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Hematopoiesis: Functional Insights by Cell Fate Conversion

Martin Wahlestedt



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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended in the Segerfalk lecture hall, BMC A10, Lund on the 14th of March
2014 at 13:00.

Faculty opponent

Hartmut Geiger, PhD, Professor

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Abstract Blood cell formation, or hematopoiesis, is maintained by rare hematopoietic stem cells (HSCs) residing in the bone marrow (BM). HSCs may self-renew (the process whereby HSCs replicate to produce new HSCs) to preserve their own numbers, as well as initiate a highly coordinated hierarchical differentiation process that results in the production of mature effector blood cells. Aging is characterized by an overall loss of cellular and organ fitness, which has been suggested to result from alterations in tissue-resident somatic stem cell function. In the blood, aging presents with several functional alterations that can be traced to cell intrinsic alterations occurring in HSCs. HSC aging has been suggested to result from accumulation of DNA damage in genomic and/or mitochondrial DNA (mtDNA). We addressed the latter in article I using mice that rapidly accumulate mtDNA mutations and display several premature aging phenotypes. These “mutator mice” displayed several hematopoietic abnormalities associated with aging, including anemia and defects in lymphopoiesis. However, several hallmarks associated with HSC aging was lacking. In addition, the observed phenotypes appeared to result from alterations in progenitor cells rather than in HSCs. Thus, we concluded that mtDNA mutations are unlikely to be the main drivers of hematopoietic aging. In article II, we investigated the relevance of epigenetic alterations for hematopoietic aging. To this end, we generated induced pluripotent stem (iPS) cells from aged hematopoietic stem and progenitor cells (HSPCs), since cellular reprogramming coincides with an epigenetic reset of the somatic donor cells. We next redifferentiated the resulting iPS cells into blood in vivo and investigated the resulting hematopoiesis for age-related parameters. This revealed that the reformed blood system displayed a balanced lineage potential, with HSC numbers and function comparable to the young setting. Therefore, we concluded that a major component of hematopoietic aging involves reversible epigenetic alterations. While mtDNA mutations appeared to impact little on somatic stem cell function per se (article I), little is known about their impact on iPS cells function. To address this, we generated iPS cells from mutator HSPCs. Although mutator iPS cells were readily formed, these displayed severe differentiation defects (article III). We traced this to a failure of mutator iPS cells to utilize mitochondrial driven oxidative phosphorylation during differentiation. In article IV, we set out to identify individual novel regulators that may impact on early hematopoietic cell fate decisions. This led to the identification of the transcription factor Hepatic Leukemia Factor (Hlf). Enforced Hlf expression could direct early cell fate decisions of multipotent GMLPs by strongly favoring myelopoiesis over lymphopoiesis. This indicates that Hlf is a key determinant of lineage fate and an important component in the regulatory networks of multipotency.			
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Table of contents

Abstract	6
Abbreviations	7
Articles and manuscripts included in this thesis	9
Background	10
1. Introduction to hematopoiesis	10
2. Towards steady-state adult hematopoiesis	12
3. The adult hematopoietic hierarchy	13
4. Regulation of hematopoietic stem and progenitor cells	16
4.1 The BM microenvironment and extrinsic influence	17
4.2 Intrinsic regulators affecting adult hematopoiesis	20
4.3 Epigenetic regulation in hematopoiesis	23
5. Aging	27
5.1 The aging hematopoietic compartment	28
5.2 Clonal dynamics of HSCs during aging	30
5.3 DNA damage and telomere attrition in hematopoietic aging	31
5.4 Mitochondria and aging	33
5.5 Epigenetics and rejuvenation of hematopoietic aging	34
Summary of articles	37
Populärvetenskaplig sammanfattning	48
Other work not included in this thesis	50
Acknowledgements	51
References	53

ABSTRACT

Blood cell formation, or hematopoiesis, is maintained by rare hematopoietic stem cells (HSCs) residing in the bone marrow (BM). HSCs may self-renew (the process whereby HSCs replicate to produce new HSCs) to preserve their own numbers, as well as initiate a highly coordinated hierarchical differentiation process that results in the production of mature effector blood cells. Aging is characterized by an overall loss of cellular and organ fitness, which has been suggested to result from alterations in tissue-resident somatic stem cell function. In the blood, aging presents with several functional alterations that can be traced to cell intrinsic alterations occurring in HSCs. HSC aging has been suggested to result from accumulation of DNA damage in genomic and/or mitochondrial DNA (mtDNA). We addressed the latter in article I using mice that rapidly accumulate mtDNA mutations and display several premature aging phenotypes. These “mutator mice” displayed several hematopoietic abnormalities associated with aging, including anemia and defects in lymphopoiesis. However, several hallmarks associated with HSC aging was lacking. In addition, the observed phenotypes appeared to result from alterations in progenitor cells rather than in HSCs. Thus, we concluded that mtDNA mutations are unlikely to be the main drivers of hematopoietic aging. In article II, we investigated the relevance of epigenetic alterations for hematopoietic aging. To this end, we generated induced pluripotent stem (iPS) cells from aged hematopoietic stem and progenitor cells (HSPCs), since cellular reprogramming coincides with an epigenetic reset of the somatic donor cells. We next redifferentiated the resulting iPS cells into blood *in vivo* and investigated the resulting hematopoiesis for age-related parameters. This revealed that the reformed blood system displayed a balanced lineage potential, with HSC numbers and function comparable to the young setting. Therefore, we concluded that a major component of hematopoietic aging involves reversible epigenetic alterations. While mtDNA mutations appeared to impact little on somatic stem cell function *per se* (article I), little is known about their impact on iPS cells function. To address this, we generated iPS cells from mutator HSPCs. Although mutator iPS cells were readily formed, these displayed severe differentiation defects (article III). We traced this to a failure of mutator iPS cells to utilize mitochondrial driven oxidative phosphorylation during differentiation. In article IV, we set out to identify individual novel regulators that may impact on early hematopoietic cell fate decisions. This led to the identification of the transcription factor Hepatic Leukemia Factor (Hlf). Enforced Hlf expression could direct early cell fate decisions of multipotent GMLPs by strongly favoring myelopoiesis over lymphopoiesis. This indicates that Hlf is a key determinant of lineage fate and an important component in the regulatory networks of multipotency.

ABBREVIATIONS

AGM	Aorta-Gonad-Mesonephros
BiPS	Blood induced Pluripotent Stem
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	Embryonic day
ES cell	Embryonic Stem cell
ETP	Early T-lineage Progenitors
FACS	Fluorescence Activated Cell Sorting
FL	Fetal Liver
GM	Granulocyte and Monocyte
GMLP	Granulocyte-Monocyte-Lymphoid Progenitor
HR	Homologous Recombination
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
iPS cell	induced Pluripotent Stem cell
LMPP	Lymphoid-primed Multipotent Progenitor
lncRNA	Long Noncoding RNA
LSK	Lin-Sca1+c-Kit+
MEF	Mouse Embryonic Fibroblast
MegE	Megakaryocyte and Erythroid
miRNA	micro RNA
MPP	Multipotent Progenitor
Msc	Mesenchymal stromal cell
ncRNA	noncoding RNA
NHEJ	Non Homologous End Joining
NK cell	Natural Killer cell
OB	Osteoblast

OC	Osteoclast
pCFU-E	pre-Colony Forming Unit-Erythroid
PcG	Polycomb Group
pGM	pre-Granulocyte Monocyte
PRC	Polycomb Repressor Complex
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
TF	Transcription Factor
Treg	Regulatory T-cell
TrxG	Trithorax Group
WT	Wild-type

ARTICLES AND MANUSCRIPTS INCLUDED IN THIS THESIS

- I. Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging.
Norrdahl GL, Pronk CJ, Wahlestedt M, Sten G, Nygren JM, Ugale A, Sigvardsson M, Bryder D.
Cell Stem Cell. 2011 May 6; (5):499-510.8
- II. An epigenetic component of hematopoietic stem cell aging amenable to reprogramming into a young state.
Wahlestedt M, Norrdahl GL, Sten G, Ugale A, Frisk MA, Mattsson R, Deierborg T, Sigvardsson M, Bryder D.
Blood. 2013 May 23;121(21):4257-64
- III. Somatic cells with a heavy mitochondrial DNA mutational load render iPS cells with distinct differentiation defects.
Wahlestedt M, Ameer A, Moraghebi R, Norrdahl GL, Sten G, Woods NB, Bryder D.
Manuscript accepted for publication in Stem Cells.
- IV. Critical modulation of hematopoietic lineage fate by the PAR/bZIP transcription factor Hepatic Leukemia Factor.
Wahlestedt M, Säwén P, Ladopoulos V, Norrdahl GL, Göttgens G, Bryder D.
Manuscript

BACKGROUND

1. Introduction to hematopoiesis

Several organ systems with independent functions act in concert to sustain appropriate function of the human body throughout life. The circulatory system is a critical component, and functions to transport blood throughout the body via a coordinated network of arteries, veins and capillaries. Blood is a liquid composed of fluid plasma and several cell types with distinct functions. For instance, the red blood cells, mediate oxygen transport to various parts of the body, while megakaryocyte-derived platelets are critical for blood coagulation and wound healing. Another major function of the blood is to circulate various components of the immune system that act as a defense barrier against foreign pathogens. The immune system can be divided into an innate part; composed of Basophils, Eosinophils, Macrophages and Neutrophils that mediates a primary defense, and an adaptive part that mediates later and more specific responses to invading pathogens and comprises B cells, T cells, Natural Killer (NK) cells as well as Dendritic Cells (DCs).

The cellular components of the blood are formed through a continuous process termed hematopoiesis, in which rare hematopoietic stem cells (HSCs) residing in the bone marrow (BM) give rise to all mature blood cell types. Hematopoiesis is an extremely high-throughput process, requiring the small population of HSCs to produce what has been estimated to be 10¹² new blood cells every day (1). In order to cope with this enormous proliferative pressure, HSCs have the potential to self-renew; that is to give rise to a new HSC through cell division. Moreover, HSCs are multipotent, meaning that they have the potential to give rise to progeny of different cellular lineages. To prevent proliferative exhaustion of the HSC pool, blood cell formation occurs in a highly coordinated hierarchical manner involving many intermediate short-lived progenitor stages with restricted differentiation potential but vast proliferation potential. Following initial maturation in the BM, immature and mature blood cells are transported to other peripheral organs, such as the spleen, thymus and lymph nodes, for further maturation.

While adult, or somatic, stem cell populations by now have been identified in a wide range of tissues including the brain, eye, hair-follicle, intestine, lung, mammary gland and skeletal muscle (2-8), blood forming HSCs are by far the most well-studied adult stem cell population (9). Moreover, HSCs represent the only somatic stem cell population routinely utilized in the clinic when performing bone marrow transplantation (BMT) to treat various hematological malignancies (9).

The current knowledge about HSCs and hematopoiesis is the combination of about six decades worth of research and observations, and stems from work aimed at preventing radiation sickness. An important early observation in this regard was the fact that survivors following the World War II nuclear bombings at Hiroshima and Nagasaki died over an extended period of time, which was later attributed to be caused by a compromised hematopoietic system (for a review see (10)). In 1950, Jacobson and colleagues found that shielding the spleen with lead was sufficient to protect mice from radiation sickness (11), and in 1951 two independent studies by Jacobson et al and Lorentz et al found that the transplantation of bone marrow or spleen cells was sufficient to rescue irradiated mice (12, 13). Moreover, Till and McCulloch observed in 1960 a linear relationship between the survival of lethally irradiated mice and the amount of transplanted BM cells (14). Collectively, these experiments suggested the bone marrow and spleen to contain proliferating, blood-forming cells. In later experiments, it was also observed that the transplantation of BM cells resulted in the generation of small colonies on the spleen of recipient mice that contained proliferating hematopoietic cells of donor origin, termed Colony Forming Unit-Spleen (CFU-S) (15). Again, a linear relationship between the number of formed spleen colonies and the number of transplanted BM cells was observed. This relationship was further refined by Till and coworkers who used donor specific labeling by sublethal irradiation prior to transplantation and found that each spleen colony originated from a single hematopoietic cell (16). These findings proposed the existence of rare hematopoietic cells with the potential to regenerate mature blood cells of the different lineages. Interestingly, the isolation of such colonies in combination with serial transplantation revealed the existence of self-renewing cells, since the isolated spleen colony cells were able to form new colonies on the spleens of secondary recipient mice (17). The CFU-S therefore seemingly contained cells with all properties of a stem cell by today's standards, including self-renewal and multilineage differentiation potential. However, direct studies of these cells were complicated by their rareness and unknown identity. In 1978, Schofield and coworkers were able to establish that CFU-S were in fact not stem cells per se, but rather derived from immature progenitor cells (18).

Groundbreaking efforts in the 1980's by several research groups utilizing retroviral marking of donor cells prior to transplantation revealed the existence of single hematopoietic cells with long-term multilineage reconstitution potential. This was revealed since the tracking of retroviral integration sites post transplantation revealed clonal integrations present in all blood cell lineages (19-22). Although, these studies indicated the existence of HSCs, the lack of unique HSC markers hindered the isolation and study of these cells. To overcome this, extensive developments into Fluorescence Activated Cell Sorting (FACS) based methodologies involving the functional characterization of cells with different cell surface phenotypes have been performed. These endeavors have allowed for the prospectively isolation not only HSCs, but also multiple progenitor fractions and mature cells to near homogeneity

from mixed samples using a combination of cell surface specific markers coupled to fluorescence-conjugated antibodies and FACS. Using such approaches, the existence of long-term multilineage repopulating cells was finally indisputably shown by Smith and colleagues, who were able to rescue irradiated mice by isolating and transplanting limiting dilution doses of hematopoietic cells (23). It was later estimated that these cells are indeed very rare in the BM, representing only about 0.004% of the total bone marrow cellularity (24). Collectively, the aforementioned studies along with many others, have not only provided knowledge surrounding the existence of HSCs and hematopoietic precursor cells, but will likely also be of importance for the study of other cellular hierarchies and stem cell populations (9).

