Early Blood Cell Formation "in sickness and health, 'till death do us part"

Pronk, Kees-Jan

2008

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Early Blood Cell Formation

“in sickness and health, ‘till death do us part”

Cornelis JH Pronk

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Faculty opponent:
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Department of Cell Biology, Section Stem Cell Biology,
UMCG, University of Groningen, The Netherlands.

With the approval from the Faculty of Medicine, Lund University, this thesis will be
defended on December 11th, 2008, at 13.00 in Segerfalksalen, BMC A10, Lund
Voor mijn allerliefste
Linda, Mathilda en Nelis
"Every portrait that is painted with feeling is a portrait of the artist, not of the sitter."
Oscar Wilde, The picture of Dorian Gray (1891)

"Een mens begint maar verstand van vrijen te krijgen als het te laat is om er verstand van te hebben."
Louis Paul Boon (1912-1979)

"Härmed förklarar jag den nya Djurgårdsfärjan invigd som ska gå här mellan ... eh ... hållplatserna!"
Kung Carl XVI Gustaf (1985)
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<tr>
<td>AGM</td>
<td>Aorta-gonad mesonephros</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloblastic leukemia</td>
</tr>
<tr>
<td>B</td>
<td>B cell/lymphocyte</td>
</tr>
<tr>
<td>Bio</td>
<td>NHS-biotin labeled cells</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BS</td>
<td>Bisulfite genomic DNA sequencing</td>
</tr>
<tr>
<td>CAFC</td>
<td>Cobble-stone area forming cell</td>
</tr>
<tr>
<td>CB</td>
<td>Cord blood</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CFU-S</td>
<td>Colony forming unit-spleen</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphatase-guanine</td>
</tr>
<tr>
<td>CRU</td>
<td>Competitive repopulation unit</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dKO</td>
<td>Double KO</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>E</td>
<td>Erythrocyte</td>
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<tr>
<td>ELP</td>
<td>Early lymphoid progenitor</td>
</tr>
<tr>
<td>EP</td>
<td>Erythroblast</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>ETP</td>
<td>Early thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activate cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FL</td>
<td>Fetal liver</td>
</tr>
<tr>
<td>FL</td>
<td>Flt3 (or Flk2) ligand</td>
</tr>
<tr>
<td>G</td>
<td>Granulocyte</td>
</tr>
<tr>
<td>G–csfr</td>
<td>Granulocyte-colony stimulating factor receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte-monocyte progenitor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft-versus-leukemia</td>
</tr>
<tr>
<td>H3K4/9/27me</td>
<td>Histone H3 lysine 4/9/27 methylation</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>KL</td>
<td>cKit ligand (or SCF)</td>
</tr>
<tr>
<td>KLS</td>
<td>cKit+Lineage-Scal+</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LCR</td>
<td>Locus control region</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid primed multipotent progenitor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long-term culture initiating cell</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long-term HSC</td>
</tr>
<tr>
<td>M</td>
<td>Monocyte/macrophage</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocyte/macrophage-colony stimulating factor receptor</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblasts</td>
</tr>
<tr>
<td>Meg</td>
<td>Megakaryocyte (or Mk)</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte-erythrocyte progenitor</td>
</tr>
<tr>
<td>MkP</td>
<td>Megakaryocyte progenitor</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OP9-DL1</td>
<td>Delta-1 ligand expressing OP9 cells</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb group protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRC</td>
<td>Polycomb-repressor complex</td>
</tr>
<tr>
<td>pre CFU-E</td>
<td>Pre colony forming unit-erythrocytes</td>
</tr>
<tr>
<td>pre GM</td>
<td>Pre granulocyte/ monocyte</td>
</tr>
<tr>
<td>pre MegE</td>
<td>Pre megakaryocyte/erythrocyte</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase PCR</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor (or KL)</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short-term HSC</td>
</tr>
<tr>
<td>T</td>
<td>T cell/lymphocyte</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin (also THPO)</td>
</tr>
<tr>
<td>TrxG</td>
<td>Trithorax group protein</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-SCID</td>
<td>X-linked severe combined immunodeficiency</td>
</tr>
<tr>
<td>YS</td>
<td>Yolk sac</td>
</tr>
</tbody>
</table>
Articles included in this thesis


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Preface

It is with great pleasure that I hereby present my doctoral thesis. This thesis consists of two parts. The first part gives a general introduction to the field, followed by a summary and discussion of the studies that are included in this thesis. The second part displays the reprints of the articles and manuscript on which this work was built.

The process of blood cell formation (hematopoiesis) has traditionally been subject to intense experimental investigation, including a great body of work on hematopoietic stem cells (HSC); the ancestors of all mature blood cells. Not only have these studies given insights into the biological processes governing hematopoiesis, they have also created increased understanding of hematological diseases and aided to create a window of therapeutic opportunities. In addition, due to their accessibility and relatively uncomplicated transplantability, studies on the hematopoietic organ have often served as a role model for other organ systems that are characterized by the presence of organ specific stem cells with the capacity to generate all cell types contained within these organs in a hierarchical fashion.

Although direct experimentation on human blood cells would be most valuable to understand biological processes in human biology and disease, technical and ethical issues complicate this work. However, the use of animal models, such as mouse-models, has shown itself to be of great value and to a large degree translational to human biology. Using these animal models allows for accessibility to a large number of individuals, for the use of genetically modified study objects, and for good means for functional evaluation. The questions raised in this thesis were therefore addressed by using mouse-models.

The work on the role of tumor necrosis factor on HSC activity (Article I) was performed under the supervision of Prof S.E. Jacobsen. The work on identifying early myeloid development (Articles III and IV) and some processes underlying HSC regulation in aged individuals (Article II) was performed under the supervision of Dr D. Bryder. I hope you enjoy reading this thesis.

Cornelis JH Pronk
INTRODUCTION TO THE HEMATOPOIETIC ORGAN

MATURE HEMATOPOIETIC CELLS

The hematopoietic organ, or blood cell system, is probably one of the most complicated and also dynamic organ systems. This is due to the diversity of different cell types and cellular functions, as well as the immense cellular turnover contained within this organ. In men, an astonishing $10^{12}$ blood cells are produced each day in steady-state (Ogawa 1993). In cases such as bleeding or infection the requirement and output of certain cell types can be highly increased. However, improper overproduction of either the wrong cell type or at the wrong time could lead to unwanted consequences. Therefore, high demands for a stringent and dynamic regulation of blood cell production is a prerequisite. The hematopoietic system can functionally be divided into three major classes: i) oxygen transportation, ii) coagulation, and iii) immune surveillance; and up to at least ten different blood cell types have been identified to perform these tasks.

![Figure 1. Schematic overview of the different cell types in the hematopoietic system. Depicted are the estimated life length (in parenthesis) and the main functional characteristics of these cells. hrs: hours, wks: weeks, mths: months, yrs: years](image-url)
Transportation of oxygen (O₂) is conducted by the red blood cells, or erythrocytes. Erythrocytes are enucleated, concave cells that contain hemoglobin (Hb), the protein responsible for the binding of both O₂ and carbon dioxide (CO₂). Upon passing through the capillary network within our respiratory system (lungs), CO₂ bound to Hb is exchanged for O₂. As the erythrocytes migrate to the peripheral tissues, the opposite process takes place. Improper erythrocyte production, for instance due to iron-deficiency, or increased turn-over in cases such as spherocytosis, subsequently leads to low erythrocyte counts (anemia) and presents clinical symptoms such as fatigue.

Coagulation, or blood clotting is the process in which platelets, or thrombocytes, interact with a network of coagulation factors to prevent spontaneous bleedings, or to stop bleeding upon inflicted injury. Thrombocytes are formed and shedded from bone marrow (BM) residing megakaryocytes and released into the blood stream (Junt et al. 2007). Many thousand thrombocytes can be released from one single megakaryocyte. Abnormalities in either platelet numbers or function can lead to life threatening events. Too few or dysfunctioning platelets increase the risk of bleedings. Too many can cause thrombosis and can cause events such as stroke and myocardial infraction (Patel et al. 2005).

Our immune surveillance is an intricate and complicated process involving many different blood cell types and factors to protect us against invading pathogens including bacteria, viruses, parasites and fungi. This process is complicated by the fact that we cannot function without the presence of a large number of bacteria present on, for example, our skin and in our digestive system. Therefore, a certain degree of immune-tolerance is required. Also, the ability to distinguish between foreign and self-components is crucial to avoid induction of auto-immunity (i.e. self-destruction) while dysregulation could cause diseases such as rheumatoid arthritis. However, recognition of aberrant over normal self-components is imperative for the control of cancer development. Our immune system can largely be divided into the innate and adaptive immune system and collaboration between these two is required to confer optimal immune responses. The innate system is considered the more primitive system. In general, cells within the innate system, such as granulocytes, macrophages, mast cells, dendritic cells (DC) and natural killer (NK) cells use germ line encoded receptors (Toll-like receptors) for pathogen recognition. This allows for a rapid, though less specific and more limited recognition of invading pathogens and is therefore considered as the “first line” of defense (Akira et al. 2006). Pathogen recognition is often followed by elimination through either phagocytosis or induction of
cytotoxicity. Phagocytosis subsequently mediates both degradation and presentation of pathogenic antigens. Macrophages, neutrophilic granulocytes and mast cells are the major players of the innate immune system and are predominantly involved in the defense against bacterial infections, mainly through phagocytosis. DCs are the main antigen-presenting cells and together with macrophages the main source of pro-inflammatory cytokine production (Itano and Jenkins 2003). Eosinophilic and basophilic granulocytes are involved in parasitic infection. NK cells are important for the control of tumorgenesis, viral infections and intracellular bacteria (Di Santo 2006) and confer cytotoxicity through the release of cytotoxic granules such as perforins. In addition, NK cells can potently produce pro-inflammatory cytokines. Antigen presentation, as well cytokine production by the innate system subsequently instructs the initiation and activation of the adaptive immune system. The adaptive immune system is considered as the “second line” of defense; here, too, a high level of fine-tuning and control is required to avoid processes such as auto-immunity and allergy (Germain 2001). Upon activation, B and T lymphocytes rearrange their antigen receptor genes and mature into effector cells. This is a time-consuming process and therefore does not allow for a rapid immune response. However, it can develop a targeted defense against an almost infinitive number of antigens. In addition, upon repeated exposure to the same pathogenic antigen, a rapid and precise immune reaction can be generated. This is achieved through a process called immunological memory. In the process of T cell maturation, naive T cells encounter antigen-presenting cells followed by proliferation and differentiation of cytotoxic T cells. These cytotoxic T cells are primarily involved in the lysis of virus-infected cells. B cells on the contrary do not possess cytotoxic capacities. Antigen binding/presentation to the surface of a B cells promotes clonal expansion and differentiation into plasma cells with the ability to produce antigen specific antibodies (immunoglobulins, Igs). Not only upon primary but also upon repeated exposure to this antigen (i.e. subsequent infections), Igs are excreted from these plasma cells to activate the innate immune system to rid the infectious pathogens. Since a part of these B cell clones are very long-lived, they confer protection to repeated infection by a certain pathogen over many years. This feature has been taken advantage of in the development of vaccines. When vaccinated, an individual is injected with either a dead or dysfunctional pathogen. These pathogenic antigens are presented to immature B-cells and subsequently B-cell clones producing specific Igs are generated to confer a hopefully life-long immune protection. A large number of clinical syndromes and immune diseases have been described that are characterized by improper maturation of immune
cells, deficiencies in immunoglobulin production, deficient cytokine production, etc. In these cases, the subject is more susceptible to infection or tumor formation.

Of the different cell types described above, erythrocytes, thrombocytes, granulocytes and macrophages are traditionally referred to as the myeloid or myeloerythroid cells. B cells, T cells and NK cells are referred to as lymphoid cells. This “classification” is not only based on functional, but also on developmental differences. All mature blood cells arise from one common ancestor, the hematopoietic stem cell (HSC) that through a series of events can differentiate into all cell types. In the more traditional view, cells within the myeloerythroid lineages are developmentally more related than cells within the lymphoid lineages. This latter observation has, however, been questioned. The subsequent chapters will therefore discuss in more detail some aspects of HSCs and of the early blood cell differentiation towards these different lineages.
IMMATURE HEMATOPOIESIS

The scientific birth of the hematopoietic stem cell field

Mature blood cells all have a very varying life span, ranging from just days to several years. As the hematopoietic system has such high regenerative capacities, a long-standing thought has suggested the existence of one or more precursor cell types that “feed” the blood system throughout life. This could imply the existence of a self-sustaining mechanism for these cells, commonly referred to as self-renewal. These assumptions eventually led to the identification of the HSC and the development of bone marrow replacement therapies (i.e. bone marrow transplantation). This treatment is nowadays a relatively common and often life-saving clinical feature and its developement by (amongst others) Dr E. Donall Thomas was awarded the Nobel Prize in 1990.

We can already find reports on bone marrow (BM) transfers early in the last century. In the 1930s and 1940s, Osgood et al. treated unconditioned patients with aplastic anemia by repeated BM infusions without conditioning, though this treatment was without positive effects (Osgood et al. 1939). Also, Reckers et al. injected BM into irradiated dogs, without successful engraftment (Rekers et al. 1950). The reason as to why these experiments failed is most likely due to the absence or suboptimal conditioning or immunosuppression of the host prior to transplantation. Therefore, these experiments suggested the existence of an immunologic reaction against donor BM cells when transplanted into another recipient. Subsequently, Lorenz et al. performed successful BM transplantation and showed that radioprotection could be achieved upon BM transplantation into a lethally irradiated host between mice from the same strain (Lorenz et al. 1951). Indeed, in 1959 Thomas et al. reported the first successful BM transplantation between two identical twins, of which one suffered from refractory leukemia (Thomas et al. 1959). Following lethal irradiation and BM transplantation, this patient showed full hematological recovery. These findings suggested amongst others the presence of BM located cells that ultimately give rise to mature blood cells, although the possibility of stimulation on endogenous cells by the transplanted graft could not be ruled out fully. Also, as large quantities of cells were transplanted, these experiments did not show direct proof for BM derived cells with the potential to generate more than one cell type, a feature called multipotentiality.
Figure 2. Transplantation of increasing doses of BM cells into lethally conditioned mice gave increasing frequencies of (A) surviving mice and (B and C) CFU-S. Adapted from Till and McCulloch (McCulloch and Till 1960; Till and Mc 1961).
Together, these findings strongly suggested the presence of a **small subset** of BM residing cells that possessed 1) **great proliferative** potential, 2) **multi-lineage** potential, and 3) **self-renewal** capacity; all characteristics that can be attributed to the **HSC**. Yet later studies showed that CFU-S have limited self-renewing capacity and do not contain combined myeloerythroid and lymphoid potential, but rather only myeloerythroid potential (Magli et al. 1982; Jones et al. 1989). Therefore, they cannot be ascribed to as being a true HSC.

Between 1980 and 1990, a series of studies was conducted that proved the existence of single hematopoietic cells with the capacity to generate multi-lineage (myeloerythroid and lymphoid) offspring over longer periods of time (Dick et al. 1985; Keller et al. 1985; Lemischka et al. 1986; Capel et al. 1989). In these studies, retrovirally transduced BM cells were transplanted, and clonal integration sites were detected in multiple hematopoietic organs in the reconstituted host. The studies that used labeling to trace multipotentiality and self-renewal, either by retroviral integration (Dick et al. 1985; Keller et al. 1985; Lemischka et al. 1986; Capel et al. 1989) or by irradiation induced chromosomal aberration (Becker et al. 1963), have been criticized in that the experimental procedures themselves might have induced a genetic imbalance that made non-HSC gain “HSC-like” properties. Nevertheless, these studies have created great insight and paved the way for further investigation and identification of the HSC. This lead in the 1990s to some seminal proof-of-principle experiments in which one single, genetically unaltered HSC was able to long-term multi-lineage reconstitute a lethally conditioned host (Smith et al. 1991; Osawa et al. 1996).
Birth of the hematopoietic system - before birth

Besides the hematopoietic system, a range of other adult solid organs and tissues possess regeneration capacities with varying cellular turnover rates. Many of these organs were shown to contain a cellular subset that replenishes the different cellular functions of these organs life-long. These cells are generally designated as adult or somatic stem cells. Although subject to debate (Blau et al. 2001), a large body of experimental evidence has shown that the cellular potential of these adult stem cells is often restricted to the organ in which they reside, with little or no capacity to generate cells of other tissues (Wagers et al. 2002b; Wagers and Weissman 2004). This raises the possibility that these cells are generated only once in a lifetime, presumably pre-natal. Efforts have been made to establish the exact time and location at which the first blood cells (and HSC) appear in order to understand the underlying and supporting mechanisms. Due to the migratory properties of the blood system and the multiple embryonic sites in which blood cells emerge, some controversies still exist within this field of research. As many of the advances in this field have been made in mouse-models that are largely translatable to human (Tavian et al. 2001), a general overview will be given based on mouse biology.

The hemangioblast is the cell type proposed as common precursor for hematopoietic and endothelial (vascular) cells, partly due to the proximity and synchronous occurrence of these cells in the yolk sac (YS) blood islets; the site believed to initiate hematopoiesis. Studies using Flk1-/- mice (Shalaby et al. 1997) and clonal differentiation analysis of mouse ES cells have supported this view (Choi et al. 1998). Yet this idea has been challenged in studies using blastocysts injection of different labeled ES cells resulting in blood islets that consisted of more than one original ES cell, in addition to the fact that not all blood cells were generated through Flk1+ precursor cells (Ueno and Weissman 2006). Moreover, it has been found that in vitro derived ES cells containing blood and vessel potential can give rise to smooth muscle cells (Ema et al. 2003) and that the transcription factor Runx1 is required for definitive, but not primitive hematopoiesis (North et al. 1999; North et al. 2002), suggesting the existence of different cell types or perhaps a hierarchical positioning of these cells.

The initial wave of blood cell formation, called primitive hematopoiesis, is considered to take place in the YS at 7.5 dpc (days post-coitum) and is characterized by the production of mainly red blood cells and can therefore not be assigned as the occurrence of HSCs. Its primary function is oxygen transportation through the developing embryo. Primitive hematopoiesis is soon followed by definitive
hematopoiesis, with the capacity to long-term (throughout life) generate all cells of the blood system.

