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Molecular Mechanisms in Hematopoietic Stem Cell Aging

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Abstract The blood is composed of many different cell types that through tightly regulated mechanisms are derived from hematopoietic stem cells (HSCs). In addition, HSCs are able to undergo self-renewing divisions whereby new HSCs are produced. This is an extremely important feature of HSCs in order to ensure the existence of the HSC pool that is paramount to provide life-long hematopoiesis. Aging is in general characterized by reduced ability to sustain tissue homeostasis and return to a homeostatic state after stress or trauma. Several alterations arise in the hematopoietic system with advancing age and several of these have been suggested to originate at the level of HSCs. With advancing age a bias toward myeloid cells arises within the hematopoietic system characterized by a reduced production of lymphoid cells. Despite an accumulation of HSCs in aged mice it has been suggested that aged HSCs display a decreased proliferation that depends on p16Ink4a activity. Although we observed (article I) a decreased replicative activity in physiologically aged HSC we found no evidence for increased p16Ink4a activity in these cells. In article II we demonstrated several hematopoietic defects reminiscent of premature HSC aging including anemia, lymphopenia, and myeloid lineage skewing in mice that rapidly accumulate mitochondrial DNA (mtDNA) mutations. This however, was due to distinct differentiation blocks and/or disappearance of downstream progenitors in the absence of several hallmarks of physiological HSC aging such as epigenetic alterations and accumulation of a myeloid biased subset of HSCs. These findings highlight the necessity of intact mitochondrial function for multilineage hematopoiesis but argue against mtDNA mutations as primary drivers of HSC aging. How growth factors and the relevant signaling pathways dictate HSC lineage specification is not fully understood. Aged mice, deficient in the signal adaptor protein LNK (article III), that acts to dampen several extrinsic signaling pathways, did not display repopulating defects otherwise observed in physiologically aged HSCs. This argues that enhanced cytokine signaling can counteract several key aspects of age-associated HSC decline.		
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Vits er þörf
þeim er víða ratar.
Dælt er heima hvað.
Að augabragði verður
sá er ekki kann
og með snotrum situr.
-Hávamál

“If I have seen further
it is by standing on
the shoulders of
giants.”
-Isaac Newton

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Table of contents

Table of contents	5
List of abbreviations	7
Articles and manuscripts included in this thesis	9
Background	11
1. Hematopoiesis	11
2. The discovery of the hematopoietic stem cell	12
3. Embryonic origin of hematopoietic stem cells.....	14
4. Early hematopoietic differentiation.....	15
5. Regulation of HSC homeostasis.....	18
5.1 The microenvironment	18
5.2 Intrinsic regulation	20
6 Metabolism.....	21
6.1 Hypoxia and reactive oxygen species.....	22
6.2 Liver kinase B1.....	24
7. Aging.....	25
7.1 Stem cells and aging.....	26
7.2 Hematopoietic aging.....	27
7.3 What lies beneath? Heterogeneity of the HSC compartment. .	31
7.4 Life span and cycling of HSCs	32
8 Molecular mechanisms involved in aging.....	34
8.1 DNA damage.....	34

8.2 Telomere shortening and defective mitochondrial function...	35
8.3 Tumor suppressors and epigenetic regulation.....	36
Discussion of articles	39
Populärvetenskaplig sammanfattning.....	49
Articles and manuscripts not included in this thesis	51
Acknowledgements	53
References	57

List of abbreviations

AGM	Aorta-gonad-mesonephros
Atm	Ataxia telangiectasis mutated
ATP	Adenosine triphosphate
BM	Bone marrow
CFU-E	Colony forming unit erythroid
CFU-S	Colony forming unit spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DC	Dendritic cell
dHSC	Definitive hematopoietic stem cell
E	Erythroid
FACS	Fluorescence activated cell sorting
Gfi-1	Growth factor independent 1
GM	Granulocyte-macrophage
GMP	Granulocytic-macrophage progenitor
Hox	Homeobox
HR	Homologous recombination
HSC	Hematopoietic stem cell
IFN- γ	Interferon- γ
Lkb1	Liver kinase B1
LMPP	lymphoid-primed multipotent progenitor
LSK	Lin ⁻ Sca1 ⁺ cKit ⁺
MegE	Megakaryocytic/erythroid

MEP	Megakaryocyte-erythroid progenitor
Mk	Megakaryocyte
MMP	Mitochondrial membrane potential
MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
NEHJ	Non homologous end joining
Nk	Natural killer
OB	Osteoblast
p38 MAPK	p38 mitogen-activated protein kinase
PTEN	Phosphatase and tensin homologue
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SP	Side population
ST-HSC	Short term hematopoietic stem cell
TFN- α	Tumor necrosis factor- α
TGF- β	Transforming growth factor- β
Vhl	Von hippel lindau protein
WT	Wild type

Articles and manuscripts included in this thesis

- I. Attema JL, Pronk CJ, **Norrdahl GL**, Nygren JM, Bryder D. Hematopoietic stem cell ageing is uncoupled from p16^{INK4A}-mediated senescence. *Oncogene*. 2009 Jun 4;28(22):2238-43.

- II. **Norrdahl GL**, Pronk CJ, Wahlestedt M, Sten G, Nygren JM, Ugale A, Sigvardsson M, Bryder D. Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. *Cell Stem Cell*. 2011 May 6;8(5):499-510.

- III. **Norrdahl GL**, Wahlestedt M, Gisler S, Sigvardsson M, Bryder D. Enhanced cytokine responsiveness can counteract age-induced decline in hematopoietic stem cell function. *Manuscript* (2011).

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Background

1. Hematopoiesis

The human body can be seen as several biological systems that each serves a particular function in order to sustain life. One system, the circulatory system, circulates blood throughout the entire body via a complex network of arteries, veins, and capillaries. Blood is a fluid made up of liquid (plasma) and several cell types. Mature blood cells perform various indispensable tasks. Red blood cells transport oxygen to cells and platelets derived from megakaryocytes take part in blood coagulation during wound healing. A major function of the blood is to provide protection against foreign pathogens. This is mediated by the immune system, composed of T cells, B cells, Natural killer cells (Nk cells), Dendritic cells (DC), Neutrophils, Eosinophiles, Basophiles, and Macrophages.

Hematopoiesis, or the formation of blood cells, is an ongoing process throughout life. All mature blood cells originate from hematopoietic stem cells (HSCs), a cell type that resides in the bone marrow (BM) at low frequencies (Figure 1), by stepwise differentiation through successively lineage restricted progenitor cells. The life span of the different mature blood cells varies dramatically, from days to weeks or even years. In humans it has been estimated that each day 10^{12} new blood cells are formed (Ogawa, 1993). Bleeding and other forms of trauma or illness increase the demand for specific types of mature blood cells. The hematopoietic system has to respond rapidly to such changes. This response is amongst others mediated by immature progenitors that give rise to mature cells. The great proliferative potential of the hematopoietic system requires strict control mechanisms to ensure that neither too many nor few cells are produced and failure to maintain this balance is manifested in various hematological disorders. Abnormal differentiation, proliferation, or apoptosis can give rise to leukemias or lymphomas while defective production of white blood cells can result in a reduced immunological response.

HSCs maintain blood formation throughout life, and are routinely used in transplantation therapies aimed to restore normal hematopoiesis in patients with hematological disorders. Identifying the challenges and possible

changes to HSC function is highly important in order to understand how hematological diseases arise and with the aim of refining current therapeutic strategies for more successful outcomes.

Although experimental use of human hematopoietic stem cells would give results that more exactly reflect human hematopoiesis, this is often difficult due to technical, practical, and ethical issues. Therefore all experiments in this thesis have been performed on murine hematopoietic cells and the following discussion refers to hematopoiesis in the murine setting unless otherwise indicated.

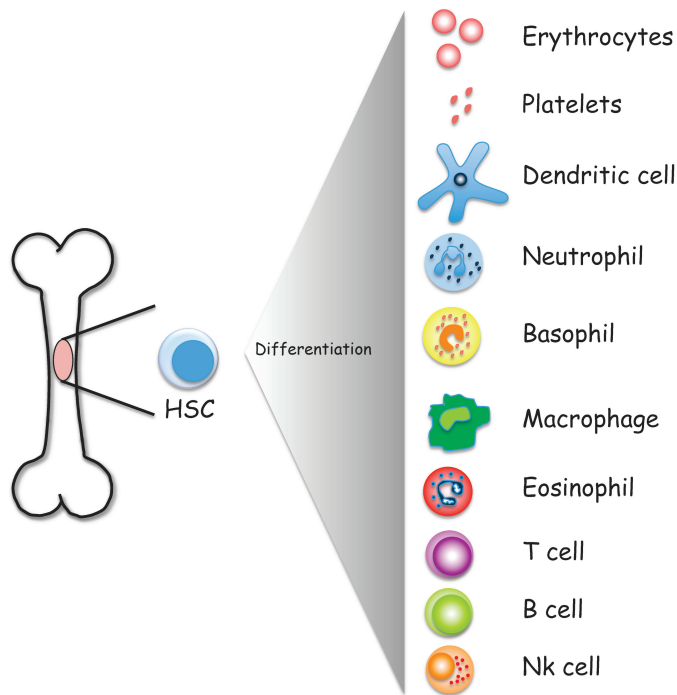


Figure 1. Schematic overview of hematopoiesis with all mature blood cells generated from HSCs.

2. The discovery of the hematopoietic stem cell

To understand the reasons behind various ailments in the hematopoietic system, it has from a clinical perspective been necessary to understand how blood cells are produced. It has been established that our blood cells originate from a small number of self-renewing HSCs capable of producing all cell types

of the blood at the single cell level. However, the firm demonstration of this knowledge is the result of extensive studies dating some 60 years back.

An important historical observation that affected the field of hematopoiesis was that victims of the Hiroshima atomic bombing, who received the lowest lethal dose of radiation died with delayed kinetics. These victims suffered from a range of hematopoietic defects, including severe anemia (for a review see (Weissman and Shizuru, 2008)). It was later shown to be sufficient to shield the spleens of irradiated mice with lead in order to protect them from radiation syndrome (Jacobson et al., 1950). This indicated that a long-lived progenitor cell type existed in the hematopoietic system capable of sustaining hematopoiesis. Indeed, mice were rescued after radiation by injection of spleen or BM cells (Jacobson et al., 1951; Lorenz et al., 1951). Lorenz and coworkers hypothesized in 1951 that the injection of BM cells might function by creating new centers of blood formation, which had not been affected by radiation (Lorenz et al., 1951). However they could not exclude that BM transplantation resulted in production of a circulating factor responsible for the successful hematopoietic rescue.

In 1960 Till and McCulloch observed that a linear relationship existed between the number of BM cells transplanted and increased survival after radiation (McCulloch and Till, 1960). In later work they discovered the formation of spleen nodules of proliferating hematopoietic cells, named colony forming unit spleen (CFU-S), in recipient mice after BM transplantation (Till and McCulloch, 1961). Interestingly, a relationship existed between the number of these nodules, and the number of cells injected (Till and McCulloch, 1961). In a series of elegant experiments Till and coworkers induced clonogenic markers in donor cells by sub lethal radiation and observed that each spleen colony originated from a single transplanted cell, demonstrating the existence of a rare cell type able to give rise to different hematopoietic cells (Becker et al., 1963). The colony forming cells were able to give rise to new colonies by transplantation of single colonies into a new recipient demonstrating a self-renewal capacity of colony forming cells (Siminovitch et al., 1963). Properties attributed to CFU-S included their ability to proliferate, the ability to give rise to multiple lineages of differentiated offspring as well as to self-renew, all are hallmark features used today to define any type of stem cell. Although it seemed plausible that the CFU-S represented a primitive hematopoietic cell, direct studies of the properties of these cells were not possible due to their rare and unknown identity. Later it became clear that the CFU-S were derived from progenitor cells instead of HSCs (Schofield, 1978).

