experiments on human BM hematopoietic cells, we chose a population of $CD34^+$ stem/progenitor cells. These cells were magnetically separated from low-density mononuclear cells using a Direct CD34 Progenitor Cell Isolation Kit and $MS^+$ Column (from Miltenyi Biotec, Bergisch Gladbach, Germany). 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 routinely for clinical

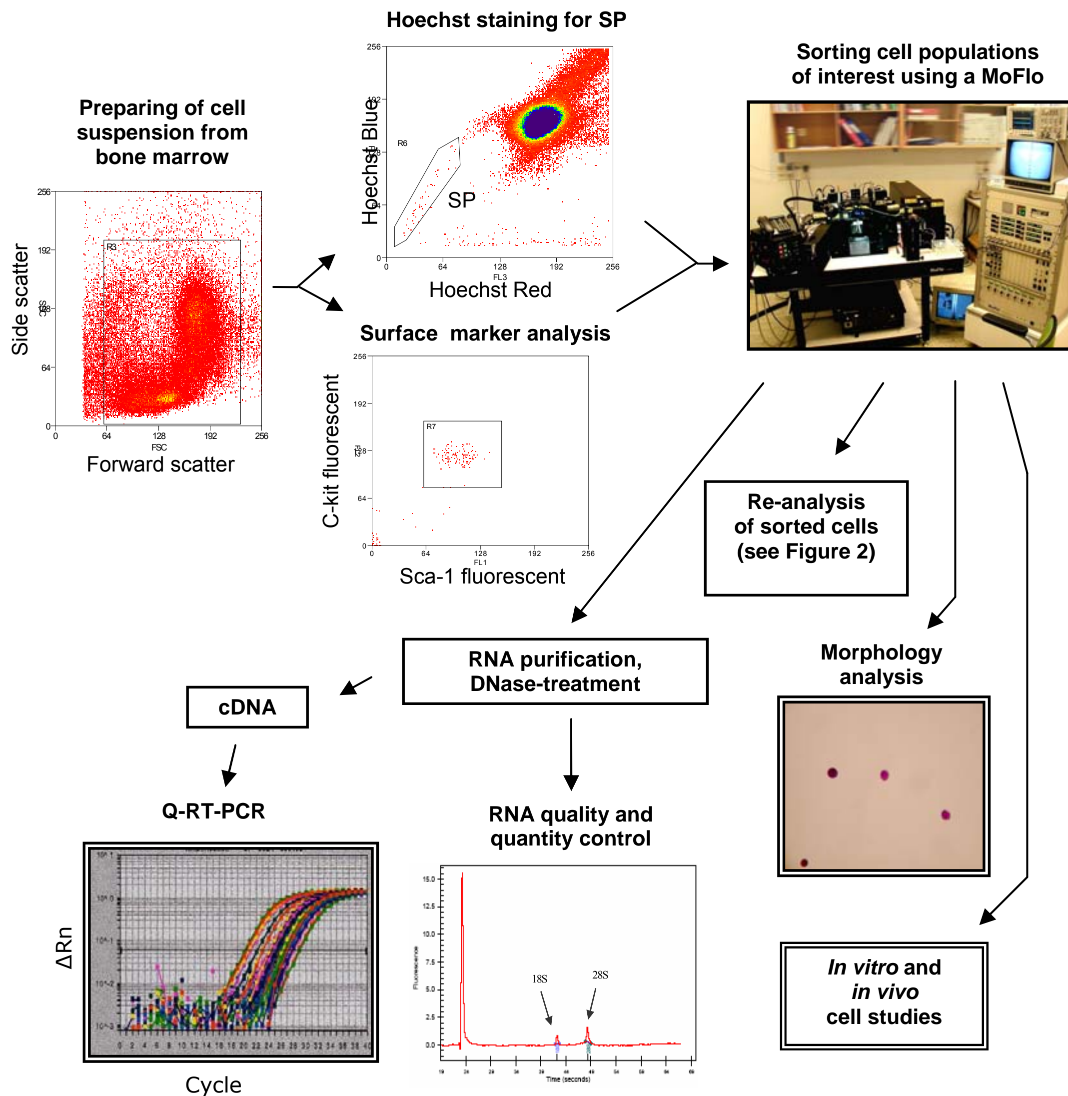


**Figure 5. Methodological approach for combination of flow cytometric, morphological and molecular analysis of hematopoietic stem cells.** Sorting of cells based on surface marker expression and Hoechst efflux revealing side population (SP) allows fast collection of pure cell fractions for further morphological and morphological analysis, as well as for *in vitro* and *in vivo* studies. By increasing the velocity of the cell flow by a magnitude of several fold, automated cytometric cell analysis and sorting can be used for preparative purposes and not only as an analytical tool.