Following the YS, the next site containing hematopoietic potential (definitive) is located around the dorsal aorta, called the aorta-gonad mesonephros (AGM) region at 10.5-11 dpc (Muller et al. 1994). These cells have the potential to give long-term reconstitution and were therefore considered the first site of HSC generation. There is some controversy as to whether the YS contain definitive HSC activity. Back in the 1970s, some experiments supported this notion whereby removal of the YS resulted in the absence of hematopoietic cells in the fetal liver (FL) (Moore and Metcalf 1970). These findings were supported in a recent study where pulse labeled 7.5 dpc HSC gave FL and adult hematopoiesis (Samokhvalov et al. 2007). Indeed, the number of HSCs present in the FL is more than can be accounted for from HSCs generated in the AGM alone (Kumaravelu et al. 2002). However, cultured mouse AGM cells give long-term reconstitution upon transplantation, whereas cultured cells from YS do not (Cumano et al. 1996; Medvinsky and Dzierzak 1996). This supports the idea that definitive hematopoiesis and HSC arise first in the AGM. More recently, the placenta has been suggested as a source for HSC generation (Gekas et al. 2005; Ottersbach and Dzierzak 2005) that arises almost coincidently with the appearance of HSCs in the AGM region. This led to debate whether placenta HSCs were generated de novo, or if they colonized the placenta upon circulation. However, a recent report demonstrated that in Ncx1-/- embryos lacking heartbeat and therefore circulation, HSC emerge in the placenta, indicating that the placenta is also a site for HSC generation (Rhodes et al. 2008).

Subsequently, fetal circulation is established 8.0 dpc (McGrath et al. 2003) and the FL is seeded 11.0 dpc becoming the major site to support fetal hematopoiesis (Yokota et al. 2006), followed by seeding of the thymus, spleen and eventually the BM. There is however some experimental proof for similar seeding of FL and BM (Delassus and Cumano 1996). In FL, HSCs cycle and expand rapidly during the following days (Ema and Nakauchi 2000); a characteristic seemingly different to the slower diving post-natal adult HSC (Nygren et al. 2006). Towards the last stage of the pregnancy, BM becomes the main site of hematopoiesis and will remain so through adult life.

Clearly, there have been and still are contradictions in this field of research. Often it is difficult to experimentally address the posited questions. For instance, the ultimate test for reading out HSC activity is to transplant and determine long-term
reconstitution abilities. This might be difficult, since cells at different embryonic stages and sites might need different external (microenvironmental) stimuli to develop with full potential. Furthermore, isolation of HSC from embryonic tissue is technically challenging, although advances in flowcytometric based phenotypic identification have been a useful tool (Bertrand et al. 2005).

Figure 3. Schematic overview of pre-natal hematopoiesis at indicated sites and dpc (days post-coitus). HB: hemangioblast, YS: yolk sac, PrE: pro-erythrocytes, AGM: aorta-gonad mesonephros, FL: fetal liver, BM: bone marrow.
HEMATOPOIETIC STEM CELLS IN THE ADULT MAMMALIAN

Assays in hematopoietic stem and progenitor cell characterization

As mentioned before, the stringent definition of HSC properties was posed decades ago. However, due to technical limitations, many questions regarding HSC function have been difficult to address. Indeed, true HSC function can be defined as by (1) one single HSC showing simultaneous the capacity (2) throughout life to (3) self-renew, (4) differentiate into all blood cell lineages and (5) functionally replace the whole hematopoietic organ. This definition implicates the demands to experimentally:

1. identify one single stem cell;
2+3. show that this cell contains long-term self-renewing potential;
4. demonstrate the capacity to differentiate into all blood cell lineages;
5. transfer this single HSC to a host with a deficient/lacking blood cell system.

In the 1950s and 1960s, in vivo transplantation experiments into irradiated recipients (McCulloch and Till 1960) and experiments for in vitro clonal evaluation of BM cells (Bradley and Metcalf 1966) were developed and formed the foundation for defining hematopoietic stem and progenitor characteristics. Nowadays, these experimental strategies are refined, allowing one to address the demands posited above. I will hereafter present a brief overview of the experimental approaches commonly used to assay HSC and their direct progeny (hematopoietic progenitors/precursors), many of which were used in the experimental body of this thesis.

Immunophenotypic evaluation

The phenotypic identification of cellular subsets (or populations) in early hematopoiesis is based primarily on the presence or absence of cell-surface marker (protein) expression on individual cells. Fluorochrome conjugated monoclonal antibodies that bind to these markers are visualized by fluorescence activated cell sorting (FACS) that allows for cells to be identified and viably isolated (sorted) for further evaluation. Already in the early days of FACS it was suggested that no single cell surface markers exist that define HSCs or progenitors to high purity (Goldschneider et al. 1978; Spangrude et al. 1988), but rather the combined expression of several markers allow the isolation of cellular subsets. Great technical advances have been made over the last decades in flow cytometry/FACS.
Today, it is possible to simultaneously evaluate the expression pattern of up to 17 different cell surface markers on one single cell (Chattopadhyay et al. 2006) and the availability and quality of fluorochrome conjugated antibodies has also increased. In addition, FACS based isolation has improved and gives the possibility to clonally sort single cells, even of very infrequent populations, with almost 100% purity.

Furthermore, FACS has been used more functionally to identify HSCs taking advantage of a functional characteristic of HSC; the presence of a multi-drug resistant pump that efficiently excludes a range of chemical compounds from its cytoplasm and thereby often confers relative resistance to chemotherapeutic treatment (de Jonge-Peeters et al. 2007). Cells retaining low levels of Rhodamine 123 (Phillips et al. 1992) and Hoechst 33342 (Goodell et al. 1996) following treatments with these dyes are thought to constitute the more immature cells.

**Functional evaluation**

Functionally, hematopoietic stem and progenitor cells are evaluated using both *in vivo* and *in vitro* assays. Roughly, these assays are used to assess proliferation and differentiation potentials of the examined cells. Bonafide long-term HSC activity is primarily evaluated using *in vivo* experiments. *In vitro*, as compared to *in vivo* experiments, provide only surrogate long-term assays and are more applicable to evaluating lineage and proliferation potentials of more mature progenitors. In many cases, the assay itself has not changed so much over the years, only the purity of the input population has increased.

In both man and mouse, long-term *in vitro cultures* have identified two (probably overlapping) cell-types: the long-term culture initiating cell (LTC-IC) (Dexter et al. 1977) and the cobble-stone area forming cell (CAFC) (Ploemacher et al. 1989). Both assays are based on co-cultivating of hematopoietic cells with supporting feeder cells. These assays can evaluate both human and mouse cells, are not confounded by possible homing defects, give read-out earlier as compared to *in vivo* experiments, and ease the simultaneous screening of a large number of cells. However, the heterogeneity of these cells and the limited erythroid and lymphoid readout are some major drawbacks of these assays.

*Short-term in vitro assays* identify more mature, often (oligo-) lineage-restricted progenitors. Typically, semi-solid cultures like methylcellulose and agar are
used, which give clonal information on characteristics such as size, color and composition of the individual colonies (Broxmeyer 1984; Metcalf 2004). These assays are efficient and useful to read out granulocytic/monocytic, erythroid, megakaryocytic and to some extend B cell potential. Also, these cell types read out efficiently in liquid cultures using myeloid cytokines stimulation. B-lymphoid and especially T-lymphoid potential has been more difficult to assess. However, recent advances using the OP9 and OP9-DL1 (Delta-1 Notch ligand expressing OP9 cells) based co-cultures to support B-cell or T-cell development, respectively has very much improved assessment of these lineage potentials (Schmitt and Zuniga-Pflucker 2002). In articles III and IV, we have to a large extent taken advantage of methylcellulose, agar and OP9 cultures to determine lineage potentials and clonal liquid cultures to evaluate clonogenic potentials of several myeloerythroid progenitor populations.

*In vivo* assays are mainly used to assess multipotentiality and the longevity of the more immature hematopoietic progenitor and stem cells. Typically, these assays read out over a time-period of several months. Due to the availability of large number of mouse-specific monoclonal antibodies for flowcytometry as well as the existence of a large number of genetically modified mouse strains (Guasch and Fuchs 2005), most experiments traditionally have been exercised in mice.

The competitive repopulation unit (CRU), or limiting dilution assay has been used for quantification of long-term reconstituting HSC (Szilvassy et al. 1990). In this assay, lethally conditioned recipients are transplanted with decreasing numbers of donor test cells which by different means are distinguishable from endogenous recipient cells and “helper cells” that are co-transplanted with the test cells. Co-transplantation with helper cells is often required, as the test cells alone often do not confer radioprotection during the first weeks (Jones et al. 1990). Three to four months following transplantation, recipients are analyzed for test-cell derived reconstitution, and based on the number of positive and negative reconstituted mice, the frequency of HSC in the original graft can be calculated (Figure 4).

The competitive transplantation assay allows, in addition to “semi-quantification”, for the qualification of test HSCs (or output per CRU). In this assay, donor test cells are transplanted against a pre-defined competitor fraction. Whereas helper cells in the limiting dilution assay are often artificially compromised in their HSC activity (Szilvassy et al. 1990; Miller and Eaves 1997), the competitor fraction in this assay should have normal HSC activity. At given time-points following transplantation, including serial-transplantation, the contribution of each of the fractions
to multi-lineage reconstitution is measured and indicative of the potential of the tested HSC (Figure 4). This assay is very useful to compare HSC potential of genetically modified cells with normal wild-type (WT) cells and was used in article I in this thesis.

For obvious reasons, *in vivo* long-term experiments in humans for identification of human HSCs have great limitations. Therefore, different xenograft models were developed, which allowed for the transplantation of human cells into a diversity of immune-compromised mice (Kamel-Reid and Dick 1988; McCune et al. 1988; Larochele et al. 1996).

![Figure 4](image)

**Figure 4.** Schematic overview of a competitive transplantation experiment using the CD45.1/CD45.2 congenic mouse system to distinguish different sources of hematopoietic cells.

**Molecular evaluation**

Technical advances and means to analyze output data have created an explosive growth in the knowledge of genetic and epigenetic regulation of cellular characteristics and fates. In the studies presented herein, some of these techniques were applied on highly purified populations (article II-IV). Quantitative RT-PCR (or qRT-PCR) and global microarray analysis allow for the evaluation of gene activity of some or several thousands genes, respectively, using a population of cells. **Multiplex single-cell RT-**
PCR provides insight into the gene expression status of multiple genes occurring in a single cell.

More recently, epigenetic signatures of cells have been identified as essential components for the regulation of gene activity. For instance, DNA methylation, a typical mark for transcriptional repression can be tracked with \textit{bisulfite genomic DNA sequencing} (BS) analysis (DeAngelis et al. 2008). Modulation of chromatin structure, representing either up- or down-regulation of the associated gene can be monitored by chromatin immunoprecipitation (ChIP), or by miniChIP in the case of analysis of limited numbers of input cells (Attema et al. 2007).
FUNCTIONAL CHARACTERISTICS OF THE HSC

Prospective isolation of the HSC

HSCs reside in the BM and early experiments from Till & McCulloch suggested that these cells are infrequent (McCulloch and Till 1960; Till and Mc 1961). Later, more precise quantification using limiting dilution experiments estimated the HSC in the murine system to constitute about 1 in 10,000-20,000 whole BM cells (Szilvassy et al. 1990; Rebel et al. 1996). The infrequent nature of the HSC has created difficulties for further characterization and created the need either to purify or at least to enrich this cell. Purification would not only clear the road for studying HSC properties, but it would also give further insights into lineage potentials and the identification of preceding steps in early blood cell development.

Attempts to separate HSC based only on size gave rather poor enrichment (Jones et al. 1990) and it wasn’t before the use of FACS that HSC could be isolated to near functional homogeneity (Figure 5). However, it should be taken into account that FACS isolation is based on cell surface expression of proteins and that this expression can alter during, for instance, stress, proliferation, development and aging (Spangrude et al. 1995; Morrison et al. 1996; Ogawa 2002). In mice, the first reports on the purification of HSC by FACS were based on the expression of WGA (wheat germ lectin) and H-2K (Visser et al. 1984) or on the expression of Sca1, low expression of Thy1.1 and the absence of lineage marker expression (Spangrude et al. 1988; Uchida et al. 1994). Further enrichment was achieved by excluding cells that lack expression of cKit (Ikuta and Weissman 1992). These cells constitute about 0.05% of total BM cells, but still contain only about 10% bonafide HSCs. Later, cKit+, lineage- and Sca1+ (KLS) cells that are CD34-/low further enriched for HSC activity (Osawa et al. 1996), with until now still almost unprecedented purity. As illustrated in Figure 5A, combined expression patterns of CD34 and Flt3 (or CD135/Flk2) within the KLS compartment allowed for the isolation of so-called long-term HSC (KLSCD34+Flt3-), short-term repopulating HSCs (KLSCD34+Flt3-), and multi-potent progenitors that lack self-renewing potential (KLSCD34+Flt3+) (Adolfsson et al. 2001; Yang et al. 2005); these subsets are described in more detail later. Cell isolation using drug efflux properties as described earlier, has identified a population of cells named “side population” (SP) that efficiently exclude Hoechst 33342 upon treatment (Goodell et al. 1996). This method
can be used alone or in combination with cell surface marker analysis (Matsuzaki et al. 2004), but has the disadvantage of being sensitive to minor procedural changes. More recently, by usage of expression patterns of some SLAM family proteins, CD150+CD48-, cells within the KLS compartment were identified and gave around 50% positive reconstitution upon single cells transplantation (Kiel et al. 2005). In addition to this rather high purity, expression profiles of these SLAM markers seem unaltered in processes like mobilization, transplantation and aging (Yilmaz et al. 2006) and make these attractive candidates to use when studying cell behavior under such conditions.

In most of the murine studies, a long-term multi-lineage repopulation assay was used to measure HSC activity. In the human system this has been more difficult and in vitro assays and xenograph transplantation models were used to isolate HSC activity. Human HSC activity resides within the Lin-CD34+CD38- compartment (Petzer et al. 1996) and low expression Thy-1 (Baum et al. 1992) or absence of CD19 expression (Castor et al. 2005) seems to further purify this compartment.

Figure 5. Phenotypic identification of HSC. (A) Example of a FACS based evaluation of some immature BM precursor populations as proposed by Jacobsen and coworkers (Adolfsson et al. 2001; Yang et al. 2005). (B) A selection of cell surface markers identified by their present or absent expression on mouse (red box) and human (blue box) HSC.
The niche – home of the HSC

The stem cell niche is considered the physical site (microenvironment) where a stem cell resides for support of its different cellular functions including proliferation, quiescence and self-renewal. Already in the 1970s it was suggested that HSCs are not randomly spread throughout the BM, but rather concentrate along the endosteal surface of long bones (Lord et al. 1975). Dexter et al. were first to describe long-term cultures supported by BM derived feeder cells, stressing the importance of the microenvironment (Dexter et al. 1977). One year later, the idea of the stem cell niche hypothesis was launched (Schofield 1978). Direct visualization of BM niches has been a challenge. Even though combinations of markers have enabled HSC purification of whole BM by flow cytometry, it has been problematic to apply these for immunofluorescent microscopic evaluation; at least until more recently (Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Adams et al. 2006), although these report have been questioned based on the low frequencies of the their “HSC-positive events”.

Two separate studies reported on mutant mice that were characterized by increased frequencies of osteoblasts as well as HSC and showed co-localization of these cells lining the bone surface (Calvi et al. 2003; Zhang et al. 2003). This indicated the existence of an osteoblastic (or subendosteal) HSC niche. In support of this, angiopoietin (Araki et al. 2004) and THPO (Wright et al. 2001) appear to regulate HSC maintenance and quiescence, respectively, and are expressed by osteoblasts. Also, increased local concentrations of calcium near the endosteum, caused by osteoclastic activity, promote HSC maintenance (Adams et al. 2006).

Using intravital microscopy, transplanted HSCs were shown to lodge to another site within the BM: a microdomain surrounding the BM vasculature (Sipkins et al. 2005) at the same location as phenotypically defined HSC (Kiel et al. 2005). This indicated a second site for resident HSCs, the vascular niche. There is some debate as to whether the osteoblastic niche would support mainly quiescent HSC (Zhang et al. 2003), whereas the HSC within the vascular niche are more replicatively active (Kiel et al. 2005; Adams et al. 2006). Also, it is unclear if these niches are two distinct regulatory units, or whether they are mutually dependent. Sugiyama et al. proposed a mechanism for HSC maintenance in the vascular niche and a functional link between the two niches. They found that HSC were usually located adjacent CXCL12 expressing cells present in both niches, although CXCL12 expression is much higher in the
vascular niche (Sugiyama et al. 2006). As they found most HSCs to reside in the vascular niche and to lose HSC activity upon conditional deletion of CXCL12-CXCR4 signaling, they suggest that the vascular niche also plays a role in HSC maintenance. On the other hand, as HSC do present with a level of recirculation in steady state (Wright et al. 2001) and as HSC home to the BM through the blood stream following in transplantation, it seems tempting to appoint different functions to the vascular and the endosteal niches.

Clinically, the niche concept is of high relevance. It was, for instance, shown that antibody induced clearing of the host niches facilitates engraftment of donor cells (Czechowicz et al. 2007). This could implicate that there indeed is a physical limitation in “niche space” throughout the body. Upon stress, extramedullary hematopoiesis can take place in the spleen and liver, opening possibilities to experimentally further expand stem cells. The niche concept is also of great importance in light of neoplastic transformation. First, mutations that affect the BM microenvironment can induce myeloproliferative disease in genetically unmodified hematopoietic cells (Walkley et al. 2007). Also, in some hematopoietic malignancies, cancer stem cells have been identified that resemble normal stem cells of the original tissue (Bonnet and Dick 1997; Castor et al. 2005). Regulatory cues from the respective niches would not only allow for better understanding of local malignant invasion, but would also open for opportunities to understand (and prevent) metastasis.

**Figure 6.** Schematic overview of the HSC within the osteoblastic and vascular niches and its interaction with a selection of cell-intrinsic regulators. Adapted from Orkin and Zon (Orkin and Zon 2008).
**HSC cellular fates**

In order to maintain a functional hematopoietic organ, the HSC can adapt to a number of cellular fates: 1) **quiescence**, in order to not unnecessarily obtain mutational events during replication, 2) **self-renewal**, as a means to maintain the HSC pool throughout life, 3) **differentiation** to induce production and replacement of mature blood cells, 4) **apoptosis** to control HSC pool size and to minimize the risk for transforming events, and 5) **mobilization** in and out of the BM (Figure 7). This latter feature is debated as to whether it does occur in steady state and for what purpose. All of these cellular fates are governed by a fine balance and the interplay of regulatory processes including (i) cell extrinsic signaling, (ii) epigenetic function and (iii) transcriptional control. These regulatory processes will be discussed later.