2. Towards steady-state adult hematopoiesis.

While adult hematopoiesis takes place in the bone marrow, this is not the case during embryonic development. The hematopoietic system has its origins in the mesodermal germ layer (25), and the “birth” of the blood system is characterized by two waves in the embryo. The first wave, primitive hematopoiesis, mainly generates erythroid progeny from lineage-restricted progenitors that appear in the extra-embryonic yolk sac at around embryonic day 7-7.5 (E7-7.5) (for a review see (26)). This first wave of embryonic hematopoiesis produces transitory hematopoietic cells and is not thought to contribute to adult hematopoiesis (26). Instead, the second wave of embryonic blood development, definitive hematopoiesis, generates the first cells with HSC characteristics (26). Both the exact timing and location by which these cells arise in the developing embryo is a matter of debate (Figure 1) (26). However, transplantation experiments into lethally irradiated recipient mice have revealed an absence of definitive HSCs (dHSCs) in embryos prior to E10 (27). Definitive hematopoiesis is currently thought to initiate at different sites in the embryo, and one of the most well described locations is the aorta-gonad-mesonephros (AGM) region of the dorsal aorta where dHSCs have been proposed to arise at E10.5-11 (27, 28). However, other sites where dHSCs might arise have also been described. In the placenta, dHSCs have been found to be present at E10.5-11 in the absence of an established circulatory system (29-31), indicating that the placenta may be a site for *de novo* dHSC formation. Several studies have through the transplantation and lineage tracing of yolk sac cells from E8-10 also proposed a role for the yolk sac in the formation of dHSCs (32-36). However, due to technical limitations, it remains debated whether the observed dHSCs activity in these studies were of yolk sac origin or if they arised from a predecessor to the AGM region (26). Moreover, dHSCs have been found in the yolk sac at E12.5, although this could be due to the yolk sac having a dHSC supportive environment (37) and that cells home to the yolk sac via the circulation.

Regardless of where in the embryo the dHSCs arise, they start to migrate to the fetal liver (FL) at around E11 (Figure 1) (38). In the FL, dHSCs expand their numbers (39) before again migrating to the BM, spleen and thymus during later stages of gestation. Interestingly, it has been found that HSCs in the BM of 3-week-old mice display FL HSC characteristics including increased cell cycling, self-renewal, and a tendency to give rise to myeloid progeny (40). Moreover, between weeks 3 to 4 after birth a developmental switch from fetal to adult HSC behavior occurs (40). The exact mechanisms governing this checkpoint are largely unknown but have been shown to be cell intrinsic to the HSCs (40).

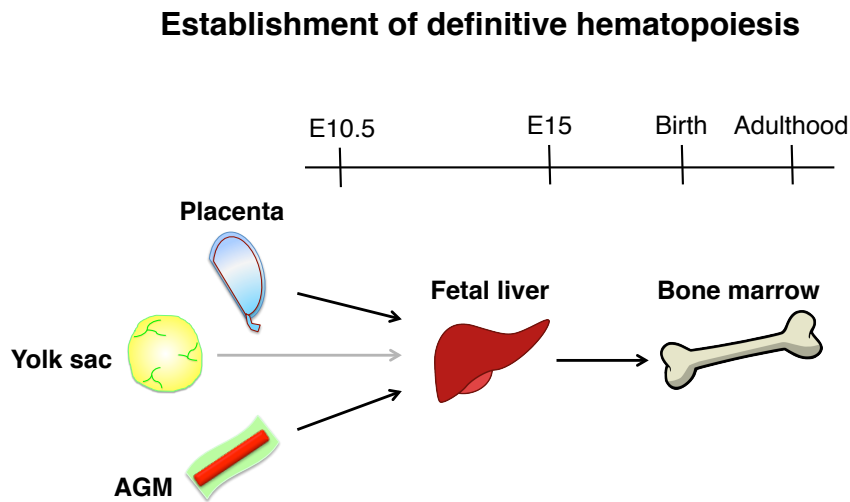


Figure 1. Schematic overview of the proposed developmental origins of definitive HSCs and their path towards colonization of the adult bone marrow. AGM: aorta-gonad-mesonephros, E: embryonic day. HSC: hematopoietic stem cell.

3. The adult hematopoietic hierarchy

The adult BM is the primary site for early blood cell formation, where adult HSCs provide life-long production of mature effector cells. As previously mentioned, extensive efforts involving the use of FACS, cell surface markers and functional assays have provided unprecedented insights into the hierarchical structure of the hematopoietic organ (Figure 2). However, no exclusive cell surface markers to isolate HSCs have been identified, instead the prospective isolation of HSCs and their downstream progenitors rely on a combination of markers. While a few different

marker combinations to isolate HSCs are utilized and the structure of the hematopoietic hierarchy is still being assembled, it is well established that all HSC activity is contained within a fraction of cells that are negative for markers associated with mature blood cells (Lineage-), while being positive for the cell surface markers c-Kit and Sca1 (41-43). This fraction of cells is commonly referred to as the LSK population and is a mixture of HSCs and progenitors. It has been found that the frequency of HSCs within the LSK compartment is about one in thirty cells (9) and to further enrich for HSCs, the cell surface markers CD34, Flt3, CD48 and CD150 are commonly used. HSCs are confined to the part of the LSK population that is negative for all these markers, but positive for CD150 (24, 44, 45). Following initial differentiation events, HSCs start to express CD34 and give rise to multipotent progenitors (MPPs) with diminished self-renewal activity but retained multilineage differentiation potential (46, 47). The immediate steps of HSC lineage commitment remain somewhat controversial. For quite some time, it was firmly thought that MPPs may give rise to either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP) with differentiation potential restricted to these two branches of hematopoiesis (48, 49). The former, having the capacity of generate all myeloid, megakaryocyte and erythroid (MegE) progeny was however found to generate a quite low frequency of mixed lineage clones under in vitro cultures (49), raising concerns about the homogeneity of this population. Later, work conducted by Pronk and colleagues revealed that the CMP population in fact is highly heterogeneous containing both myeloid restricted and MegE restricted progeny, but not both (50).

The identification of the lymphoid-primed multipotent progenitor (LMPP) population and the cell surface wise similar GMLP population (identified by transcription factor reporters) harboring combined lymphoid and myeloid differentiation potential but with largely absent MegE potential, have proposed a common ancestry of the granulocyte and monocyte (GM) and the lymphoid lineages that is separate from the MegE lineages (51, 52). However, this view has been challenged (53) and recent observations by Yamamoto et al have revealed the existence of long-term repopulating cells with combined myeloid and MegE potential residing within the phenotypically defined HSC compartment, suggesting that alternate routes of myeloid commitment and cell generation might exist (54).

At the GMLP stage, progenitors either become restricted to the GM lineages (pGM) or the lymphoid lineages via CLPs that have the ability to generate B, T and NK cells (48), while retaining only a minor myeloid potential (55-57). The loss of T and NK cell potential among CLPs has recently been attributed to the acquirement of Ly6D expression (58, 59). Moreover, while all B cell development is thought to occur via the CLP stage, T-cell development may occur via alternate routes (60-62). Within the thymus, early thymic progenitors (ETPs) have been identified (60) but while the exact ontogeny of this population remains elusive, it has been found to harbor a minor B cell potential as well as T cell and myeloid potential, making it probable that

this one of the earliest T cell committed precursor population. Evidence against CLPs as the only and main thymus seeding progenitor population include their differential cell surface phenotype compared to ETPs, the finding that ETPs persist following CLP deletion due to Ikaros deficiency, as well as the rareness by which CLPs are found to circulate (63). Although, one might speculate that CLPs could give rise to a so far unknown BM progenitor population that subsequently seeds the thymus, it has been suggested that ETPs are derived from a cell type within the LSK compartment (63).

A complicating feature for the study of early hematopoietic development is that slightly different variants of the hematopoietic hierarchy are used by different research groups. However, a reason for this (in essence a good one) is the continuous efforts aimed at resolving the cellular make up of hematopoiesis. For this reason, one may view the hematopoietic hierarchy as a working model for hematopoiesis rather than a fixed map of differentiation (Figure 2). In the end, and common for all hematopoietic lineages, further maturation into effector blood cells take place via many more lineage-restricted progenitor stages in the BM and peripheral sites, such as the lymph nodes, spleen and the thymus and ultimately sustain the immune system throughout life.

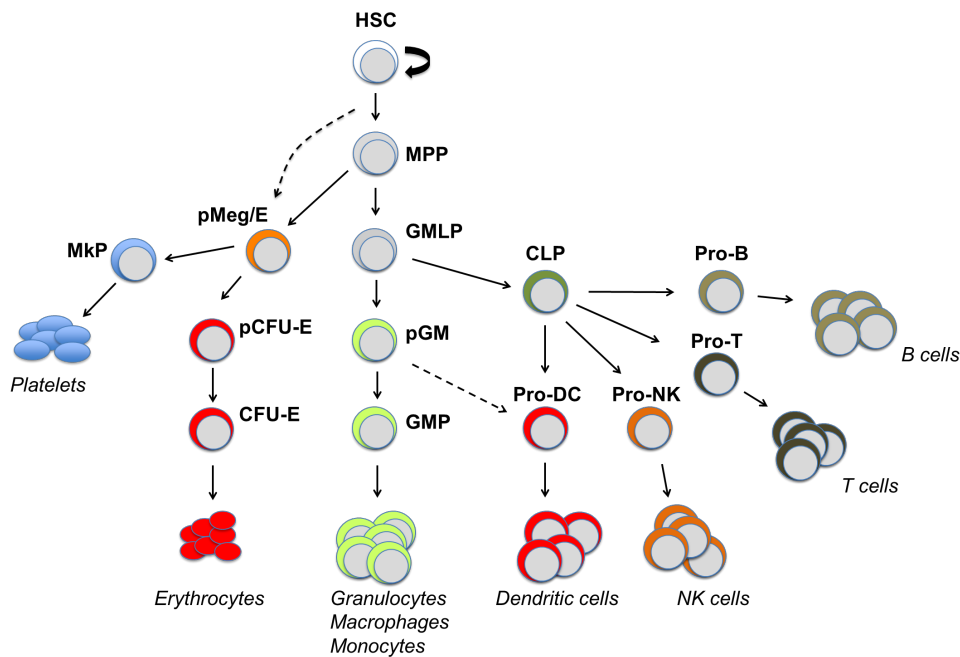


Figure 2. Schematic depiction of the proposed hematopoietic hierarchy and hematopoietic differentiation from HSCs.

4. Regulation of hematopoietic stem and progenitor cells

In order for HSCs to provide life-long generation of mature effector cells and at the same time avoid exhaustion and disease development, a tight regulation of early hematopoiesis is necessary. As opposed to FL HSCs, HSCs in the adult BM cycle infrequently at homeostatic conditions, exhibit a prolonged G1 cell cycle phase and are regarded to largely reside in a quiescent state (40, 64-66). Nonetheless, HSCs have the ability to enter the cell cycle when needed to mediate quick responses should hematopoietic homeostasis become altered, as evidenced by the fact that acute myelosuppression forces HSCs into cycle (67, 68). It appears reasonable that HSCs themselves do not largely contribute to the massive levels of proliferation associated with ongoing hematopoiesis, but rather passes on this task to various progenitor cell populations. The high proliferative capacity of hematopoietic progenitor cells also makes them prime targets for cellular transformation and leukemogenesis. In addition, although genetic aberrations can be found in HSCs, leukemic diseases usually represents by massive expansion of progenitor cells (69, 70). Thus, not only thorough knowledge of HSC biology but also that of hematopoietic progenitors is of crucial importance to understand healthy and malignant blood cell formation.

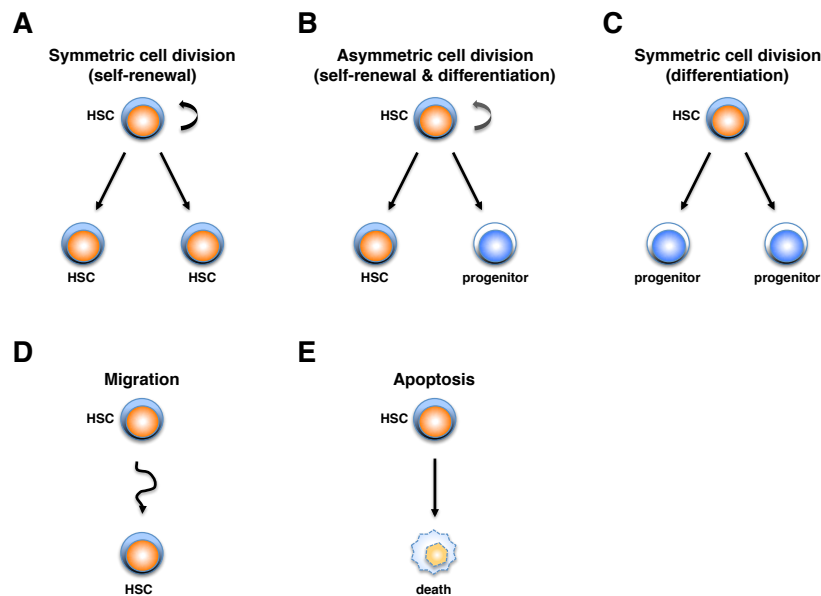


Figure 3. Schematic overview of different HSC cell fate choices. (A) Symmetric cell division giving rise to two new HSCs, (B) asymmetric division resulting in one HSC and one progenitor destined for differentiation, (C) symmetric division giving rise to two progenitor cells with loss of self-renewal, (D) an HSC could migrate via the blood stream (E) or undergo apoptosis.