The demonstration of a single hematopoietic stem cell that was capable of a long-time multi-lineage reconstitution came to light in the 1980s (Capel et al., 1989; Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). Several studies were performed, where a traceable genetic marker was introduced in BM cells prior to transplantation by retroviral integration. Subsequently, clonal integration sites were detected in several hematopoietic lineages in the transplanted recipients, demonstrating that a single cell clone could give rise to mature cells of all lineages (Capel et al., 1989; Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). This firmly demonstrated the existence of HSCs. However it still remained to prospectively isolate and functionally examine such a non-manipulated cell. To facilitate this task, markers had to be elucidated in order to isolate HSCs from other BM cells.

In order to prospectively isolate HSCs, the development of applications involving fluorescence activated cell sorting (FACS) was crucial. Using this methodology, it has been possible to isolate populations of rare HSCs and progenitor cells from large samples to a near homogeneity. In a culmination of previous efforts it was demonstrated that transplantation of single cells of a rare cell type comprising 0.004% of total BM along with a radio protective dose of cells gave a durable long time repopulation of all cell lineages upon serial transplantation (Osawa et al., 1996), ultimately demonstrating the clonal nature of HSCs. It is important to emphasize that the prospective identification of HSCs requires a large panel of cell surface markers since no known individual marker exists that marks only HSCs. Different combinations of cell surface markers have been provided that aid in the direct identification of HSCs (Kiel et al., 2005).

3. Embryonic origin of hematopoietic stem cells

HSCs reside in the BM of adult individuals and continually produce progenitor cells that eventually give rise to mature blood cells. While hematopoiesis originates from the BM in post-natal life, this is not the case during embryonic and fetal development. The exact embryonic origin of a definitive HSC (dHSC), which by definition is able to restore multi-lineage hematopoiesis in irradiated adult recipients, is still the topic of intensive research (Medvinsky et al., 2011). Whether the requirement of cells derived from an embryonic/fetal environment to function in an adult BM environment truly addresses whether these cells are indeed dHSCs remains an open question.

Hematopoietic differentiation initiates in the yolk sac when mouse embryos reach the age of 7.0 - 7.5 days (E7.0-7.5) (Moore and Metcalf, 1970; Silver and Palis, 1997). However, explant culture studies have demonstrated

that hematopoiesis occurs in the yolk sac in the absence of dHSCs before E11.5 (Cumano et al., 1996; Cumano et al., 2001; Medvinsky and Dzierzak, 1996; Muller et al., 1994). The first wave of blood cell generation (primitive hematopoiesis) is thought to take place at E7.5 and mainly produces erythroid progeny. dHSCs cannot be found in the embryo prior to E10 as transplantation of cells before that time does not result in multi-lineage repopulation in adult recipients (Muller et al., 1994).

The aorta-gonad-mesonephros (AGM) is considered to be the next site of hematopoiesis, containing hematopoietic progenitors as early as E9.5 (Medvinsky et al., 1993). The AGM has been shown to be a source of dHSCs since cells extracted from the AGM at E10.5-11 are able to repopulate adult recipients (Medvinsky and Dzierzak, 1996; Muller et al., 1994). HSC activity has also been noted in the placenta from E10.5-11 (Gekas et al., 2005). This activity has been shown to arise in the absence of an active circulatory system, which suggests that the placenta initiates dHSC development autonomously (Rhodes et al., 2008). The fetal liver displays dHSC activity around E11.5 and becomes the major site of dHSC potential in the developing embryo (Medvinsky and Dzierzak, 1996). The fetal liver does not support *de novo* dHSC development and it has been suggested that cells from the AGM region, placenta, and yolk sac seed the fetal liver (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Whether the development of dHSCs in the AGM region is dependent on cells originating from the yolk sac is the subject of an ongoing debate (Samokhvalov et al., 2007). Around birth, hematopoiesis shifts from the fetal liver to the BM that becomes major site of hematopoiesis (Ema and Nakauchi, 2000).

4. Early hematopoietic differentiation

The maturation of blood cells from HSC to mature effector cells is dependent on multiple differentiation steps that involve several intermediates referred to as progenitor cells (Figure 2). Using flow cytometry, HSCs have been defined by the absence of specific lineage cell associated surface proteins, CD34 and Flt3, and the expression of Sca1 and ckit surface proteins (Lin⁻Sca1⁺ckit⁺CD34⁻Flt3⁻) (Adolfsson et al., 2001; Adolfsson et al., 2005; Osawa et al., 1996; Spangrude et al., 1988; Uchida et al., 1994; Yang et al., 2005a). An alternative HSC phenotype was described, based on the expression of proteins that belong to the SLAM family of receptors, defined by FACS as Lin⁻Sca1⁺ckit⁺CD150⁺CD244⁻CD48⁻ (Kiel et al., 2005). In the initial steps of hematopoietic differentiation, HSCs were found to up-regulate CD34 expression and give rise to cells defined as Lin⁻Sca1⁺ckit⁺CD34⁺Flt3⁻, and referred to as short term HSCs (ST-HSCs) (Yang et al., 2005a). ST-HSCs

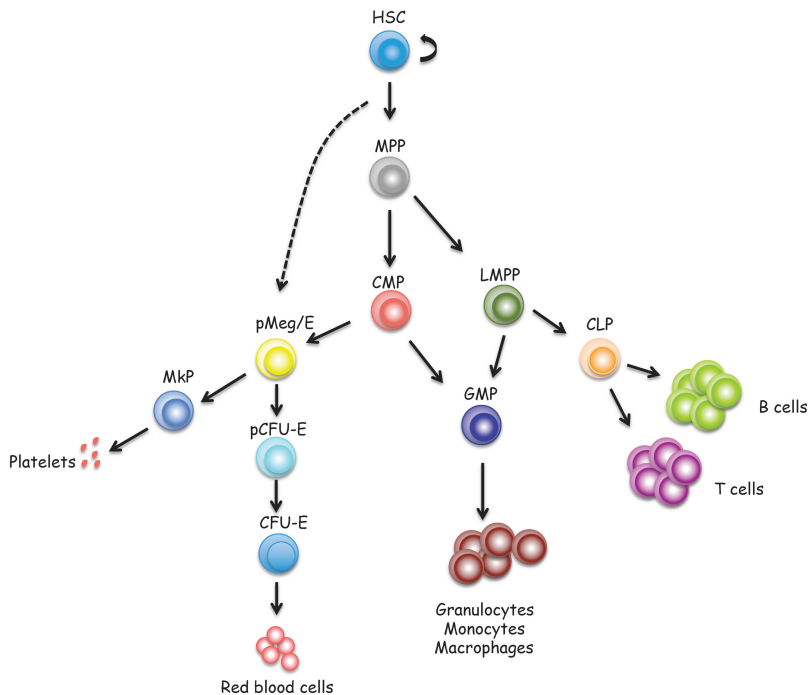


Figure 2. Schematic representation of hematopoietic differentiation from HSCs.

displayed a reduced self-renewal capacity while retaining a full multi-lineage potential, because they were able to support multi-lineage reconstitution, although not beyond 16 weeks (Morrison et al., 1997; Yang et al., 2005a). The next steps in hematopoietic differentiation are considered to entail a sequential loss of lineage choices (Bryder and Sigvardsson, 2010). ST-HSCs give rise to multipotent progenitors, following Flt3 up-regulation, which are referred to as lymphoid-primed multipotent progenitors (LMPPs; Lin⁺Sca1⁺cKit⁺CD34⁺Flt3⁺) (Adolfsson et al., 2005; Yang et al., 2005a). LMPPs harbor a robust lymphoid and granulocyte-macrophage (GM) potential whilst displaying a limited potential to give rise to megakaryocytes (Mk) or erythroid cells (E) suggesting a lineage decision between the GM/lymphoid potential and megakaryocytic/erythroid (MegE) potential, coinciding with a differential expression of Flt3 (Adolfsson et al., 2005; Mansson et al., 2007). An unresolved debate remains whether development of all blood cell types, including the MegE lineage, cells go through a common Flt3⁺ progenitor (Boyer et al., 2011).

Earlier work led to the identification of a set of progenitors downstream of HSCs/ST-HSCs/LMPPs that had lost their multipotentiality and were committed to specific lineages (Akashi et al., 2000; Kondo et al., 1997). In this view, the common lymphoid progenitor (CLP, Lin⁻Sca1^{int}cKit^{int}IL7Ra⁺) represents a common progenitor for all lymphoid cells (B, T, NK cells and DCs) (Kondo et al., 1997), while common myeloid progenitors (CMPs; Lin⁻Sca1⁺cKit⁺CD34^{high}FcγR2/III^{int}) were shown to be the precursors to the GM and MegE lineages, giving rise to megakaryocyte-erythroid progenitors (MEPs; Lin⁻Sca1⁺cKit⁺CD34^{low}FcγR2/III^{low}) or granulocytic-macrophage progenitors (GMP, Lin⁻Sca1⁺cKit⁺CD34^{high}FcγR2/III^{high}) (Akashi et al., 2000).

The CMP and the CLP suggested a binary branch point in the hematopoietic tree downstream of HSCs. However, advances in the use of additional cell surface markers in flow cytometry and subsequent functional evaluation of isolated cell types gave rise to a more detailed and complex view of the hematopoietic tree. The discovery of LMPPs for instance provided an alternative differentiation path for lymphocytes and myeloid cells. Since the resolution of the hematopoietic developmental tree continued to increase, more and more progenitors could be prospectively isolated. The originally described CMP compartment could be divided into two cellular subsets based on the expression of Slamf1 (CD150) and subsequent expression pattern analyses of integrin-α2b (CD41), endoglin (CD105), and Slamf1 (CD150) within the myeloerythroid compartment led to the identification of additional developmental intermediates (Pronk et al., 2007). Cells that lacked CD41 expression and were associated with high CD105 expression were committed to E differentiation whereas CD150 expression correlated with Mk, MegE, and E development (Pronk et al., 2008; Pronk et al., 2007). The remaining cells within this compartment that lacked expression of both CD105 and CD150 were found to be restricted to a GM fate (Pronk et al., 2008; Pronk et al., 2007). The originally described CLP compartment has undergone substantial revision as the T cell potential of CLPs was shown to be limited while more immature BM progenitors (Lin⁻Sca1⁺cKit⁺) gave rise to a robust T cell production (Allman et al., 2003; Schwarz and Bhandoola, 2004). Within the CLP compartment it has been suggested that a loss of T cell and Nk potential correlates with the expression of Ly6D with Ly6D⁺ CLPs representing B committed progenitors (Inlay et al., 2009; Mansson et al., 2010).

As informative as the hematopoietic developmental tree currently may be, it rather poorly takes the dynamic nature of the hematopoietic system into account. This is highlighted by the fact that despite the vast amount of research that has focused on revealing the developmental relationship between the different hematopoietic progenitors, a consensus

does not exist on the detailed structure of the hematopoietic tree. Nevertheless, in order to navigate, a map is needed and as the explorers of the past improved their maps, the hematopoietic map needs to be under constant revision as techniques and assays are further developed. The fact that many human blood cell diseases often are characterized by deregulation at specific stages of hematopoietic development, underlines the necessity of such detailed maps to facilitate increased understanding regarding events leading to these diseases.

5. Regulation of HSC homeostasis

In order for HSCs 1) to provide a life-long hematopoiesis, 2) to expand in numbers during embryonic/fetal development and 3) to maintain the HSC pool during adult life, HSCs undergo self-renewing divisions, which entails that at least one of the daughter cells is a HSC (Figure 3). Dysfunctional control of self-renewal can have devastating effects, as defective self-renewal would bring about a loss of HSCs and with time resulting in tissue failure and subsequent death.

At the same time as multilineage-potential and self-renewal is lost upon hematopoietic differentiation, proliferative capacity of the more differentiated progeny increases. HSCs cycle more actively during embryonic/fetal development in contrast to adult life where HSCs remain relatively proliferative inactive or quiescent (Bowie et al., 2007b; Cheshier et al., 1999; Nygren et al., 2006; Sudo et al., 2000). In adult animals an estimate of 75% of all HSCs reside in the G_0 phase of the cell cycle (Cheshier et al., 1999). HSCs are forced into the cell cycle after transplantation (Allsopp et al., 2001), and eventually lose their repopulating potential upon serial transplantation (Harrison et al., 1978; Ross et al., 1982) a phenotype reversible by the overexpression of the polycomb protein Ezh2 (Kamminga et al., 2006).