![Figure 7. Schematic overview of different HSC cellular fates choices.](image-url)
Run or duck - HSC self-renewal versus quiescence

Self-renewal is defined by the generation of at least one daughter cell that upon replication contains identical properties as the parent cell. A HSC can divide symmetrically, giving rise to two identical cells, or asymmetrically, generating two distinct types of cells. So, symmetrical divisions can give rise to either two new HSCs (self-renewal) or mature cells (differentiation). Asymmetrical division could then give rise to one new HSC and mature (or committed) cell (Figure 7). These processes require a high level of fine-tuning to ensure life-long replenishment of the blood system on one hand, without the risk for overgrowth or cancer on the other. The self-renewing activities of HSC are ultimately tested using transplantation assays.

In the developing embryo, there is a need for rapid expansion of HSCs (Ema and Nakauchi 2000) as opposed to the adult (Sudo et al. 2000), where HSC are to a higher degree in replicative silence (quiescence) and reside in G0 (Cheshier et al. 1999; Nygren et al. 2006; Bowie et al. 2007b). Interestingly, some factors that are crucial for HSC replication/maintenance during embryogenesis, including the transcription factors SCL/tal1 (Mikkola et al. 2003) or Sox17 (Kim et al. 2007), are not required during adult life; or only during stress but not in steady state adult BM, like NOTCH (Mancini et al. 2005) or Wnt (Gongdon et al. 2008).

Many factors interplay in the process of self-renewal and quiescence making these processes mutually dependent. Both cell extrinsic cues from the microenvironment and cell intrinsic factors, like transcription factors and epigenetic regulators, control these fates. In vitro experiments have suggested heterogeneity within the stem cell pool when it comes to cells converting to symmetrical versus asymmetrical divisions (Brummendorf et al. 1998), with cytokine stimulation shifting the balance towards asymmetrical (differentiating) cell divisions (Takano et al. 2004).

Most factors that regulate self-renewing activity have been identified as positive regulators that increase HSC activity. Loss of such a regulator like Bmi1, a member of the Polycomb group of transcriptional repressors, led to the eventual loss of blood cell formation (Park et al. 2003), whereas over expression caused increased HSC activity (Iwama et al. 2004). Further, Bmi1 regulates the expression of a diversity of factors, amongst others the Hox genes (van der Lugt et al. 1996; Takihara et al. 1997; Park et al. 2003). Indeed, Iwama and colleagues have also demonstrated that functional Bmi1 is required for HoxB4 induced activation of HSC (Iwama et al. 2004). In the case of HoxB4, this is of particular interest as this factor was identified as one of the strongest
positive regulators of HSC self-renewal. Overexpression of HoxB4 strongly expanded HSC in vivo and in vitro (Sauvageau et al. 1995; Antonchuk et al. 2001; Antonchuk et al. 2002; Miyake et al. 2006). Even more, simultaneous overexpression of HoxB4 and its co-factor Pbx1 (Moskow et al. 1995) resulted in vitro in over a 100-fold expansion of HSCs (Cellot et al. 2007). Unexpectedly, loss of HoxB4 signaling only mildly affected HSC activity (Brun et al. 2004), although this could be explain by redundancy, since deficient activity of several Hox genes simultaneously caused HSC defects (Bjornsson et al. 2003; Magnusson et al. 2007).

Furthermore, signaling through the canonical Wnt3A/β-actin pathway was suggested to positively regulate HSC function in in vitro experiments (Reya et al. 2003) and loss of function studies (Zhao et al. 2007). This has however been questioned (Cobas et al. 2004; Koch et al. 2008) and indeed rather the opposite has been suggested when conditionally over expressed (Kirstetter et al. 2006; Scheller et al. 2006). Also for Wnt, a link with Hox signaling was established (Kirstetter et al. 2006), as well as with Notch signaling (Duncan et al. 2005). In the case of extra-cellular Notch signaling, gain-of-function studies resulted in increased self-renewing capacities (Varum-Finney et al. 2000; Stier et al. 2002). However, inhibition of CSL, the downstream target of Notch signaling, had no effect on HSC activity (Maillard et al. 2008). Also here, the mechanism could be the same as in the case of Wnt, in that loss-of-function studies are perhaps more valuable in identifying a role for these factors, whereas gain-of-function evaluates the locally induced and perhaps supra-physiological concentrations and does not reflect normal biology. Equally, to address the role of cell extrinsic factors, like cytokines, loss-of-function studies remain superior. For instance, it was shown through total or partial loss-of-signaling studies that the cytokines thrombopoietin (Qian et al. 2007; Yoshihara et al. 2007) and cKit (Bowie et al. 2007a; Thoren et al. 2008), respectively, play important roles in maintaining HSC in a quiescent state. During quiescence, it is of vital importance to prevent HSC from choosing an apoptotic path and indeed anti-apoptotic proteins, like members of the Bcl2 family, have been identified as suppressing cellular death (Domen et al. 1998; Domen and Weissman 2000).

The quiescent state appears to be a functionally important property of HSC. Upon proliferative stress, HSC function deteriorates (Siminovich et al. 1964; van Os et al. 2007). When serially transplanted, HSC function and numbers gradually decline (Harrison et al. 1978; Ross et al. 1982), although this could be circumvented by overexpression of Ezh2 (Kamminga et al. 2006). Also, differences between mouse strains were observed with regard to proliferative activities of the HSCs, with a negative
correlation between the age, or the life span of a specific strain and HSC cycling activity (Phillips et al. 1992; de Haan et al. 1997). This, together with the fact that active cycling increases the risk accumulate mutations, makes it desirable to keep the HSC pool at a low proliferation rate. This would urge the need for both positive and negative regulators of HSC (or self-renewing) activity. From the literature, there is support that negative regulators exist, serving to restrict the HSC pool. Transplantation of myeloablated hosts with high numbers of genetically modified HSCs characterized by enhanced self-renewal capacities resulted in HSC levels within, but not beyond, levels observed in unmanipulated mice (Antonchuk et al. 2001). In addition, transplantation of high and low doses of cytostatically exposed bone marrow (BM) cells gave a maximum of 15-fold amplification in HSC numbers (Pawliuk et al. 1996), while mature blood cell subsets were fully replenished in all cases. Some factors were proposed as negative regulators in *in vivo* experiments. Loss-of-function of the cyclin-dependant kinase inhibitors p18 (Yuan et al. 2004) and p21 (Cheng et al. 2000; Ducos et al. 2000) improved HSC function, meaning that these factors normally work to inhibit HSC function. In the case of p21 however, this led to premature exhaustion of the HSC pool (Cheng et al. 2000). By contrast, a similar study performed in another mouse strain could not establish a similar role for p21 (van Os et al. 2007). Also, the Zinc-finger repressor Gfi1 seems to restrain HSC self-renewing potentials (Hock et al. 2004; Zeng et al. 2004), as well as the transcription factor CEBPalpha (Zhang et al. 2004) and the signaling adapter molecule LNK (Buza-Vidas et al. 2006). In addition, p16 has been implicated in HSC self-renewal although more in the context of ageing. Also, some cell-extrinsic factors, like tumor necrosis factor (TNF), were implied to regulate HSC activity negatively. As these two factors form the basis of articles II and I respectively, they are discussed later in more detail.

In cancer development and the discovery of cancer stem cells (CSC, see later chapter), the regulatory mechanisms of self-renewal are of high clinical interest. For instance, whereas HSC expansion by overexpression of HoxB4 does not lead to cancerous transformation (Antonchuk et al. 2001; Antonchuk et al. 2002), it does in other cases, like the mixed lineage leukemia (MLL) proto-oncogen with its fusion partners (Krivtsov et al. 2006). Interestingly, a link was observed between MLL and Hox-gene activity (Horton and Williams 2006) and also, MLL rearranged infant leukemias (ALL) often co-express increased levels of Hox-(target-) genes (Imamura et al. 2002).
It seems intriguing to speculate the mechanism that makes a cell a stem cell. There is very recent, ample evidence that introduction of certain transcription factors in terminally differentiated cells can induce multi-potentiality and seemingly reactivate the self-renewing apparatus (Hanna et al. 2008). This leaves some questions as to how important the microenvironment, or niche, is for maintaining HSC activity. As discussed previously, evidence does exist on the importance of microenvironmental signals in maintaining HSCs (Wright et al. 2001; Arai et al. 2004; Kiel et al. 2005; Adams et al. 2006; Sugiyama et al. 2006). However, it was never shown that these signals in fact are involved in self-renewing mechanisms. Moreover, complicating all work on self-renewal is the definition of self-renewal itself. Can self-renewal be defined as that upon cell division \( i \) at least one exact copy of the parent cell is generated, or \( ii \) at least one cell is generated that contains HSC properties? There seems to be a limit as to how often a HSC can divide (see above), implying that the generation of an exact copy of a parent cell is not accomplished. Also, to prove the generation of an exact copy, molecular profiles should be identical: imbedded in the assay lies a practical impossibility. Even the less stringent definition of self-renewal as per \( ii \) might be problematic. How can one prove multi-potentiality and self-renewing potential within one cell, or even its direct progeny, simultaneously? Most data remains correlative, although long-term multi-lineage reconstitution by one transplanted HSC (Osawa et al. 1996) does provide evidence that supports “definition (ii)”.

**Figure 8.** A selection of some cell-intrinsic and –extrinsic factors that were identified to regulate HSC self-renewal (and/or maintenance) as indicated.
**HSC – WHAT IS THE CLINICAL RELEVANCE?**

The hype of Stem Cell research

The field of stem cell research has gathered increasing interest in recent years. This has led to an exploding number of publications in stem cell biology. This “hype” in stem cells was amongst others made possible due to large financial support globally and public opinion and interest has been one of the driving forces behind this. Indeed, stem cell research has even forced public figures to engage in these matters, like the opposing attitudes to stem cell research by Pope John Paul II and President George W. Bush, as opposed to the positive attitude of the governor of California, Arnold Schwarzenegger. Influential foundations, like the Michael J. Fox foundation, have also shaped public opinion, and injected large financial donations into stem cell research.

Part of the public interest in stem cell research is probably explained by the therapeutic possibilities embedded in stem cell characteristics, but ethical issues, too, have created wide interest; and probably to some degree even curiosity as to how far biological limits can be stretched. In 1998, Science Magazine published the first report on the generation of a human ES cell line derived from donated *in vitro* fertilized material (Thomson et al. 1998). This was a milestone report that generated a heavy debate, as illustrated by three commentaries to this study already in the same issue of Science Magazine. As a matter of fact, only one year earlier, the technical possibility of the cloning and subsequent generation of a viable mammalian was personified by the sheep Dolly (Wilmut et al. 1997). The use of cloning, rather referred to as *nuclear transfer*, is limited by ethical and technical difficulties, although it suggested that the epigenetic state of differentiated cells was not fixed and pliable for reprogramming (Jaenisch and Young 2008). This led to the recent development of another, ethically (and perhaps technically) less controversial approach to generate “new pluripotent ES-cell-like cells”; induced pluripotent stem (iPS) cells. Retroviral introduction of several transcription factors in adult, differentiated cells created these iPS cells displaying ES cell properties, both in the mouse (Takahashi and Yamanaka 2006) and the human system (Hanna et al. 2008; Park et al. 2008). At this moment, the bridge for these cells to cross towards application in clinical practice seems still longer than the Öresund bridge. In some state of the art studies however, it was shown (using mouse models) that a gene-therapeutic approach in autologous iPS could correct some inherited diseases (Hanna et al. 2007; Wernig et al. 2008). The work on ES cells and their and clinical significance lies beyond
the scope of this thesis work. Therefore, I will hereafter focus on and give a short overview of the clinical implication of the HSCs.

**Bone marrow (or HSC) transplantation**

Bone marrow transplantation (BMT), also referred to as HSC transplantation, is nowadays probably the most widely applied treatment modality that takes advantage of the regenerative power of healthy donor stem cells. In BMT, bone marrow cells either from the patient him- or herself (autologous) or from another donor (allogeneic) are transplanted into a recipient and this is often a life-saving treatment. Prior to transplantation, the recipient receives conditioning therapy to enable engraftment of the transplanted cells. Traditionally, BMT was applied to patients suffering from a variety of blood cell malignancies (leukemia), but nowadays it is also applied in non-malignant settings, like treatment of autoimmune diseases (Burt et al. 2008) or BM failure syndromes (Pronk and Bekassy 2004) with autologous or allogeneic BMT, respectively.

Some of the early work in BMT that led to the first successful BMT between 2 identical twins (Thomas et al. 1959) has already been described above in the chapter “The scientific birth of the hematopoietic stem cell field”. These early studies used conditioning therapy with either whole body irradiation or cyclophosphamide treatment to enable engraftment of the transplanted marrow (Santos and Owens 1969). Many of these “old”, but also recent conditioning therapies are rather tough and frequently cause high morbidity. This has led to so-called “mini-transplantations”, or reduced intensity conditioning, which are often applied to the elderly and unfit (McSweeney et al. 2001).

In these initial studies, allogeneic transplantations between identical twins were successful, but they were unsuccessful between unrelated donor versus recipient, complicated by inflammation in a range of tissues. This complication is referred to as graft-versus-host disease (GVHD) and we now know this is caused primarily by HLA-unidentical (or mismatching) donor T-lymphocytes. Indeed, immunosuppressive treatment following transplantation could to some degree control GVHD (Storb et al. 1970). The pro-inflammatory cytokine tumor necrosis factor (TNF), that occupies an important role in this thesis work in regulating HSC activity, was identified as one of the major players in conferring GVHD (Schmaltz et al. 2003).

The upside of GVHD development was the observation that GVHD occurrence reduced the incidence of leukemic relapse. In fact, it was shown that donor T-lymphocytes not only gave GVHD, but could also confer cytotoxicity to resident
leukemic (or tumorgenic) cells; a graft-versus-leukemia (GVL) response (Bonnet et al. 1999). Nowadays, donor T-lymphocyte infusion to induce GVL reactions are often used in the case of leukemic relapse following transplantation, or following mini-transplantations (Peggs et al. 2004). Means to treat GVHD have improved over the years and are now one of the reasons for the increasing use of parent HSC as the donor cell source in so-called haploidentical transplantations (Aversa et al. 1998).

Whereas cells from BM aspirates were traditionally used as the transplantation source, the current cell source is often peripheral blood derived CD34+ “HSCs” following G-CSF mobilization. Mobilized CD34+ cells give a more rapid hematopoietic reconstitution, although the risk for GVHD increases (Copelan 2006). In case of the urgent need for transplantation, and in the absence of a suitable donor, cord blood (CB) derived HSCs can be an attractive source. CB transplantation requires less stringent HLA matching and confers less GVHD, while containing GVL activity, as compared to donor sources from the adult (Wagner et al. 2002). Although CB derived HSC as a transplantation source is promising, it does give slow reconstitution and also the low number of cells obtained from one donor limits transplantations into adult recipients. *Ex vivo* expansion of CB cells would circumvent this latter obstacle and is discussed later on.

**Cancer stem cells**

Leukemia is the collective name for a heterogeneous group of diseases characterized by the malignant transformation of a blood cell type. Roughly, leukemias are divided in myeloid versus lymphoid and acute versus chronic, and large therapeutical and prognostic differences are implicated with the diverse phenotypes (i.e. different leukemias). Traditionally, chemotherapy has been the treatment of first choice. Treatment outcomes have been varying, with frequently occurring relapses following initial remission. Today, we know that some types of leukemia (and also other solid tumors) are characterized by a very small subset of cells, referred to as cancer stem cells (CSCs). These cells carry stem cell features in that (i) one single CSC has the capacity to induce tumor relapse and (ii) CSCs are relatively insensitive to chemotherapeutic treatment (Reya et al. 2001). Together, this implies a risk that conventional chemotherapy would not confer a cure in leukemias that are characterized by the presence of CSCs. Therefore, it is of great value not only to identify which malignancies are
initiated by a CSC but also to characterize the CSC itself in order to design better means of treatment.

The notion that tumors might constitute a diversity of cell types was proposed many years ago (Heppner 1984). In the 1990s, John Dick and co-workers isolated within the bulk of acute myeloid leukemic (AML) cells a small CD34+CD38- population that initiated leukemia upon transplantation into NOD/SCID mice (Lapidot et al. 1994; Bonnet and Dick 1997). Interestingly, this “CSC containing population” of cells shared phenotypic similarities with normal HSC in man. This lifted discussions on the cellular origin of cancerous transformation and in addition underscored that detailed identification (for instance by cell surface markers) of early cellular subset is of great value to our study and understanding of malignant transformation. Keeping CSC properties in mind, it would clinically be most beneficial to develop treatments that specifically eliminate CSC without affecting other cell types, including normal HSC. Indeed, in 2002 some of the first reports that selectively targeted CSC in AML were published (Guzman et al. 2002) and this approach will most likely be translated to the clinic in the coming years.

![Figure 9](image)

**Figure 9.** One cancer stem cell (CSC, or leukemia initiating cell) has the capacity to give rise to all tumor cells over a longer period of time. (A) Conventional treatments often target the tumor bulk cells. CSC are relatively insensitive to this treatment and causes relapse upon treatment withdrawal. (B) CSC targeted therapy confers cytotoxicity specifically to the CSC, whereas normal HSC and tumor bulk cells survive. As tumor bulk cells are short-lived, they disappear with time, allowing for the normal HSC to replenish the hematopoietic system with normal blood cells.
Gene Therapy

Gene therapy can be described by the insertion of a gene into a cell in order to functionally replace an absent or defective gene. It is the group of monogenetic disorders that otherwise can be treated with allogeneic BMT that are especially suitable candidates for gene therapeutic approaches (Cavazzana-Calvo et al. 2005). Introduction of genetic material can be achieved by several systems, of which retroviral integration is the most common. Virus-based vectors can roughly be divided into 2 groups: oncoretroviral and lentiviral vectors. Whereas the former can only transduce dividing cells, the latter have the capacity to transduce non-diving cells (Naldini et al. 1996). In light of using non/slow dividing HSC for these purposes, this is an important issue.

HSC do form attractive candidates for gene therapy, as genetic correction of HSCs by stable integration ultimately leads to genetic correction of all its progeny. Instead of allogeneic transplantation for disease correction purposes, autologous HSC could be obtained, genetically modified ex vivo, and subsequently re-introduced (transplanted) into the patient. There are however some drawbacks to the use of gene therapy: (i) usage and handling of viral factors requires stringent safety precautions; (ii) the number of disorders eligible for gene therapeutic correction is limited; (iii) and the risk of insertional mutagenesis. A trial designed to genetically correct subjects suffering from the X-linked sever combined immunodeficiency (X-SCID) syndrome (Cavazzana-Calvo et al. 2000) was complicated by the occurrence of T cell leukemia in 3 out of 17 children (Hacen-Bey-Abina et al. 2003). Leukemic conversion was likely induced by vector integration in close proximity to the LMO2 gene (involved in T cell development) and the notion emerged that vector integration may not be as random as was previously thought (Nienhuis et al. 2006). This created a major drawback for gene therapeutic trails. Finally, (iv) the absence of effective ex vivo HSC expansion approaches is still a concern, as will be discussed hereafter. Nevertheless, as mentioned previously, the development of iPS cells has perhaps created options for a broader use of gene therapy (Hanna et al. 2007).