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applications (Handgretinger et al., 1998). Getting further enrichment in primitive stem cells (e.g., isolation of $Lin^-$ $CD34^+CD38^-$ subpopulations, rhodamine low fraction or SP) will also considerably reduce cell numbers. In the experiments with sampling of human volunteers, this was not technically possible and ethically not advisable. The flow cytometry in this study was used for evaluation of isolated cell fraction purity that in these experiments ranged from 70.3 to 95.6 %, median 82.3 % (Article III).

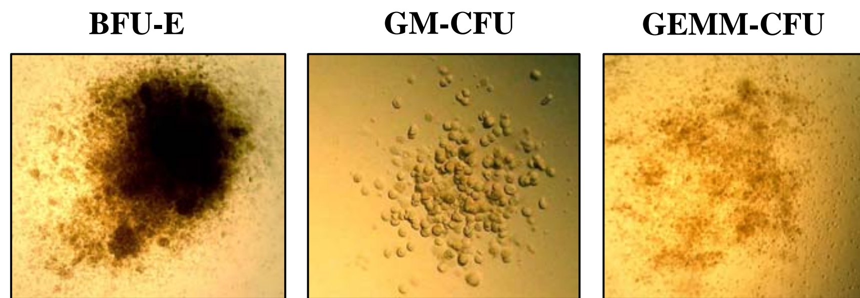


**Figure 6. Colony assay of side population (SP) stem cells**. The pictures (x 50 magnification) of burst-forming units-erythroid (BFU-E, left), granulocyte-macrophage (GM-CFU, middle) and granulocyte, erythroid, macrophage, megakaryocyte colony-forming units (GEMM-CFU, right) were taken on day 12 of growth. SP cells were plated in a concentration of 100 cells/ml in complete methylcellulose medium (MethoCult 3434, StemCell Technologies Inc, Canada). The index colony/cells plated for SP cells was 1/3, and demonstrated more than 75 times enrichment in clonogenity compared to the whole bone marrow (Article I).

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Hence, the chosen populations of hematopoietic mouse stem cells or human stem/progenitor cells, and the methods for cell isolation, were considered appropriate for the aims of the study.

### *RNA purification*

RNA quality is important for any gene expression analysis. In our studies of simultaneous analysis of all major clock genes, RNA quantity was an additional critical factor for the evaluation of clock gene interactions. In the beginning, we performed several test experiments aimed at finding the best way of preparing cells for further RNA purification. The following protocol was chosen as the most appropriate: cells were spun down (300 g, 5 minutes at + 4 $^{0}$C), supernatants discarded, pellets snap frozen in liquid nitrogen and placed at –80 $^{0}$C until use. This procedure yielded sufficient quantities of total RNA for further gene expression analysis avoiding RNA degradation. Both human and mouse samples were of high quality, confirmed by Nano LabChip tests run on an Agilent 2100 Bioanalyzer.

In the human study, we were able to proceed with the cells within 2-3 hours after BM sampling. However, in the mouse study, due to the experimental conditions, Hoechst staining and cell sorting were performed after all samples were collected. This raised a question whether a delay in cell flow cytometric analysis and sorting could increase the risk of RNA degradation. In addition, we needed to prove that (1) flow cytometric sorting itself used in experiments with mouse BM, and the long time necessary for collection of appropriate cell

numbers, would not influence the RNA quality and (2) clock gene appropriate cell numbers would not influence the RNA quality and clock gene expression.

The results of the study published in Article I indicate that enrichment by high-speed flow cytometric cell sorting (even with speeds up to 30,000 events/cells per second) induced little or no RNA degradation and did not influence the profile of clock gene expression. Thus, we confirmed that the method we used can be employed for circadian variation studies, as well as for chronobiological experiments, when collection and preparation of the samples cannot be done simultaneously and with a delay span of 12 h or even 24 h.