5.1 *The microenvironment*

The microenvironment presents an important factor in HSC regulation and the idea of a special HSC niche dates back to the 1970s (Schofield, 1978). Despite that recent studies have been aimed at visualizing the physical position of HSCs in the BM, a debate still exists on the subject (Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009). However, HSCs have been found to localize near the endosteum that is lined by cells referred to as osteoblasts (OBs; osteoblastic niche), as well as in close proximity to the sinusoidal endothelium (perivascular niche) (Ehninger and Trumpp, 2011). A drawback to these techniques is the fact that, unlike with multi-parameter flow

cytometry, it is not possible to simultaneously stain against multiple cell-surface markers to identify putative HSCs, and importantly it is not possible to functionally test candidate HSCs.

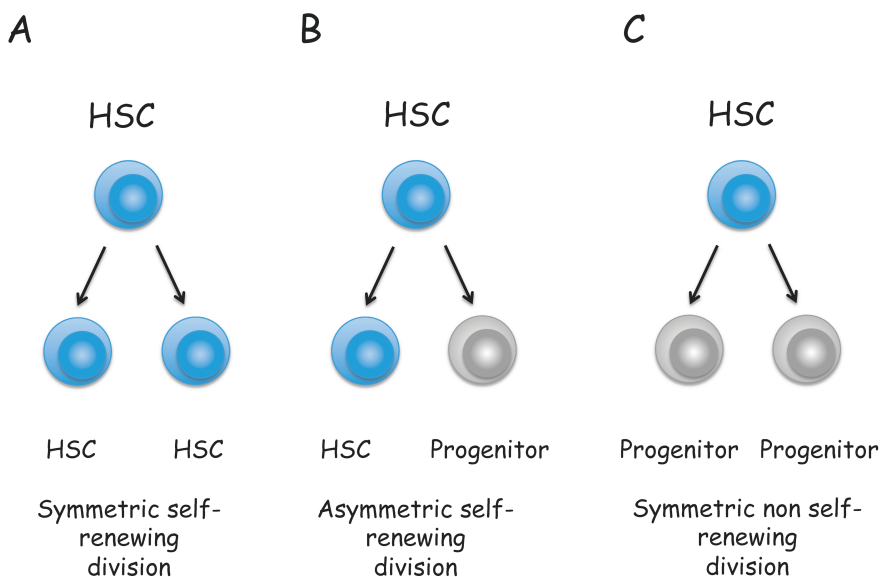


Figure 3. HSC fate upon cell division. a) Symmetric division resulting in two daughter HSCs leading to an expansion of HSCs. b) Asymmetric division resulting in a HSC and a cell destined for differentiation, thereby maintaining the HSC pool and production of mature effector cells. c) Symmetric division resulting in two cells destined for differentiation leading to a reduction of the HSC pool.

OBs were shown to be important to HSCs function, because HSCs numbers correlated with the increased number of OBs (Calvi et al., 2003; Zhang et al., 2003) illustrating the importance of micro-environmental factors in HSC regulation. Additionally, the interaction between the ligand Angiopoietin-1 expressed on OBs and the receptor tyrosine kinase Tie2 expressed on HSCs mediates quiescence and enhances survival of HSCs (Arai et al., 2004). Recently a nestin⁺ mesenchymal stem cell (MSC) was identified being in close association with candidate HSCs, and the deletion of nestin⁺ MSCs results in mobilization of approximately 50% of HSCs (Mendez-Ferrer et al., 2010). Nestin⁺ MSCs give rise to OBs and mediate the increase of HSC

numbers upon parathyroid hormone treatment, inducing proliferation and differentiation of nestin⁺ MSCs into OBs (Mendez-Ferrer et al., 2010).

Extrinsic signals in the form of cytokines have additionally been demonstrated to be imperative for HSC function; these include thrombopoietin that signals through its receptor mpl (Qian et al., 2007; Yoshihara et al., 2007) and the cytokine tyrosine kinase receptor ckit (Bowie et al., 2007a; Thoren et al., 2008). Less is known about the role of extrinsic factors that act negatively on HSC activity. The transforming growth factor- β (TGF- β) has been shown to be a negative regulator of HSCs function in vitro, however “loss of function” studies in vivo have not been able to support this role of TGF- β (Blank and Karlsson, 2011). Interferon- γ (IFN- γ) was shown to act negatively on the maintenance and function of human HSCs in vitro (Yang et al., 2005b). HSCs exhibited an increased proliferation upon IFN- γ treatment accompanied by a modest reduction in repopulation capacity (Baldrige et al., 2010). However, mice deficient in IFN- γ exhibited no difference in candidate HSCs numbers although IFN- γ ^{-/-} HSCs displayed improved repopulation upon competitive transplantation (Baldrige et al., 2010). Tumor necrosis factor- α (TNF- α) was shown to negatively regulate HSCs in vitro (Bryder et al., 2001; Dybedal et al., 2001) and recently a suppressive role for TNF- α on HSCs in vivo was elucidated in mice deficient in TNF- α receptors (Pronk et al., 2011).

5.2 *Intrinsic regulation*

Although not a universal feature, various genetic models have demonstrated a negative correlation between increased proliferation and self-renewal. The zinc finger repressor, growth factor independent 1 (Gfi-1) restricts HSC proliferation and its absence suggests a loss of self-renewing divisions as demonstrated by transplantation experiments (Hock et al., 2004; Zeng et al., 2004). Similar results were observed in mice deficient in the phosphatase and tensin homologue (PTEN) protein (Yilmaz et al., 2006; Zhang et al., 2006), as well as mice deficient in the FoxO proteins FoxO1, FoxO3 and FoxO4 (Tothova et al., 2007).

Several factors have been shown to act negatively on HSCs and thereby possibly act to limit the size of the HSC pool. This includes proteins such as the cyclin-dependant-kinase inhibitor p18 (Yuan et al., 2004), the transcription factor C/EBP α (Zhang et al., 2004), and the signal adaptor protein LNK (Ema et al., 2005).

HSC self-renewal is dependent on the activity of Bmi1 (Park et al., 2003), a member of the polycomb group of transcriptional repressors, and overexpression of this protein results in enhanced HSC function (Iwama et al., 2004). Hoxb4 is a member of the homeobox (Hox) family of transcription

factors and has been shown to be strongly inductive of self-renewing cell divisions of HSCs in vitro and in vivo (Antonchuk et al., 2001, 2002; Thorsteinsdottir et al., 1999). Somewhat surprisingly its absence does not lead to a dramatic reduction of HSC function although mild effects were demonstrated, seemingly due to the redundant effects of other Hox proteins (Bjornsson et al., 2003; Brun et al., 2004; Magnusson et al., 2007).

6 Metabolism

Although HSCs are relatively metabolically inactive (Yamazaki et al., 2006), they nevertheless require energy in order to maintain molecular integrity and biological function. The major currency of cellular energy is adenosine triphosphate (ATP), which is produced most effectively by metabolizing glucose to pyruvate (Figure 4) that is oxidized almost completely to CO₂ by the process of oxidative phosphorylation in an oxygen dependent manner in the mitochondria (Vander Heiden et al., 2009). In the absence of oxygen, cells respond by redirecting pyruvate from the mitochondria and generate lactate from pyruvate in an anaerobic reaction (Vander Heiden et al., 2009). In the context of ATP production, anaerobic glycolysis is by far less efficient and produces only 2 moles ATP/mole glucose whereas oxidative phosphorylation provides 36 moles ATP/mole glucose (Vander Heiden et al., 2009). Cancer cells, despite being highly proliferative, rely primarily on glycolysis for ATP production despite abundant oxygen levels as demonstrated by Otto Warburg early in the 19th century (Warburg, 1956). At first glance it seems counterproductive for a cell in a great need for energy to select a more energy inefficient mechanism. However, cells are in need of more than just energy, since they need to synthesize substrates for membranes, proteins and nucleic acids. Glucose provides carbon, oxygen, and hydrogen as substrates for anabolic processes as well as giving rise to NDAPH, through the pentose phosphate pathway, which is utilized in the synthesis of fatty acids and modulation of the redox potential in the cell in order to minimize the effects of reactive oxygen species (ROS) that can cause macromolecular damages (Levine and Puzio-Kuter, 2010). Therefore it can be beneficial that not all glucose is catabolized to CO₂ and H₂O through oxidative phosphorylation and depends on the functional characteristics of the cell type in question (Levine and Puzio-Kuter, 2010).

The exact nature of HSCs metabolism remains elusive and the relatively low frequency of HSCs has proved an obstacle in such analyses. However, HSC metabolism is the target of a growing research interests and recent efforts have revealed several important aspects of the metabolic characteristics of HSCs.

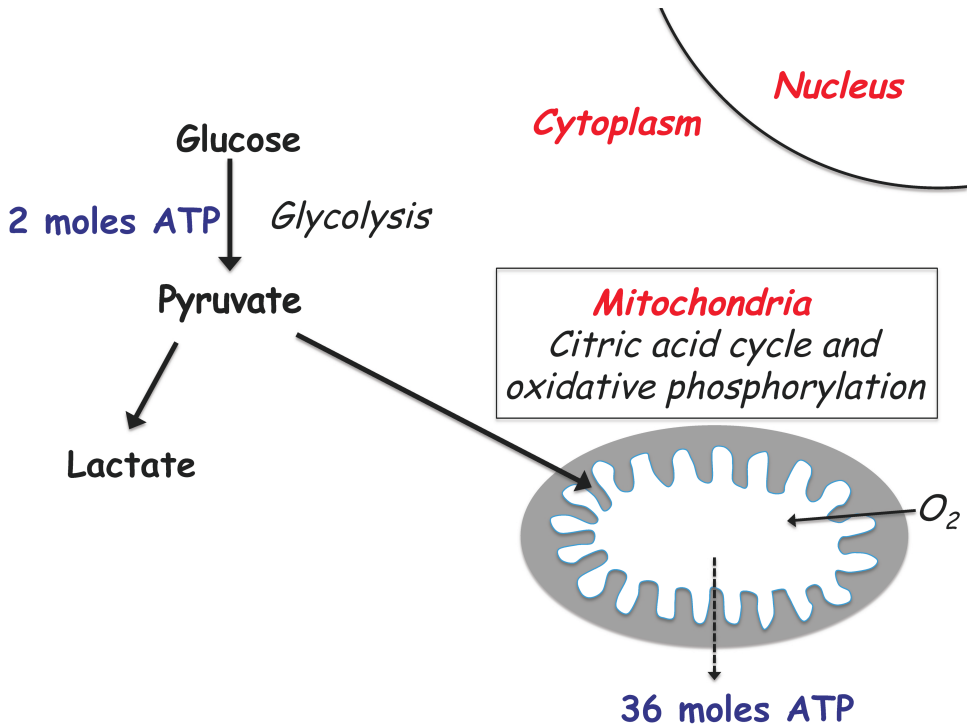


Figure 4. ATP production by glycolysis and oxidative phosphorylation. Glycolysis produces 2 moles of ATP for each mole of glucose. Lactate can be generated from the pyruvate produced by glycolysis. In the presence of oxygen pyruvate can be further metabolized in the mitochondria, giving rise to 36 moles of ATP.