To describe and model early hematopoietic development, different cell “fate” options are usually considered (Figure 3). One of the hallmark definitions of a stem cell is its ability to self-renew, a property of uttermost importance in order to prevent exhaustion of the HSC pool. HSC self-renewal may occur through either asymmetric or symmetric cell division (71). In the former, a mitotic event gives rise to one HSC and one differentiating progenitor cell, while a symmetric division instead gives rise to two daughter cells with the potential of the parental HSC (Figure 3A-B) (71). However, it also remains a possibility for an HSC to divide and give rise to two cells destined for differentiation (Figure 3C). In addition, HSCs may occasionally migrate and enter the circulation (72) (Figure 3D); however it is unclear to what extent this occurs under homeostatic conditions. Finally, like every other cell, HSCs can undergo programmed cell death, or apoptosis, to regulate the HSC pool size and to deplete HSC clones with an altered developmental fitness (Figure 3E). The same fate options, with the exception of self-renewal, also extend to different progenitor fractions. However, upon the maturation into progenitors committed towards a specific lineage, cell fate choices become more restricted as the developmental plasticity narrows. The mechanisms involved in regulating these cell fate decisions will be discussed later in this thesis.

4.1 The BM microenvironment and extrinsic influence

In 1978, Schofield postulated that HSCs reside in a specialized three-dimensional “niche” at the endosteal bone surface in the adult BM (18). Since then, a large amount of work has been aimed at trying to understand the exact whereabouts and factors that comprise these niches, in order to increase the understanding of HSC biology. However, despite having been subjected to numerous studies, the understanding of the HSC niche is far from complete. As Schofield postulated, one candidate HSC niche is the endosteal surface lining the trabecular bone, another one is the perivascular area of the sinusoidal blood vessels (18, 44). These two locations are traditionally described as two separate niches with distinct functions. The “endosteal niche” is thought to more firmly support HSC quiescence (73), while the “perivascular niche” is thought to support HSC division and/or differentiation (44, 73). In addition, the environment supporting HSCs has been proposed to be less oxygenated (hypoxic); a condition thought to protect HSCs from reactive oxygen species (ROS) induced damage (74). However, the sinusoidal vessels often reside in close contact to the endosteum (75), which indicates that a close dynamic relationship of these two candidate niche locations exists to maintain HSCs *in vivo*.

Various HSC supportive potential niche components have been identified (Figure 4). For instance, osteoblasts (OBs) lining the bone surface are thought to be components of the HSC niche for a number of reasons. First, OBs secrete multiple cytokines and express several ligands involved in hematopoietic development,

including the ligand Angiopoietin-1, which bind to the rather HSC specific receptor Tie2 and mediate HSC quiescence (76), and the cytokine Tpo that mediate signaling through the Mpl receptor present on HSCs (77). Another example includes the ligand Jagged1, which bind to Notch receptors present on hematopoietic cells (78). Moreover, two independent studies using genetic models where OB numbers were increased have further demonstrated the importance of OBs for the regulation of HSCs, since an increased amount of OBs correlated with an increase in HSC numbers (78, 79). In addition, the classical antagonist to the bone forming OBs, the osteoclasts (OCs), which are the primary liberators of calcium from the mineralized bone, have also been implicated as important for HSC-niche interactions, since HSCs express a calcium sensing receptor (CaR) which upon deletion result in the relocation of adult HSCs from the BM to the periphery (80, 81). Moreover, the inhibition of osteoclast function through bisphosphonate treatment resulted in decreased HSC numbers and engraftment (82).

In favor of a HSC supportive role for the vascular endothelial cells lining the sinusoidal vessels are findings that these cells produce several HSC associated cytokines including stem cell factor (SCF), which upon specific deletion in endothelial cells results in a reduction of HSC numbers (83). In addition, mesenchymal stromal cells have been suggested to mediate HSC retention to the BM, since the deletion of either CXCL12 (also known as SDF-1) abundant reticular (CAR) cells or Nestin positive mesenchymal stem cells (MSCs) expressing the ligand SDF-1, which is thought to be important for HSC-niche interactions (84), results in a reduction of the HSC population (85, 86). Moreover, the importance of MSCs is further reinforced by their role in the differentiation into OBs and the concomitant regulation of OB numbers, which indicate that they can contribute to HSC maintenance at multiple levels (86). Similarly, the depletion of monocytes was described in two studies to lead to decreased retention of HSCs to the BM, which associated with decreased SDF-1 levels indicating that monocytes have a role in the regulation of HSC function (87, 88). Interestingly, by specific deletion of FoxP3 regulatory T (Treg) cells that reside in proximity of the endosteal surface, it has been found that also Tregs are necessary for the BM to support HSC homeostasis, since these experiments revealed a severe reduction in HSC numbers and thus also appear to comprise important components of an HSC niche (89). Moreover, other hematopoietic progenitors and mature blood cells most likely also contribute to maintain the HSC pool by providing feedback signals to increase or decrease overall or lineage specific hematopoietic output.

Advancement in imaging techniques have revealed that presumable HSCs indeed reside near the endosteum and in close proximity to the vasculature (75). While it is clear that many components in the bone marrow do support HSC regulation, the lack of methodology combining locational imaging with functional assessment is currently hindering definitive assessment of where in the BM adult HSCs predominantly reside.

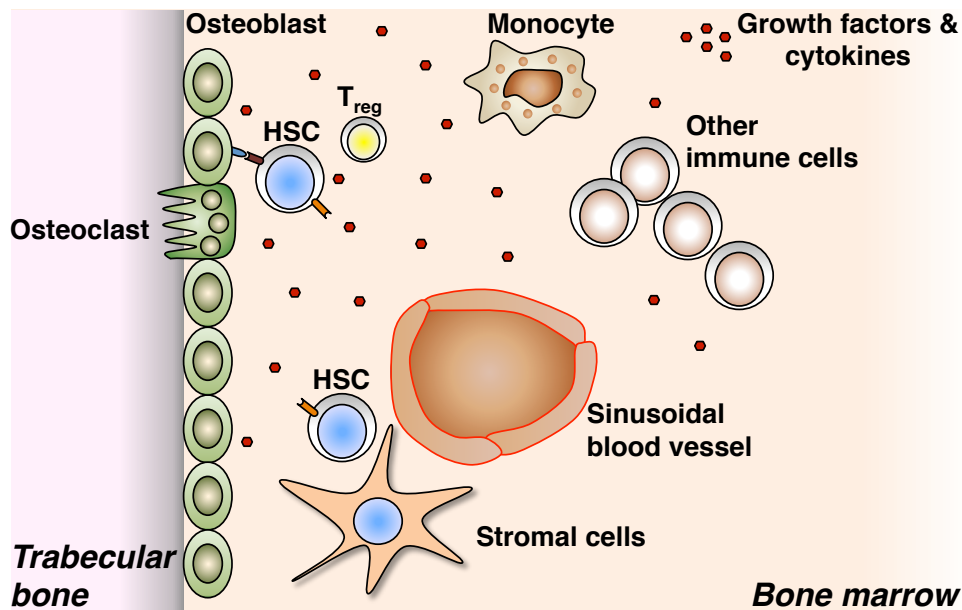


Figure 4. Schematic representation of various components that may constitute the BM HSC “niche”.

The decisive mechanisms that govern the early fate decisions of HSCs remain relatively poorly understood but are likely to occur as a consequence of signals from their immediate environment in combination with intrinsic regulatory factors. While the ability to undergo symmetric divisions is important to expand the HSC pool during for instance embryogenesis and BM transplantation, homeostatic conditions might as discussed favor asymmetric division. The latter may occur in various ways and is likely to result from either an uneven distribution of intrinsic fate-determinants in the resulting daughter cells, or alternatively that the daughter cells become exposed to differential environmental cues (71). Integral roles in these early lineage restriction events have been attributed to hematopoietic growth factors, or cytokines, however, the specific role of cytokines in these processes remains controversial. One theory postulates that cytokines act in a permissive manner, by allowing a developing cells to pursue differentiation along a predetermined path, through the supply of survival and/or proliferation signals, and the other that cytokines might also act instructively and directly initiate and direct differentiation (90, 91). Evidence for both theories has been obtained from studies using mouse models. Supporting the former theory are the findings that mice lacking the cytokines G-CSF and GM-CSF display relatively mild steady-state hematopoietic phenotypes (92, 93). These findings might in part be explained by cytokine receptor redundancy, although studies where multiple myeloid promoting cytokine receptors were ablated did not exhibit particularly enhanced abnormalities as compared to single knockout mice (94, 95). Thus, cytokine signaling

might act in a pure permissive manner. However, introduction of GM-CSFR and IL2R β expression in CLPs does lead to the generation of GM cells (96), supporting a potential instructive role for cytokines. Based on the current work performed on the subject, it seems reasonable that cytokine signaling can act both instructively and permissively for the regulation of developing hematopoietic cells.

4.2 Intrinsic regulators affecting adult hematopoiesis

While the supporting microenvironment clearly impacts on hematopoiesis, complex networks of gene expression regulators have also been implicated as important regulators for blood cell maturation. These insights have been acquired through the use of various genetic gain and loss of function models, where gene function is either enhanced or diminished to elucidate function. Although not a universal feature, models where HSC self-renewal is enhanced often lead to exhaustion of the stem cell pool. For instance, the loss of the transcriptional repressor Growth factor independence 1 (Gfi1) results in increased HSC cycling and loss of reconstitution potential (97, 98), which may be explained at least in part by Gfi1's role in regulating the expression of the cell cycle inhibitor p21 (97). Given the slow cell cycle activity of HSCs, it is not surprising that cell cycle regulators maintain HSC function, and direct loss of function models of cyclin-dependant kinase inhibitors p21 and p18 have been found to increase HSC function (99, 100), although the deletion of p21 resulted in HSC exhaustion during hematopoietic stress (101). Another group of cell cycle regulators is the Retinoblastoma (Rb) family of transcriptional repressors and cell cycle inhibitors, and compound deletion of all three members (pRb, p107 and p130) result in myeloproliferation, increased HSC self-renewal and exhaustion following transplantation (102).

Another group of genes, the Hox genes, initially identified in *Drosophila* as important regulators of body patterning pathways and are also important for mammalian embryogenesis (for a review see (103)). In addition, several Hox genes have been implicated in HSC regulation; *Hoxa9* deficient HSCs display severely compromised reconstitution ability (104), while the overexpression of *Hoxb4* can greatly expand HSC numbers in vitro and in vivo (105-107). Moreover, the simultaneous overexpression of *Hoxb4* and its co-factor *Pbx1* (108) resulted in a striking 100-fold expansion of HSCs in vitro (109). A bit confusingly, the loss of *Hoxb4* does not result in a dramatically reversed phenotype, which could be explained by functional redundancy of other Hox genes (110, 111). Moreover, mice deficient for the Hox co-factor *Meis1* (112) display increased HSC cycling and loss of stem cell activity (113, 114).

An important role for appropriate regulation of apoptosis has also been linked to HSC maintenance. For example, deletion of the anti-apoptotic factor *Mcl1* leads to a complete failure of hematopoiesis (115), while forced expression of another anti-apoptotic gene, *Bcl2*, increases HSC competence (116). Through similar work, many

other transcription factors have also been implicated in the HSC maintenance machinery including Cebpa, E2A, Mecom, Ikaros, Myc and PU.1 (Sfpi) (117-122). Perhaps not unexpectedly, most of these genes are also important for appropriate progenitor cell maintenance, indicating the complex regulation of early hematopoietic development.

HSCs not only express transcriptional regulators associated with their immediate self-renewal and quiescence machinery, but have also been found to express various lineage-affiliated genes at low levels. This, “lineage priming”, has been suggested to reflect the multipotency of HSCs (123-125), but may also reflect the existence of HSC clones with different developmental potential (which will be more discussed in a later section). For instance, the MegE associated gene Vwf is expressed at high levels in HSCs (125) and was recently found to label HSCs with increased platelet potential (126). Interestingly, this lineage priming was observed to be more pronounced for myeloid- and MegE affiliated gene expression programs than for lymphoid transcripts (123, 125). This finding could reflect a closer relationship of HSCs to the myeloid cells than the lymphoid lineages, however, whether these findings reflects expression levels of lineage-affiliated genes that may regulate cell fate on a broad scale, or whether the active lineage-associated transcriptional programs merely function to prime HSCs for differentiation, remains an interesting question to be addressed.