Ex vivo expansion of HSC

Ex vivo expansion of HSC is desirable for a number of reasons. For example, expansion of autologous HSC for gene therapy would allow for safety screening of clones prior to transplantation. Even more, ex vivo expansion of CB derived HSC would circumvent
the issue of low cell numbers contained within the transplanted graft. Expansion of HSC has to a degree been successful.

As described previously, introduction of certain transcription factors, such as HoxB4 (Antonchuk et al. 2001; Antonchuk et al. 2002; Miyake et al. 2006), augmented *ex vivo* expansion of murine HSC. However, this requires retroviral infection. Therefore, the use of cell extrinsic stimuli may clinically be more beneficial. Different protocols were developed, like stimulation with a soluble TAT-HoxB4 (Krol et al. 2003) or combinations of different cytokines, like cKit ligand/Flt3-ligand/IL-11 (Miller and Eaves 1997). Also, serum free cultures with the addition of fibroblast growth factor-1 (FGF-1) alone expanded HSC activity in BM cell cultures (de Haan et al. 2003). FGF-1 was later used in combination with other factors such as insulin-like growth factor (IGF)-2 (Zhang and Lodish 2004) and angiopoietin-like 2&3 proteins, and induced a 24-30 fold HSC expansion (Zhang et al. 2006).

For human HSC, attempts to expand have in general been disappointing, although some claim robust and long-term *ex vivo* expansion as reviewed by Piacibello (Piacibello et al. 2005). Cytokine stimulation protocols, like in Bhatia et al. (Bhatia et al. 1997), only gave mild and temporary expansion. As in mouse, HoxB4 overexpression (Schiedlmeier et al. 2003) or stimulation (Amsellem et al. 2003) was performed and gave *ex vivo* expansion, although moderate. Overexpression of BMI1 in human CD34+ cells was more promising (Rizo et al. 2008), although disadvantaged by the need for retroviral infection.

Taken together, studies in both human and mouse have shown the possibility to expand HSC *ex vivo*. However, these protocols need further development to permit clinical applicability. As the family of Hox genes seems highly preserved throughout evolution, it is interesting and not unexpected that these genes were shown to play a role in both human and mouse HSC self-renewal. Also, the relatively simple approach to promote *ex vivo* expansion by FGF stimulation entails clinical potential. A recent study that observed HoxB4 mediated expansion through inhibition of FGF receptors therefore seems controversial (Schiedlmeier et al. 2007). However, this study also linked HoxB4 with TNF signaling and showed results in agreement with other publications as well as the conclusion we draw in article I. The following chapter will describe the role of TNF in early hematopoiesis and HSC homeostasis.

43
BACKGROUND TO ARTICLES

TUMOR NECROSIS FACTOR AND HSC HOMEOSTASIS
(background to article I)

More than a century ago the observation was made that bacterial infections induce tumor regression (necrosis) and in 1944 lipopolysaccharide (LPS), isolated from bacterial extracts, was assigned as a mediating factor (Shear and Turner 1943). Later, it was actually shown that the effects of LPS were not direct, but mediated through a factor contained in the serum (O’Malley et al. 1962). This factor was referred to as tumor-necrotizing factor, later renamed as tumor necrosis factor - alpha (TNF). In 1984-85, TNF was isolated and cloned (Aggarwal et al. 1985b); in addition, it was shown to signal through two distinct cell surface receptors, the Tnfrsf1a (TNFR-p55) and the Tnfrsf1b (TNFR-p75) receptors (Aggarwal et al. 1985a). Today, we know that these TNF receptors are part of a larger TNF-receptor superfamily containing over 30 different receptors that mediate signaling through binding of an almost equally high number of activating ligands (Aggarwal 2003). TNFR-p55 and Fas receptors have a common intracellular death-domain (Itoh and Nagata 1993; Tartaglia et al. 1993) and have mostly been implicated in apoptosis signaling, whereas TNFR-p75 has been implicated in proliferation (Figure 10) (Aggarwal 2003).

Figure 10. TNF signaling through its distinct TNFR-p55 and TNFR-p75 receptors. TNF is involved in many cellular processes. TNFR-p55 shares a FADD (Fas associated death domain) with Fas and has primarily been implicated in apoptosis. TNFR-p75 has primarily been implicated in survival/apoptosis. However, some redundancy, ligand passing and cross-talking between the two TNF receptors has been suggested.
TNF is a pleiotrophic factor that is involved in a large number of biological processes throughout the body (Aggarwal 2003). Also, it is involved in the pathogenesis of a large number of diseases like rheumatoid arthritis, inflammatory bowel diseases, graft-versus-host disease (GVHD), and some BM failure syndromes (Younes and Aggarwall 2003). The widespread expression of TNF receptors on a variety of cells and the activation of different signaling pathways upon ligation to the different TNF receptors hampers detailed characterization of TNF induced cell fates. This is further complicated by proposed mechanisms such as cross talking, redundancy and ligand passing between TNFR-p55 and -p75 receptors (Wajant et al. 2003), as well as the potential requirement for both receptors to elicit maximal TNF signaling (Weiss et al. 1998; Mukhopadhyay et al. 2001). In addition, it should be kept in mind, in experiments using TNF stimulation, that soluble TNF was shown to primarily ligate to TNFR-p55, whereas membrane bound TNF has a higher affinity for the TNFR-p75 receptor (Grell et al. 1995).

Several in vivo studies have described the effects of TNF on blood cell development as a consequence of signaling through each of the receptors. For instance, the anemia that is often observed in patients with rheumatoid arthritis is thought to be TNF induced (Papadaki et al. 2002) and this was supported in studies that induced anemia in mice by TNF injections (Clibon et al. 1990). Along the same line, a large body of evidence points to TNF induced repression of blood cell formation in both in vivo and in vitro studies, in both human and mouse systems (Johnson et al. 1988; Ulich et al. 1990; Jacobsen et al. 1992; Fahlman et al. 1994; Rusten et al. 1994a; Rusten et al. 1994b; Snoeck et al. 1996; Maguer-Satta et al. 2000; Bryder et al. 2001; Dybedal et al. 2001; Quentmeier et al. 2003). Induction of differentiation (Maguer-Satta et al. 2000; Dybedal et al. 2001), induction of apoptosis (Selleri et al. 1995; Weiss et al. 1998; Papadaki et al. 2002), as well as delayed commitment to active cell cycle (Beyne-Rauzy et al. 2004) have all been suggested as explanations for this TNF-induced growth inhibition. However, a number of studies provide evidence for both stimulatory and inhibitory actions of TNF in hematopoiesis, dependence on TNF concentrations, TNFR-p55 or -p75 receptor dependent activation, collaboration with other cytokines, and the types of targeted cellular subsets (Johnson et al. 1988; Ulich et al. 1990; Jacobsen et al. 1992; Fahlman et al. 1994; Rusten et al. 1994a; Rusten et al. 1994b; Snoeck et al. 1996; Quentmeier et al. 2003).

Most work in studying the effects of TNF on blood cell formation was performed using more committed blood cells. Not so much work has been done on the
effects and roles of TNF on HSC homeostasis and some of these reports are seemingly conflicting. For instance, Zhang et al. used a knockout mouse deficient in TNFR-p55 expression and found increased levels of phenotypically defined HSC (Zhang et al. 1995), whereas Rebel et al. found that such mice are characterized by decreased HSC activity following transplantation (Rebel et al. 1999). In agreement with Zhang et al., mice deficient for TNF ligand production have increased frequencies of several immature hematopoietic progenitor subsets (Drutskaya et al. 2005). Also, it was demonstrated that TNF suppresses HSC activity in vitro (Bryder et al. 2001; Dybedal et al. 2001) and that this reduction in HSC numbers was preferentially observed when HSC were induced to actively cycle (Bryder et al. 2001). None of the above mentioned studies have investigated the effects on HSC homeostasis following in vivo TNF stimulation. In addition, a role for TNF signaling in HSC that are deficient for both TNFR-p55 and –p75 signaling was never established. To address these questions, we performed a series of experiments that are presented in article I. We found that in vivo treatment of TNF has suppressive effects in HSC activity in a cell cycle dependent manner. Also, by loss-of-function studies we established a negative role for TNF in HSC maintenance in non-steady state hematopoiesis and that signaling through both TNF receptors is required to elicit full inhibitory actions.
The process of aging has lately gained increasing attention. This is partly due to the emergence of a large cohort of elderly in the Western world causing an increasing prevalence in age-related diseases. But also, recent insights have taught us that the process of “normal” aging is often not totally dissimilar from pathogenetic events in diseases like cancer. The process of aging is intriguing, considering the large differences in lifespans between different species, but also between different cell types within one organism. The use of mouse models has shown to be of great help in trying to understand these processes, but they also entail some considerations. For instance, as the lifespan of a mouse is around 2.5 years, would a 2.0 year old mouse’s stem cell compare to a 2.0 year old, or say a 65 year old human’s stem cell?

Early reports on the process of aging were purely descriptive, but the development of new techniques allowed for a more mechanistic approach to study the “aging phenotype”. For instance, the insulin/IGF-1 pathway was shown to be involved in the aging process and seems evolutionarily conserved; it was shown to be important in worm, fly and mammalian aging (Tatar et al. 2003). Even more, defective insulin/IGF-1 signaling did not only slow down the aging process, but also tumor formation, supporting a link between these processes (Pinkston et al. 2006).

The longevity of stem cells and the consequential risk of acquiring transforming events makes HSC an interesting and important target to study in aging biology. In addition, as elderly often present with skewed blood cell formation (anemia, thrombocytosis and decreased adaptive immune responses), the aging model also provides for clues on lineage commitment regulation. In agreement with the latter clinical observations, HSC from young mice have greater lymphoid potentials as compared to old HSC (Sudo et al. 2000; Kim et al. 2003; Rossi et al. 2005). Indeed, children are more prone to develop lymphoid leukemias, whereas myeloid leukemias and myeloproliferative diseases occur more frequently at higher age. This suggest the existence of differential regulation of HSC based on their age, as was illustrated when comparing global gene expression signatures of fetal and young adult HSC (Ivanova et al. 2002) and between young and old adult HSC (Rossi et al. 2005). A range of additional clinical observations implicated in aging have been confirmed and investigated using mouse models. Some of these will be discussed hereafter shortly.
(1) *In vitro* studies show decreased proliferative potentials of old, human HSC when compared to young HSC (Landsdorp et al. 1993) and also in BMT; using elderly as donors is disadvantageous (Kollman et al. 2001). In support of this, serial transplantation in mice diminishes both **HSC function and numbers** (Harrison et al. 1978; Ross et al. 1982) and HSC from old mice have decreased peripheral blood reconstitution abilities in a cell-autonomous manner (Kim et al. 2003; Rossi et al. 2005). Interestingly, the HSC pool seems increased in aged mice (Morrison et al. 1996; de Haan et al. 1997; Sudo et al. 2000), probably due to the increased likelihood of undergoing self-renewing cell divisions (Rossi et al. 2005). The reasons as to why this occurs can only be speculated upon, although it could be due to clonal expansion of some of the HSC.

(2) **Telomeres** represent the “ends” of our chromosomes and function to confer genetic stability. Upon cell division, telomere length decreases and this can be counteracted through mechanisms such as telomerase or alternative telomerase lengthening. These mechanisms are therefore most active in cycling cells. When telomere shortening reaches a critical point, the cells often commit to senescent or apoptotic cell fates, although it can also increase the risk for malignant transformation. Even though telomerase activity is detected in HSC (Harrington 2004), telomere shortening does occur in aging HSC in humans (Vaziri et al. 1994) and mice (Allsopp et al. 2001). In addition, following stress such as BMT, telomerase activity cannot prevent telomere shortening (Wynn et al. 1998; Allsopp et al. 2003a). In agreement with this, mice deficient for telomerase activity are characterized by accelerated HSC exhaustion (Allsopp et al. 2003a) and decreased reconstitution abilities with age (Rossi et al. 2007a). Also, patients bearing mutations in genes involved in the telomerase machinery present with BM failure (Yamaguchi et al. 2005; Calado et al. 2007; Xin et al. 2007). However, whereas constitutive activation of telomerase activity could rescue telomere shortening following serial transplantations, it could not prevent HSC exhaustion over WT levels (Allsopp et al. 2003b). This would indicate that telomerase-independent barriers also exist that limit HSC transplantation capacities. Signaling through the cell cycle inhibitor p21 was suggested as a mechanism for telomere attrition induced cellular dysfunction, since defective p21 expression rescued proliferation and reconstitution potentials in telomerase deficient HSC (Choudhury et al. 2007). Also, TNF is implicated in induction of cellular senescence by inhibiting telomerase activity (Beyne-Rauzy et al. 2004; Beyne-Rauzy et al. 2005).
(3) Back in 1975, the “immortal stand hypothesis” was proposed, indicating that stem cells upon asymmetric cell division retain the old DNA strands and segregate the newly synthesized chromosomes to differentiated progeny as a means to prevent the accumulation of mutations (Cairns 1975). However, this hypothesis was shown to be unlikely, as reviewed by Lansdorp (Lansdorp 2007), and evidence has pointed to the presence of **nuclear DNA-damage** even in HSC. This gradual accumulation of DNA-damage in the HSC genome is considered to contribute to the aging phenotype. A variety of sources that inflict this damage has been suggested, such as reactive oxygen species (ROS), telomere shortening, DNA replication, etc. (Rossi et al. 2008). Accurate control of these lesions is of particular importance for HSC, as these cells are both long-lived and pass on all genomic defects to their progeny. However, DNA-repair is not perfect and DNA lesions accumulate with age (Hamilton et al. 2001). That DNA-damage contributes to the aging phenotype is illustrated in diseases with defective DNA-repair mechanisms (Bohr 2002). Moreover, mice with defective DNA-repair machineries have demonstrated reduced HSC potentials and these hematological defects were accentuated during aging and replicative stress (Rossi et al. 2007a; Rossi et al. 2007b).

(4) Apart from the nucleus, mitochondria are the only organelles to contain DNA (mtDNA). Due to the proximity to ROS, a lack of histone protection and limited DNA repair system, **mtDNA mutation** rates are drastically higher compared to nuclear DNA and accumulate with age (Yakes and Van Houten 1997). As mitochondria play a role in normal hematopoiesis (Fontenay et al. 2006), mitochondrial dysfunction has been suggested for a variety of hematological diseases, such as leukemia, lymphoma and those affecting the erythroid lineage such as dyserythropoiesis, sideroblastic anemia and myelodysplastic syndrome (Penta et al. 2001). Interestingly, these conditions prevail mainly in the aged individual. Indeed, mice with defective mtDNA proof reading obtain accelerated mutation rates and age prematurely (Trifunovic et al. 2004). Increased ROS levels were suggested to play an important role in this and were also shown to upregulate p16\(^{INK4A}\) expression leading to HSC failure (Ito et al. 2004).

(4) **p16\(^{INK4A}\)** is a cyclin dependent kinase inhibitor that has been implicated to confer replicative senescence in aged stem and progenitor cells (Janzen et al. 2006; Molofsky et al. 2006). In concordance with this, p16\(^{INK4A}\) expression was found to be upregulated in old HSC compared to young (Janzen et al. 2006; Pearce et al. 2007) although at low levels. Using mouse models, it was shown that enforced expression of p16\(^{INK4A}\) induced proliferative arrest and cell death (Park et al. 2003; Janzen et al.
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However, these experiments were performed under non-
steady state hematopoiesis and p16INK4A induced senescence might therefore not
represent a physiological cell fate in the aging HSC. This notion is supported in some
other reports (Morrison et al. 1996; Sudo et al. 2000; Iwama et al. 2004) and will be
discussed in more detail in the context of article II.

Figure 11. Proposed mechanisms of HSC aging and their relationship to
blood aging phenotypes. Genomic instability through DNA damage and
telomere attrition, and an altered transcription program leads to dysregulation
of a number of cellular processes. This can cause some of the phenotypes
often observed in the aged individual. Adapted from Warren and Rossi
(Warren and Rossi 2008).
DIFFERENTIATION; LEAVING THE “HSC BOX” (Background to articles III&IV)

HSC possess the ability to generate all cell types of the blood system. Through a series of events, immature hematopoietic cells gain proliferative potential and commit to the different lineages at the expense of self-renewing and multi-lineage capacity. In this chapter, I will discuss some of the work that has been done on identifying the hierarchical composition of lineage development (or differentiation). High resolution profiling of the developmental stages is of great value to understand and study the underlying mechanisms that guide these cell fate decisions. In addition, it would create greater insights in leukemic development, as a developmental block at a certain stage in the hematopoietic tree characterizes many of the leukemic blasts. In this chapter, I will focus primarily on mouse studies as the vast majority of the work was performed using mouse models. However, the similarities between mouse and human differentiation (Hao et al. 2001; Manz et al. 2002; Edvardsen et al. 2006; Majeti et al. 2007) make these studies of direct interest also for human biology.

The prospective, FACS based isolation of cells at different stages of development was primarily achieved by cell-surface marker profiling; in some cases aided by reporter-mouse strains. Interpretation of FACS results has to be done carefully as expression profiles of many commonly used markers in FACS can be altered upon induction of cellular stress, or in gain- or loss-of-function models. For instance, the LMPP population (see below) is phenotypically characterized by cKit+Lin-Sca1+Flt3+ expression. As Flt3 expression is down-regulated in the absence of Ikaros signaling, it is difficult to study the LMPP in Ikaros null mice (Yoshida et al. 2006). Most developmental steps are driven by the interplay of cell-extrinsic and cell-intrinsic factors; some of these regulators will be discussed in more detail in a following chapter.

The first steps

As mentioned in the chapter “Prospective isolation of the HSC”, no single marker has been identified to define HSC and this also holds true for its preceding immature progenitors. All HSC activity in adult mice resides within the cKit+, lineage-, Sca1+ (KLS) cell compartment that constitutes about 0.1% of total BM cells (Spangrude et al. 1988; Ikuta and Weissman 1992; Morrison and Weissman 1994). Despite the infrequency of the KLS population, only a minor fraction within this subset possess
long-term multi-lineage reconstitution potentials (LT-HSC). As a **LT-HSC** loses "stemcellness", long-term self-renewing capacity is considered the first property to diminish. Upon upregulation of Flt3 (or Flk2) (Adolfsson et al. 2001; Christensen and Weissman 2001) or CD34 (Osawa et al. 1996), or down-regulation of Slamf1 (CD150) (Kiel et al. 2005), LT-HSC differentiate into multi-potent progenitors (**MPP**) devoid of long-term self-renewing capacity. This transition was later refined and intermediate short-term reconstituting cells, like the KLSCD34+Flt3- HSC (**ST-HSC**) were identified. ST-HSC possesses HSC qualities but can only give transient reconstitution (Yang et al. 2005). These cells are of great clinical importance, as these cells can efficiently confer conditioning protection in the first weeks following transplantation.