6.1 Hypoxia and reactive oxygen species

HSCs have been suggested to reside within a hypoxic BM environment (Parmar et al., 2007). This hypoxia has been coupled to the relative quiescence and function of HSCs. In line with this, HSCs would respond to hypoxia by directing their metabolism towards anaerobic glycolysis, and consequently lead to a reduction in ROS originating from mitochondrial metabolism. Constant exposure to ROS can lead to accumulation of macromolecular damage, such as DNA damage, thus considering the long life span of HSCs, minimizing ROS levels should be preferable. In support of this, increased repopulation potential was shown to correlate with low levels of ROS upon sub-fractionation of the hematopoietic compartment based on different ROS levels (Jang and Sharkis, 2007). Additionally, global proteomic analysis demonstrated that immature hematopoietic cells (Lin⁻Sca1⁺cKit⁺; LSK) express high levels of glycolytic enzymes and the glucose transporter,

Glut1 (Unwin et al., 2006). In more detail, HSCs have been shown to harbor low levels of ATP when compared to whole BM and the ATP production in HSCs was further demonstrated to arise from non-mitochondrial metabolism (Simsek et al., 2010). HSCs exhibited lower oxygen consumption, increased glycolytic-dependent lactate production and elevated levels of the hypoxic inducing factor Hif1- α (Simsek et al., 2010). In support of this, glycolytic pathways are dominant in the energy metabolism of wild type (WT) HSCs and Hif1- α deficiency leads to a down-regulation of Glut1 (Takubo et al., 2010). Although HSCs deficient in Hif1- α are capable of maintaining steady state hematopoiesis, defects arise upon hematopoietic stress, such as transplantation, with decreased donor contribution originating from Hif1- α deficient HSCs (Takubo et al., 2010). In the opposite setting, increased stabilization of Hif1- α mediated by the absence of one allele of the Von Hippel-lindau protein (Vhl), resulted in increased quiescence and expansion of HSCs upon transplantation while over-stabilization of Hif1- α mediated by the absence of both alleles of Vhl led to dramatically reduced output of donor cells (Takubo et al., 2010). Combined, these data indicate that HSCs are predisposed to anaerobic glycolysis that in turn provides an additional protection from oxidative damage.

If HSC function is dependent upon low ROS concentration resulting from a low oxygen microenvironment, how do they respond to the highly oxygenated environments as encountered in transplantation experiments? During isolation, HSCs maintain their low ROS status and upon cell culture, cells derived from the ROS^{low} fraction of the BM still maintained a lower ROS level when compared to ROS^{high} originating cells, although both subsets displayed an overall increase in ROS after 6 weeks in culture (Jang and Sharkis, 2007). This indicated that additional mechanisms other than hypoxia act to protect HSCs from ROS. Transcripts of the tumor suppressor p53 are enriched in HSCs when compared to more mature hematopoietic progenitors, and its expression has been implicated in maintenance of HSC quiescence (Liu et al., 2009b). Furthermore, analyses in other organs have revealed a role for p53 in the expression of various antioxidant proteins that results in a decrease of ROS (Sablina et al., 2005) and interestingly, higher concentrations of antioxidant proteins have been noted in LSK cells in comparison to more mature cells (Unwin et al., 2006). Bmi1 deficient mice display an increase in ROS in mature thymocytes that results from a defective mitochondrial function (Liu et al., 2009a). This resulted in increased nuclear DNA damage and induced cell death of Bmi1^{-/-} thymocytes and deletion of Chek2, which is involved in DNA damage response, inhibited the ROS induced cell death without altering ROS levels (Liu et al., 2009a). Despite that Chek2 deletion resulted in increased LSK cell number it however did not rescue the

transplantation defect observed in *Bmi1*^{-/-} mice (Liu et al., 2009a). Furthermore, in the absence of the ataxia telangiectasia mutated (*Atm*) gene, self-renewal of HSCs is reduced due to increased levels of ROS, which prevents multilineage reconstitution upon transplantation (Ito et al., 2004). In response to the increase in ROS, the p38 mitogen-activated protein kinase (p38 MAPK) was activated and mediated a LSK specific expression of p16^{Ink4a} and p19^{Arf} (Ito et al., 2006). Treatment with an inhibitor against p38 MAPK subsequently rescued HSCs (LSK-side population) in *Atm* deficient mice. Additionally antioxidant treatment improved hematopoietic reconstitution of *Atm* deficient HSCs and prevented the exhaustion of WT LSK cells upon serial transplantation (Ito et al., 2004; Ito et al., 2006). In connection to these observations, absence of the forkhead box proteins FoxO1, FoxO3, and FoxO3 (*FoxO*) is accompanied by a reduced expression of *Atm* in LSK cells and leads to a loss of candidate HSCs and reduced long-term repopulation potential (Tothova et al., 2007). This correlated with elevated ROS levels and decreased quiescence of LSK cells with a concomitant increase in apoptotic cells (Tothova et al., 2007). Antioxidant treatment of *FoxO* deficient LSK cells, which showed lower expression of genes involved in oxidation protection, resulted in restoration of the LSK pool with simultaneous increase in quiescence and reduced apoptosis (Tothova et al., 2007). The cell cycle and ROS phenotypes of *FoxO* deficient mice were restricted to the LSK compartment (Tothova et al., 2007). Myeloid progenitors displayed a similar increase in apoptosis in the absence of altered ROS levels, suggesting that the apoptotic effects of *FoxO* deficiency might be ROS independent in these progenitors (Tothova et al., 2007). Collectively this highlights the importance of exact regulation of HSCs metabolism and ROS levels in order to preserve HSC quiescence and function.

6.2 *Liver kinase B1*

Although HSCs seem to utilize glycolysis to larger degree than that of oxidative phosphorylation it does not entail that mitochondria are not important to HSCs function. Studies addressing the role of the liver kinase B1 (*Lkb1*), considered to be a central regulator of cellular metabolism (reviewed in (Shackelford and Shaw, 2009)), highlight the importance of intact metabolism for HSC function (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). Upon deletion of *Lkb1*, HSCs were forced into cycle resulting in an initial increase in HSC numbers with a subsequent increase in apoptosis and loss of HSCs (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). *Lkb1* deficient HSCs were incapable of multilineage reconstitution as demonstrated upon transplantation of HSCs into irradiated recipients (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). These defects were somewhat surprisingly shown to be independent of AMPK

and mTorc1 (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010), which are regulated by Lkb1 (Krock et al., 2011). Additionally Lkb1 deficiency resulted in decreased mitochondrial membrane potential (MMP) and an increase in mitochondrial mass in HSCs accompanied by increased glucose uptake (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). Interestingly, Lkb1 deficient Lin⁻ cells displayed elevated levels of long-chain fatty acids and several essential fatty acids (Gurumurthy et al., 2010). Global gene expression analyses of Lkb1^{-/-} HSCs revealed altered expression of genes involved in the PPAR γ metabolic pathway, with down-regulation of PPAR γ coactivators and regulators of mitochondrial biogenesis, Pgc-1 α and Pgc-1 β (Gan et al., 2010).

7. Aging

As unicellular organisms reproduce by a division of a mother cell, giving rise to two daughter cells, a separation of a germ line (the genetic information that flows from one generation to the next) and a soma (the body) does not exist. This suggests that unicellular organisms should not age, as it would eventually lead to a loss fitness of the whole species and a subsequent extinction. Consequently in higher animals, a deterioration of the parental soma does not affect the offspring negatively as procreation entails a reproduction of the soma. Although most unicellular species (as well as metazoans with no clear separation between the germ line and soma (Hydra)) show no signs of aging, the budding yeast displays proliferative senescence after certain number of divisions (Vijg, 2007). In the case of budding yeast, cell divisions are asymmetric with the aging phenotypes arising in the mother cells, thus the detrimental effects of aging in budding yeast are confined to the parent such as in most metazoans (Vijg, 2007). *Escherichia coli* that morphologically displays symmetrical divisions also exhibits signs of aging, in this case however one of the daughter cells inherits the old end of the mother cells that was not created during the division with the cell inheriting the old part acquiring a decrease in fitness (Stewart et al., 2005).

The German scientist August Weissman proposed that cells of higher animals had a limited capacity to proliferate (Kirkwood and Cremer, 1982). He also noted that the only importance regarding the length of the life span of an individual is that he should be enabled to do his work towards maintenance of the species, which is reproduction (Kirkwood and Cremer, 1982). Aging in general is accompanied with declining tissue function and diminished regeneration capacity upon injury or stress (Rando, 2006). Thus, aging is not advantageous to an organism since it tends to decrease its

reproductive fitness. It has been the topic of much debate how such a trait has been able to evolve. Early explanation included that it was paramount to species to possess a mechanism that could limit population size (reviewed in (Kirkwood and Austad, 2000)). This is however unlikely, since aged animals are infrequent in the wild such a mechanism would be useless. Another theory put forward by Medawar suggests that aging is due to an accumulation of mutations that are disadvantageous late in life, and therefore no selective pressure will exist against such mutations (Medawar, 1952). A related theory postulated by Williams argues for the existence of pleiotropic genes, that is to say genes that are advantageous early in life but harmful later on (Williams, 1957). However, the idea of late acting genes relies on an early and a late stage in the life span of the individual, thus late acting genes would be a reinforcing consequence but not the cause of aging (Kirkwood and Holliday, 1979). According to the “disposable-soma” theory of aging, a separation is made between the mortal soma and the immortal germ line (Kirkwood and Holliday, 1979). Life span is regulated to a large extent by extrinsic factors, thus individuals need to survive and remain healthy during that time in order to reproduce and extend the germ line (Kirkwood and Holliday, 1979). Thus, organisms would not gain much from investing in mechanisms that would ensure “healthy” survival of the soma beyond that time frame (Kirkwood and Holliday, 1979).

7.1 Stem cells and aging

Tissue specific stem cells represent an interesting intermediate between the disposable soma and the germ line. They function to replenish tissue with new cells to replace worn out or damaged ones throughout life. As noted above, diminished capacity to respond to injury and stress is one of the hallmarks of aging. This places tissue specific stem cells in a potential central role in the aging of tissues. Post mitotic tissues such as the heart are less likely to depend on constant generation of new cells, and aging of such a tissue is more likely to reside at the level of post mitotic effector cells. However, the hematopoietic system represents a highly proliferative tissue that is dependent on constant output of new cells.

Several scenarios of stem cell aging (Figure 5) are conceivable due to changes that arise at the stem cell level (Jones and Rando, 2011). First, alteration(s) arise that affect the self-renewal capacity of the stem cell pool leading to a reduction in stem cell numbers. Second, the lineage potential of stem cells is affected leading to a skewed ratio of mature cells. Third, the function of mature cells is diminished. Fourth, apoptosis or senescence downstream of stem cells leading to reduced number of mature cells.

Accumulation of such damaging alterations over time would ultimately lead to a diminished functional potential of the residing tissue.

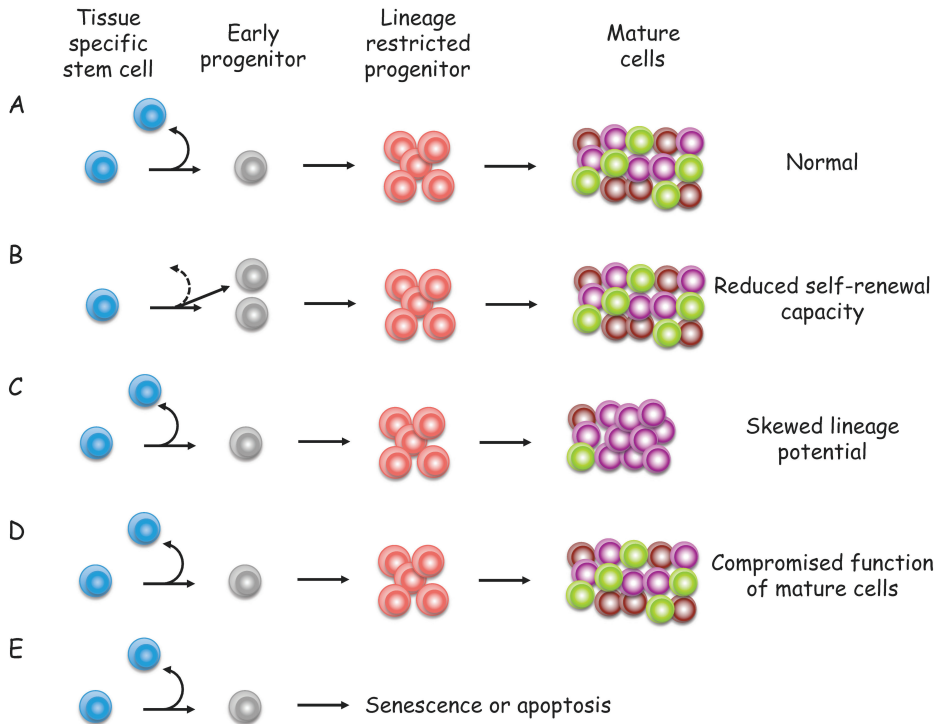


Figure 5. Models of stem cell aging. a) Normal functional capacity of stem cells. b) Reduced stem cell self-renewal capacity resulting in a gradual depletion of the stem cell pool. c) Altered stem cell lineage potential resulting in an altered cellular composition of the residing tissue. d) Alterations arising at the level of stem cells that diminishes mature effector cell function. e) Alterations arising at the level of stem cell that lead to apoptosis or senescence upon differentiation.