Following initial HSC maturation into multipotent progenitors, various transcriptional regulators with diverse functions further dictates cell fate (Figure 5) (127). An early developmental example of this is the antagonistic relationship between the transcription factors Gata1 and PU.1. Here, high expression levels of Gata1 can inhibit PU.1 activity and vice versa and directly instruct hematopoietic progenitor cells to commit towards erythroid and myeloid fates respectively (128-131). This indicates that differential levels of transcription factors can instruct cell fate decisions. Moreover, through the use of FACS and promoter reporters, it has been found that multipotent progenitors expressing higher levels of Gata1 have robust MegE potential as well as GM potential, whereas cells expressing PU.1 instead retain lymphoid and GM potential (52). Further maturation towards the erythroid or megakaryocytic lineages has been found to depend on another antagonistic relationship between Klf1, that promotes erythropoiesis, and Fli-1 which favors platelet generation (132). Multipotent progenitors with higher expression of PU.1 (GMLPs/LMPPs) can develop into the lymphoid or myeloid lineages and this fate decision may depend on differential levels of PU.1 expression, where higher levels likely promote differentiation into myeloid committed progenitors (133). At this stage, pGMs and the more mature GMPs depend on PU.1 and Cebpa to further direct maturation into macrophages and granulocytic cells via secondary factors depending on an antagonistic relationship of Egr and Gfi1, where the former favors development of macrophages and Gfi1 granulocytic cells (134, 135).

Lymphoid specification is thought occur in GMLPs with lower expression PU.1 (133) and requires the concomitant expression of Ikaros (120, 136), which in turn is

thought to upregulate Gfi1 that suppresses PU.1 and a myeloid fate (137). Besides its role in lymphoid specification, Ikaros has also been implicated in HSC self-renewal and experiments using a fluorescent Ikaros reporter have revealed that Ikaros is also required for the generation of GMLPs, since its loss not only result in a failure to upregulate Flt3 expression but also diminishes the lymphoid potential of these cells (120, 138). Lymphoid specification further depends on E2A proteins that also have diverse functions throughout hematopoiesis, including a role in HSC self-renewal as well as regulating the differentiation of the MegE and myeloid lineages (118, 139, 140). Moreover, E2A and PU.1 are also implicated in the expression of the IL7 receptor, which is crucial for the initiation of lymphoid development (141, 142). The first identified progenitor subset to be specified towards a lymphoid fate is the CLP stage. Here, B-lineage commitment can occur through the E2A dependent induction of a B cell transcriptional program involving genes such as Ebf1, Pax5, Foxo1, in turn sealing the B lymphoid fate (143).

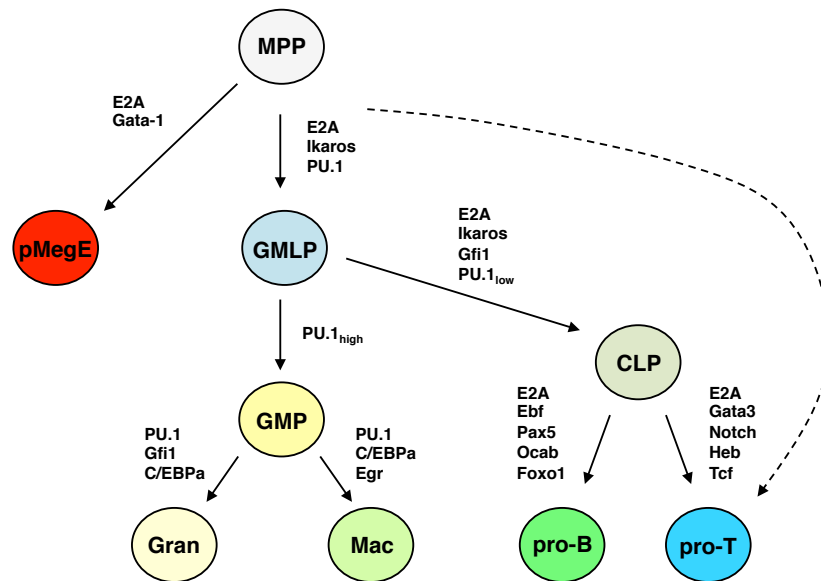


Figure 5. Schematic diagram of transcriptional regulators that may influence on cell fate decisions during hematopoietic differentiation.

T cell fate specification is thought to occur in early thymic precursors that initially retain myeloid and a minor B cell potential (60) and, as opposed to B lymphopoiesis, T cell specification relies on external stimuli via the Notch receptors to extinguish alternate fates which subsequently induces a T cell specific transcriptional program involving E2A, Gata3, Heb and Tcf (144). The extreme potency of Notch signaling to support T lymphopoiesis can be demonstrated in vitro

on OP9 stromal cells stably expressing the Notch ligand Delta-like, since T cell competent progenitor subsets efficiently develop into T lymphocytes using this system (145).

4.3 Epigenetic regulation in hematopoiesis

One of the more favored explanations for how lineage potential becomes restricted throughout hematopoietic differentiation is that progressive epigenetic alterations result in the silencing and/or inability to induce the expression of genes associated with alternate lineages. Epigenetics is a broad term and can be explained as stable modifications that lead to changes in overall DNA and chromatin structure without affecting the underlying DNA sequence. Epigenetic mechanisms are crucial for maintaining cell identity at various stages of development (146). Although different epigenetic regulatory mechanisms have been known for quite some time, their importance for cell identity have been thoroughly demonstrated in recent years, when reprogramming of somatic cells have become a hot topic of cell biology. This “sub field” of cell biology was fueled by pioneering work from Shinya Yamanaka and colleagues, who found that the induced expression of several embryonic stem (ES) cell associated TFs (Oct3/4, Sox2, Klf4 and c-Myc) in somatic fibroblast cells could respecify the cells to become induced pluripotent stem (iPS) cells; stem cells with the potential to differentiate into all the different tissues and cell types of an organism (147). This field has received much attention since its manifestation, due to the promise it holds for regenerative medicine. The induction of these pluripotent iPS cells relies on the “resetting” of a cell’s epigenetic patterns (148) and, since the initial findings described above, the ability to induce iPS cells have expanded to involve a range of different somatic cell types using various methodologies to achieve reprogramming (149). In addition, more recent work has revealed the potential of various lineage associated TFs to directly convert a terminally differentiated somatic cell into another specialized cell of an alternate lineage (149). For instance, in the blood, it was recently found that the forced expression of sets of TFs in fibroblasts could directly re-specify the cells toward a hematopoietic fate (150, 151), although these lacked HSC activity (150, 151). Since somatic cell reprogramming in most cases rely on the exogenous expression of a set of given genes without any specific alteration of the underlying DNA sequence, these studies demonstrate that cell identity is closely connected to a cells’ epigenome.

Several layers of epigenetic modifications that may affect a cells chromatin compaction and transcriptional activity have been uncovered (Figure 6). One of the most well known examples of this is the methylation of cytosines (C) that are followed by guanine (G) residues (Figure 6B). These so called CpG stretches, where the “p” stands for the phosphate group positioned in between the two bases, may occur as long repeats known as CpG islands and are often present near a gene’s promoter (152). Consequently, the general consensus holds that increased amount of

CpG methylation coincides with transcriptional inactivity (152). Moreover, it has been estimated that 5' CpG islands are involved in the regulation of about 60% of all human genes (153) and aberrant methylation patterns are commonly observed in various cancers (154). A striking example of how changes in CpG methylation may impact on developmental processes can be seen during embryogenesis. Here, the methylation level transiently decreases from the 70-80% observed in somatic cells (155) to about 1/3, but is restored at the time of implantation (155). There are also multiple lines of evidence for a role of CpG methylation in the regulation of hematopoiesis. Two independent studies have disrupted *de novo* DNA methyltransferase function in HSCs and it was originally found that simultaneous knockout of both Dnmt3a and Dnmt3b abolished HSC reconstitution potential (156). Challen et al on the other hand found that single loss of Dnmt3a lead to an increased *in vivo* self-renewal of HSCs, however, by qualitative measurements the Dnmt3a^{-/-} HSCs displayed a decreased reconstitution potential in serial transplantation assays (157). Moreover, Dnmt3a loss lead to altered DNA methylation patterns, gene expression changes, and a tendency to generate B lymphoid progeny (157). In addition, the loss of the DNA methylation maintenance enzyme Dnmt1 has been shown to negatively affect HSC self-renewal and favor the generation of myeloerythroid progeny (158). Consistent for a role of DNA methylation in hematopoiesis, genome-wide methylation mapping in various hematopoietic progenitor fractions have revealed distinct patterns of methylation coinciding with different developmental stages (159). Interestingly, the hemimethylated DNA generated after DNA synthesis is the preferred substrate of Dnmt1 and DNA methylation has therefore been proposed to be of key importance for an "epigenetic memory" following cell division (160).

Although DNA methylation is a powerful mechanism for gene silencing that seems involved in hematopoietic maintenance, other layers of epigenetic regulation exists that corroborate to maintain higher order chromatin and transcriptional status. This includes posttranscriptional modifications to the amino acid tails' of the histones (Figure 6A) (161, 162). The histones are proteins residing in the nucleus that in the form of an octamer wind approximately 147 base pairs of DNA to compose a structure known as the nucleosome, which act as the primary DNA "packaging" unit (163). Each histone contains a protruding amino acid tail that has been found to be subject for a range of posttranscriptional modifications, including acetylation, methylation, phosphorylation, sumoylation and ubiquitinylation (152). Interestingly, the presence, or absence, of many of these modifications has been found to correlate with transcriptional competence by affecting DNA accessibility (152, 161, 162). The two perhaps most well studied histone modification marks that may impact transcriptional activity occur on the tail of histone 3 (H3), and include trimethylation of lysine 4 (H3K4me3) which often associate with actively transcribed loci, and trimethylation of lysine 27 (H3K27me3) often correlating with transcriptionally silent genes (164). In addition, studies from ES cells have revealed that the presence

of both these modifications near a particular promoter may be acting to “pose” the gene for active transcription during differentiation and exit for pluripotency (165). This indicates that histone modifications may be implicated in the regulation of developmentally important genes. Similar observations have also been seen in hematopoiesis (166, 167), suggesting that these mechanisms may be involved in lineage priming and/or commitment of hematopoietic stem and progenitor cells (HSPCs). Many factors involved in the maintenance of histone modifications have been identified from work performed in *Drosophila* and the factors regulating the H3K27me3 and H3K4me3 marks can be grouped into either the Polycomb group (PcG) or the Trithorax group (TrxG) proteins respectively (152). The former compose the Polycomb Repressor Complex 1 and 2 (PRC1 and PRC2), responsible for the maintenance and de novo application of the H3K27me3 modification respectively (152, 161). Experiments using one of the core components of PRC1, Bmi1, have shown crucial roles for PRC mediated silencing in hematopoiesis; Park et al found that the deletion of Bmi1 resulted in loss of BM and fetal liver HSCs’ long-term repopulation capacity (168). In addition, the reverse experiments where Bmi1 expression was retrovirally enforced, resulted in increased reconstitution potential (169). Further support for an important role of polycomb repressor complexes in hematopoiesis comes from studies of the histone methyl transferase Ezh2. Similarly to Bmi1, forced expression of this factor was shown to increase the ability of LSK cells to repopulate conditioned hosts (170). Conversely, deletion of Ezh2 in adult HSCs does not appear to negatively affect their function (171), although the deletion of the closely related histone methyltransferase Ezh1 has been proposed to induce HSC growth arrest and senescence (172). In addition, the deletion of essential PRC component, Eed, resulted in HSC exhaustion (171). The TrxG proteins form similar complexes that can act to oppose polycomb mediated silencing by instead introducing activating histone marks (173). In hematopoiesis, one of the most well studied TrxG protein is the methyl transferase Mixed Lineage Leukemia (Mll), which was originally identified as a gene commonly occurring in leukemic oncofusion proteins (173). Mll is involved in the introduction of the activating H3K4me3 histone modification (173), and although loss of Mll is embryonically lethal, conditional deletion in bone marrow results in hematopoietic failure which was suggested to depend on abnormal proliferation of HSPCs (174). Collectively, these findings indicate essential roles for appropriate maintenance of histone modifications to maintain normal hematopoietic development.

Another class of epigenetic modifiers that may impact on hematopoietic regulation involves non-coding RNAs (ncRNAs) (Figure 6C). ncRNAs are a diverse group of RNA molecules that are non-protein coding and different types of ncRNAs have been found to comprise the major part of the transcribed genome (175). One type of ncRNAs are micro RNAs (miRNAs). These ncRNAs are small single-stranded RNA molecules of approximately 20-24 bp in length that function to mediate mRNA stability (175). By now, several miRNAs have been identified that act during various

stages of hematopoiesis (for a review see (176)). Another group of ncRNAs, long non-coding RNAs (lncRNAs), represents non-coding RNAs above 200 nucleotides in length (175). Although a currently expanding field, several lncRNAs have been identified that may impact on transcriptional outcome and a growing body of evidence has highlighted the importance of lncRNAs for appropriate epigenetic regulation (175, 177). For instance, both PRC1 and PRC2 have RNA binding properties and it has been estimated that 20% of all lncRNAs are in direct association with polycomb proteins (178), indicating that they are important for appropriate higher order chromatin structures. A classic example involves the inactivation of the X chromosome during early female embryogenesis. Here, polycomb proteins binds to the lncRNA Xist, which is expressed on the target X chromosome, and initiates polycomb mediated silencing and heterochromatin formation (described in (177)).