### *Choice of reference genes for evaluation clock gene expression: a "geometric mean" approach*

In the studies of circadian clock gene expression when at least some of the target genes are expected to vary, it was important to find appropriate reference genes with a constant level of expression over 24 h. For both mouse and human studies, we chose the normalization strategy described previously (Vandesompele et al., 2002), using the geometric mean of three (mouse study) or four (human study) endogenous control genes. Therefore, 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. This approach allowed us to reduce the possible error related to the use of only one housekeeping gene as an endogenous control for normalization.

## Rhythms of main body markers as an indicator of a circadian coordination in mice/human during the sampling of BM

To ensure that experimental animals or human subjects maintained circadian coordination during sampling of BM, we tested the levels of so called "body marker rhythms". High amplitude, significant hormonal rhythms were observed in corticosterone and melatonin in the mouse study, and in cortisol, growth hormone, testosterone in the human study. In addition, core body temperature in the mouse study, and total white blood cells, neutrophils, and lymphocytes in the human study also showed significant rhythms. These results indicate that the groups of mice and human subjects had a good circadian coordination over the 24 h period, when BM was sampled for gene expression analysis.

## Relative level of clock gene expression (stem/progenitor cells vs. whole BM, and mouse vs. human) is dependent on cell population and species

Before our studies, clock genes had never been analyzed in hematopoietic stem cells. Therefore, when we found that these genes are expressed in mouse SP cells or human CD34$^+$ cells it was important to compare the level of relative expression of key clock genes in these cell populations and the whole BM.

The most pronounced difference in mouse hematopoietic cell populations was found for m*Per1* expression. In SP stem cells, it was almost 3 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 to the other clock genes, depending on circadian time (Article II). It should be noted here (and will be discussed in the next section) that m*Per1* was not rhythmic in SP cells, contrary to the whole BM.

In humans, clock gene relative mRNA expression showed similar levels in CD34$^+$ and whole BM cells, 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. Expression of h*Bmal1* was as low as h*Per1* in CD34$^+$ cells, while it was the highest of all clock genes in the whole BM (Article III).

Thus, the overall results demonstrated differences in relative levels of clock gene expression in hematopoietic cells corresponding to both the studied BM cell populations and species. However, we were not able to find any correlation between the level of clock gene expression and the rhythmic or non-rhythmic character of its circadian variations (see next section) at this time.

## The 24-h variations of clock gene expression are different in mouse stem cells and whole BM, which indicates that circadian variations of clock genes in mouse BM are developmentally regulated

With the exception of m*Per2,* the majority of the key clock genes in mouse hematopoietic SP stem cells were not expressed rhythmically over 24-h periods during the constant conditions of the study. The pattern of clock gene circadian variations in the stem cells was different from that in the whole BM, where circadian rhythms were also observed for m*Per1* and

m*Rev*-erb$\alpha$. Even the peaks of m*Per2,* the only gene which showed 24-h rhythmic expression in both cell populations, did not coincide (although acrophases and their 95% limits were not significantly different, P=0.145).

A quite unexpected finding in this study was that circadian variations of m*Per1* in mouse SP stem cells were weak and/or undeveloped. *Per1* is known to be a core circadian gene and is regarded as a key marker of the circadian molecular clock. Rhythmic expression of this gene has been identified in both central and peripheral tissues. In contrast, we found a robust circadian rhythm for m*Per1* in the whole BM, where high-amplitude variations of this gene were both large and consistent in all three experiments.

Recently, Morse et al, 2003 hypothesized that clock gene expression is developmentally regulated in testis based on their results that clock genes were constant over 24 h in this organ. Later, similar pattern of clock gene variations were demonstrated in thymus (Alvarez and Sehgal, 2005). These data led authors to a conclusion that the circadian clock does not operate in immature cells undergoing differentiation (Alvarez and Sehgal, 2005). As was discussed earlier, the SP fraction of mouse BM is highly enriched for primitive stem cells with long-term reconstitution ability (Goodell et al., 1996), and is comprised mostly of quiescent hematopoietic stem cells (Arai et al., 2004). Our findings that circadian expression of most of the key clock genes appeared to be non-rhythmic in this cell population can indicate that the activity of molecular clocks in mouse hematopoietic stem cells is also developmentally regulated.

## Mouse and human hematopoietic BM stem/ progenitor cells have a specific profile of circadian clock gene expression that is different as compared to other tissues

In the mouse study we were able to collect liver samples in parallel to BM samples, and thus could compare patterns of clock gene expression in hematopoietic cells and liver. This also gave the advantage of having liver as a reference tissue, where the circadian patterns of molecular clocks are well-documented. In our experiments, 24-h clock gene expression variations in liver were similar to that reported by several others, including one in $B6D2F_1$ mice (Filipski et al., 2004).