7.2 Hematopoietic aging

The hematopoietic system is an example of a highly proliferating tissue and its functionality is directly dependent on HSC function for the constant production of mature cells. With advancing age, several age-associated alterations arise within the hematopoietic system (Figure 6). These include 1) a diminished function of the adaptive immune system, 2) an increased incidence of myeloid diseases including leukemia, and 3) an increased incidence of anemia (Rossi et al., 2008). Additionally, advancing age of donors has been shown to significantly correlate with reduced overall and disease-free survival of patients after BM transplantation (Kollman et al., 2001).

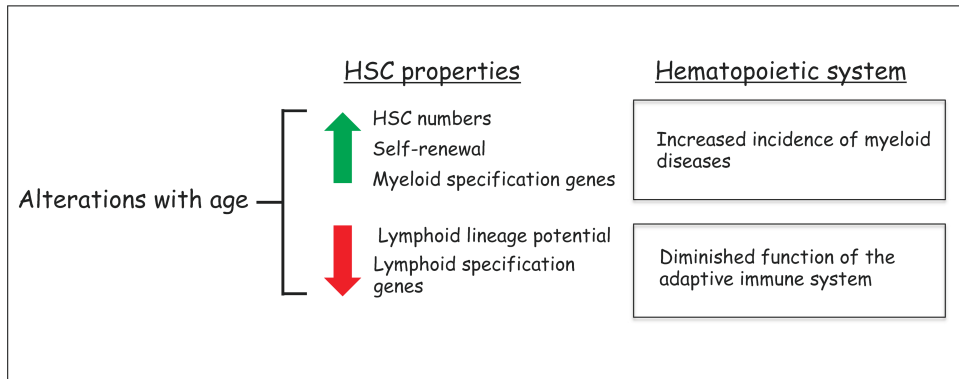


Figure 6. Alterations in the hematopoietic system upon HSC aging. Molecular and functional changes arising at the level of HSCs resulting in alterations of the hematopoietic system with advanced age. Adapted from Rossi and coworkers (Rossi et al., 2007b).

The normal lifespan of C57Bl/6 mice is between 27-29 months (Harrison and Archer, 1987). Following transplantation, HSCs are estimated to outlive their host by multiple life spans (Harrison and Astle, 1982). This does not entail that HSCs function is fixed during aging. HSCs increase in numbers with age when assayed by phenotype and/or function (de Haan et al., 1997; Morrison et al., 1996; Rossi et al., 2005; Sudo et al., 2000). Aged HSCs have a greater capacity for self-renewal, a cell autonomous feature since it is preserved upon transplantation of aged HSCs into a young BM environment, despite performing far worse in overall repopulation when compared to their young counterparts (Pearce et al., 2007; Rossi et al., 2005). The age-dependent increase in HSCs number might represent a compensatory mechanism, given the decline in function and altered lineage output upon aging.

It has been the topic of some debate whether similar increase of HSCs is observed in humans, although an increase has been observed in the number of Lin-CD34⁺CD38⁻CD90⁺ cells (Beerman et al., 2010b; Kuranda et al., 2011). The recent discovery of a novel human HSC marker facilitated the isolation of a single HSC capable of reconstituting immuno-compromised mice (Notta et al., 2011). This finding should make it possible to address changes of human HSC numbers during aging with more accuracy.

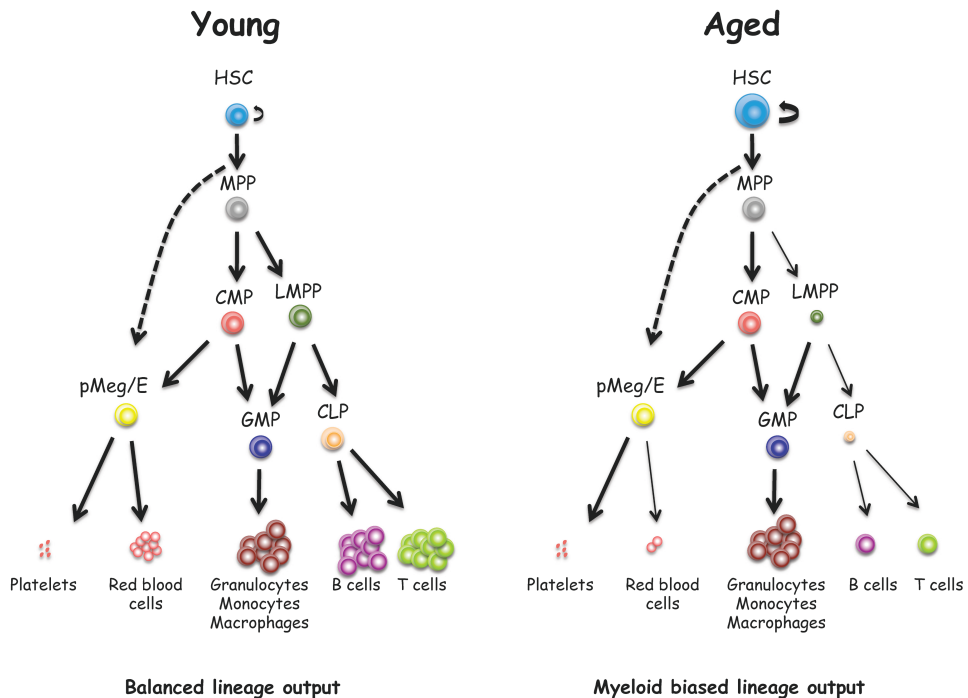


Figure 7. Schematic representation of aging in the HSC and progenitor cell compartment. A schematic overview of the effects of advancing age on the generation of mature effector cells by differentiation from HSCs through progenitor cells. Left panel) An young and lineage balanced hematopoietic system. Right panel) An aged hematopoietic system displaying an increase in HSC numbers, a reduction in lymphoid output and generation of red blood cells. Adapted from Beerman and coworkers (Beerman et al., 2010b).

An interesting observation was made when aged HSCs were transplanted at limiting doses into young recipients (Sudo et al., 2000). Several recipients displayed reconstitution of the myeloid lineages in the absence of a significant lymphoid rescue (Sudo et al., 2000). Initially explained as an accumulation of defected HSCs, this altered lineage potential in favor of myelopoiesis (Figure 7) was later confirmed and shown to be one of the hallmarks of hematopoietic aging (Muller-Sieburg et al., 2004; Rossi et al., 2005). The loss of lymphoid potential with advanced age in humans coincides with the simultaneous decrease in incidence of leukemia of lymphoid origin and increase in leukemia of myeloid-origin (Beerman et al., 2010b). Moreover, global gene expression analyses of young compared to aged HSCs revealed an increased expression of myeloid-associated transcripts with a concomitant reduction of lymphoid related genes with age

(Rossi et al., 2005). This is represented functionally by an intrinsically reduced output of lymphoid progenitors by aged HSCs and diminished responsiveness of aged lymphoid progenitors to the cytokine IL-7 in vitro (Miller and Allman, 2003; Rossi et al., 2005). Intriguingly, young and aged BM cells respond differently to the potent oncogene BCR-ABL as transformation of young BM cells initiated either a myeloproliferative disorder or a lymphoid leukemia with a B cell phenotype whereas in contrast, aged BM cells gave primarily rise to a myeloproliferative disorder (Signer et al., 2007).

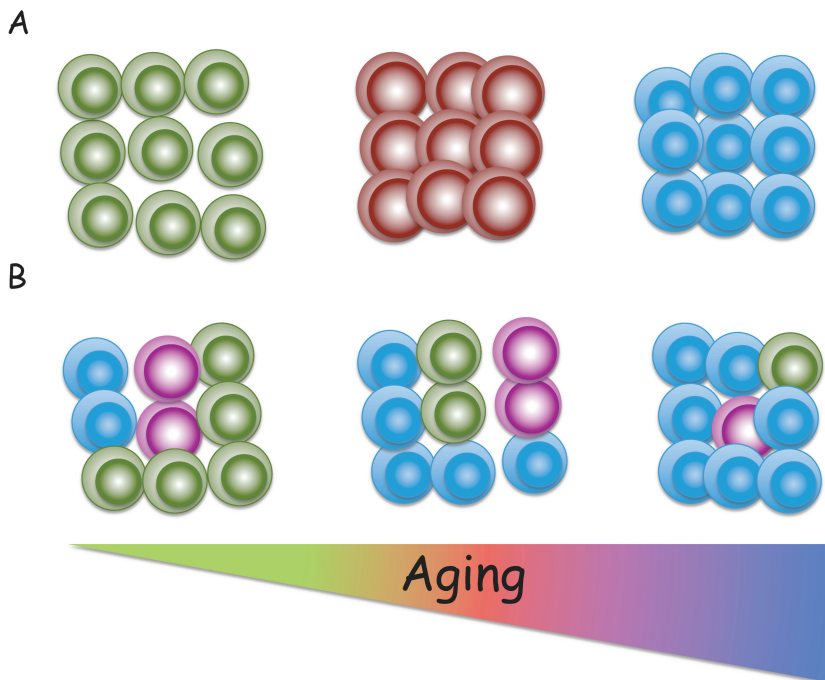


Figure 8. HSC aging models. a) One model presumes that the young HSC pool is composed of a functionally homogeneous collection of HSC clones. Aging results in an altered functional capacity of the whole HSC pool. b) Another model presumes that the young HSC pool is heterogeneous and due to clonal selection a specific functional clone becomes dominant with age.

Thus, a scenario emerges where the altered composition of mature cells within the hematopoietic system is a direct consequence of an altered stem cell function. This is represented at the molecular level of HSCs as well in the differentiation capacity that is independent of an aged BM microenvironment.

7.3 What lies beneath? Heterogeneity of the HSC compartment.

An intriguing question is how the HSC pool changes as the individual ages. HSCs arise and undergo vast expansion during embryonic development. Thereafter, the HSC pool has to maintain itself by a mechanism of self-renewal as no new HSCs are thought to be derived from a more primitive cell type in adult life. Due to the self-propagating nature of HSCs, at least two scenarios have been proposed to explain aging in the hematopoietic compartment (reviewed in (Rossi et al., 2008)). One suggests that the HSC pool is a homogeneous collection of cells that gradually changes upon aging, a scenario where all cells are equally competent in their lineage potential (Figure 8a). The other assumes that early in life, the HSC pool is a combination of functionally separate clones with different lineage potentials (Figure 8b). The existence of a selective advantage and/or different life spans would thereafter lead to an expansion or decline of distinct subtypes during aging. These two scenarios however, are not mutually exclusive and hematopoietic aging might in fact be represented by a combination of these two models (Rossi et al., 2008).