![Diagram](image)

*Figure 12.* Pathways of early hematopoiesis as proposed by **A** (Kondo et al. 1997; Akashi et al. 2000), **B** (Adolfsson et al. 2005), **C** (Pronk et al. 2007) and **D** (Arinobu et al. 2007). Adapted from Murre (Murre 2007).
About ten years ago, Weissman and co-workers proposed a model in which the first steps of lineage restriction mutually excluded myeloerythroid from lymphoid potential through the identification of a common myeloid progenitor (CMP) (Akashi et al. 2000) and common lymphoid progenitor (CLP; Figure 12A) (Kondo et al. 1997). The CLP was originally defined as Lin-Sca1<sup>+</sup> cKit<sup>+</sup> IL7Ra<sup>+</sup> cells that at a population level gave rise to T cells, B cells, NK cells, DCs but not to granulocytes (G) monocyte/macrophages (M), erythrocytes (E) or platelets/megakaryocytes (Meg) (Kondo et al. 1997; Karsunky et al. 2005). Recently, the CLP compartment was further subdivided into CLPFlt3+ cells with combined B, T, NK and DC potential and CLPFlt3- cells which primarily gave B cells; both devoid of myeloerythroid potential (Karsunky et al. 2008). Also Stephen Nutt Pu.1 reporter mice showed this in 2005.

However, some studies claim that the CLP only has limited T cell potentials (Allman et al. 2003; Schwarz and Bhandoola 2004); moreover, a number of reports detected myeloid output from these cells (Allman et al. 2003; Balcünaitė et al. 2005; Rumfelt et al. 2006; Mansson et al. 2008). These studies shed some doubt on the strict divergence of myeloerythroid versus lymphoid lineage restriction. In support of this idea, Jacobsen and co-workers identified an LKSCD34+Flt3+ cell population that contained combined myeloid (GM) and lymphoid (B/T/NK) potential, but lacked significant Meg and E potential, which was named lymphoid primed MPP (LMPP; Figure 12B) (Adolfsson et al. 2005). Although this was recently challenged (Forsberg et al. 2006), additional reports have suggested loss of MegE potential to be the first lineage restricting event. For instance, KLS cells that express high levels of Ikarsos (a zinc finger DNA-binding protein) overlap greatly with the LMPP and exhibit G/M/B/T/NK potential without MegE output (Yoshida et al. 2006). Also, Lai and Kondo subdivided the KLS compartment based on Flt3 and VCAM-1 (vascular cell adhesion molecule-1) expression and found KLSFlt3+VCAM-1+ cells that functionally resembled LMPP and LKSIkarsos+ cells (Lai and Kondo 2006).

The first stage of lymphoid restriction, upon loss of myeloid potential remains debated. Igarashi et al. used a Rag1-GFP reporter and identified KLSCD27+Rag1+ ELp (early lymphoid progenitor) cells with lymphoid and no myeloerythroid potential (Igarashi et al. 2002). However, since the CLP, possessing some myeloid potential (Allman et al. 2003; Balcünaitė et al. 2005; Rumfelt et al. 2006; Mansson et al. 2008) lies downstream of the ELp, it could be difficult to combine these findings. In addition, since KLS cells, but not CLPs give robust T cell progeny (Schwarz and Bhandoola 2004; Benz and Bleul 2005), the question is raised as to where T cell restriction occurs.
A complicating factor here is the fact that T-cells are thought to mature in the thymus (Heinzel et al. 2007); thus raising the question as to whether the earliest T-cell restricted progenitors reside in the BM, the thymus or in the blood stream while traveling from BM to thymus? Allman et al. identified a Lin-Kit-CD25-CD44+IL7Ra<sub>low</sub> early thymic progenitor (ETP) cell that resides intra-thymically and possesses robust T potential with delayed B and very limited myeloid potentials (Allman et al. 2003). In addition, these ETP were shown to develop independently from a CLP. Benz and Bleul used a CCR9 reporter strain and identified intra-thymic Lin-Kit+CD25-CCR9<sup>high</sup> thymic multipotent precursor (TMP) cells that contained T and some B, NK, DC and myeloid potential (Benz and Bleul 2005). Loss of CCR9 expression coincided with loss of B cell potential and could therefore be seen as the first step of B cell potential exclusion. Signaling through the Notch pathways was shown to be of importance for T cell development from these early thymic progenitors, at the expense of B cell development (Sambandam et al. 2005; Tan et al. 2005).

B cell specification and maintenance is regulated by a number of transcription factors, including Ikaros, EBF, E2A, PU.1 and Pax5 that interplay in a finely regulated network as reviewed by Nutt and Kee (Nutt and Kee 2007). B cell restriction, upon loss of T cell potential is believed to occur within the BM residing CD19+B220+ pro-B cell population that expresses high levels of the B-cell specific gene Pax5 (Rumfelt et al. 2006; Fuxa and Busslinger 2007). Mansson et al. recently found lambda-5 expressing CLPs with robust B cell and limited T cell potential (Mansson et al. 2008). These lambda-5 expressing CLPs were however devoid of myeloid potential, as opposed to lambda-5 negative CLPs that gave myeloid readout. Upregulation of lambda-5 within the CLP compartment could therefore be the first stage of myeloid versus lymphoid divergence. These lambda-5 negative CLPs probably overlap largely with pre-pro-B cells that were ascribed similar lineages potentials (Rumfelt et al. 2006) and present with a similar cell-surface phenotype except for B220 expression.

With regard to the establishment and generation of the myeloerythroid lineages, some controversies also exist. In defining the branch-point of early lymphoid versus myeloerythroid divergence, a CLP (see above) and CMP (Akashi et al. 2000) were isolated. This CMP was shown to have combined G/M/Meg/E potentials and to be the direct ancestor of the more restricted GMP (granulocyte-macrophage progenitor) and MEP (megakaryocyte-erythocyte progenitor); all three residing within the cKit+Lin-Sca1- (KLS-) compartment (Figure 12A). Within the KLS- compartment, CMP were CD34<sup>high</sup>FcgRII/III<sup>int</sup>, GMP were CD34<sup>high</sup>FcgRII/III<sup>high</sup> and MEP were
CD34<sup>low</sup> FcγRII/III<sup>int/low</sup>. The MEP gave only Meg/E progeny and the GMP only G/M whereas the CMP had all these lineage potentials combined. This latter feature has been challenged in a number of reports, including in articles III and IV of this thesis (Figure 12C). First, it is important to appreciate that only a small fraction of the cells within the originally described CMP compartment actually possesses combined G/M/Meg/E potentials (Akashi et al. 2000; Nakorn et al. 2003; Iwasaki et al. 2005; Terszowski et al. 2005; Yoshida et al. 2006). Secondly, other studies have demonstrated the establishment of the Meg/E and G/M lineages without proof for a CMP intermediate. For instance, Takano et al. performed a series of in vitro clonogenic pair daughter experiments and found no cell that gave combined G/M/Meg/E output (Takano et al. 2004). Also, Nutt et al. took advantage of a PU.1 reporter mouse and found PU.1 expression in CMP to be associated with G/M potential, whereas PU.1 negative CMP mainly produced Meg/E (Nutt et al. 2005). In similar studies, Terszowski et al. could functionally subdivide the CMP based on IL-3Ra expression (Terszowski et al. 2005). These studies strongly indicate a heterogeneous composition of the CMP compartment and shed doubt on the existence of a real CMP. A recent study however, also by Akashi and co-workers, described “another” CMP that is contained within the KLS compartment (Arinobu et al. 2007). They found GATA-1 expressing KLSCD34+ cells to display G/M/Meg/E (CMP) potential, whereas PU-1 expressing KLSCD34+ cells gave G/M and lymphoid progeny. The latter cell type was referred to as GMLP (Figure 12D). Unfortunately, this study does not describe the relationship of this new CMP to the previously described CMP. As the KLS- CMP is heterogeneous, it is possible to constitute cells that are already restricted to either the G/M or Meg/E cell fates even prior to the GMP and MEP stage. We proved these assumptions to hold true and identified, based on differential expression of Slamlf1 (CD150), Endoglin (CD105) and CD41 (Integrin alpha 2b), a pre GM and pre MegE compartment (Pronk et al. 2007; Pronk et al. 2008) that will be discussed below (Figures 12C and 17). As these cellular subsets mature, they give rise to more restricted offspring. We found the pre MegE to generate a uni-potent megakaryocytic progenitor (MkP) that largely overlaps with the previously described MKP (Nakorn et al. 2003) or that generates an uni-potent erythroid progenitor (pre CFU-E: colony forming cells - erythrocytes). The pre CFU-E subsequently matures through a series of intermediate stages and gradually loses proliferative capacities. Based on expression levels of Ter119 and CD71, pre CFU-E mature through the CFU-E, proerythroblast (pro-EB) (Terszowski et al. 2005), basophilic EB, late basophilic/polychromatophilic EB to the orthochromatophilic EB
stage (Figure 13) (Socolovsky et al. 2001) to ultimately give rise to reticulocytes/erythrocytes.

Figure 13. Illustration of a flow cytometric approach to identify all stages of erythroid maturation. *: cellular subset identified in this thesis. EB: erythroblast, Baso: basophilic, Chro: chromatophilic, Ortho: orthochromatic, Retic/Ery: reticulocyte/erythrocyte.
REGULATION OF DIFFERENTIATION

The cues that guide HSC to ultimately differentiate into functional effector cells are both diverse and interdependent. Roughly, cell regulation is guided by external stimuli, like cytokines, and internal processes such as transcription factors, epigenetic and post-translational regulation. In the following chapters, I will discuss these mechanisms and their involvement mainly in HSC, early multipotent and myeloproliferative progenitors as these cells are the focus of this thesis. That said, regulatory mechanisms work in a concerted manner to guide cellular identity. For instance, for a cytokine to bind to the cell-surface, receptor expression is required. Expression of this receptor is dependent on an epigenetic state that allows transcription factor binding to a certain promoter to induce gene transcription and translation, which subsequently requires the correct post-translational processing for the product (e.g. cell surface receptor) to be produced and translocated to the cell surface.

Cytokine regulation

Cytokines are a group of proteins, either membrane-bound or soluble, that act through binding to specific receptors on target cells. In the absence of a specific cell-surface receptor, the cytokine cannot bind and can therefore not elicit direct targeting of this cell. In the hematopoietic field, a large number of cytokines have been identified, cloned (Metcalf 1993; Ogawa 1993) and shown to play a role in both immature and mature hematopoiesis in processes such as survival, proliferation and differentiation (Zhu and Emerson 2002; Kaushansky 2006).

For most hematopoietic lineages, cytokines have been identified that play important roles in the establishment of these lineages, like EPO for erythrocytes, TPO for platelets (Kaushansky and Drachman 2002), G-SCF for granulocytes, M-CSF for macrophages (Metcalf 1993), IL-7 for B cells (Maraskovsky et al. 1998) and Notch-ligand for T-cells (Sambandam et al. 2005). Importantly, many cytokines interact with other cytokines in a complementary, synergistic or antagonistic manner. In addition, one single cytokine may be important but not crucial for inducing/maintaining a certain cell fate (i.e. redundancy) or could play different roles at different stages within the hematopoietic tree. Below, I will give some examples of different cytokines that have similar roles, or single cytokines that have context-dependant diverse effects.
One such cytokine is **TPO** (thrombopoietin), with its ligand-binding receptor mpl. TPO was shown to play a role not only in the generation of platelets (Kaushansky and Drachman 2002), but also at more immature stages like the HSC. Stimulation with TPO alone supports HSC survival, whereas it induces HSC proliferation in combination with IL-3 or SCF (stem cell factor or cKit ligand) (Sitnicka et al. 1996). Indeed, mpl is expressed in HSC (Buza-Vidas et al. 2006), TPO deficient signaling negatively affects HSC function in mice (Qian et al. 2007) and patients with Mpl dysfunction present with thrombocytopenia and BM failure (Ballmaier et al. 2003).

**SCF, or KL** (cKit ligand) also has multiple roles. It was shown to be involved in lymphopoiesis, erythroidopoiesis and megakaryopoiesis (Lyman and Jacobsen 1998) and cKit was highly expressed in HSC, MPP and myeloid progenitors (Ikuta and Weissman 1992; Akashi et al. 2000). As was the case with TPO, some reports showed that stimulation with SCF alone promotes survival of HSC (Li and Johnson 1994; Keller et al. 1995), whereas it stimulates HSC to cycle in combination with other growth factors (Jacobsen et al. 1996). Studies using partial or complete loss-of-function models supported a role for cKit in HSC functioning (Miller et al. 1996; Bowie et al. 2007a; Sharma et al. 2007; Thoren et al. 2008), as well as in lymphopoiesis (Waskow et al. 2002).

Like cKit, Flt3 (or Flk2) is a member of the family of tyrosine kinase receptors and is ligated by **FL (Flt3 ligand)**. Although initially suggested to play a role in HSC regulation (Mackarehtschian et al. 1995), Flt3 is not expressed on HSC (Adolfsson et al. 2001; Christensen and Weissman 2001) and FL deficient mice have normal HSC levels (Sitnicka et al. 2002), suggesting a redundant role of this cytokine in HSC homeostasis. However, evidence clearly points to a role of FL in early lymphoid development (Lyman and Jacobsen 1998; Sitnicka et al. 2002) and as well does **IL-7** (Milne and Paige 2006).

A genetic transcriptional network that guides early **myeloid** specification has been established (see later), and some cell-extrinsic stimuli were shown to be involved in stimulation or specification of these myeloid lineages. The role of TPO in platelet development was already discussed above. Together with TPO, **EPO** and **IL-3** (interleukin 3) were also shown to stimulate megakaryocyte production *in vitro*, whereas G-CSF (granulocyte-colony stimulating factor) had negative effects (Metcalf et al. 2002; Metcalf et al. 2005). EPO has, apart from playing a role in megakaryopoiesis, primarily been implicated in erythropoiesis. By using loss-of-function models, both EPO and also
SCF play non-redundant roles in erythroid specification in vivo, although EPO alone is sufficient to generate erythroid cells in vitro (Munugalavadla and Kapur 2005). Transforming growth factor-β (TGF-β) has been implicated in negatively affecting early hematopoiesis (Ducos et al. 2000; Larsson and Karlsson 2005), although only in in vitro systems (Larsson et al. 2005). By contrast, positive effects of TGF-β were described for the establishment of the granulocytic lineage (Keller et al. 1991; Keller et al. 1994). Otherwise, the factors that are most implied in granulocyte-macrophage formation are GM-CSF (granulocyte/macrophage-CSF), G-CSF, M-CSF (macrophage-CSF) and IL3. **M-CSF** is mainly involved in monocyte/macrophage production and **G-CSF** mainly in granulocyte production (Metcalf 1993), although some level of redundancy and synergy of these two factors was described in more immature hematopoietic precursors (Barreda et al. 2004). However, G-CSF is indispensable for terminal differentiation and functioning of neutrophiles (Lieschke et al. 1994). Interestingly, G-CSF receptors (as well as EPO receptors) are already expressed on KLSCD34-Flt3- LT-HSC (Mansson et al. 2007), treatment with G-CSF mobilizes HSC in human (Barreda et al. 2004) and mice (Wagers et al. 2002a), and human cord blood Lin-CD34+CD38- HSC are responsive to G-CSF stimulation (Nishi et al. 1996). Therefore, G-CSF is another good example of one single cytokine having redundant, essential, synergistic, positive or negative actions dependant on the context and the cell type its actions are pertained. **IL-3** and **GM-CSF** are involved in the generation of both G and M colonies and are considered to act on more immature progenitors (Metcalf 1993; Bryder and Jacobsen 2000). However, some redundancy exists between these factors, as mice deficient for either of these factors present with only marginal reductions in blood cell formation (Stanley et al. 1994; Nicola et al. 1996).

The exact modes as to how cytokines guide or direct cell fates have been the subject of discussion. Different models, like stochastic versus deterministic and instructive versus permissive have been proposed and evaluated in different experimental settings as reviewed by Robb (Robb 2007). However, with current knowledge on the context dependant multiple actions of single cytokines and, even more, increasing understanding of intrinsic transcriptional and epigenetic networks, these models seem like an oversimplification and are probably not as “black and white” as sometimes proposed.
Transcriptional regulation

Transcription factors (TF) are proteins that interact with coding and non-coding regions of genes, which in a stage and lineage dependent manner interact with other factors to promote or repress expression of genes that specify or direct a certain cellular fate. In hematopoiesis, transcriptional networks that guide HSC maintenance/survival (Orkin 2000) or differentiation towards the different blood cell lineages have been established and are discussed in a number of excellent reviews (Kim and Bresnick 2007; Nutt and Kee 2007; Rosenbauer and Tenen 2007; Rothenberg 2007). Some TF, like the previously described HOX factors, can modulate the function of chromatin-associated factors and vise versa and illustrate the interdependence of genetic and epigenetic regulation.

Many of the initially identified TF in hematopoiesis were discovered in the context of human hematopoietic malignancies as translocations of these TF often cause deregulation and can result in proto-oncogenic activities. For instance, TF like MLL, Runx1, TEL/ETV6, SCL/tal1 and LMO2 were implied in the regulation of normal hematopoiesis, but account also for a large part of the TF that are involved in leukemogenesis (Orkin and Zon 2008).

As mentioned, there exists a temporal and stage specific requirement of TF to pertain certain actions. This is illustrated, for instance, in HSCs; Sox17 (Kim et al. 2007) and SCL/tal1 (Mikkola et al. 2003) are required for HSC generation and specification, respectively, during fetal but not adult life. For SCL/tal1 however, a role during adult life was established in the maturation of erythroid and megakaryocytic lineages (Mikkola et al. 2003) and similar findings were observed for Runx1 (Ichikawa et al. 2004). Also, adult aged HSCs, as compared to young adult HSC, display both lineage skewing as well changes in gene expression levels of a large number of genes (Sudo et al. 2000; Rossi et al. 2005; Nijnik et al. 2007) and exemplify temporal gene expression differences within a certain cell type.