We found that the circadian organization in the BM was different from liver. Thus, m*Per1* (only in whole BM), m*Per2* and m*Rev-erb*$\alpha$ in hematopoietic cells oscillated with amplitudes several-fold lower as compared to liver. In both hematopoietic cell fractions,

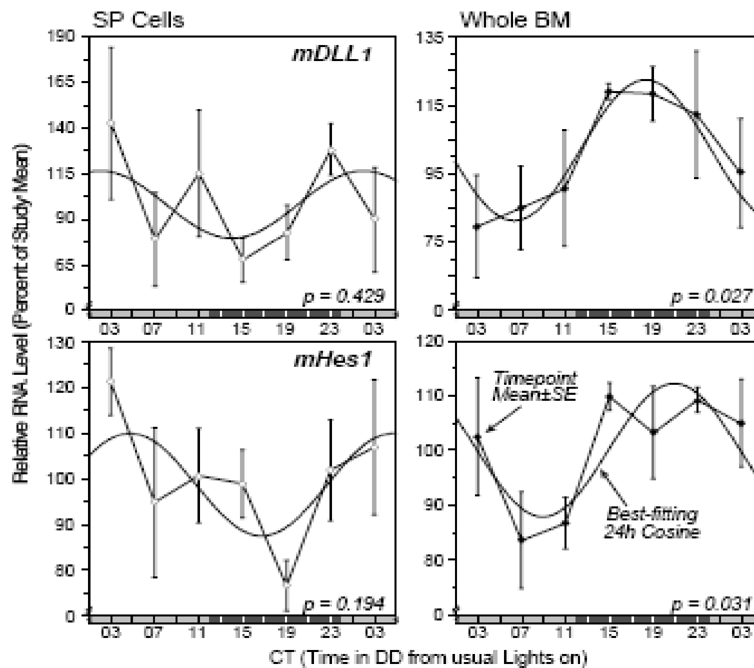


**Figure 7. m*Dll1* and m*Hes1* express circadian rhythms in whole bone marrow (BM, right panel) but not significantly in side population (SP) stem cells (left panel, see *p* values)**.

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circadian expression of m*Cry1*, known to be transcribed rhythmically in liver (Bustin, 2002), was more or less constant. Perhaps one of the most interesting findings was that m*Bmal1* is expressed rhythmically and with a high amplitude in liver, but did not oscillate significantly in mouse SP stem cells or in whole BM. This result was so unexpected that we repeated the analysis of m*Bmal1* expression in the whole BM using another primer/probe set (Tamaru et al., 2003). This reproduced a nonrhythmic m*Bmal1* expression profile and thus confirmed that the absence of m*Bmal1* rhythmicity in the BM populations was not due to the primers/probe design. In addition, we showed that h*Bmal1* was not significantly rhythmic over 24 h in human $CD34^+$ cells.

*Bmal1* is a positive regulator of the molecular clock, and was shown to be expressed rhythmically in anti-phase to *Per* genes in some of the peripheral tissues, such as mouse liver (Yamamoto et al., 2004) or human oral mucosa (Bjarnason et al., 2001; Bustin, 2002; Yamamoto et al., 2004). Recent studies have demonstrated that the *Bmal1* rhythm could also be unstable in other tissues, and have an unusual phase relationship with *Per2* in human peripheral blood mononuclear cells (Teboul et al., 2005), or mouse BM (Granda et al., 2004). It was recently suggested that *Bmal1* regulation may be tissue-specific (Emery and Reppert, 2004), since RORs proteins that (with REV-ERB A) control *Bmal1* transcription (Akashi and

Takumi, 2005) showed strikingly different patterns across peripheral tissues with varying peak times (Guillaumond et al., 2005).