Several studies have demonstrated, either by transplantation of highly purified candidate HSCs or BM cells at limiting dilution frequency, an inherent functional heterogeneity in the HSC pool of young mice (Beerman et al., 2010a; Challen et al., 2010; Cho et al., 2008; Dykstra et al., 2007; Muller-Sieburg et al., 2004). With regard to repopulation kinetics and lineage output, it was proposed that the HSC compartment could be divided into four functionally distinct subsets (Dykstra et al., 2007). One subset referred to as α -cells, displayed an initial lag in reconstitution and produced predominantly myeloid cells in combination with an extensive self-renewal potential. Furthermore, serial transplantation experiments suggested the existence of a hierarchy between these different subsets. However, this work did not reveal any markers making it possible to prospectively isolate these different subsets of HSCs (Dykstra et al., 2007). A significant progress was made recently by the publication of three independent studies wherein distinct subtypes of HSCs from young mice were prospectively isolated by a differential expression of the cell surface protein CD150 or by different dye efflux activities (Beerman et al., 2010a; Challen et al., 2010; Morita et al., 2010). Candidate HSCs defined by FACS as Lin-Sca1+cKit+(Flt3-)CD34-CD150^{High} displayed a myeloid biased lineage potential in combination with a higher self-renewal capacity, when compared to cells having the same phenotype differing only in levels of CD150 expression (Beerman et al., 2010a; Morita et al., 2010). The CD150^{High} HSCs were suggested being capable of generating both CD150^{Med} and CD150^{Low} HSCs, thereby residing on the top of a cellular hierarchy within the HSC compartment (Morita et al., 2010).

Interestingly around 10% of the CD150^{high} cells displayed a myeloid limited phenotype after transplantation with a lag over 3 months in repopulation kinetics upon primary transplantation (Morita et al., 2010). The proportion of CD150^{high} HSCs increased dramatically during aging and aged CD150^{high} HSCs gave rise to a myeloid dominant hematopoietic reconstitution upon transplantation, similar to their young CD150^{high} counterparts (Beerman et al., 2010a). Comparable lineage bias was observed using different dye efflux activity within the side-population (SP; Lower-SP with high activity and Upper-SP with low activity) as a marker for cell isolation (Challen et al., 2010). Interestingly, HSCs that display the highest dye efflux activity also express the highest levels of CD150 within the side population (Challen et al., 2010). The lineage bias was somewhat magnified when Upper- and Lower-SP HSCs were transplanted together which lead the authors to suggest that in the presence of one HSC subtype, the other subtype is more biased towards the fate that it is already predisposed to (Challen et al., 2010).

Collectively, these findings point toward a clonal selection model of aging. However, as the reconstitution potential of the CD150 subsets is diminished during aging, deficiencies seem to arise within the clonal subtypes while the lineage potential of each subtype is retained, indicating that a clonal selection model is not sufficient alone in explaining the aging of HSCs (Beerman et al., 2010a).

7.4 Life span and cycling of HSCs

Within weeks after birth, HSCs “switch” from a highly self-renewing and proliferative fetal phenotype to a more quiescent one, a process that has been shown to be a cell intrinsic feature in development (Bowie et al., 2007b). This transmigration of the HSC population results in the generation of a heterogeneous pool of adult HSCs. The heterogeneous clonal composition of the HSC compartment becomes progressively homogeneous during aging. What sort of mechanism is responsible for one subtype prevailing throughout adult life while another disappears? Is it purely a stochastic process or is it in some way a deterministic or pre-programmed feature of the hematopoietic system? Intuitively it seems unlikely that such a feature could be written off as purely stochastic, as the myeloid dominance of the aged hematopoietic system has been reported in numerous independent publications. If deterministic, how is it controlled?

What if each clonal HSC subtype carries an internal timer, with the only difference being the time that each subtype is given until it reaches the end of its life? Myeloid biased HSC clones have previously been shown to display longer life spans compared to lymphoid biased clones, when measured as the total donor contribution to peripheral blood after

transplantation (Muller-Sieburg and Sieburg, 2008; Muller-Sieburg et al., 2004). The donor repopulation resulted in ballistic shaped curves (with donor contribution on one axis and time on the other) with lymphoid biased clones repopulating rapidly post transplantation contrasting the delayed response from myeloid biased clones that are able to sustain donor contribution for an extended period of time (Muller-Sieburg and Sieburg, 2008; Muller-Sieburg et al., 2004). It was recently suggested that individual HSC clones are programmed with finite life spans, a phenomenon which is independent of external signals mediated by the microenvironment (Sieburg et al., 2011). This claim was supported by experiments demonstrating that daughter HSCs derived from a single transplanted HSC clone, all displayed very similar repopulating kinetics (Sieburg et al., 2011).

If HSCs have different life spans, what is the measurement of cell age? The age generation hypothesis states that the fewer generations (divisions) HSCs have undergone the more capable of self-renewal and the production of mature progeny they are, indicating that cell divisions are a measurement of cellular aging (Rosendaal et al., 1976). HSCs display slow proliferation kinetics in steady state *in vivo* and candidate HSCs with a more active proliferation history show inferior long-term repopulating competence (Foudi et al., 2009; Nygren and Bryder, 2008). Thus if there exists an inherent difference in the cycling profile of different HSCs subset, this might offer an explanation to the disappearance of lymphoid biased HSCs during aging. In a study analyzing the dilution of a histone 2B-green fluorescence protein in HSCs, it was suggested that two subpopulations exist with one population (around 20% of HSCs) proliferating at a slower rate ($\leq 0.8-1.8\%$ per day) and the other (around 80% of HSCs) proliferating at a faster rate (5.3-11.1% per day) (Foudi et al., 2009). An additional study using a similar approach, reported the existence of a small subset of HSCs that lie dormant and proliferate with extremely slow kinetics in order to preserve their function, and contribute minimally to steady state hematopoiesis only becoming active upon hematopoietic emergency before returning to dormancy (Wilson et al., 2008). The authors further suggested that these cells represented the most active HSCs that upon adulthood become dormant, thereby marking the end of the expansion phase that began at the embryonic/fetal stage (Wilson et al., 2008). It was suggested that this proliferative heterogeneity might be reflected in the heterogenic lineage potentials observed in the HSC pool (Raaijmakers and Scadden, 2008). This view was contested by Takizawa and coworkers that reported that the long-term multilineage repopulating potential lies both in HSCs that had frequently cycled and in quiescent HSCs that did not cycle over a period of 14 weeks. They further showed that aged HSCs were more inclined to return to quiescence after transplantation than

young HSCs (Takizawa et al., 2011). Based on these results a model emerged wherein some HSCs dominate hematopoiesis at a given time and subsequently return to quiescence with the potential of contributing again upon reactivation, and this results in a similar turnover of the HSCs compartment (Takizawa et al., 2011).

8 Molecular mechanisms involved in aging

8.1 DNA damage

The nuclear genome of eukaryotic cells contains the blueprint for most cellular components, and it is imperative for intact cellular function to preserve the integrity of the DNA. Cells are confronted with multiple insults to their DNA on daily basis. ROS, a natural by-product of metabolic activity, can result in oxidative DNA damage in addition to other sources of mutations such as UV irradiation and the inherent error rate upon DNA replication. Furthermore, by estimation, several thousand lesions arise in the genome of each cell daily merely by spontaneous depurination and hydrolysis of DNA (Lindahl, 1993). This places an enormous amount of importance on the DNA repair machinery of the cell. According to the disposable soma theory of aging, less importance is placed on the maintenance of somatic cells. In this context, aging would occur because of a somewhat compromised maintenance of DNA, proteins and other constituents of somatic cells. Patients suffering from certain defects in DNA maintenance and repair pathways display in many cases various degrees of premature aging (Martin, 2005). If DNA mutations arise in stem cells without an appropriate repair response, all downstream progeny will be likely to inherit these genomic alterations.

Certain features of HSCs are presumed to aid in the protection against accumulation of DNA mutations. One is the fact that the majority of HSCs reside in the G_0 phase of the cell cycle (Cheshier et al., 1999) thus reducing the risk of replicative errors. Additionally, HSCs generate low levels of ROS compared to other hematopoietic progenitors (Tothova et al., 2007) and are capable of expelling out toxic compounds (Goodell et al., 1996). However, it was not until recently that it was addressed how HSCs respond to DNA lesions. HSCs were shown to be more tolerant to irradiation and the subsequent formation of double strand breaks, whereas myeloid progenitors are poised to undergo apoptosis (Mohrin et al., 2010). Furthermore, due to the quiescent nature of HSCs they have been suggested to employ the more error prone repair mechanism or nonhomologous end joining (NHEJ) while surviving myeloid progenitors use the high fidelity homologous

recombination (HR) mechanism for DNA repair (Mohrin et al., 2010). When HSCs were forced to proliferate they retained their irradiation tolerance but utilized HR instead of NEJ for DNA repair thus reducing the risk of further alterations to their DNA (Mohrin et al., 2010). This scenario suggests that HSCs are somewhat protected against DNA lesions due to their quiescent nature with the disadvantage of having to utilize a suboptimal mechanism of DNA repair and thus presenting a mechanism how HSCs accumulate DNA lesions during aging.

It has been proposed that accumulation of DNA mutations is responsible for the age-related phenotypes of stem cells (Park and Gerson, 2005) with both candidate murine and human HSCs harboring increased number of γ -H2AX foci with advancing age indicative of double strand breaks of the DNA (Rossi et al., 2007a; Rube et al., 2011; Yahata et al., 2011). Additionally, transplantation procedures were found to cause ROS-induced accumulation of DNA damage in human HSCs with a subsequent functional deterioration (Yahata et al., 2011). In support of this, diminished stem cell function has been reported in several strains of mice with defective DNA maintenance (Morales et al., 2005; Navarro et al., 2006; Nijnik et al., 2007; Reese et al., 2003; Rossi et al., 2007a). Transplantation of HSCs with compromised DNA maintenance revealed an age-associated decline of HSC function with reduction in self-renewal, repopulation capacity and increased apoptosis (Nijnik et al., 2007; Rossi et al., 2007a). Yet, the myeloid bias of physiologically aged HSCs was not observed upon diminished DNA maintenance, which indicates that it is unlikely that DNA lesions are the sole cause of hematopoietic aging (Rossi et al., 2007a; Rossi et al., 2008).

8.2 Telomere shortening and defective mitochondrial function

Regions of repetitive sequences (telomeres) characterize the ends of chromosomes. With each cell division these sequences shorten unless they are maintained by telomerase. Telomeres in humans become shorter with increased age and BM recipients display shorter telomeres in donor cells than observed in the original donor (Wynn et al., 1998). In murine studies, telomere shortening arises in HSCs when serially transplanted and HSCs lacking telomerase have a reduced serial transplantation capacity (Allsopp et al., 2001; Allsopp et al., 2003a). This scenario is complicated by the fact that the same authors reported that although overexpression of telomerase reverse transcriptase did maintain telomere length it did not extend the serial transplantation capacity indicating that other mechanisms take part in the exhaustion upon serial transplantation (Allsopp et al., 2003b). In aged animals, all mitotic cells lacking telomerase activity should display altered telomere length and not only hematopoietic cells. In accordance with this,

transplantation of WT BM cells into mice deficient in the telomerase RNA component resulted in reduced generation of B cells, however with retained myeloid potential (Ju et al., 2007). This coincided with reduced numbers of BM mesenchymal cells and altered plasma cytokine levels (Ju et al., 2007). Contrary or additionally to this, the loss of lymphoid potential was shown to mainly be due to systemic alterations and not cell-autonomous changes in the thymic or BM niches (Song et al., 2010). Recently, a differential response of HSC subtypes in young mice to cytokine signaling was reported, implicating changes in BM cytokine concentrations to the aging of HSCs (Challen et al., 2010). When considering the effects of altered telomere dynamics it is important to keep in mind that big differences exist between species in this aspect. Mice have long telomeres compared to humans and display moderate expression of telomerase in somatic tissues (Geiger and Rudolph, 2009). Given the data obtained from murine experiments, it is intriguing to speculate whether telomere shortening arises in human HSCs with increased age.