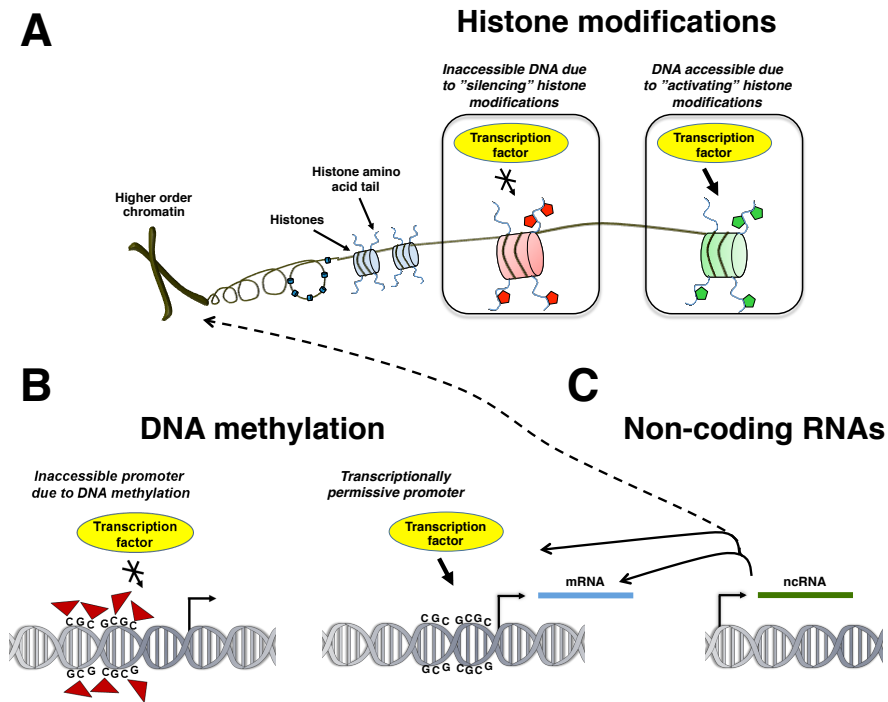


Figure 6. Schematic representation of different epigenetic mechanisms that can impact on transcriptional competence and cell identity. (A) Histones act as the primary DNA packaging unit and contain protruding amino acid tails that can be subjected to various covalent modifications that can affect chromatin compaction and transcriptional activity. (B) Cytosine nucleotides followed by guanines (CpG) may be subjected to methylation and often negatively correlate with transcription. (C) Various forms of non-coding RNAs can impact on transcription, promoter accessibility and chromatin compaction. In concert, epigenetic mechanisms maintain higher order chromatin structures and are decisive for cell identity.

Another example involves the lncRNA HOTAIR, which is expressed in the locus containing the HOXC cluster, and is essential for polycomb mediated silencing of the HOXD cluster during development (179). Similarly, the lncRNA HOTAIRM1 present in the Hoxa locus between HOXA1 and HOXA2 has been implicated in HOX gene regulation and retinoic acid induced myelopoiesis (180). Moreover, Mira, the gene for the lncRNA Mistral, is located between Hoxa6 and Hoxa7 and its expression is activated during retinoic acid induced differentiation of mouse ES cells, which leads to Mll recruitment, H3K4me3 establishment at the Mistral gene and subsequent activation of Hoxa6 and Hoxa7 expression (181). Further bridging the connection between lncRNAs and epigenetics is the recent finding that the hematopoietic-associated gene CEBPA contain a lncRNA directly involved in preventing DNA methylation and silencing of the locus (182). Specifically, it was found that the lncRNA, ecCEBPA, interact with DNMT1 to prevent DNA methylation and silencing of the locus (182). In addition, the investigators identified similar RNA-DNMT1 interactions on a global scale and proposed that this mechanism most likely extends also to numerous other loci (182), thereby implicating lncRNA-Dnmt1 binding as an important epigenetic regulatory mechanism.

In summary, several layers of epigenetic regulatory mechanisms act to mediate both locus specific, as well as global, chromatin formation states which may impact on transcriptional activity. However, further in-depth studies are needed to understand the interplay between these regulatory mechanisms to ultimately control cell identity and fate decisions. Unfortunately, such molecular experiments have often been hindered in the case of HSCs, due to their rare nature. Despite, it is evident that many intrinsic regulatory mechanisms act to regulate hematopoiesis and it is equally clear that extrinsic signals may influence on these developmental processes. An outstanding question currently left unresolved is how the different extrinsic and intrinsic signals complement each other to achieve this.

5. Aging

The physiological process of aging is a common feature for most organisms and has always been a mysterious and curious phenomenon of biology. Aging may be broadly defined as a time-dependent decline of organismal function and, while the underlying causes of organismal aging remains debated, it is a research area with huge potential benefits for society (70, 183). The overall lifespan of the human population is increasing; however, aging often associates with decreased life quality and the increased prevalence of various diseases, such as cancers (70). Thus, to gain an increased understanding of how aging manifests might not only aid in the understanding disease development, but perhaps allow for “healthier” late stages of life.

From an evolutionary view, aging can be explained to result from selective pressures and might therefore have underlying genetic components. This would result in the preservation of necessary resources for the greater population through the removal of individuals who are past their reproductive prime (discussed in (183)). However, most animals in nature often succumb to premature death due to for instance disease or predation and generally do not exhibit aging-associated characteristics and this view of aging has therefore been challenged (184). In 1952, Medawar proposed that aging arises from the accumulation of mutations that may impact organismal fitness later in life. These would therefore be irrelevant from a reproductive standpoint, and would thus escape a negative selection pressure (185). Some years later, Williams proposed a closely related theory that stipulates that aging may arise from pleiotropic genes; genes that are advantageous early in life, but disadvantageous with increased age (186). Moreover, strong support for an intrinsic genetic component for aging can be drawn from the vastly different lifespan of many species in nature (187). However, aging may also be regulated by extrinsic factors. Therefore, according to the “disposable soma” theory, a separation should be made between the “disposable” soma and the “immortal” germ line, because an organism needs to remain fit long enough to reproduce, thereby ensuring the longevity of the germ line (188). In this sense, an organism would not benefit from the survival of the disposable soma after reproduction has been achieved (188).

However, the disposable soma theory fail to take into account adult stem cells, which due to their multipotential and self-renewal might be seen as an intermediate between the immortal germ line and the soma. Aging is associated with difficulties in maintaining organ and tissue homeostasis, as well as to return to homeostatic conditions following stress (70). A primary function of tissue-specific stem cells in many organs is to maintain tissue homeostasis through the replenishment of cells that may be lost through normal wear and tear processes or other insults (9). Therefore, in tissues dependent on adult stem cell function, aging may be seen as a gradual failure of various tissue specific stem cell populations to maintain organ and tissue function later in life (70).

5.1 The aging hematopoietic compartment

One organ heavily dependent on adult stem cell function is the hematopoietic system. Like other tissues, the hematopoietic system is subjected to several changes with increasing age (Figure 7) (189). For instance, there is a clear association of age and a decreased potential to mount adaptive immune responses (70). In part explanatory for this, is the involution of the thymus that starts around puberty (190). In addition, the elderly populous respond worse to vaccination treatments and have an increased incidence various hematological diseases with origins in the myeloid lineages (70). There is also an increased incidence of developing age-associated anemias (70) and a major limitation for successful bone marrow transplantation is donor graft age (191).

Although, HSCs retain the capacity to generate abundant numbers of mature blood cells far exceeding the lifetime of a C57Bl/6 mouse (192), HSCs are nonetheless subjected to age-associated alterations (189). In fact, many of the age-associated alterations in the blood can be directly linked to cell intrinsic alterations occurring at the level of HSCs (189).

One of the hallmark definitions of the aged hematopoietic system is its' skewed output of mature effector cells. This can be visualized by the transplantation of HSCs from aged mice (22-26 months old) into lethally irradiated young hosts, which results in a skewed reconstitution pattern in strong favor for myeloid cells at the expense of cells of the lymphoid compartments (66, 193-195). Importantly, the transplantation of young HSCs into aged recipient mice does not result in a skewed blood cell output, indicating that these age-associated alterations are intrinsic to the aged HSCs (193). In association to the skewed output of mature cells, and in relation to the previously discussed lineage priming, aged HSCs express increased levels of transcripts associated with myeloid differentiation and a concomitant decrease of lymphoid transcripts (193). Moreover, hematopoietic aging associates with an increased frequency of myeloid progenitors and a severe decrease of lymphoid committed progenitor cells (193, 196, 197). Another interesting connection to the increased prevalence of myeloid leukemias and age was obtained from experiments where young and aged BM cells were transformed with BCR-ABL, a potent leukemogenic oncogene (196). This showed that young BM cells give rise to either a myeloproliferative or a B lymphoid leukemia, whereas aged BM cells primarily gave rise to a myeloproliferative disorder (196). Thus, by multiple accounts, the decreased competence of adaptive immune responses and increased incidence of myelogenous disease in older humans may be directly linked to changes in the immature hematopoietic compartments.

Another common feature of hematopoietic aging is the greatly expanded HSC compartment in aged mice compared to their young counterparts (66, 193, 198-201). Interestingly, the expansion of the HSC compartment occurs independently of the aging microenvironment, since this feature is maintained following transplantation into young hosts (193, 200). However, aged HSCs perform significantly worse than young HSCs in competitive transplantation assays (193-195, 201), perhaps indicating that the expansion of the aged HSC compartment is a compensatory mechanism in an attempt to sustain the decreased cellular fitness with an increased quantity of stem cells.

Collectively, these findings indicate that the altered composition and competence of the aging immune system is a direct consequence of age-associated cell-intrinsic alterations in the HSC compartment.

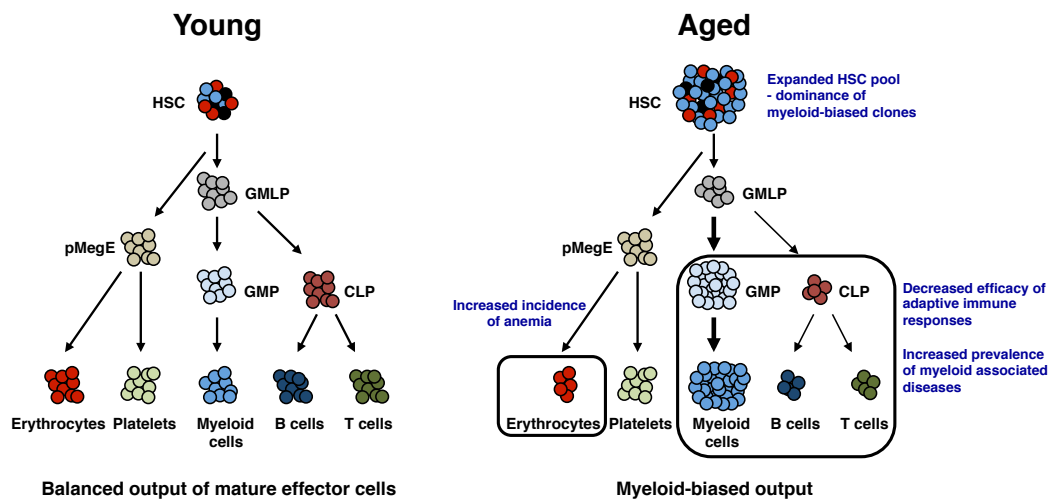


Figure 7. Schematic hierarchical diagrams depicting age-related alterations in the hematopoietic system

5.2 Clonal dynamics of HSCs during aging

One might speculate that the HSC compartment is homogenous early in life with every HSC possessing equal potential and that increasing age gradually changes every HSC clone in a similar manner to mediate the functional decline. Another interpretation could be that the HSC pool is composed of various clones with different potentials and that the composition of the HSC compartment is altered with age. Support for the latter interpretation has arisen from several studies where limiting dilution amounts of highly purified BM cells have been competitively transplanted. These studies have revealed that the HSC pool of young mice is indeed composed of a mixed population of HSC clones with different properties (201-207). For example, certain HSC clones termed β -cells, were in a study by Dykstra et al shown to result in a balanced reconstitution pattern upon competitive transplantation (205). Another type, α -cells, primarily gave rise to myeloid progeny and, although they displayed an initial lag in reconstitution, inherited robust self-renewal potential (205). Unfortunately, this study did not reveal any additional cell surface markers to allow for the discrimination of a certain HSC subset other than via transplantation readout. However, several independent studies have been carried out that used varying levels of the CD150 cell surface antigen, as well as differential dye efflux capabilities, to isolate functionally distinct HSC clones (203, 204, 208). Using transplantation assays, it was found that HSCs with high CD150 expression gave rise to a myeloid biased reconstitution pattern (203, 208). In addition, these cells appeared to be inherently primed towards myelopoiesis, as evident by differential expression levels of myeloid

and lymphoid transcripts (203). The study by Morita and colleagues further suggested that there is an inherent hierarchy among HSCs, where CD150 high cells can give rise to CD150 medium and low expressing cells suggesting these to be the most primitive HSC subtype (208). Interestingly, it was found that CD150 high HSCs increased vastly with age and gave rise to a similarly biased reconstitution pattern as their young CD150 high counterparts (203). A comparable lineage bias was observed when HSCs was isolated based on differential dye efflux capabilities within the so called “side population” (SP) (204). Of note, the cells with the highest dye efflux activity within the SP did express the highest levels of CD150 (204). An alternative approach was recently taken where the authors utilized the cell surface marker CD41 to subdivide CD150 expressing HSCs (207). This revealed both that CD41 expressing HSCs increase with age, but also that they give rise to a myeloid lineage bias following competitive transplantation (207).

These findings clearly suggest that hematopoietic stem cell aging coincides with an expansion of certain HSC clones with age that may be isolated via differential cell isolation procedures. However, apart from the skewed lineage output, a dominant feature of aging HSCs is also their overall lowered reconstitution potential on a per cell basis compared to their young counterparts (193-195, 201). While it seems reasonable that myeloid biased HSCs do expand over time, other alterations common to the different HSC subtypes must also be taken into consideration to be able to fully explain the HSC aging. It has been suggested that reduced homing of aged HSCs may contribute to the worsened reconstitution potential (201). However, as this decrease was measured to be around 2-fold (201), a reduced homing capacity cannot fully explain the lowered reconstitution potential of aged HSCs.