Our results that *Bmal1* expression was not rhythmic in tested BM populations both in mice and humans suggest that it could be undeveloped in rapidly proliferating hematopoietic tissues. These data also showed that organization of gene time regulation in BM is different from other tissues. This concept is confirmed by our findings that m*Dbp* and m*Wee1*, closely related to the clock system in liver, were not rhythmic in mouse BM cells. Both m*Dbp* (Lopez-Molina et al., 1997) and m*Wee1* (Matsuo et al., 2003) displayed a robust circadian rhythm in mouse liver and were used as classical examples of clock-controlled genes. Interestingly, we found that m*Dll1*, coding a Delta-like ligand for Notch receptor (Karanu et al., 2000), and m*Hes1,* a transcription factor and one of the major target of Notch signaling (Davis and Turner, 2001), were expressed rhythmically in whole BM (**Figure 7, right**). Notch signaling plays an important role in regulating differentiation and lineage specification in hematopoiesis, and the genes related to that system and expressing circadian rhythms could be candidate clock-controlled genes in BM. However, additional experiments have to be performed (e.g., with clock-gene knockouts) to prove that these genes are directly regulated by the clock gene(s). It is of special interest that m*Dll1* and m*Hes1* did not oscillate in SP stem cells (**Figure 7, left**) contrary to whole BM. This result confirms that rhythms in hematopoietic stem cells are developmentally-regulated. Notable during hematopoiesis, Notch signaling is especially important for stem cell compartmentalization, and has the propensity to expand the stem cells, promote their self-renewal, and influence their survival (Allman et al., 2002). Thus, *Hes1* plays a significant role in mouse primitive hematopoietic stem cell expansion, significantly preserves the long-term reconstituting activity of these cells during culture, and induces a several fold increase in mouse SP (Kunisato et al., 2003). This may address the question of possible correlations between the regulatory function of Notch signaling in stem cells and non-rhythmic expression of at least some of the genes involving in this pathway.

From the point of physiological need, the specific organization of the molecular clock in hematopoietic tissue has to be related to its specific functions (see Introduction). Markedly different distributions of circadian phases in peripheral tissues (Storch et al., 2002), as well as minor overlap of cycling (clock-controlled?) genes between tissues (Panda et al., 2002), suggest that the clock system in general is organized in a tissue-specific manner. This property of the clock system is physiologically relevant, and useful in the process of adaptation of peripheral tissue(s) to particular conditions. This also supports the concept that a

molecular clock plays an important role in the regulation of specific tissue functions (Gachon et al., 2004).

Further studies aimed on defining molecular links between circadian clocks and gene/protein expression in BM may help to understand how and to what extent hematopoiesis is controlled by endogenous rhythms.

# CONCLUSIONS

1. The developed methodological approach combining flow cytometric high-speed sorting (in mice) and magnetic beads isolation (in human) of stem/progenitor cell populations with Q-RT-PCR is useful for clock gene expression analysis and circadian variation studies in small hematopoietic cell fractions

2. We demonstrated, for the first time, clock gene expression in purified hematopoietic stem cells in mice and stem/progenitor CD34$^+$ cells in human over a 24-h period, with a relative level of expression different from that in whole bone marrow.

3. Most clock genes (with the exception of m*Per2*) studied in mouse hematopoietic SP stem cells were not oscillating in a fully organized circadian manner, which could indicate that circadian clock gene expression variations in mouse bone marrow are developmentally regulated.

4. We demonstrated that three of eight key clock genes expressed in human bone marrow CD34$^+$ progenitor/stem cells h*Per1,* h*Per2* and h*Cry2* exhibit a significant circadian rhythm.

5. *Bmal1* does not oscillate significantly in any of the studied fractions of mouse or human hematopoietic cells, which could indicate that *Bmal1* is not rhythmic in hematopoietic tissues.

6. In general, clock gene expression in human and mouse hematopoietic bone marrow stem and progenitor cells has a specific circadian profile with some differences from the patterns in other tissues.

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# ERRATA

The following typographical and grammatical corrections have been made after the submission of the thesis.

Page 17, paragraph 1 after the subtitle, has been added: "…systematic 24-h (circadian) variations."

Page 19, paragraph 1, has been added: "…an important statistical tool for biological studies of rhythms."

Page 19, the last paragraph: "…period (PER) gene…" has been changed to: "…period (*per*) gene…"

Page 22, the last paragraph: "…accumulate or controlled…" has been changed to: "…accumulate or are controlled…"

Page 27, III, "…(2006) Circadian variations in clock gene expression of human bone marrow CD34-positive cells (submitted to *J Biol Rhythms*)." has been changed to: "…(2007) Circadian variations in clock gene expression of human bone marrow CD34-positive cells (*J Biol Rhythms* **in press**)."

Page 37, Conclusion 2: "...total bone marrow" has been changed to: "…whole bone marrow."

# ARTICLES