Mice with telomere dysfunction displayed several metabolic alterations such as impaired mitochondrial biogenesis and function, decreased gluconeogenesis and increased ROS levels (Sahin et al., 2011). Specifically, HSCs exhibited a reduction in mitochondrial DNA (mtDNA) copy number, decreased expression of gene networks downstream of PGC-1 α / β that control various features of mitochondrial biology and cellular metabolism (Sahin et al., 2011). Furthermore, oxidant treatment did not rescue the transplantation defects of telomere dysfunctional HSCs (Sahin et al., 2011). Defective mitochondrial function has been implicated in the process of organismal aging (Balaban et al., 2005; Kujoth et al., 2005; Trifunovic et al., 2004). Moreover, a study on *Saccharomyces cerevisiae* has revealed a role for intact mitochondrial function in maintaining genomic stability (Veatch et al., 2009). The role of intact mitochondrial function in hematopoietic stem cell aging is addressed in more detail in Article II of this thesis.

8.3 Tumor suppressors and epigenetic regulation

The hematopoietic system is as aforementioned highly proliferative. This aspect is advantageous as the whole system is dynamic and well equipped to respond to altered cellular requirements. The proliferative capacity can however, be a blessing in disguise. Renewable tissues run the risk of acquiring and propagating mutations that can lead to malignant transformation of cells. Thus there is a need for surveillance mechanisms that monitor the genomic integrity of the cells. Cells have several choices upon insult, to repair, undergo apoptosis, or cellular senescence (mitotic arrest). Effective cancer prevention at young age is an attractable feature and this is

mediated in part by proteins with tumor suppressor function. However, this aspect might fit the theory of antagonistic pleiotropy (Williams, 1957), as too active tumor suppression might put too much restriction on cellular integrity and thereby leading to a loss of stem cell and tissue functionality with increasing age (Campisi, 2003).

Bmi1 is a part of the Polycomb group of transcriptional repressors that represses gene expression of their target genes, as mentioned in chapter 5.2, by introducing a trimethyl group on lysine 27 of histone 3 (Ringrose and Paro, 2007). HSC function is dependent on the correct expression of Bmi1 as deregulation results in aberrant expression of the two splice variants of the Ink4a locus p16^{Ink4a} and p19^{Arf}, that inhibit cell proliferation and induce apoptosis, respectively (Park et al., 2003). However, the derepression of p16^{Ink4a} and p19^{Arf} did not impart a senescent phenotype on clonally cultured Bmi1^{-/-} as HSCs actively entered the cell cycle and gave rise to daughter cells upon first cell division similarly to WT HSCs (Iwama et al., 2004). This indicated that the effect of Bmi1 was mediated through additional target genes and raised questions about the role of cellular senescence in aged HSCs, which is the focus of article I in this thesis. Of note, Bmi1 binds to genomic regions marked both by H3K27me3 (repressive) as well as H3K4me3 (active), in hematopoietic progenitors (Oguro et al., 2010), characteristic of transcriptionally poised genes to be expressed during differentiation (Bernstein et al., 2006). Repression of B cell lineage factors was lost in Bmi1^{-/-} immature progenitors (HSC/MPPs) and coincided with a reduction of H3K27me3 and consequently increased B cell generation (Oguro et al., 2010). Thus, Bmi1 has been suggested as a candidate for regulating HSCs lineage potential during aging (Pollina and Brunet, 2011). The Ink4a locus has additionally been implicated in aging of the hematopoietic system downstream of HSCs. Expression of p16^{Ink4a} is increased during B lymphopoiesis in aged mice whereas myeloid progenitors display no expression at all (Signer et al., 2008). Ectopic expression of Bmi1 rescued the proliferative defect of aged pre and pro B cells and transformation of aged pre and pro B cells was only possible with the BCR-ABL oncogene in the presence of Bmi1 overexpression (Signer et al., 2008). This story is yet more complex, because a pleiotropic effect of p16^{Ink4a} deletion was observed between two lineages within a tissue (Liu et al., 2011). B-lineage specific deletion of p16^{Ink4a} did not rescue the age-dependent decline in B cell lymphopoiesis and caused an increase in the development of B-lineage cancers (Liu et al., 2011). In contrast, T-lineage specific deletion resulted in a slower thymic involution and rescue of age-associated phenotypes in peripheral T cells (Liu et al., 2011).

Increased activity of the tumor suppressor protein p53 limits the repopulating ability of aged HSCs whereas young cells show normal engraftment potential (Dumble et al., 2007). In contrast, diminished p53 activity increased the engraftment potential of aged HSCs in addition to increased susceptibility to cancer formation (Dumble et al., 2007). This is in agreement with pleiotropic effects of a tumor suppressor pathway as alterations of the p53 pathway might delay aging in the hematopoietic compartment, however this anti aging effect might entail an increased risk for cancerous transformation. Recently a role for p53 activity was revealed in a competition between candidate HSCs clones (Bondar and Medzhitov, 2010). Clones with lower p53 activity had a competitive advantage upon low dose of irradiation without undergoing apoptosis or mitotic arrest, suggesting that a mechanism is in place in hematopoietic progenitors and HSCs that remembers past damage and results in the replacement of these cells with more “healthier” clones if they are available (Bondar and Medzhitov, 2010).

Discussion of articles

ARTICLE I

Attema JL, Pronk CJ, Norddahl GL, Nygren JM, Bryder D. Hematopoietic stem cell ageing is uncoupled from p16^{INK4A}-mediated senescence. *Oncogene*. 2009 Jun 4;28(22):2238-43.

Aim:

To investigate the putative role of the tumor suppressor protein p16^{Ink4a} in aged HSCs.

Questions addressed in Article I:

Do aged HSCs display altered in vivo proliferation kinetics?

Utilizing an in vivo tracking assay (Nygren and Bryder, 2008) we followed cell division of HSCs in vivo. These analyses revealed that most young and aged HSCs had divided within a two-week period. Despite that heterogeneity existed in the proliferative profile of aged HSCs, on a population basis they displayed slower proliferation kinetics in comparison to young HSCs.

Is the slower proliferation kinetics of aged HSCs due to activation of the tumor suppressor p16^{Ink4a}?

To address the role of p16^{Ink4a} in HSC aging, we analyzed mRNA expression in HSCs isolated from young and aged WT HSCs. In single cell reverse transcriptase polymerase chain reaction (RT-PCR) assays we failed to detect expression of p16^{Ink4a} transcripts both in young and aged HSCs, despite some signs of a low level expression when aged HSCs were assayed in bulk by quantitative RT-PCR (qRT-PCR). Further analyses revealed that p16^{Ink4a} expression was only detected in 0.32% of aged HSCs, demonstrating that p16^{Ink4a} expression is a very rare event upon HSC aging.

Is p16^{Ink4a} subjected to differential epigenetic regulation in aging?

To address whether aged HSCs are predisposed to undergo p16^{Ink4a}-mediated senescence, we analyzed the expression of various regulators of p16^{Ink4a} in young and aged HSCs. qRT-PCR analyses performed in bulk and single cell RT-PCR analyses demonstrated only modest alterations in HSCs across age. Because we observed no expression of p16^{Ink4a} in the vast majority of aged HSCs we investigated whether a difference existed in epigenetic marks that are associated either with transcriptional activation (H3K4me3) or repression (H3K27me3 or CpG methylation) across the Ink4a locus. No signs of differential promoter methylation was observed between young and aged HSCs. Additionally, our data showed that H3K27me3 enrichment at the Ink4a/Arf locus does not change across age demonstrating that p16^{Ink4a} remains epigenetically silenced in aged HSCs.

Discussion:

When cells lose their ability to divide while maintaining viability, they are said to undergo cellular senescence. Although our data indicated that aged HSCs show slower proliferation kinetics when compared to young HSCs, the majority had divided within a 14-day period, in agreement with a previous report from our laboratory demonstrating a complete turnover of the whole HSC pool within three weeks, whereas studies using histone 2B GFP mouse models have suggested a heterogenic proliferation of the HSC pool (Foudi et al., 2009; Nygren and Bryder, 2008; Wilson et al., 2008). This proliferative potential suggests that cellular senescence is not a central mechanism mediating HSC aging. Collectively, we failed to detect a differential regulation of p16^{Ink4a} in the aged HSCs pool when compared to young HSCs, suggesting that p16^{Ink4a} does not play a major role in the aged phenotype of aged HSCs. This is in contrast to previous studies that reported increased expression of p16^{Ink4a} in aged HSCs (Janzen et al., 2006; Pearce et al., 2007) and an improved repopulation capacity of aged HSCs deficient in p16^{Ink4a} (Janzen et al., 2006). However, p16^{Ink4a} deficiency did not correct the myeloid bias of aged HSCs upon transplantation and the observed differences in apoptosis and increased cycling were noted in non-steady state hematopoiesis upon transplantation (Janzen et al., 2006). Importantly it was not observed that p16^{Ink4a} induction leads to a cellular senescence in HSCs (Janzen et al., 2006). Interestingly, derepression of p16^{Ink4a} and p19^{Arf} is not sufficient to impart senescence on HSCs (Iwama et al., 2004) and clonal evaluations have revealed that physiologically aged HSCs are equally capable as young HSCs in giving

rise to progeny in vitro (Morrison et al., 1996; Sudo et al., 2000). Nevertheless, our data do not exclude a role for p16^{Ink4a} activation in hematopoietic aging as increased expression has been observed in B lymphopoiesis as discussed previously in chapter 8.3 of this thesis.

All analyses in this study were performed on non-modified young and physiologically aged WT animals. This is worth mentioning because it can be difficult to draw direct conclusions from transgenic animals when addressing complex phenotypes such as aging. The importance of this is highlighted when research efforts on several mouse models of aging are taken into account as they have revealed that although these models display certain aging phenotypes they fail to mimic the multifactorial aspects of aging (Morales et al., 2005; Nijnik et al., 2007; Rossi et al., 2007a).

ARTICLE II

Norrdahl GL, Pronk CJ, Wahlestedt M, Sten G, Nygren JM, Ugale A, Sigvardsson M, Bryder D. Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. *Cell Stem Cell*. 2011 May 6;8(5):499-510.

Aim:

To investigate the importance of intact mitochondrial function in HSCs and the putative role of diminished mitochondrial function in HSC aging.

Questions addressed in article II:

Does a differential dependence on intact mitochondrial function exist in early hematopoietic differentiation?

To investigate the potential role of mitochondrial regulation in early hematopoietic differentiation we set out to quantify mitochondria in hematopoietic stem and progenitor cells. A reduction of mitochondrial content was observed upon differentiation of HSCs to the bipotent megakaryocytic/erythroid precursor (pre Meg/Es). This was followed by an increase in cellular mitochondria content upon further erythroid development. Additionally, we observed increased frequency of erythroid-committed progenitors (pre CFU-Es) and a selective reduction of Pro B cells in the BM of transgenic mice (mutator mice) that express a proofreading defective form of the mitochondrial DNA polymerase gamma (Trifunovic et al., 2004). Despite that mutator mice have been described as a model for premature aging these phenotypes were distinct from physiological aging, as aged WT mice displayed an overall reduction of lymphoid progenitors and failed to exhibit an accumulation of erythroid progenitors.

Is the phenotype of mutator mice autonomous to HSCs?

Given the altered BM composition of mutator mice, we wanted to address the functional capacity of HSCs in mutator mice. Transplantations of HSCs revealed severe defects in the output of mature lymphoid cells from mutator HSCs, whereas myelopoiesis or HSC regeneration was not affected. Serial transplantation of young mutator HSCs revealed a diminished self-renewal capacity accompanied by a decrease in total donor contribution. Additionally,

we observed a diminished output of erythroid progenitors starting already upon primary transplantation indicating that the observed lineage defects of mutator mice arise at the levels of lineage restricted progenitors rather than at the stem cell level.

What are the cellular consequences of accumulation of mtDNA mutations?

In light of the hematological defects observed in mutator mice we decided to investigate the cellular consequence of accumulated mtDNA mutations in more detail. By sequencing mtDNA isolated from single cells we observed a similar mutation rate in HSCs and pre CFU-Es isolated from mutator mice. Furthermore, mutator HSCs, GM, and pre CFU-Es exhibited lower mitochondrial potential when compared to their WT counterparts and in contrast to all cells investigated, mutator pCFU-E were not able to raise their MMP in vitro. Despite the lowered MMP of mutator cells, no change in cellular ATP levels or ROS levels was observed. Nonetheless, we observed a lineage restricted increase in apoptosis in mutator mice including pre CFU-Es and B cells, whereas HSCs were not affected.