In addition to the importance of stage and temporal TF activity, transcriptional levels, interaction/competition with other TF and even the order of expression of different TF (Iwasaki et al. 2006) were shown to be highly relevant for cell fate determination. Some of these factors are illustrated in the differentiation from HSC to more mature blood cell lineages. One such example is the interplay between GATA-1 (associated with Meg/E specification) and PU.1, (an Ets family transcription factor that
is involved in HSC self-renewal and early lymphoid and myeloid specification). High levels of expression of PU.1 were shown to inhibit GATA-1 activity and vise versa (Nerlov and Graf 1998; Rekhtman et al. 1999; Zhang et al. 1999; Nerlov et al. 2000; Rhodes et al. 2005) and guided multipotent cells to convert to a myeloid or erythroid cell fate, respectively (Figure 14). It was indicated that these factors bind to each other to prevent interaction with their respective binding sites and by these means are mutually inhibitory. This implies that changes or imbalance in TF activity could “flip” cellular fates. Against this background, Akashi and co-workers used reporter mice for PU.1 and GATA-1 and found within the KLS compartment CD34+Flt3-GATA-1+ cells that possess G/M/Meg/E (or CMP) potential and CD34+Flt3+PU.1+ cells that possess G/M/B/T (or GMLP) potential, which indicates existence of a CMP and at least two different routes of G/M potentiation (Arinobu et al. 2007).

![Figure 14. The interplay between the transcription factors (TF) PU.1 and GATA-1 exemplifies how two TF can act antagonistically and how different levels of the respective TF can guide cellular differentiation](image)

More examples for direct mutual antagonistic actions of TF in cell fate determination are described in the myeloerythroid lineages; C/EBPalpha and PU.1 in respect to granulocyte versus monocyte development, respectively (Reddy et al. 2002; Dahl et al. 2003), PU.1 and Gfi1 for monocyte and neutrophil development, respectively (Arinobu et al. 2007), and EKLF and Fli-1 in erythroid and megakaryocytic development, respectively (Starck et al. 2003). A model of protein complexes in which TF directly interact has been proposed to accommodate the observations of direct antagonism and context-dependence of TF (Orkin 2000). Within these protein complexes, the recruitment of chromatin remodeling factors is considered
to be an additional step in gene expression regulation. For instance, GATA-1 is described to both activate erythroid associated genes and repress proliferation associated genes (like cKit and c-myc). GATA-1 mediated repression of genes does not only need direct interaction with the TF FOG-1 (friend of GATA-1), but also needs recruitment of the nucleosome remodeling and histone deacetylase (NuRD) complex (Hong et al. 2005) that acts to induce epigenetic repression. Moreover, NF-E2, another erythroid affiliated TF, associates with the MLL2 complex that induces H3K4 (histone 3 lysine 4) methylation and thereby allows for gene transcription (Demers et al. 2007).

There is some debate as to how TF are involved in the differentiation and multi-lineage potentials of immature cells. (i) Does the expression of lineage associated TF reflect the lineage potentials of a cell? (ii) And when has the cellular architecture changed to a degree that a cell is “locked” into a certain cell fate, irrespective of the quality of TF activity at that time-point? With regard to the first question, HSC express low levels of genes associated with most lineages, and upon lineage commitment, some lineage associated genetic programs are inactivated whereas others are upregulated (Bruno et al. 2004; Mansson et al. 2007). However, HSC also express a number of non-hematopoietic genes (Akashi et al. 2003), but do not generate cells of non-hematopoietic origin (Wagers et al. 2002b). In addition, about 60% of the originally described CMP co-expresses Meg/E and G/M associated genes at a single cell level (Miyamoto et al. 2002). However, only a minor fraction of these cells actually generate mixed Meg/E/G/M colonies (Akashi et al. 2000; Na Nakorn et al. 2002; Iwasaki et al. 2005). These findings question whether the presence of low levels of transcriptional products reflects true lineage potential. Instead, lineage commitment could rather be regarded as a continuum in which transcriptional programs are gradually shut down. In this case, remnants of the transcribed product can still be detected even though transcription itself is already inactivated. In support of this and as an example, we found the myeloid associated gene product MPO (myeloperoxidase) expressed in a number of MkP; a compartment fully restricted to the megakaryocytic fate (Prönk et al. 2007). The discussions on the mechanisms of lineage priming should, in addition, be put in the context of an epigenetic state that is set to allow or prevent actual gene transcription to occur, and will be discussed shortly.

With regard to the second question stated above, there are some theories as to what degree plasticity occurs within the hematopoietic system. Today, there is a body of evidence that shows that more or less committed cells can convert to another cell fate upon overexpression of one or more specific genes and is indicative for the possibility of
cellular reprogramming. For instance, MLL-ENL overexpression converts T cell progenitor fates and generates myeloid cells with T cell receptor rearrangements (Drynan et al. 2005). Similarly, C/EBPα overexpression in pre-T cells induced a switch to the macrophage lineage (Laiosa et al. 2006) and its enforced expression in committed B cells converted these cells into macrophages that did present with immunoglobulin rearrangements (Xie et al. 2004). Also, upon overexpression of GATA-1, CLP and G/M progenitors produced Meg/E colonies in vitro (Iwasaki et al. 2003). Additional experiments have shown that, upon expression of certain TF, cells within the hematopoietic system can also convert to a non-hematopoietic fate, like the switch of committed B cells to iPS (induced pluripotent stem) cells upon the enforced overexpression and repression of a number of TF (Hanna et al. 2008). Although these experiments illustrate the plasticity within the hematopoietic system, as well as an epigenetic state that allows for these lineage switches, it should be kept in mind that the enforced overexpression of these TF is an artificial infliction. An interesting question is whether these events would also occur in normal biology. Upon hematopoietic stress, such as bleedings and infections, we know that the output of the required lineages increases robustly and promptly. However, it is (to my knowledge) unclear if this increase always follows “normal hematopoietic development”, or whether lineage redirection can take place in these cases. An argument in favor might be that cell extrinsic stimulation with G/M associated cytokines alone can redirect lymphoid cells to a myeloid fate (Kondo et al. 2000; King et al. 2002; Iwasaki-Arai et al. 2003) although this is only correlative.
**Epigenetic regulation**

The field of epigenetic regulation has recently been fueled with increasing interest and impact. A PubMed search on the query “epigenetic” in the “title” gave 30 hits in 1997 and 495 hits in 2007, of which many were in high impact journals, thus illustrating the increasing development of this field. Epigenetics can be defined as a process that regulates the capacity of TF to bind to regulatory regions through modifications and alterations in chromatin structures. Consequently, epigenetic regulators can promote or repress the ability for a certain gene to be transcribed. This regulation is pertained through a variety of processes like DNA methylation, histone variants, nucleosome repositioning and posttranslational histone modifications (Reik 2007). The importance of epigenetic regulation in hematopoiesis is illustrated in that many hematopoietic malignancies are characterized by improper functioning of chromatin modifying enzymes (Rice et al. 2007). This has potential clinical implications, as the activity of some of the regulators of chromatin structure and also epigenetic marks can be modulated, thus creating a window of therapeutic opportunity (Fazi et al. 2005; Vitoux et al. 2007; Ito et al. 2008). In this section, I will shortly describe and focus on some of these processes that are probably best described and were used in article II in this thesis.

Chromatin is constituted of DNA and histone proteins that form nucleosomes; the basic unit of chromatin. Nucleosomes comprise ~146bp of DNA wrapped ~1.8 turns around an octamer core of histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4). The physical proximity of the nucleosomes identifies the transcription accessibility of gene regulatory regions (promoters and enhancers) to initiate transcription: a dense chromatin structure (heterochromatin) is associated with “inactive chromatin”, whereas a more open structure (euchromatin) is generally associated with active or “open” chromatin. Importantly, a chromatin state, or structure in a certain cell is often preserved in the progeny upon cell division and thereby also determines the cell identity of the daughter cells; this is called “epigenetic memory”. The protruding N-terminal domain (histone tail) of the H3 protein has been shown to have important regulatory properties. For instance, methylation of lysine 4 (H3K4me) is associated with transcriptional activity (Schneider et al. 2004), whereas methylation of lysine 9 (H3K9me) and 27 (H3K27me) correlate with a repressive state. These processes are regulated by a group of histone methyltransferase (HMT) and histone demethylase (HDM) enzymes (Shi and Whetstine 2007). In addition to the methylation state, acetylation status of the histone H3 and H4 tails is also of
importance; it is generally associated with transcriptional activity and is controlled by histone acetyltransferase (HAT) and acetyl-deacetylase (HDAC) activity (Hess-Stumpf 2005). Occasionally, active and repressive histone marks can be detected simultaneously within the regulatory region and are referred to as bivalent domains. These observations underscore the balance of both positive and negative transcriptional regulators to guide transcriptional activity.

In addition to the histone modification mentioned above, **methylolation** status of the **DNA** itself also directs chromatin structure. Methylation of cytosine-phosphate-guanine (CpG) dinucleotides is mediated by DNA methyltransferases (DNMT) (Miranda and Jones 2007). Methylated promoter regions generally disable gene transcription, either through proteins that bind to methylated DNA or by induction of histone deacetylation. However, the impact of DNA methylation regulation is debated, as many control regions do not contain CpGs. A study from Sullivan et al. illustrated the impact of the epigenetic state in the promoter region of the TNF-alpha gene in different cell types (Figure 15); the epigenetic state became increasingly permissive as an immature cell type differentiated towards a TNF-alpha producing cell (Sullivan et al. 2007).

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Figure 15. Activated monocytes produce TNF, whereas hematopoietic precursors, or precursors with hematopoietic potential do not. This figure illustrates the epigenetic changes at the TNF locus during differentiation/maturation. The epigenetic state at the TNF locus becomes increasing "transcriptional permissive" as immature cells differentiate towards TNF producing cells. Adapted from Sullivan et al. (Sullivan et al. 2007).
The epigenetic state described above is dependent and regulated by a group of proteins, and vise versa. Some of these are the trithorax group of proteins (TrxG), implicated in gene activation and the polycomb group of proteins (PcG), implicated in gene repression (Schwartz and Pirrotta 2008). Many of these proteins have been ascribed roles both in the regulation of HSC activity as well as in differentiation processes. The polycomb-repressor complex (PRC)-2 comprises Suz12, Eed1/2/3 and the HMT Ezh2. These trimethylate H3K27 (“repressive mark”) and thereby allow recruitment of PRC-1 members like the previously described Bmi1, with consequent gene silencing. Deficient Bmi1 signaling led to the inability of HSC to give long-term reconstitution (Park et al. 2003), probably due to their inability to permit a quiescent state leading to premature exhaustion.

By contrast, members of the TrxG family, like the MLL (mixed lineage leukemia) protein complexes, induce trimethylation of H3K4 (“active mark”) and thereby sustain or induce transcriptional activity (Ruthenburg et al. 2007). Known targets of MLL include the family of Hox genes (Ernst et al. 2004) and MLL is thereby thought to play a role in normal hematopoiesis. In addition, MLL is known to frequently translocate with numerous fusion partners, thereby inducing leukemic conversion (Dou and Hess 2008). These fusions often cause a reduced ability for MLL to induce H3K4 methylation. Interestingly however, the increased transcriptional activity observed in MLL-translocated leukemias is thought to be conferred through the fusion partners, rather than MLL itself (Okada et al. 2005).

Apart from playing a role in hematopoiesis (see below), epigenetic regulation, or dysregulation, has been shown to play a role in many processes, such as aging. More specifically, the p16INK4A protein is encoded by the INK4A-ARF locus and has been proposed to act as a tumor suppressor and mediates replicative senescence in aging cells (Zindy et al. 1997; Janzen et al. 2006; Molofsky et al. 2006). It was shown that p16INK4A expression is under direct (negative) control of factors within the PRC-1 and PRC-2 complexes, like Bmi1 and Ezh2, respectively (Ohtani et al. 2001; Bracken et al. 2007; Kotake et al. 2007). How these processes are regulated in aging cells in the blood system is unknown and this question formed the background to the experimental work presented in article II.

The role of epigenetic regulation in hematopoietic differentiation was illustrated in a study by Attema et al. (Attema et al. 2007). As mentioned previously, HSC express low levels of a diversity of lineage-affiliated genes. It was shown, in HSC and MPP cells, that the regulatory regions of these genes where characterized by a “permissive” epigenetic
state: dimethylated H3K4, acetylated H3 and under methylated CpGs. In more
differentiated progeny, high levels of H3K4 were found in promoters of genes that
associate with its cell type, whereas control regions of “lineage-unrelated” genes where
characterized by DNA methylation and H3K27 (repressive marks). A similar study
assaying human multipotent and committed cells confirmed these findings (Maes et al.
2008). This adds, besides cell-extrinsic stimulation and TF activation, an extra level of
cell fate regulation in that genes can be “primed” or kept in a permissive state in order to
commence transcription upon proper stimuli. What the exact determinants are that
eventually “tip” a cell to a certain cell fates remain to be shown. Given the ‘chicken-and-
the-egg’ character of this question (epigenetics regulating TF activity regulating
epigenetics), it will be a challenge to provide a quick answer.
AIMS OF THIS THESIS

Traditionally, the hematopoietic system is among our most well characterized organ systems which has in many aspects served as a role model for other cellular systems (Bryder et al. 2006). However, many questions remain unanswered. Detailed knowledge of the mechanisms that govern HSC homeostasis and that guide differentiation through a series of developmental stages, enable us to understand some of the basic regulators involved in these processes. In addition, such knowledge would create increased insights into conditions such as leukemia development and the process of aging. These conditions are characterized by improper activity of regulatory processes leading to altered HSC function and/or differentional progression. The specific aims in this thesis were therefore as follows:

Specific aims

Article I
What are the effects and roles of tumor necrosis factor (TNF) on HSC activity, and which of the two TNF receptors accounts for this?

Article II
What are the proliferative properties of unmanipulated aged versus young adult HSC \textit{in vivo}? Does p16\textsuperscript{INKA} signaling play or role in these processes?

Article III & IV
Does profiling of additional cell surface markers enable us to further subfractionate the earliest myeloerythroid progenitor compartments? What are the functional and molecular characteristics that are associated with the newly identified cellular subsets? How do they compare to previous characterized hematopoietic cell types?
SUMMARY AND DISCUSSION OF THE ARTICLES

Article I

Tumor Necrosis Factor negatively regulates hematopoietic stem cell maintenance in vivo: requirement for two distinct receptors


An overview of TNF and its role in hematopoiesis is already described herein on pages 44-46. In short, TNF has in numerous studies been implicated to negatively regulate a number of hematopoietic cells, including HSCs. However, no study has by lack-of-function models actually shown a negative regulatory role of TNF on HSC activity. Furthermore, although a number of cell-intrinsic regulators were found to negatively regulate HSC function, no single cell-extrinsic factor has to date been shown to do so. In this light, we set off with a series of experiments in an attempt to establish a role for TNF in HSC regulation.

TNF receptors are widely expressed throughout the hematopoietic system (Aggarwal 2003). Under ex vivo self-renewing conditions, purified HSC upregulate cell surface expression of TNF receptors and TNF stimulation causes compromised reconstitution abilities of HSC in such settings (Bryder et al. 2001). We confirmed these findings using an in vivo approach (Article I: Figure 4) to stimulate HSC with TNF in a more physiological environment. We found that TNF treatment caused a mild reduction in total bone marrow cellularity (Article I: Figure 4B) in the number of CRUs (competitive repopulating unit; Article I: Figure 4C) and in cells within the phenotypically defined LSKCD34- HSC compartment (data not shown). As HSC upregulate TNF receptors upon cycling (Bryder et al. 2001), we aimed to induce HSC cycling in vivo by 5-FU injection, prior to TNF treatment. Upon cycling of HSC, TNF induced selective decreases in CRU activity (Article I: Figure 4E). These findings therefore strongly suggest that actively cycling HSC are increasingly susceptible to TNF induced repression as compared to HSC in steady state, which are typically characterized by relative quiescence (Nygren et al. 2006). It seems plausible that the effects of TNF treatment on HSC are caused by its direct actions on HSC, rather than alternative cell-extrinsic mechanisms, as is suggested both from previous in vitro approaches (Bryder et al. 2001) as well as from our studies of mice lacking TNF receptors (see below).
Although we clearly showed a reduction in total numbers of CRUs following TNF treatment in vivo, it was unclear what mechanisms underlay these findings. Previous studies have both suggested induction of differentiation (Maguer-Satta et al. 2000; Dybedal et al. 2001), induction of apoptosis (Sellier et al. 1995; Weiss et al. 1998; Papadaki et al. 2002) as well as delayed commitment to active cell cycle (Beyne-Rauzy et al. 2004) as explanations for TNF induced growth inhibition. On the other hand, a number of studies provide evidence for both stimulatory and inhibitory actions of TNF in hematopoiesis, dependent on TNF concentrations, TNFR-p55 or -p75 receptor dependent activation, collaboration with other cytokines and target cell types (Johnson et al. 1988; Ulich et al. 1990; Jacobsen et al. 1992; Fahlman et al. 1994; Rusten et al. 1994a; Rusten et al. 1994b; Snoeck et al. 1996; Quentmeier et al. 2003).

Artificial TNF stimulation, as performed in Article I: Figure 4, often results in temporary and super-physiological concentrations and also does not necessarily mimic the physiological presence of both soluble and membrane-bound TNF; a potentially important aspect as soluble TNF was shown to primarily ligate to TNFR-p55, whereas membrane bound TNF has a higher affinity for the TNFR-p75 receptors (Grell et al. 1995). For these reasons, we considered lack of function models using mice deficient for either or both of TNFR-p55 and TNFR-p75 receptors to be of great interest. Whereas Rebel et al. found mice deficient for TNFR-p55 receptor expression to represent with decreased HSC activity following transplantation (Rebel et al. 1999), Zhang and colleagues found similar mice to have increased levels of phenotypically defined HSC (Zhang et al. 1995). In agreement with Zhang et al, mice deficient for TNF ligand production have increased frequencies of several immature hematopoietic progenitor subsets (Drutskaya et al. 2005). Lack of function studies using single TNF receptor knockout mice are however complicated by proposed mechanisms such as cross talking, redundancy and ligand passing between TNFR-p55 and -p75 receptors (Wajant et al. 2003), as well as the potential requirement for both receptors to elicit maximal TNF signaling (Weiss et al. 1998; Mukhopadhyay et al. 2001). In addition, assessment of HSC activity in TNF ligand knockout mice (Drutskaya et al. 2005) by means of transplantation is complicated by the endogenous TNF production in such mice.