Interestingly, it was in a recent study proposed that the phenotypically defined HSC compartment is a mixture composed of HSCs and various myeloid committed progenitors with long term reconstitution potential (54). It was further suggested that these myeloid repopulating cells mature independently of the “traditional” hematopoietic hierarchy (54). Although the authors did not look into this specifically and highly speculative, it remains a possibility that these cells expand with age, which would lead to an overestimation of the HSC pool size in the aged setting. However, it is currently unclear how these myeloid progenitors relate to the myeloid biased HSCs defined in the other described studies.

5.3 DNA damage and telomere attrition in hematopoietic aging

Since genomic DNA contains the information necessary to construct most necessary cellular components, it is of uttermost importance that its integrity is appropriately maintained. However, the inherent DNA damage repair machinery is constantly challenged due to various insults. For instance, it has been estimated that each cell is exposed to several thousand lesions each day due to spontaneous DNA hydrolysis and depurination (209). Other potent DNA damage inducing agents include reactive

oxygen species (ROS), formed during normal metabolic activity, UV irradiation and the spontaneous error-rate of the DNA replication machinery. In a hierarchical organ system such as hematopoiesis, one can envision that harmful mutagenic events occurring in the somatic stem cells can have profound effects in the entire system as it would also spread to downstream progeny. Additionally, since the pool of HSCs needs to provide an individual with the life time supply of new blood cells, appropriate measures to reduce DNA damage is of special importance in these primitive cells. As mentioned, HSCs reside predominantly in the G0 cell cycle phase during steady-state, which reduces the risk of replicative errors (64). In addition, it has been proposed that HSCs rely more on glycolysis than oxidative phosphorylation (OXPHOS) for their energy production (210). While much more inefficient at generating ATP than mitochondrial dependent OXPHOS (2 vs 36 moles ATP per glucose), active glycolysis also result in a lower ROS production (211). Indeed, it has been found that HSCs produce lower amounts of ROS than downstream myeloid progenitors (212). In addition, HSCs have a very potent machinery to pump out toxic compounds (213). HSCs have also been found to withstand irradiation induced DNA double-stranded breaks to a better extent than myeloid progenitors, which were more prone to undergo apoptosis (214). In the same study, it was further suggested that quiescent HSCs and proliferating myeloid progenitors employ differential DNA repair mechanisms (214). In this regard, HSCs were proposed to utilize the more error-prone non-homologous end joining (NHEJ) repair mechanism, whereas myeloid progenitors use the more stringent homologous recombination (HR) machinery for DNA damage repair (214). Interestingly, upon induced proliferation HSCs appeared to retain their irradiation tolerance, but instead made use of HR to repair the induced damage (214). Collectively, these findings suggest that several HSC properties actively function to reduce the formation of DNA damage. However, since the quiescent state of HSCs appear to coincide with the need to use a more error-prone DNA damage repair pathway, this could potentially lead to the accumulation of lesions during aging.

DNA damage has been proposed to be a major feature of somatic stem cell aging (215) and aging HSCs have been found to harbor an increased amount of double-stranded breaks compared to their young counterparts, as measured by the detection of γ -H2AX foci (216, 217). In further support for a role of DNA damage during HSC aging are findings that several mouse models with defective DNA repair components harbor stem cell defects (216, 218-221), and the transplantation of cells from such animal models revealed multiple age-associated defects including reduced self-renewal, reconstitution potential and an increased apoptosis (216, 218). However and importantly, the myeloid lineage bias observed during normal hematopoietic aging was not observed in such models indicating that, although aging is probable to coincide with the accumulation of DNA damage, this cannot alone explain the shortcoming associated with hematopoietic aging (70, 216).

The ends of the chromosomes contain regions of repetitive sequences that protect the chromosomes from being recognized as double stranded breaks and other deterioration (222). With the exception of certain cell types, such as ES cells, the telomeres shorten with each cell division (222). After reaching a critically low point, cells may submit to growth arrest or apoptosis (222). Therefore, age-associated telomere attrition has been proposed to be involved in the functional decline associated with aging by limiting the number of cell divisions a given cell can undergo (222). Although HSCs have been reported to inherit telomerase activity to some extent (223), this activity is not sufficient to shield HSCs from telomere attrition during enforced proliferation in associated with serial transplantation(224). In addition, using aged telomerase deficient mice, it has been found that critically shortened telomeres can result in accelerated HSC exhaustion (225) and age-associated decreases of HSC reconstitution ability (216). However, a complicating finding is that the reverse experiment, where telomerase was enhanced, did not increase HSC serial transplantation capacity despite mediating protection from telomere attrition (226). Thus, it appears as if mechanisms other than telomere attrition act to limit HSC transplantability and replicative potential. Moreover, it must be stressed that C57Bl/6 mouse telomeres are much longer than their human counterparts (227). Therefore, it was necessary to breed telomerase deficient mice for several generations to allow for the telomeres to become critically shortened in order to observe the age-associated decreased reconstitution potential (216). To conclude, the above findings along with the vastly shorter life span of laboratory mice compared to humans argue against a major role for telomere shortening in murine hematopoietic aging.

5.4 Mitochondria and aging

In the middle of the 20th century, Denham Harman proposed that aging results from deleterious damage induced by free radicals, and that an organisms life span is decided by its ability to cope with cellular damage induced by ROS (228). The mitochondria, which are cellular organelles with diverse functions including metabolism and regulation of apoptosis (229), are the primary source for free radical (ROS) production through their OXPHOS activity (211). This places them in close proximity to potentially damaging ROS molecules, hence a refined variant of the original theory; the mitochondrial theory of aging, was proposed (230). This is an appealing theory since the mitochondria have independent genomes that lack protective histones and the fact that mitochondrial DNA (mtDNA) has been suggested to be more mutagenesis-prone than nuclear genomic DNA (231). This theory also suggests that an initial accumulation of oxidative damage to mtDNA in turn can result in further ROS accumulation due to a lowered and/or altered mitochondrial function, which would result in a “vicious cycle” of continuous ROS

buildup, lowered mitochondrial function and cell death (230). However, the generality of this hypothesis has been questioned (232).

Further implicating the mitochondria in the aging process is the connection between increased organism age and an increase in mtDNA damage in most tissues (233-236). To study the potential role of mtDNA damage in aging, mouse models expressing a proof-reading defective form of the nuclear encoded mtDNA polymerase gamma (Pol γ) have been generated (237, 238). Such mice accumulate mutations successively and progressively in their mtDNA with each replication and ultimately develop premature aging phenotypes in several organs that include anemia, osteoporosis, reduced fertility and heart enlargement, weight loss and premature death (237, 238). These studies, not only support a role for the mtDNA mutations in aging, but also show that mtDNA damage accumulation can be causative for the acquisition age-associated phenotypes. However, the role of mtDNA mutations in aging primitive stem cell and progenitor cell populations has not been extensively studied, which is the basis for the work performed in papers I and III of this thesis.

5.5 Epigenetics and rejuvenation of hematopoietic aging

As discussed, various intrinsic components governed by epigenetic regulatory patterns play a key role in the regulation of hematopoiesis. Therefore, the shortcomings observed during hematopoietic aging could result from altered epigenetic and transcriptional patterns that would affect stem cell behavior and the outcome of hematopoiesis. Support for this comes from multiple studies, including recent studies where the DNA methylation profile of young and aged HSCs was investigated (195, 239). In the study by Beerman et al, it was found that HSCs displayed an age-associated increase in DNA methylation at many distinct loci (195). Interestingly, many of these loci were implicated in lymphoid and erythroid differentiation (195). In addition, excessive proliferation of young HSCs by 5-fluoro uracil (5-FU) challenge resulted in an altered methylation pattern resembling that of aged HSCs (195). Thus, not only does HSC aging appear to coincide with an altered DNA methylation pattern that may affect downstream progeny, but some of these alterations may be replication dependent (195). Many of the age-associated methylated regions were also found to be direct polycomb target genes, indicating both, a dynamic relationship between DNA methylation and PcG complexes in the regulation of HSC gene expression, and that polycomb target genes might change during HSC aging (195, 239). Furthermore, genome wide gene expression profiling of young and aged HSCs has revealed many age-associated transcriptional alterations (193, 240, 241). Importantly, many of the identified differentially regulated genes are common among these studies, indicating that distinct alterations in the transcriptional landscape is yet another hallmark of HSC aging (see paper II). In association, differential expressions pattern of several epigenetic regulators and

chromatin modifiers has been reported in aging HSCs (193, 240), which further supports a role for an altered epigenome in hematopoietic aging.

The polycomb member *Bmi1* has been indirectly implicated in the aging process, even though its expression remains unchanged in aging HSCs. This is due to its role in repressing the expression of the cell cycle inhibitor p16 and tumor suppressor p19 (168). P16 and p19 are two splice variants of the *Ink4a* locus and their expression has been observed to increase in an age-associated manner in a variety of different tissues (242). The expression of p16 can lead to cellular senescence; a cellular state that has been associated with aging (242). Indeed, the loss of *Bmi1* in HSCs leads to derepression of these genes and a concomitant hematopoietic failure (168). However, the findings that p16 expression is a rare event in aging HSCs (243), in combination with the fact that overexpression of *Bmi1* in p16 and p19 compound mutant animals still exhibit increased HSC function *in vitro* (244), argues against a role for p16 dependent senescence in hematopoietic aging and suggests that *Bmi1* must have other important target genes in hematopoiesis. On this subject, mice deficient for *Bmi1* display loss of repression of several B lineage specifying factors in HSCs and GMLPs, hence *Bmi1* appears to be involved in maintaining HSC multipotentiality (167). Because of this, *Bmi1* has been proposed to be a candidate regulator of HSC lineage potential during aging (245). In addition, another previously discussed polycomb component, *Ezh2*, has been reported to become down regulated in aging HSCs (193, 243). In summary, a dysregulation of epigenetic and transcriptional regulatory machinery seems to be a prominent feature of HSC aging. However, whether these alterations occur as a consequence of other alterations, such as DNA damage, or whether they may be a reversible driving force for the maintenance of the hematopoietic aging phenotypes has not been established, which was the focus of paper II of this thesis.

If aging results from altered epigenetic and transcriptional patterns another outstanding question relates to whether aging coincides with a more general “epigenetic drift” that affects multiple genes that collectively lead to the establishment of aging phenotypes, or whether the altered expression of a select few factors may alone be responsible for the establishment of hematopoietic aging. Naturally the latter would be more attractive, since this alternative would simplify any rejuvenation attempts. Although the likely scenario is that hematopoietic aging is a multiparameter setting, a few factors have been identified that may directly influence the aging process. For instance, HSCs devoid of the signal adaptor protein *Lnk*, which dampens many extracellular signaling pathways, are shielded against the age-associated decline in differentiation and reconstitution potential (246). While *Lnk* is a cell intrinsic protein, this finding potentially links the aging environment to the cell autonomous changes in aging HSCs. In addition, aged HSCs display reduced expression of the chromatin organizer *Satb1*, and overexpression of this gene was shown to enhance the lymphoid differentiation potential of aged LSK cells *in vitro* (247). Aging hematopoietic stem and progenitor cells have also been shown to display reduced

expression of the histone deacetylase (HDAC) Sirt3 and lentiviral overexpression of this gene resulted in increased reconstitution potential of aged BM cells compared to untransduced cells; an effect which was suggested to depend on an enhanced oxidative stress response (248). Additionally, aging HSCs has been found to harbor enhanced activity of the small RhoGTPase Cdc42 which results in loss of cell polarity (249). Moreover, elevated Cdc42 activity in aging HSCs appeared to associate with global loss of H4K16 acetylation (249). Interestingly, the restoration of polarity via pharmacological inhibition of Cdc42 activity in aged HSCs ameliorated several hematopoietic aging associated phenotypes and restored global AcH4K16 abundance (249). In a follow up study, it was suggested that the underlying reason for these findings was an age-associated shift from canonical to non-canonical Wnt signaling due to increased Wnt5a expression in aged HSCs (250).

SUMMARY OF ARTICLES

Article I

Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging.

Norrdahl GL, Pronk CJ, **Wahlestedt M**, Sten G, Nygren JM, Ugale A, Sigvardsson M, Bryder D. *Cell Stem Cell*. 2011 May 6; (5):499-510.8

In the present work, we investigated the importance of intact mitochondria for HSC function and whether compromised mitochondrial function is a contributing factor in HSC aging. To aid us in these studies, we utilized a mouse model harboring a proofreading defective Poly enzyme (237). These “mutator” mice progressively acquire seemingly random mtDNA mutations and develop several premature aging phenotypes at around 7-9 months of age and have reinforced the view that lesions to mtDNA may contribute to the aging process (237).

We initially investigated whether HSCs and downstream progenitor subsets are differentially dependent on proper mitochondrial function. Although the mitochondrial content remained quite stable in the cell fractions investigated, a marked reduction of mitochondrial content could be observed upon differentiation into the bipotent pMegE subset. Upon further commitment of this subset into the erythroid lineages, we instead observed an increase in mitochondrial content. Interestingly, in the BM of the mutator mice, we observed an increased frequency of early erythroid progenitors (pCFU-Es). In addition, the mutator mice displayed a marked reduction of Pro-B cells. Based on these phenotypes it appeared as if the premature aging associated with the mutator mice is distinct from physiological aging, since aged WT mice displayed an overall reduction of lymphoid precursors and did exhibit an expansion of erythroid progenitors.