How relevant are the molecular basis of the mutator phenotypes to physiological aging?

As mutator mice have been described as a model for premature aging, we compared various parameters known to change upon physiological aging. Both young and middle-aged mutator HSCs gave rise to a hematopoietic system skewed in favor of myelopoiesis, somewhat reminiscent of aged HSCs. However, in the case of young mutator HSCs this occurred without an expansion of a myeloid biased Slamf1^{hi} HSC population, as observed upon physiological aging (Beerman et al., 2010a). Additionally premature aging in the HSCs compartment of mutator mice did not involve global transcriptional changes typical of aged HSCs.

Discussion:

Our results demonstrate a differential dependence on intact mitochondrial function within the hematopoietic cell compartment. The relative tolerance of HSCs to the mutator setting is in concordance with recent studies indicating that HSCs rely on anaerobic glycolysis as a primary source of ATP production (Simsek et al., 2010; Takubo et al., 2010; Unwin et al., 2006). Nevertheless, HSCs are not entirely independent of mitochondrial function as extensive

proliferation of mutator HSCs leads to a decline in function, in agreement with previous data showing that correct mitochondrial regulation is important for HSCs (Gan et al., 2010; Gurumurthy et al., 2010; Liu et al., 2009a; Nakada et al., 2010; Sahin et al., 2011).

Despite the accumulation of mtDNA mutations we did not observe decreased levels of cellular ATP, a feature that has previously been observed in other tissues in mutator mice (Hiona et al., 2010; Trifunovic et al., 2004). Due to the proliferative nature of the hematopoietic system, we hypothesize that a selection of cells with adequate ATP levels might occur mediated by increased apoptosis of cells in the affected lineages, which is not a possibility in post mitotic tissues.

Mitochondria fulfill an array of functions including synthesis of ATP, steroids, lipids, and the breakdown of sugars and long chain fatty acids (McBride et al., 2006). Additionally and interestingly, the electron transport chain is involved in pyrimidine biosynthesis and defects in this process leads to a p53 mediated induction of apoptosis (Khutornenko et al., 2010). Thus, it might be an over-simplification to ascribe the mutator phenotype only to reduced ATP levels. It remains to be elucidated precisely by which cellular mechanisms the mutator phenotype arises within the hematopoietic system. The possibility remains that separate aspects of mitochondrial function are responsible for defects in the hematopoietic lineages affected by the mutator genotype. Since mutator mice have a gradual and multifactorial phenotype, identifying the affected molecular pathways should allow for more detailed studies directly addressing perturbed pathways in the affected lineages.

The close proximity of mtDNA to the source of oxidants (electron transport chain) and the fact that mtDNA is not associated with histones, makes mtDNA sensitive to ROS (Balaban et al., 2005). According to the mitochondrial theory of aging a vicious cycle arises within the mitochondria, with ROS originating in mitochondria resulting in mtDNA damage and subsequently altered function of the electron transport leading to an increased ROS production (Harman, 1972). Because, we (and others) do not observe increased ROS levels in mutator cells (Hiona et al., 2010), we conclude that a vicious cycle of mtDNA mutations and increased ROS production is not a prominent feature of hematopoietic aging. Nonetheless, mutator mice do display various aging-like phenotypes such as anemia, osteoporosis, thymic involution, and weight loss (Trifunovic et al., 2004). Despite this we conclude that the observed lineage defects in hematopoietic differentiation are distinct from normal aging, as mutator HSCs do not exhibit epigenetic changes or accumulation of a myeloid biased subpopulation of HSCs, typical of physiological hematopoietic aging (Beerman et al., 2010a;

Rossi et al., 2005). However, the possibility that mtDNA mutations are a contributing factor in aging cannot be excluded.

ARTICLE III

Norrdahl GL, Wahlestedt M, Gisler S, Sigvardsson M, Bryder D. Enhanced cytokine responsiveness can counteract age-induced decline in hematopoietic stem cell function. Manuscript (2011).

Aim:

To investigate whether altered cytokine signaling might modulate HSC aging.

Questions addressed in article III:

How does LNK deficiency affect steady state aging of HSCs?

In order to address how LNK deficiency affects steady state hematopoiesis with advanced age, we analyzed the hematopoietic compartment of both aged WT and LNK^{-/-} mice. Aged LNK^{-/-} mice displayed elevated numbers of mature white blood cells in peripheral blood in addition to an altered cellular composition of the BM, with increased frequency of HSCs and megakaryocytic progenitors.

Is HSCs function preserved upon aging of LNK deficient HSCs?

To address the functional capacity of aged LNK^{-/-} HSCs we performed competitive transplantation experiments. Aged LNK^{-/-} HSCs performed far better upon competitive transplantation compared to aged WT HSCs with a substantial contribution to all lineages investigated, in contrast to both the diminished overall- and lymphoid contribution of aged WT HSCs. This was further demonstrated upon serial transplantation, with no significant lymphoid contribution observed in hosts transplanted with aged WT HSCs, whereas recipients of aged LNK^{-/-} HSCs displayed high levels of donor derived myeloid and lymphoid cells.

What are the molecular consequences of LNK deficiency?

We performed global gene expression analyses to address how HSC function and self-renewal is maintained in the absence of LNK. These analyses identified an altered gene expression profile in LNK^{-/-} HSCs with an overrepresentation of genes involved in cell cycle progression that were repressed in the absence of LNK. To investigate whether the phenotype of

LNK^{-/-} HSCs was accompanied by changes in telomere length we evaluated telomere length in single HSCs. This comparison did not reveal any differences in telomere length between aged WT and LNK^{-/-} HSCs, indicating that differential telomere maintenance is not a critical feature of the aged LNK^{-/-} HSC phenotype.

Discussion:

As HSCs reside at the top of the cellular hierarchy in the hematopoietic system and give rise to all mature blood cells, it should be of clinical interest to identify cellular mechanisms that could be potentially manipulated in order to address hematological ailments that arise with advanced age.

Although, the number of HSCs increases with age they display a loss of function including an altered lineage potential (Beerman et al., 2010a; Cho et al., 2008; Morrison et al., 1996; Rossi et al., 2005; Sieburg et al., 2006; Sudo et al., 2000). Aged LNK^{-/-} HSCs did not recapitulate the transplantation-associated phenotypes of aged WT HSCs because they displayed a robust potential to reconstitute both the lymphoid and myeloid lineages. As discussed in chapter 7.3, it has been suggested that the HSC pool in young animals consists of functionally distinct clones, whereas upon aging this heterogeneity is decreased (Beerman et al., 2010a; Challen et al., 2010; Cho et al., 2008; Dykstra et al., 2007; Morita et al., 2010; Muller-Sieburg et al., 2004; Sieburg et al., 2006). In light of this and our data demonstrating the preservation of HSC function in aged LNK^{-/-} mice, it would be interesting to address the clonality of the HSC compartment in LNK deficient mice. If the HSC pool in young LNK^{-/-} mice is composed of functionally distinct clones and that heterogeneity is preserved upon aging, it would imply that an aged-associated loss of lymphoid biased clones does not occur in the absence of LNK. However if the LNK deficiency leads to a homogeneous HSC compartment composed of HSC clones that are capable of a robust multilineage differentiation, it raises the question whether it would be possible to modulate the lineage potential of physiologically aged HSCs by altered cytokine signaling.

The high frequency of aged LNK^{-/-} HSCs in addition to our data indicating that as a population they are more quiescent when compared to their WT counterparts, might suggest that progenitors downstream of HSCs in LNK deficient mice might possess a higher proliferative potential. This would lead to a reduced demand of cellular output from LNK^{-/-} HSCs and a subsequent reduction in the proliferation of HSCs. Thus, further investigations detailing the mechanisms responsible for the preserved HSC

function upon LNK deficiency should present an exciting avenue in the pursuit for therapies aimed at diseases where HSC dysfunction plays a central role such as BM failure and various leukemias.

Populärvetenskaplig sammanfattning

Blodet är sammansatt av många olika typer av mogna celler som är ansvariga för specialiserade roller inom blodsystemet. Röda blodceller transporterar syre till kroppens celler, vita blodceller försör oss med försvar mot mikroorganismer och blodplättar ser till att stoppa blödningar. Nybildning av blodceller behövs kontinuerligt eftersom vissa celler lever bara i några dagar medan andra kan leva i månader eller år. Dessutom när vi blir sjuka eller blöder då uppstår det ett ändrat behov av mogna celler som blodsystemet måste kunna svara omedelbart på. Var kommer alla de mogna cellerna i blodet ifrån? Det finns en väldigt sällsynt celltyp, som sitter i benmärgen och kallas för blodbildande (hematopoetiska) stamceller (HSC). HSC har den unika förmågan att kunna ge upphov till alla blodceller (differentiering) och dessutom att skapa en exakt kopia av sig själv i en process kallad självförnyelse. För att kunna skapa mogna celler då genomgår HSC en differentieringsprocess som i början ger upphov till olika stadier av allt mognare celltyper som succesivt förlorar förmågan att bilda de olika blodcellerna tills slutligen en viss mogen blodcell har skapats. Att HSC kan självförnyas, försäkras att HSC kan stå för blodbildning livet ut. Men det finns ett problem. När vi blir äldre ökar risken för vissa blodrelaterade sjukdomar, bland annat cancer och dessutom minskar blodets förmåga att kämpa emot infektioner. Dessa åldersrelaterade ändringar i blodet tror man kan vara orsakade av förändringar i de blodbildande stamcellerna. Denna avhandling är fokuserad på molekylära mekanismer som tros kunna orsaka åldersrelaterade förändringar i HSC funktion.

Trots att antalet HSC ökar i åldrande möss har det hävdats att gamla HSC inte delar sig lika ofta som unga. Ökad aktivitet av ett celldelnings hämmande protein p16, har föreslagits orsaka denna egenskap samt att vissa gamla HSC delar sig inte alls. I artikel I i denna avhandling undersökte vi aktiviteten av p16 i HSC från normalt åldrade möss. Vi kom fram till att trots gamla HSC delar sig inte lika ofta som unga HSC har de en omfattande celldelnings förmåga och samtidigt kunde vi inte hitta något bevis på p16 aktivitet i gamla HSC.

I våra celler finns olika organeller och en av de, mitokondrier, förser celler med bland annat molekyler (ATP) vilka används som energikälla för att driva diverse cellulära processer. Mitokondrier har sitt eget DNA (mtDNA) och uppsamlad skada på det leder till uppkomst av olika kropps och cellulära förändringar i möss som vanligt vis kopplas till åldrande. I artikel II undersökte vi om förändringar som uppstår i blodet, på grund av ökad frekvens av mtDNA skada, orsakas av tidigt åldrande av HSC. Vi kom fram till att intakt funktion av mitokondrier är nödvändig för normal blodbildning men att mtDNA skada på egen hand driver inte åldrande av HSC.

I artikel III har vi analyserat stamcellsfunktion i möss som saknar genen LNK, vilken är involverad i omvandling av signaler från signalmolekyler (cytokiner). HSC från normala gamla möss visade flera funktionella defekter medan gamla HSC som saknar LNK förvånande uppförde sig som unga stamceller. Dessa resultat ger därför en viktig insikt i vilka mekanismer är involverade i att bevara stamcellfunktion i blodsystemet.

Articles and manuscripts not included in this thesis

Pronk CJ, Rossi DJ, Månsson R, Attema JL, **Norrdahl GL**, Chan CK, Sigvardsson M, Weissman IL, Bryder D. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell*. 2007 Oct 11;1(4):428-42.

Wahlestedt M, **Norrdahl GL**, Sten G, Ugale A, Mich Frisk MA, Mattson R, Deierborg T, Sigvardsson M, Bryder D. An Epigenetic Component of Hematopoietic Stem Cell Aging Amenable to Reprogramming Into a Young State. Submitted manuscript (2011).

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