Therefore, we took the approach to assay HSC numbers and activity in TNFR-dKO mice. In addition, we generated TNF receptor single knockout mice to establish a possible role for each of these receptors. Single receptor knockout mice were generated directly from the TNFR-dKO to exclude genetic background differences as a possible explanation for the observed differences. In agreement with TNF receptor levels below
detection level on resting HSC, we did not observe differences in phenotypic and functional HSC numbers in steady state TNFR-dKO mice (Article I: Figure 1A and 1B), although the proportion of actively cycling HSC was somewhat increased in TNFR-dKO mice (Article I: Figure 1C and 1D). Only in non-steady state BM, using transplantation assays, did we observed a striking advantage for HSC lacking TNFR-p55 and –p75 receptor expression to reconstitute conditioned recipients (Figure 2A) with BM LSK levels that mimic PB reconstitution levels (Article I: Figure 2A and 2C). The latter indicates these observations to be a result of competitive advantages of cells within and not outside the HSC compartment. This was confirmed by the absence of long-term reconstitution following transplantation with TNFR-dKO LSKCD34+ cells (Article I: Suppl figure 1A), the close progeny of LSKCD34- long-term HSC (Yang et al. 2005).

In similar experiments, BM cells from TNFR-p55KO and TNFR-p75KO (single receptor KO mice) were in vitro assayed in the presence or absence of TNF (Article I: Figure 2A and 2B) or competitively transplanted with WT BM (Article I: Figure 2C-F). These experiments point to required signaling through both receptors to elicit full inhibition as the absence of either of these receptors could not recapitulate (either partially off fully) the observations made in the TNF-dKO mice.

Irradiation (Xun et al. 1994) and 5-FU treatment (Okamoto et al. 2000) cause increased TNF blood levels and this could be a possible confounding factor in our experiments. However, as TNF treatment of 5-FU conditioned BM caused an additional decrease in HSC numbers, we propose these mechanisms to be of limited relevance to the conclusions drawn from the present studies. Also, we found that TNF induced growth inhibition in vitro of purified HSC could not be abrogated in cells over expressing the anti-apoptotic protein Bcl2 (Figure 3B), suggesting at least that this inhibition could be rescued by Bcl2 overexpression.

TNFR-dKO cells provide multi-lineage reconstitution following an increasing amount of serial transplantation, beyond that observed from wild type cells (Harrison et al. 1978). To exclude possible neoplastic conversion of the transplanted cells, limiting dilution experiments were performed transplanting 2x10^5 or 2x10^6 cells from five times serially transplanted TNFR-dKO BM. Only 2/12 and 6/12 recipients, respectively, were reconstituted with TNFR-dKO cells (Article I: Suppl figure 1B and 1C), whereas transplantation with these cell doses using fresh BM usually gives multi-lineage reconstitution in all recipients. These findings demonstrate that although TNFR-dKO cells maintain reconstitution abilities after several rounds of transplantation, the number
of HSC still decreases for each round of serial transplantation. An alternative explanation is that TNFR deficient HSC accumulate a competitive disadvantage to reconstitute recipient following several rounds of transplantation, similar to WT cells (Harrison et al. 1978; Mauch and Hellman 1989; Yu et al. 2006). This interpretation is supported by our observation that PB white blood cell levels were decreased after 4-5 rounds of serial transplantation (data not shown).

A recent study by Schiedlmeier at el. exposed a connection between TNF and HoxB4 (Schiedlmeier et al. 2007), a transcription factor previously demonstrated to positively regulate HSC cycling and self-renewal (Antonchuk et al. 2001; Antonchuk et al. 2002). This, taken together with our findings that steady state TNFR-dKO HSC reside in active cell cycle at higher frequencies as compared to WT cells, could indicate changes in cell cycle and/or self-renewing HSC fates following transplantation as a possible explanation for our findings.

To our knowledge, TNF would be the first, one single cell-extrinsic factor to negatively regulate HSC activity. This has important impact in understanding how HSC are regulated. In addition, it also has clinical implication as TNF is shown to be involved in numerous clinical syndromes (Bradley 2008), including hematological disorders (Younes and Aggarwall 2003). As such, blocking of TNF signaling is widely used in clinical practice nowadays (Gatto 2006) and applications and indications for such therapies are increasing. For instance, TNF induced graft-versus-host disease (GVHD) following allogeneic bone marrow transplantation (Ferrara 2007) is currently treated with amongst others TNF blocking agents. There are several examples where such therapies have shown to modulate serious side effects. Increased understanding of the exact mechanisms by which TNF, or the absence of TNF signaling, would interfere with biological processes such as blood cell formation, would allow for more tailor made treatment strategies as well as increased awareness of its adverse side effects.

We conclude:

1. TNF is a single, non-redundant cell-extrinsic factor that negatively regulates HSC activity
2. TNF negatively regulates HSC in a cell cycle dependent manner
3. Signaling through both TNF receptors is required to elicit full inhibition
Hematopoietic stem cell ageing is uncoupled from p16\textsuperscript{INK4A}-mediated senescence


Hematopoietic homeostasis becomes increasingly altered as an individual ages. On pages 47-50 some of the functional characteristics of aging are discussed and on pages 64-67 some aspects of epigenetic regulation in the aged individual are already touched upon.

Elderly often present with suboptimal functioning of the blood cell system and frequently present with anemia, thrombocytosis, deteriorated adaptive immune responses and increased frequencies of myeloproliferative diseases (Rossi et al. 2008). These observations point to an aged hematopoietic system characterized by lineage skewing that causes “overproduction” of some blood cells (myeloid) and “underproduction” of other cells (B cells) (Sudo et al. 2000; Kim et al. 2003; Rossi et al. 2005). There has been some discussion as to whether overproduction of one cell type is at the expense of the other and at what level in the hematopoietic tree this lineage skewing originates. Some studies indicate that this lineage skewing already occurs at the earlier stages of blood cell development (Min et al. 2006; Cho et al. 2008; Guerretta et al. 2008). Indeed, we made similar observations with regard to megakaryocytic skewing in aged KLSSlamf1+ HSC (Figure 16B, right bars) and myeloid versus B cell skewing in aged KLSSlamf1- MPP (Figure 16C).

The proliferation or reconstitution capacity of HSC diminishes as they age or undergo several rounds of serial transplantation (Harrison et al. 1978; Ross et al. 1982; Lansdorp et al. 1993; Kim et al. 2003; Rossi et al. 2005), although HSC numbers increase (Morrison et al. 1996; de Haan et al. 1997; Sudo et al. 2000). In an attempt to explain these findings, some studies have indicated that aged HSC confer to a more quiescent state (Janzen et al. 2006), as was shown also in other cellular systems (Zindy et al. 1997; Molofsky et al. 2006) and that this replicative senescence is enforced by upregulation of the cyclin dependent kinase inhibitor p16\textsuperscript{INK4A}. However, p16\textsuperscript{INK4A} levels in aged HSC where only marginally upregulated (Janzen et al. 2006; Pearce et al. 2007) and urged us to further elucidate p16\textsuperscript{INK4A} involvement in these processes.

In our laboratory we recently developed a NHS-Biotin \textit{in vivo} labeling assays to track HSC cell division \textit{in vivo} (Nygren and Bryder, \textit{accepted for publication}). Taking
advantage of this assay, we found that the majority of the young HSC had divided after 1 or 2 weeks (Article II: Figure 1). The same held true for old HSC, although these did present with overall higher NHS-Biotin levels and demonstrates lower proliferation rates compared to young HSC. However, great heterogeneity existed, meaning that some HSC had divided more and some much less frequently. Previous reports assayed p16\(^{INK4A}\) expression in old HSC by quantitative RT-PCR (qRT-PCR) based on several thousand cells (Janzen et al. 2006; Pearce et al. 2007). Considering the heterogeneous proliferative composition of the HSC compartment, this raises the question if all/most cells express low p16\(^{INK4A}\) levels, or if some cells express p16\(^{INK4A}\) and some do not? Therefore, we performed a series of single cell RT-PCR (SC RT-PCR) and limiting dilution RT-PCR experiments (Article II: Figure 2) and found only a very minor fraction of old HSC to express p16\(^{INK4A}\).

Given these findings, we sought to find possible differences across age for upstream regulators of p16\(^{INK4A}\). qRT-PCR and multiplex SC RT-PCR analysis for positive regulators of p16\(^{INK4A}\) like Ets1 and Ets2, and negative regulators like Id1, Ezh2 and Bmi1 (Jacobs et al. 1999; Ohtani et al. 2001; Kotake et al. 2007) gave no obvious differences, except for a minor decrease in Ezh2 levels (Article II: Figure 3A and 3B).

As discussed in before, epigenetic marks have been ascribed important roles in the regulation of gene transcription activity. As no p16\(^{INK4A}\) expression was observed in the vast majority of HSC, we investigated some epigenetic marks that are associated with transcriptional repression as an explanation for the proliferative differences in young versus old HSC. Highly methylated promoter regions mediate gene silencing, but DNA-methylation analysis across the p16\(^{INK4A}\) promoter region in aged HSC showed no signs of this (Article II, Figure 3C). Also, analysis of histone modifications in young and old HSC did not show large differences as both presented with high levels of H3K27me3 (repressive mark) and low levels of H3K4me3 (active mark; Article II: Figure 4C). This was in strong contrast to the changes in the histone methylation patterns in murine embryonic fibroblasts (MEF) upon serial passaging (Article II: Figure 4A). These findings demonstrate that p16\(^{INK4A}\) maintains epigenetically silenced also in old HSC.

Collectively, we confirm decreased proliferative potentials of HSC across age, but could not establish a role for the p16\(^{INK4A}\) tumor suppressor gene in mediating this process. These results are seemingly contradictory to previous reports where enforced expression of p16\(^{INK4A}\) induced proliferative arrest and cell death (Park et al. 2003; Janzen et al. 2006) and in which the age-associated phenotypes in old p16\(^{INK4A}\)-deficient
HSC were rescued (Janzen et al. 2006). However, these experiments were performed mainly in non-steady state hematopoiesis and p16\(^{INK4A}\) induced senescence might therefore not be a natural outcome in the "normal" aging HSC. In support of this, Bmi1 deficient HSC in which p16\(^{INK4A}\)-activity is "derepressed" maintain proliferative capacities (Iwama et al. 2004). Also, clonal evaluation of aged HSC showed comparable colony formation (Morrison et al. 1996; Sudo et al. 2000) suggesting that old cells have not lost intrinsic capacities to enter cell cycle and generate offspring. We confirmed these result in vitro comparing young and old HSC (Figure 16B, left bars) and, in agreement with Article II: Figure 1, we found colony sizes from old HSC or MPP cells to be smaller compared to young cells (data not shown). In addition, clonogenic evaluation of old HSC that had proliferated at high (HSCBio\(^{low}\)) or low (HSCBio\(^{high}\)) rates during the preceding 7 days showed comparable colony forming capacities (Figure 16A), suggesting that proliferation history is no direct determinant for the capacities of old HSC to generate colonies in vitro.

Figure 16. Functional characteristics of old stem (HSC) and multipotent progenitor (MPP) cells. Cells from young and old mice were treated with biotin, isolated and cultured as described (Attema et al.; Pronk et al. 2007). (A) Old mice were treated with NHS-Biotin. After 7 days, biotin-high (low proliferative history) and biotin-low (high proliferative history) HSC were single cell sorted and cultured. These results indicate that recent proliferation history is no determinant for clonogenic capacities of the evaluated HSC. (B and C) Young and old HSC and MPP were single cell sorted into liquid cultures and evaluated for clonogenic potential and indicated lineage output. Whereas clonogenic abilities are unaltered with age, do old HSC and MPP present with linear skewing towards the megakaryocyte and myeloid lineages, respectively, at the expense of B-lymphopoiesis.

We conclude:

1. The proliferation activity of HSC decreases upon aging.
2. The epigenetic mediated silencing of p16\(^{INK4A}\) activity in HSC is maintained across age
3. p16\(^{INK4A}\) activity is uncoupled from senescence in aged HSC
Articles III and IV

Article III: **Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy.**

Article IV: **Deciphering developmental stages of adult myelopoiesis.**

As the Articles III and IV very much touch upon similar questions, I will discuss both of them within this chapter. A large part of the concepts and background to these studies, with regard to previous work in the identification of developmental stages of early hematopoiesis and regulatory mechanisms guiding these processes, have already been discussed in previous chapters.

Improvements in FACS based isolation of cellular subsets within the hematopoietic organ have led to a rather detailed understanding of the developmental steps in the differentiation from HSC, through intermediate cell types, to mature effector cells. Knowledge of these developmental stages is important to understand underlying mechanisms that guide these cell fate decisions. In addition, as hematopoietic diseases, like leukemias, often present with a developmental block at a certain stage, prospective isolation of these stages creates insight in cues leading to leukemic conversion. Most work in prospective isolation of cellular subsets was performed in mouse models and these have showed to be highly translatable to human hematopoiesis (Hao et al. 2001; Manz et al. 2002; Edvardsson et al. 2006; Majeti et al. 2007).

Also in these studies, we have used a FACS based approach to isolate and identify a number of cellular subsets within the myeloerythroid lineages. As discussed before, a proposed model has been around that describes the first lineage restriction events to be the divergence of myeloerythroid versus lymphoid development (Figure 12A). In this model, the multipotent progenitors (MPP) give rise to common lymphoid progenitor (CLP) cells containing B, T, DC, and NK cell potentials (Kondo et al. 1997) and common myeloid progenitors (CMP) that are restricted to G, M, Meg and E potentials (Akashi et al. 2000). An alternative model was launched through the
identification of the LMPP that contained B, T, NK, M and G potentials but no Meg and E potentials (Adolfsson et al. 2005) (Figure 12B). The disproving of a divergence of myeloerythroid versus lymphoid lineages was supported by others (Allman et al. 2003; Balciunaite et al. 2005; Lai and Kondo 2006; Rumfelt et al. 2006; Yoshida et al. 2006; Mansson et al. 2008).

Similarly, the existence of the CMP, phenotypically defined as LS-KCD34<sup>hi</sup>·FcgRII/III<sup>hi</sup>, was questioned. First, a number of studies showed that the actual frequency of cells within this population that possess combined G/M/Meg/E potential was very limited (Akashi et al. 2000; Nakorn et al. 2003; Iwaseki et al. 2005; Terszowski et al. 2005; Yoshida et al. 2006) or absent (Takano et al. 2004). Secondly, some studies demonstrated heterogeneity within the CMP fraction (Nutt et al. 2005; Terszowski et al. 2005). Therefore, these studies pointed to the establishment of the G, M, Meg and E lineages without “passing through” the CMP stage.

**Slamf1** (or CD150) and **Endoglin** (or Eng/CD105) are two cell surface proteins that were shown to be expressed on HSC in mice (Chen et al. 2002; Kiel et al. 2005). By using FACS, we found these markers not only to be expressed within the KLS (HSC and MPP containing) compartment, but also highly expressed within the KLS- compartment that contains myeloerythroid progenitors (Article III: Figure 1A-C). Given the discussion concerning the heterogeneity of the CMP, we decided to investigate if Slamf1 and Endoglin could functionally subfractionate the CMP and other cells within the KLS- compartment. We designed a prospective isolation strategy that is presented is articles III and IV that allowed us to identify both bi- and uni-potent cellular subsets, though without proof of robust CMP activity.

By evaluating Slamf1 and Endoglin expression, we found phenotypic (Article III: Figure 1D) and functional heterogeneity within the previously described CMP and MEP (Akashi et al. 2000) which aided us to design an isolation protocol as depicted in Article III: Figure 2 and Figure 12C. Through functional evaluation, both *in vitro* (Article III: Figure 3) and *in vivo* (Article III: Figure 4 and Table 1), we could functionally discriminate a number of cellular subsets:

- the **pre GM** (granulocyte/monocyte) with mainly G/M potential,
- the **GMP** (granulocyte/monocyte progenitor) with restricted G/M potential. This GMP overlaps with the previously described GMP (Akashi et al. 2000),
- the **pre MegE** (megakaryocyte/erythroid) containing both Meg and E potential and some limited combined Meg/E potential
• the MkP (megakaryocyte progenitor) with restricted Meg potential and highly overlapping with the previously identified MKP (Nakorn et al. 2003)
• The pre CFU-E (colony forming unit- erythrocyte), CFU-E and Pro Ery (erythroblast) owing restricted erythroid potentials of which the latter two were described previously (Terszowski et al. 2005).

Assuming that more immature cells generate more progeny (i.e. bigger colonies) compared to more mature cells, we performed a series of experiments assaying colony size (Article III, Figure 6A-C and 6E). Also, we performed a series of short-term experiments in which we phenotypically evaluated cellular offspring from a number of plated cell types. Together, these experiments allowed us to determine the hierarchical relationships of the cell types mentioned above: these data indicate (i) that pre GM lies upstream of the GMP, (ii) that pre MegE generates pre CFU-E that in its turn progresses to generate CFU-E and (iii) that pre MegE lies upstream of the MkP compartment (Figure 12 C and Article III, Figure 7D).

The kinetics of the HSC to generate erythrocytes and platelets are interesting. As shown in Article III: Figure 6E and Table 1, HSC produce large Meg colonies in vitro and are also in vivo responsible for the vast majority of platelet production already 14 days following transplantation. By contrast, erythrocyte production 12 days after transplantation is mainly derived from progenitor cells (Article III, Figure 4B), without contribution from the HSC. Would this mean that the HSC has a “short-cut” pathway to generate platelets? Or is the progression from HSC to platelets much faster when compared to erythrocyte formation? Regardless, this is an observation with clinical relevance considering the many situations that require replenishment of either platelets or erythrocytes. Knowledge on these kinetics would allow for more patient adjusted therapy.

In our functional evaluation, we found little evidence for cells within the KLS-compartment with combined G/M/Meg/E (CMP) potential. However, the original CMP (Akashi et al. 2000) was described using Bcl2 transgenic mice that are characterized by constitutive activity of this anti-apoptotic protein; one could argue this to explain the seemingly contradictory data. Also, in Article III we used the cytokine stimulation with “only” cKit ligand (KL or SCF), IL-3 (interleukin 3) and EPO (erythropoietin) throughout our studies. Even though this combination was shown to potently stimulate G/M/Meg/E formation (Metcalf 2004), Akashi et al. used a “stronger” cytokine cocktail including additional cytokines. To exclude difference in Bcl2 activity and cytokine stimulation as an explanation for our diverging findings, we performed a
series of experiments to address this. Even though Bcl2 transgenic cells gave higher cloning efficiency, no major differences were observed in lineage output of the tested cells; perhaps with the exception of some limited Meg/E readout from pre GM and GMP (Article IV; Figure 2A and 2B). Also, stimulation of these double-sorted pre GM with an expanded cytokine cocktail did not alter linear output (Article IV; Figure 2A and 2B). Therefore, these results are in agreement with the conclusions drawn in Article III. Recently, Akashi and co-worker found a “new” CMP that resides within the KLS compartment (Figure 12D) (Arinobu et al. 2007). Unfortunately, this work did not describe the relationship of this new CMP to the old CMP. However, as this new CMP was shown to express low levels of the Sca1 antigen (Arinobu et al. 2007), it is possible that the observed CMP activity in the KLS-compartment in the original paper rather originated from KLS contaminating cells.