Next, we wondered whether the hematopoietic phenotypes observed in the mutator mice were due to alterations intrinsic to the HSCs. To this end, we performed primary and secondary transplantations of mutator HSCs into WT recipient mice. In the primary transplants, mutator HSCs had marked difficulties in reconstituting the lymphoid lineages, while myeloid reconstitution appeared unaffected. Common to both the primary and secondary transplant experiments was that mutator HSCs displayed a reduction in erythropoiesis similar to the steady-state transgenic mutator mice. In addition, secondary transplant recipients of young mutator HSCs revealed a reduced self-renewal potential and a concomitant reduction in overall donor reconstitution. Collectively, these findings indicate that the observed phenotypes arise at different stages of ontogeny affecting both progenitor subsets and HSCs independently.

Our results thus far indicated a differential impact of mtDNA mutations at specific stages of hematopoietic differentiation. Therefore, we next investigated the consequences of accumulated mtDNA in different hematopoietic subsets in more detail. Interestingly, using a Sanger sequencing approach, we found that mutator HSCs and pCFU-Es harbored similar amounts of mtDNA mutations. When next investigating whether the increased mtDNA mutational burden affected the cells' mitochondrial membrane potential (MMP), we found that HSCs, GM, and pCFU-E derived from the mutator mice all displayed a similar reduction compared to their WT counterparts. However, pCFU-Es were the only analyzed cell type that displayed a decreased ability to increase their MMP when stimulated with oligomycin. Despite this, pCFU-Es had comparable ATP and ROS levels compared to WT cells. Nevertheless, mutator pCFU-Es and pro-B cells displayed a selective increase in apoptosis, whereas HSCs and GM cells were unaffected.

Our findings provide an insight into the differential dependence on mitochondrial function at various stages of early hematopoiesis. HSCs have been described to be dependent on glycolysis to supply their energy demands (210) and perhaps this is why, in relative terms, mutator HSCs are somewhat less affected than other subsets. Nonetheless, the fact that vigorous proliferation of mutator HSCs leads to a severely decreased reconstitution potential indicates that appropriate mitochondrial function is a necessity for HSCs, which is in agreement with previous reports (251-255). We did not observe a decrease in cellular ATP content in HSCs, which has previously been described in other tissues of these mice (237, 256). Although speculative, we think this difference might be due to the proliferative nature of blood cell development spurring a selection event in favor for cells with an appropriate ATP amount along with a concomitant increase in apoptosis.

However, it is important to emphasize that the mitochondria are involved in other cellular processes than ATP production and a decreased mitochondrial function could therefore impact on various other cellular processes (229). For instance, apart from ATP synthesis, the electron transport chain is involved in pyrimidine synthesis and defects in this process has been described to lead to p53 mediated apoptosis (257). Moreover, as the compromised proof-reading ability of the Pol γ enzyme leads to random mutagenic events in mtDNA (237, 258), one might envision that various mitochondrial components involved in an array of different processes may be affected and give rise to the observed phenotypes of these mice. Additional experiments would be needed uncover the exact molecular mechanisms that give rise to the different hematopoietic phenotypes observed in the mutator mice.

As mentioned, the mitochondrial theory of aging argues that a continuous buildup of ROS is particularly harmful for mtDNA and might explain many of the occurrences observed during aging (230). However, since we and others do not observe an increased ROS production in somatic mutator cells (256, 259), we conclude that a vicious cycle of increased ROS production and a concomitant acquisition of mtDNA mutations is not a hallmark feature of hematopoietic aging.

Although both aged WT and mutator mice gave rise to a hematopoietic system skewed in favor for myelopoiesis, this appeared to occur in the absence of an expansion of mutator myeloid biased CD150 high expressing HSCs. Additionally, we found that the transcriptome of mutator HSCs was vastly different than that of aged WT HSCs. Therefore, our findings demonstrate that the premature aging features of the mutator mice arise by different means than for WT hematopoietic aging. However, we cannot rule out that mtDNA lesions can be a contributing factor in aging.

Article II

An epigenetic component of hematopoietic stem cell aging amenable to reprogramming into a young state.

Wahlestedt M, Norddahl GL, Sten G, Ugale A, Frisk MA, Mattsson R, Deierborg T, Sigvardsson M, Bryder D. *Blood*. 2013 May 23;121(21):4257-64

In this work, we aimed to investigate the functional importance of the epigenetic and transcriptional alterations observed during hematopoietic aging.

From previous studies, it has been established that aging HSCs associate with an altered transcriptome compared to their young counterparts (193, 240, 241). However, less is known about the stability of such alterations. To investigate this, we transplanted young and aged BM cells into young recipients and isolated the transplanted HSCs four months post transplantation and subjected these to genome wide transcriptional profiling. When intersecting the resulting information with data acquired from young and aged HSCs isolated from steady-state animals (non-transplanted), we found that although transplantation itself seemed to confer certain transcriptional changes, there was a remarkable age-associated overlap between the differentially regulated genes in steady-state and transplanted HSCs. Based on this, we conclude that the age-associated transcriptional differences between young and aged HSCs are of a relatively stable nature and, since these changes persist following multiple rounds of cell division, they likely have epigenetic underpinnings and might be involved in the maintenance of the hematopoietic aging state. If so, a reversal of these alterations might be beneficial for normalizing some of the phenotypes associated with hematopoietic aging.

To address this, we utilized somatic cell reprogramming of aged HSPCs, since the induction of a pluripotent iPS state coincides with the removal of epigenetic marks that maintain cell identity (148). We therefore retrovirally overexpressed the transcription factors Oct3/4, Sox2, Klf4 and c-Myc in aged HSPCs and found that the resulting aged blood iPS (BiPS) cells had a highly similar global gene expression profile regardless of original somatic cell donor age. Next, to allow for the reestablishment of a hematopoietic system, we injected our blood-derived iPS cells into blastocysts that subsequently were implanted into pseudo pregnant mothers. Since the donor iPS cells and the recipient blastocyst were of different CD45 genotypes, we could next specifically analyze the iPS derived blood for a number of parameters commonly altered during aging. One such parameter is the myeloid lineage bias (66, 193-195). Interestingly, we found no evidence for such a skewing in the hematopoietic system derived from aged BiPS cells. Hematopoietic aging also coincides with an expanded HSC compartment (66, 193, 198-201), however, this phenomenon was not present in the aged BiPS derived hematopoietic systems. Another striking feature of aging HSCs is their markedly reduced reconstitution potential (193-195, 201) and we speculated that extensive proliferation associated

with transplantation might reveal potential shortcoming of the aged BiPS derived HSCs. Strikingly, not only did the aged BiPS derived HSCs have reconstitution potential comparable to young HSCs, but the regenerated recipient animals inherited a balanced output of mature effector cells. Thus, by multiple functional accounts, we conclude that somatic cell reprogramming can ameliorate several functional parameters normally altered with age thereby establishing an altered epigenome as a prominent feature of hematopoietic aging.

Hematopoietic aging also associates with a lowered production of naïve T cells and this has largely been attributed to the ongoing age-associated progressive thymic atrophy (189). Therefore, to assess the inherent ability of HSCs from various sources to give rise to naïve T cells, we investigated the extent by which each HSC gives rise to newly formed T cells in a transplantation setting. This revealed a striking reduction in aged HSCs ability to produce naïve T cells, while aged BiPS derived HSCs generated naïve T cells at comparable levels of young HSCs. Therefore, a reduced potential to produce naïve T cells is a strong feature of HSC aging that is independent of thymic atrophy.

One of the most widely accepted molecular features of aging in general is shortening of the telomeres residing at the ends of the chromosomes (260). We were interested in investigating this, not only in steady-state HSCs, but also in aged BiPS derived HSCs that had undergone vigorous proliferative challenges both in vivo and in vitro. To allow for the investigation of this in a detailed and sensitive manner, we developed a single cell qPCR method and found that aging steady-state HSCs displayed a small reduction in telomere length compared to their young counterparts. In contrast, aged BiPS derived HSCs contained telomeres approximately twice the size of young HSCs. While the iPS induction process previously had been reported to lead to telomere extension in mouse embryonic fibroblasts (MEFs) (261, 262), we were found that this phenomenon occurs also when using hematopoietic donor cells. However, it was not known whether this elongation is appropriately maintained following iPS cell differentiation. When investigating this, we found that BiPS HSCs retained long telomeres that ultimately required genetic dilution in the form of mouse breeding to come close to normal levels.

Hematopoietic aging has been suggested to result from multiple underlying alterations that ultimately lead to an overall reduction in cellular and organ fitness (70). In addition, most of the hallmark features of hematopoietic aging have been found to be cell intrinsic alterations at the top-most level of the hematopoietic hierarchy that result in an altered hematopoietic competence (189). In the present work we extend on this view and show that HSC aging coincides with stable, but reversible, epigenetic and transcriptional parameters that appear to have immediate consequences. While it has been speculated that stem cell aging results from the lifelong acquisition of various insults to the genomic or mitochondrial DNA (215, 236), our findings that the reprogramming of aged hematopoietic cells can yield hematopoietic cells with a “young” appearance, strongly favors a view in which

prominent epigenetic and transcriptional alterations are major contributors to hematopoietic aging. Although, we cannot rule out that the iPS reprogramming process selects for “undamaged” clones, we suggest this to be highly unlikely since young and aged HSPCs generated iPS cell lines at similar efficacies and because of the strong dominance of myeloid biased HSCs in the aged setting.

Epigenetic regulatory mechanisms may have direct impact on gene expression patterns and several components have been found to impact on hematopoietic cell function (see background section). Therefore, it is probable that the epigenetic reset coinciding with iPS reprogramming of aged cells results in a normalized regulation of certain loci that become dysregulated with age. Investigations into the nature of such loci will be highly interesting in order to pinpoint the molecular networks involved in the aging process. Moreover, it remains to be seen if our findings can be extended also to other organ systems and whether one eventually can achieve a similar epigenetic and transcriptional reversal of the aging state in a more controlled physiological setting.

Article III

Somatic cells with a heavy mitochondrial DNA mutational load render iPS cells with distinct differentiation defects.

Wahlestedt M, Ameer A, Moraghebi R, Norrdahl GL, Sten G, Woods NB, Bryder D. *Manuscript accepted for publication in Stem Cells.*

Aging has been proposed suggest to depend on an increased amount of genomic and mitochondrial DNA damage (215, 236). Our findings in article I suggested that a heavy burden of mtDNA mutations have relatively mild effects on somatic stem cell function, however, little is known about the impact of these on other stem cell populations.

One such stem cell population is iPS cells, that represent promising tools for the study of disease mechanisms, developmental processes and as future potential therapeutics. However, little focus has been aimed at studying the impact of mtDNA mutations for iPS cell generation and functionality. To investigate this, we utilized the mutator mouse strain (237) and attempted to generate iPS cells from mutator HSPCs. This revealed that mutator HSPCs were amenable for reprogramming at similar efficiencies as their WT counterparts, although the former presented with a slower growth rate. When addressed for a number of iPS associated characteristics, including immunophenotypic and morphological appearance and telomere extension, we found that mutator iPS cells were comparable to WT iPS cells in all investigated aspects. Therefore, we concluded that mtDNA mutations are largely dispensable for the generation and maintenance of pluripotent stem cells.

In the hematopoietic system, mtDNA mutations had a strong negative influence on certain differentiation pathways (article I). Thus, we were inclined to investigate the differentiation potential of the mutator iPS cells. Interestingly, these experiments revealed a severe incompatibility of a heavy load of mtDNA mutations on iPS cell differentiation. In vitro, this presented as a failure to generate appropriate embryoid bodies (EBs); an in vitro surrogate for embryonic development. Another assay to test iPS cell differentiation potential is to investigate their ability to generate teratocarcinomas; tumors comprised of cells from all three germ layers. Remarkably, when performing these experiments with mutator iPS cells, we found that they either failed to generate or generated extremely small tumors. In addition, when exposed to the most vigorous assay to test for murine iPS cell pluripotency - the ability to contribute to chimeric mice via blastocyst complementation - mutator iPS completely failed to contribute to the resulting pups. Therefore, we conclude that while a heavy load of mtDNA mutations appeared dispensable for the maintenance of iPS cells in their undifferentiated form, they resulted in a complete differentiation failure of mutator iPS cells.

Next, we were interested in investigating why the mtDNA mutational load is dispensable for iPS cell maintenance but incompatible with differentiation.

Experiments aimed at resolving this revealed that undifferentiated mutator iPS cells were comparable to WT iPS cells in terms of overall mitochondrial content, mitochondrial morphology and ATP content. On the contrary, mutator iPS cells displayed with a lower ROS content compared to their WT counterparts. Previous reports have suggested that iPS cells rely heavily on anaerobic respiration via the glycolysis for energy production (263-268). When investigating this aspect in the mutator iPS cells, we found them to inherit comparable glycolytic activity to their WT counterparts suggesting that an appropriate anaerobic respiration compensates for the reduced mitochondrial competence of the mutator iPS cells. However, during early iPS cell differentiation a metabolic switch from glycolysis to mitochondrial dependent OXPHOS is thought to occur (267, 269, 270). In light of this, we hypothesized that the observed differentiation failure was the result of the inability of the mutator iPS cells to switch to OXPHOS in an appropriate manner. Indeed, we found that newly formed mutator EBs contained reduced ATP content and a vastly enhanced glycolytic activity. This suggests not only a failure to initiate OXPHOS during differentiation, but also that the mutator iPS cells tries to compensate for their compromised mitochondrial function by an elevated glycolytic activity.