Next, we decided to assess some of the genetic signatures that coincide with the different developmental stages. We performed multiplex SC RT-PCR for some G/M, Meg and E related genes and found these genes to dominate within their corresponding cell types (Article III, Figure 5). However, we observed “promiscuous” presence of some lineage unrelated transcripts, like the presence of MPO (myeloperoxidase; G/M affiliated) transcripts in MkP and pre MegE cells that both fully lack G/M potentials. Importantly, in the vast majority of cells, these promiscuous transcripts were co-expressed with other, lineage affiliated genes. Therefore, as discussed before, these could represent remnants from transcriptional programs that are or have been shut down, rather than reflect true lineage potentials. Importantly, simultaneous expression of genes associated with all lineages within one single cell was not observed, supporting our functional data pointing to absent CMP activity.

In addition to the single cell PCR experiments, we analyzed expression levels of a selection of genes, or at more global level (Article III, Figure 7 and Article IV, Figure 3). Somewhat surprising, hierarchical clustering of the genome-wide expression patterns revealed a close relationship of the CLP and pre GM cells, as opposed to the populations possessing Meg and/or E potentials. The proximity of cells within the adaptive and innate immune system is in agreement with the divergence of Meg/E versus G/M/lymphoid potential as first lineage restricting event (Adolfsson et al. 2005). In addition to this, we found some G/M associated genes (like M-csfR and G-csfR) to be expressed in CLP and detected expression of lymphoid genes (like sterile IgM, Notch1 and Dnnt) in pre GM (Article III, Figure 7C). Indeed, CLP cells were shown to possess myeloid potential (Allman et al. 2003; Balciana et al. 2005; Rumsfelt et al.)
2006; Mansson et al. 2008) and we observed substantial B lymphoid potential in Flt3 expressing pre GM cells (Article IV, Table 1).

We conclude:
1. We have described the first progenitor subsets that are mutually exclusive for G/M (pre MegE) and Meg/E (pre GM) potential.
2. We describe a uni-potent pre CFU-E fraction that serves as the “missing link” between previously described Meg/E progenitors (MEP) (Akashi et al. 2000) and the more mature CFU-E and erythroblast stages (Terszowski et al. 2005).
3. We found functional and genetic evidence for a close proximity of cells from the adaptive (CLP) and innate (pre GM) immune system, as opposed to precursors that eventually generate platelet and erythrocytes.
Figure 17. Composite, schematic model of the earliest steps in lineage differentiation based on the work in this thesis and previous reports that were discussed herein. Indicated is a selection of markers/factors that were identified by their presence or absence at different stages of hematopoietic development. *: uni-potent, but low proliferative capacities, T/B/NK prog: T/B/NK progenitor cells.
Summary (English)

Hematopoietic stem cells (HSC), the ancestors of all blood cells, have the ability to give rise to mature effector cells through a series of developmental steps. This is a tightly regulated, though a highly dynamic process: on the one hand, dysregulation in steady-state could lead to under- or overgrowth of certain blood cell lineages, whereas, on the other hand, demands for certain mature blood cells can change dramatically in cases like bleeding and infections. In addition to the production of differentiated offspring, HSC possess the ability to undergo self-sustaining (or self-renewing) cell divisions to ascertain life-long replenishment of the hematopoietic system. High resolution profiling of the mechanisms that guide these cellular fates does not only allow to further increase our knowledge in normal cell biology, it can also provide with clues on possible pathogenetic events in cases of altered hematopoiesis like leukemia or in the aging individual. In Article I, we have investigated the role of tumor necrosis factor (TNF) in the regulation of HSC homeostasis. We found TNF to negatively regulate HSC activity in a cell cycle dependent manner, with required signaling through both TNF receptors to elicit full inhibitory actions. Thereby, this study identifies one single, cell extrinsic factor to negatively regulate HSC activity. Blood cell production in elderly is characterized by myeloid skewing at the expense of lymphoid output, in addition to increased HSC frequencies in aged mice. However, HSC replicating activity was suggested to be decreased through increased p16\(^{INK4A}\) signaling. To determine regulating factors underlying these observations, we assayed HSC in aged mice (Article II). We found evidence for decreased proliferative kinetics of old versus young HSC, though without proof of replicative senescence. In agreement with this, we found no evidence for increased p16\(^{INK4A}\) signaling in aged HSC, nor for a permissive epigenetic state at the p16\(^{INK4A}\) control region. Differentiation from a multipotent HSC to mature blood cells occurs through a series of commitment steps, generating progenitors with increasing lineage restricted potentials. The earliest lineage restricting events have been subject to debate and in Article III and IV we describe our efforts to create increased insights in the developmental steps of early myeloerythroid development. By means of flow cytometric isolation, we have identified the early granulocyte/monocyte, erythroid and megakaryocytic lineage restriction steps within the cKit+Lineage-Scal- myeloerythroid progenitor compartment. Subsequently, functional and molecular analysis of these cellular subsets has allowed us to place these cells in a hierarchical order and understand some of the genetic events that occur during lineage specification.
Populärvetenskaplig sammanfattning (Svenska)


Artikel I: Det finns en del faktorer beskrivna som har en "positiv påverkan" på HSC funktion. Det finns även, fast i mindre utsträckning, beskrivet några faktorer som har en "negativ" (eller hämmande) påverkan på HSC funktion. "Negativ reglering" av HSC är viktigt av ett antal anledningar: (i) den ser till att man inte får en "överväxt" av HSC som kan ger upphov till ex leukemi; (ii) om negativ reglering inte finns, finns det en risk att man "tröttar ut" sina HSC för tidigt, vilket kan leda till benmärgssviktssjukdomar; (iii) om HSC skulle dela sig ofta (vilket de normalt inte gör), har de ökad risk att förvärra genetiska mutationer (felaktiga ändringar av det genetiska materialet) vilket i sin tur kan leda till sjukdom. Vi har tittat på tumor nektosfaktor
(TNF) och dess roll i regleringen av HSC. TNF är ett äggviteämne som är inblandat i bl.a. immunreaktioner, men som även har inverkan på blodbildningen. TNF har mest varit relaterad till negativ reglering av mer mognna blodceller. Dock är inte så mycket känt kring TNFs påverkan på HSC. Kunskap om detta är viktigt, då anti-TNF-behandling numera används i hög utsträckning i behandlingen av reumatiska sjukdomar, inflammatoriska tarmsjukdomar, graft-versus-host-sjuka (en komplikation efter benämärstransplantation), mm. Med hjälp av möss som saknar de två receptorer (mottagare) som TNF binder till, och genom TNF-behandling av möss, har vi kunnat påvisa att TNF reglera HSC funktionen på ett negativt sätt. Vi visar även att signalering genom båda TNFreceptornas krävs för att uppnå en bra TNF-inducerad hämning.


I Artikel III och IV har vi tittat närmare på de olika steg som celler tar i utmognaden från en omogen, multipotent cell till vuxna erytrociter (E), trombocyter (Meg), granulocyter (G) och makrofagar (M). Kunskap om hur denna utmognad sker och är reglerad är av stor vikt. Till exempel karakteriseras många blodcancersjukdomar (leukemier) av en blockering i utmognaden av en viss blodcelltyp. Denna omogna blodcelltyp kan sedan expandera kraftigt och ”trycka bort” normala blodceller och därmed orsaka sjukdom. Vissa gener och ändringar i vårt genetiska material (mutationer, translokationer, mm) har visat sig vara inblandat i utvecklingen av sådana sjukdomar, men även i vanlig blodbildning. Ökad kunskap om exakt hur cellerna i vårt blodsyttem mognar och vilka faktorer som är inblandade i dessa processer ger därför inte bara ökad
insyn i den "normala" biologin, utan kan även hjälpa oss att bättre förstå vad som kan orsaka blodcellsjukdomar. Vi har tittat på en cellpopulation i benmärgen (KLS-) som utgörs av omogna blodceller med potentialen att producera E, Meg, G, och/eller M (se ovan). Hur denna utveckling exakt sker har man inte varit helt överens om. Vi har använt oss av vissa celllytemarkörer och har med hjälp av flödescytometri (en teknik som möjliggör sortering av olika celltyper) kunnat isolera några "nya" celltyper. Genom att analysera funktion och genuttryck har vi kunnat kartlägga olika egenskaper hos dessa celler. På så sätt har vi identifierat de första celler som har tappat förmågan att utvecklas till flera olika celltyper och som har begränsad G&M (pre GM), Meg&E (pre MegE) eller endast E (pre CFU-E) potential. Vi har kunnat identifiera gener som blir antingen aktiva eller passiva när en mer omogen cell "väljer" att blir en viss mer mogen cell. Vi har även påvisat att omogna celler i det myeloida immunsystem (granulocyter och makrofager) och omogna celler i det lymfoida systemet (B celler, T-celler och NK celler) har fler gemensamma egenskaper än man tidigare trott. Det skulle kunna vara en förklaring till varför patienter ibland insjuknar i (bifentypiska) leukemier med omogna celler som har båda lymfoida och myeloida egenskaper; celler som vi tror normalt inte finns i vårt blods system.
Populair wetenschappelijke samenvatting (Nederlands)

Hematopoëse beschrijft het proces van de productie van de cellen in ons bloedsysteem. Ons bloedsysteem bestaat uit een aantal verschillende soorten cellen: rode bloedlichaampjes (erytrocyten) die zorgen voor zuurstoftransport, witte bloedlichaampjes (leucocyten) die betrokken zijn bij ons afweersysteem en bloedplaatjes (trombocyten) die bloedingen tegengaan. Al deze "rijpe" bloedcellen worden geproduceerd door één type voorlopercel; de hematopoëtische stamcel (HSC). HSC verblijven in ons beenmerg, maar representeren alleen een zeer klein deel van het totaal aantal bloedcellen in het beenmerg. Het zijn deze HSC die uiteindelijk verantwoordelijk zijn voor de productie van al onze bloedcellen, gedurende ons hele leven. Terwijl de meeste rijke bloedcellen een beperkte levensduur hebben (soms slechts enkele uren tot dagen), kunnen HSC vele maanden oud worden. HSC bezitten het vermogen om tijdens een celdeeling een of twee exacte kopieën van zichzelf te maken; dit wordt zelfvernieuwing genoemd. Dit zelfvernieuwend vermogen zorgt er uiteindelijk voor dat de HSC gedurende ons hele leven in ons beenmerg aanwezig zijn. Als een HSC niet een exacte kopie van zichzelf maakt, dan differentieert hij. Differentiatie kan worden beschreven als het proces waarin een cel verschillende stappen (of veranderingen) ondergaat, waarbij hij geleidelijk het vermogen kwijt raakt om verschillende soorten cellen te produceren, om uiteindelijk in één type rijpe bloedcel te veranderen. Omdat we niet te veel of te weinig productie van bepaalde bloedcellen willen hebben, worden zowel zelfvernieuwing als differentiatie nauwkeurig gereguleerd. Anderzijds is het soms nodig om de productie van bepaalde bloedcellen sterk te vergroten, zoals bij infecties en bloedingen. Daarom moet het hematopoëtische proces dus ook een grote mate van flexibiliteit bezitten. In Artikel I&II hebben we naar bepaalde aspecten gekomen die betrokken zijn bij de regulatie van HSC in jonge (Artikel I) en oude (Artikel II) muisen. In Artikel III&IV hebben we verschillende stappen in de differentiatie van onrijpe bloedcellen tot erytrocyten, trombocyten en bepaalde witte bloedcellen (granulocyten en macrofagen) nader bestudeerd.

**Artikel I:** Er is in eerder onderzoek een aantal factoren beschreven die de functie van HSC op een "positieve" manier beïnvloeden. We weten echter minder over factoren die HSC activiteit op een "negatieve" (of remmende) manier beïnvloeden. Deze negatieve regulatie van HSC is echter belangrijk om een aantal redenen: (i) overproductie van HSC vergroot de kans op het krijgen van ziektes als bijvoorbeeld bloedkanker (leukemie); (ii) als HSC niet geremd worden, bestaat de kans dat ze voortijdig uitgeput
raken met het risico van het ontwikkelen van beenmergfaalen (aplasie), (iii) indien HSC regelmatig celdelingen ondergaan (wat ze in het algemeen niet doen), zal dat de kans vergroten op het verwerven van genmutaties (veranderingen in ons genetisch materiaal), wat ook tot bepaalde ziekten kan leiden. Wij hebben nader gekeken naar de rol die tumor necrose factor (TNF) speelt bij de regulatie van HSC. TNF is een eiwit dat onder andere betrokken is bij ons afweersysteem. Ook hebben eerdere studies laten zien dat TNF een negatief effect heeft op de meer rijpe cellen in ons bloedsysteem. We weten echter niet zo veel van de rol die TNF speelt in de activiteit van de onrijpe HSC. Kennis hierover is echter van groot belang, omdat anti-TNF therapie tegenwoordig regelmatig wordt gebruikt in de behandeling van reuma, inflammatoire darmziekten, graft-versus-host ziekte (een complicatie na beenmerg transplantaties), etc.. Met behulp van een bepaalde muizenstam die de receptoren voor TNF op hun celmembraan mist, en door middel van TNF-therapie bij muizen, laten we zien dat TNF de activiteit van HSC op een negatieve manier beïnvloedt. Ook tonen we aan dat activering van de beide types TNF receptoren een vereiste is om een maximaal negatief effect te verkrijgen.

In Artikel II hebben we oude muizen bestudeerd. Eerder is aangetoond dat oude muizen minder erytrocyten en lymfoïde witte bloedcellen hebben, terwijl het aantal myeloïde witte bloedcellen en bloedplaatjes vergroot is. Hetzelfde fenomeen zien we ook bij oudere mensen en bekend is dat dit bepaalde ziekten kan veroorzaken. Ook is eerdere aangetoond dat het aantal HSC in oude muizen vergroot is. Het vermogen van oude HSC om na transplantatie rijpe bloedcellen te produceren, is echter verminderd vergeleken met jonge HSC. Om deze waarneming te kunnen verklaren, is geopperd dat oude HSC minder vaak celdelingen ondergaan, of zich soms helemaal niet meer delen; en dat deze “delingsstilte” veroorzaakt wordt door verhoogde activiteit van p16 (een bepaald eiwit dat de celdeling remt). Ook in onze studies laten wij zien dat oude HSC zich niet in dezelfde mate delen als jonge HSC, al vinden we geen HSC die zich helemaal niet meer delen. Daar komt bij dat we ook geen bewijs vinden voor verhoogde p16-activiteit bij oude HSC. Dit betekent dat onze resultaten mogelijk niet volledig overeenkomen met eerdere publicaties. Kennis over de regulatie van HSC bij oudere dieren (en ook mensen) is zeer relevant, omdat HSC van oudere mensen vaak gebruikt worden bij beenmergtransplantaties. HSC die afkomstig zijn van een oudere donor zullen uiteindelijk ouder dan normaal worden, indien deze in een jonger iemand getransplanteerd worden, die daar weer de rest van zijn/haar leven mee zal doen. Inderdaad, het succes van beenmergtransplantaties waar HSC van oudere donoren gebruikt worden, is vaak minder groot dan bij het gebruik van HSC van jonge donoren.
In Artikel III en IV hebben we enkele onvolwassen, multipotente cellen (met het vermogen om meer dan één type rijpe bloedcel te produceren) nader bestudeerd en gekeken en hoe deze cellen uiteindelijk differentiëren (of uitrijpen) tot een erytrocyt (E), trombocyt (Meg), granulocyt (G) en/of een macrofaag (M). Het is van groot belang om te weten welke “rijpingsstappen” deze cellen ondergaan en hoe deze gereguleerd worden. Zo worden bijvoorbeeld de meeste leukemiën gekarakteriseerd door een blokkering (of stop) van een bepaald tussenstadium in de celripping. Deze onvolwassen, intermediaire cellen expanderen vervolgens en drukken de normale bloedcellen uiteindelijk weg, leidend tot het klinische beeld van een leukemie. Het is eerder aangetoond dat bepaalde genen en ook bepaalde veranderingen in ons genetisch materiaal (mutaties, translocaties, etc.) betrokken kunnen zijn bij de ontwikkeling van deze bloedziekten, maar ook betrokken kunnen zijn bij de productie van normale bloedcellen. Meer kennis over de exacte tussenstadia in de ontwikkeling van onrijpe tot rijpe bloedcel en kennis over welke factoren hierbij betrokken zijn, is daarom niet alleen waardevol om normale biologische processen te begrijpen, maar het helpt ook om het ontstaan van bepaalde ziekten beter te kunnen begrijpen. Wij hebben gekeken naar een bepaalde celpopulatie in ons beenmerg (KLS-) welke bestaat uit onvolwassen bloedcellen met het vermogen om E, Meg, G en/of M cellen (zie hierboven) te produceren. Er bestaat discussie over hoe de differentiatie tot deze volwassen cellen precies verloopt en in onze studies hebben wij geprobeerd hier meer duidelijk in te brengen. Wij hebben gebruik gemaakt van een aantal celmembraan gebonden eiwitten en met behulp van flowcytometrie hebben we een aantal “nieuwe” celtypes (intermediairen in de celripping) kunnen isoleren. Door het bestuderen van de functie en genetische activiteit hebben we bepaalde eigenschappen van deze cellen in kaart kunnen brengen. Op deze manier hebben we cellen gevonden die het vermogen hebben verloren om meerdere celsoorten te genereren en de potentie hebben om of alleen G+M (pre GM), of alleen M+E (pre Meg) of alleen E (pre CFU-E) cellen te produceren. We hebben bepaalde genen geïdentificeerd die meer of minder actief worden als een onvolwassen cel zich differentieert in de richting van een bepaalde rijpe bloedcel. Ook hebben we aangetoond dat onvolwassen cellen van het myeloïde immuunsysteem (granulocyten en macrofagen) en onvolwassen cellen van het lymfoïde immuunsysteem (B-, T- en NK-cellen) grotere overeenkomsten vertonen dan voorheen werd gedacht. Dit zou een verklaring kunnen zijn waarom sommige patiënten een bepaalde (bifenotypische) leukemie ontwikkelen met cellen die zowel lymfoïde als myeloïde eigenschappen vertonen; cellen die normaal gesproken eigenlijk niet in ons bloedsysteem voorkomen.
Articles not included in this thesis


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