A main consensus from previous work using the mutator mouse model is that the progressive accumulation of point mutations to mtDNA results in the decreased stability of respiratory complexes and an overall decreased respiratory function (258, 259). This is in line with our findings that both undifferentiated and differentiated mutator iPS cells produced little ROS that likely represent an overall lowered mitochondrial activity. In addition, the finding that somatic cells with an immense mtDNA mutational burden can be maintained as undifferentiated iPS cells fits well with the notion that iPS cells depend heavily on the glycolysis for their metabolic needs. However, as development progresses during embryogenesis and in vitro for pluripotent stem cells there is an increased need for mitochondrial respiratory activity (267, 271), which might explain why homozygous mutator mice are infertile (237) and why mutator iPS cells are unable to differentiate.

Although somatic cell reprogramming can reset many of the epigenetic and transcriptional patterns of the parental cells, mitochondrial rejuvenation did not coincide with the reprogramming process. When investigated, we found the mtDNA mutational burden to be largely maintained in the resulting mutator iPS cells compared to the originating somatic cells. Despite this, our finding that somatic cells with a heavy mtDNA mutational burden are amenable for reprogramming into iPS cells is promising both from a basic science and a regenerative medicine perspective but also flags for the necessity to carefully investigate the integrity of the mitochondrial genome. This is because eventual lesions may not present as major obstacles during the maintenance phase but can instead strongly impact on downstream differentiation processes, which in the end is the long-term goal for iPS cells in regenerative medicine.

Article IV

Critical modulation of hematopoietic lineage fate by the PAR/bZIP transcription factor Hepatic Leukemia Factor.

Wahlestedt M, Säwén P, Ladopoulos V, Norddahl GL, Göttgens B, Bryder D.
Manuscript

The maintenance of the hematopoietic hierarchy and its' different cellular subsets depend to a large extent on coordinated networks of transcription factors, with transcriptional regulators being essential mediators for cell fate decisions and cell identity (272). Although a fair amount of work has been devoted to identify intrinsic regulators of hematopoiesis, as well as their interplay, a thorough understanding of these is lacking.

To aid in this matter, we set out to identify novel regulators involved in early hematopoietic development and multipotency. We began by analyzing genome-wide expression datasets of highly purified HSCs, GMLPs and other more downstream progenitor cell subsets. We were particularly interested in identifying TFs with selectively high expression in both HSCs and GMLPs, since these genes would be likely to be involved in the maintenance of multipotency. These analyses lead to the identification of the TF hepatic leukemia factor (Hlf), a factor belonging to the proline acidic amino acid-rich basic leucine zipper (PAR bZIP) family. Hlf has previously been implicated in circadian rhythm regulated functions in the brain, (273, 274), liver (275) and kidney (276). Critical regulators of early hematopoiesis are often found in oncogenic fusion proteins in leukemic disease and, interestingly, Hlf has been found fused to the TF E2A in a subset of acute lymphoblastic leukemias (ALLs) via the translocation t(17;19) (277, 278). In addition, Hlf is commonly upregulated in NUP98-HOX driven myeloid leukemias (279). In the blood, forced expression of Hlf has been associated with increased self-renewal (280, 281) and its' promoter has been found differentially methylated at various stages of hematopoiesis (159). Based on this, we chose to detail the normal role of Hlf in hematopoiesis.

To allow for studies on Hlf, we developed a doxycycline (DOX) inducible mouse model that allows for selective and high expression of Hlf in a controllable manner (282). Next, we investigated the hematopoietic impact of enforced in vivo Hlf expression in the transgenic mice. These experiments revealed a rapid expansion of pGM and GMP myeloid progenitors, while the HSC and GMLP populations appeared less affected. On the contrary, B lymphopoiesis was severely compromised, with a rigorous decrease of most stages of B cell progenitors and a concomitant expansion of a yet unknown B220+IL7Ra+CD19+c-Kit+ presumable immature B cell population virtually nonexistent in control mice. Similarly, T lymphopoiesis was gravely affected with the enforced Hlf expression associating with rapid thymic degeneration. From these experiments, we speculated that Hlf is an important regulator of early differentiation decisions in multipotent hematopoietic progenitors

by negatively acting on lymphoid specification and instead promoting myelopoiesis. However, since Hlf expression was induced in all cells of the transgenic mice, the formal possibility remained that some of the effects were of a non-hematopoietic nature. Therefore, to address the cell autonomous effects of enforced Hlf expression, we transplanted Hlf inducible multipotent GMLPs into WT recipient mice under continuous DOX treatment. These experiments confirmed the cell autonomous nature of the observed phenotypes originally observed in the Hlf transgenic mice and indicated that Hlf is involved in lineage fate decisions in multipotent GMLPs.

In an attempt to address whether Hlf acts in an instructive manner to promote myelopoiesis over lymphopoiesis in GMLPs, we isolated Hlf transgenic GMLPs and cultured them on OP9 and OP9-DL1 stromal cells that strongly promote B and NK cell, and T cell development respectively. These experiments revealed that enforced Hlf expression was completely incompatible with B and NK cell development, while T cell development was strongly negatively affected. Concomitant with these phenotypes was a simultaneous increase in the generation of myeloid cells. Although these findings strongly suggested Hlf to be an instructive determinant of lineage fate in GMLPs, we could not rule out that Hlf acts in a differential manner on alternative subsets of GMLPs with different potential towards lympho- and myelopoiesis. To address this, we performed similar in vitro cultures initiated with single Hlf transgenic GMLPs. Remarkably; these experiments revealed that nearly all initiated cultures were exclusively composed of myeloid cells without any evidence for an increase in cell death. Based on these findings, we conclude that Hlf is a key instructive intrinsic determinant of lymphoid versus myeloid cell fate decisions in differentiating GMLPs. In further support of this, we found that several key lymphoid associated transcripts failed to become upregulated in Hlf enforced GMLPs cultured on OP9 stroma.

There are several aspects of our findings worth investigating further. For instance, since E2A-Hlf driven ALL presents as a pro-B cell leukemia (277, 278), investigations into the expanded B220+IL7Ra+CD19+c-Kit+ population observed following enforced Hlf expression might provide valuable insights into E2A-Hlf driven disease development. It is tempting to speculate that E2A-Hlf driven leukemia could arise as a consequence of a prolonged expression of Hlf during hematopoietic development. However, the findings that Hlf and E2A-Hlf appears to inherit different in vitro transcriptional activities perhaps argue against this (283, 284).

Multiple studies have suggested that myelopoiesis rather than lymphopoiesis is more closely related to HSCs (285) and our findings that Hlf is highly expressed in HSCs and GMLPs and simultaneously promote myeloid cell development suggests that Hlf is a key component of this relationship. Although, we did not obtain any evidence for an upregulated myeloid associated transcriptional program, our experiments were limited by the number of genes analyzed. To gain further insights into the mechanisms behind our observed phenotypes, genome-wide transcriptional profiling of Hlf overexpressing cells combined with chromatin immunoprecipitation experiments to determine the direct target genes of Hlf is needed.

In summary, our findings indicate that Hlf is an essential regulator of hematopoietic maintenance by strongly promoting the production of myeloid cells at the expense of lymphoid cell development. An increased insight into the transcriptional programs under control of Hlf is likely to lead to an increased understanding of early cell fate decisions of healthy and malignant multipotent hematopoietic progenitors.

POPULÄRVETENSKAPLIG

SAMMANFATTNING

Varje dag produceras miljardtals nya blodceller, vilka exempelvis har som uppgift att transportera runt syre och försvara kroppen mot olika invaderande bakterier och virus. Dessa olika blodkomponenter har sitt ursprung i sällsynta blodbildande stamceller som återfinns i benmärgen. Dessa är så kallade vävnadsspecifika stamceller och har både förmågan att återbilda sig själva (självförnyelse) och att initiera en differentieringskaskad som till slut leder till bildandet av de mogna immunförsvarscellerna. Det sistnämnda involverar många intermediära celltyper (progenitorceller) som succesivt förlorar sin differentieringspotential innan en given progenitorcell "läses" mot att endast ge upphov till en mogen blodcellstyp. Denna process är därför strikt ordnad i ett hierarkiskt system och möjliggör livslång tillförsel av mogna blodceller. Trots detta kan dessvärre olika sjukdomar med sitt ursprung i blodet uppkomma och en del av dessa är dessutom tydligt associerade med att man blir äldre. Studier i möss har visat att detta kan relateras till förändringar i de blodbildande stamcellerna. Exempelvis blir celler av den medfödda delen av immunförsvaret vanligare på bekostnad av celler av det adaptiva immunförsvaret. Det förstnämnda har som funktion att förmedla snabba, förhållandevis ospecifika svar medan det adaptiva immunförsvaret ger mer specifika svar på invaderande patogener. Denna åldersrelaterade förändring kan bland mycket annat relateras till att blodsjukdomar hos äldre individer oftare har sitt ursprung i just det medfödda immunförsvaret.

Mycket forskning har riktats mot att förstå hur sjukdomsförlopp och normal blodcellsbildning i yngre och äldre personer går till, men trots detta saknas fortfarande många viktiga pusselbitar för att nå en helhetsförståelse av dessa processer. I denna avhandling har jag därför fokuserat på att dra mitt strå till stacken och försökt identifiera olika molekylära mekanismer som är inblandade i dessa processer.

Åldersprocessen har föreslagits bero på skador och mutationer i cellernas DNA som återfinns både i cellkärnan och i cellernas mitokondrier. De sistnämnda är viktiga cellkomponenter, som bland annat har den livsnödvändiga uppgiften att producera energi, och skador i mitokondriernas DNA skulle därför kunna påverka dessa funktioner. I artikel I undersökte vi därför om åldrandet av blodet är kopplat till uppkomsten av mitokondriemutationer och använde oss av en musmodell som succesivt leder till en ökning av mitokondriemutationer, vilket resulterar i flera symptom som förknippas med åldrande. Dessa studier ledde till insikten att en intakt mitokondriefunktion är viktig för en adekvat blodcellsbildning, men att en nedsatt

sådan inte på egen hand kan förklara de åldersrelaterade förändringar som uppkommer i blodet.

Utöver permanenta skador i cellernas DNA har åldrande också föreslagits bero på förändringar i cellers genuttrycksmönster och i de faktorer som reglerar detta. I artikel II adresserade vi huruvida sådana förändringar är stabilt förekommande i blodet och om detta skulle kunna förklara åldersprocessen. Vi fann att sådana genuttrycksförändringar sker i åldrande blodstamceller och intressant nog fann vi att dessa är direkt involverade i de funktionella förändringarna som är associerade med åldrandet i blodet. Detta eftersom en återställning av dessa ledde till att blodsystemet från en gammal mus uppträdde som det hos en ung mus. Därför drog vi slutsatsen att åldrande i blodet uppkommer som följd av förändringar i de åldrande blodcellernas genuttrycksmönster och de faktorer som reglerar dessa.

För att adressera frågeställningarna i artikel II använde vi oss av så kallad "cellreprogrammering", vilket omvandlar en given kroppslig cell till en pluripotent stamcell; en typ av cell som kan ses som ett slags embryonalt förstadium med förmågan att återbilda alla olika celler i kroppen. Eftersom detta ledde till en återställning av de åldersrelaterade förändringarna i blodet var vi i artikel III intresserade av att utvärdera om denna process även skulle kunna mildra ålderssymptom som uppkommer som en följd av mitokondriemutationer. För att svara på detta omvandlade vi blodceller med mitokondriemutationer till samma typ av pluripotenta stamceller. I dessa studier fann vi att mitokondriemutationer i de resulterande pluripotenta stamcellerna ledde till en oförmåga i dessa cellers möjlighet att bilda kroppens olika mogna celltyper. Vi drog således slutsatsen att mitokondriemutationer kan ha starka negativa effekter på pluripotenta stamcellers funktion och att förekomsten av dessa inte kan "rejuveneras" med hjälp av cellreprogrammering.

I artikel IV var vi intresserade av att nå en ökad förståelse för enskilda faktorer som är inblandade i att styra blodcellsbildningen mot olika typer av mogna celltyper. Dessa mekanismer är viktiga att nå detaljerad förståelse kring eftersom de förändras vid olika blodsjukdomar men också, som nämnts ovan, vid åldrande. På basis av detta identifierade vi genen Hlf som visade sig kunna styra utvecklingen av omogna progenitorceller mot att nästan exklusivt bilda celler av det medfödda immunförsvaret på bekostnad av adaptiva immunförsvarsceller. Vi drog därför slutsatsen att Hlf är en viktig komponent i det komplexa nätverk som styr nybildningen av mogna celler i immunförsvaret.

OTHER WORK NOT INCLUDED IN THIS THESIS

Reduced repression of cytokine signaling ameliorates age-induced decline in hematopoietic stem cell function.

Norrdahl GL, **Wahlestedt M**, Gisler S, Sigvardsson M, Bryder D.

Aging Cell. 2012 Dec;11(6):1128-31

SOCS2 is dispensable for BCR/ABL1-induced chronic myeloid leukemia-like disease and for normal hematopoietic stem cell function.

Hansen N, Ågerstam H, **Wahlestedt M**, Landberg N, Askmyr M, Ehinger M, Rissler M, Lilljebjörn H, Johnels P, Ishiko J, Melo JV, Alexander WS, Bryder D, Järås M, Fioretos T. *Leukemia.* 2013 Jan;27(1):130-5

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