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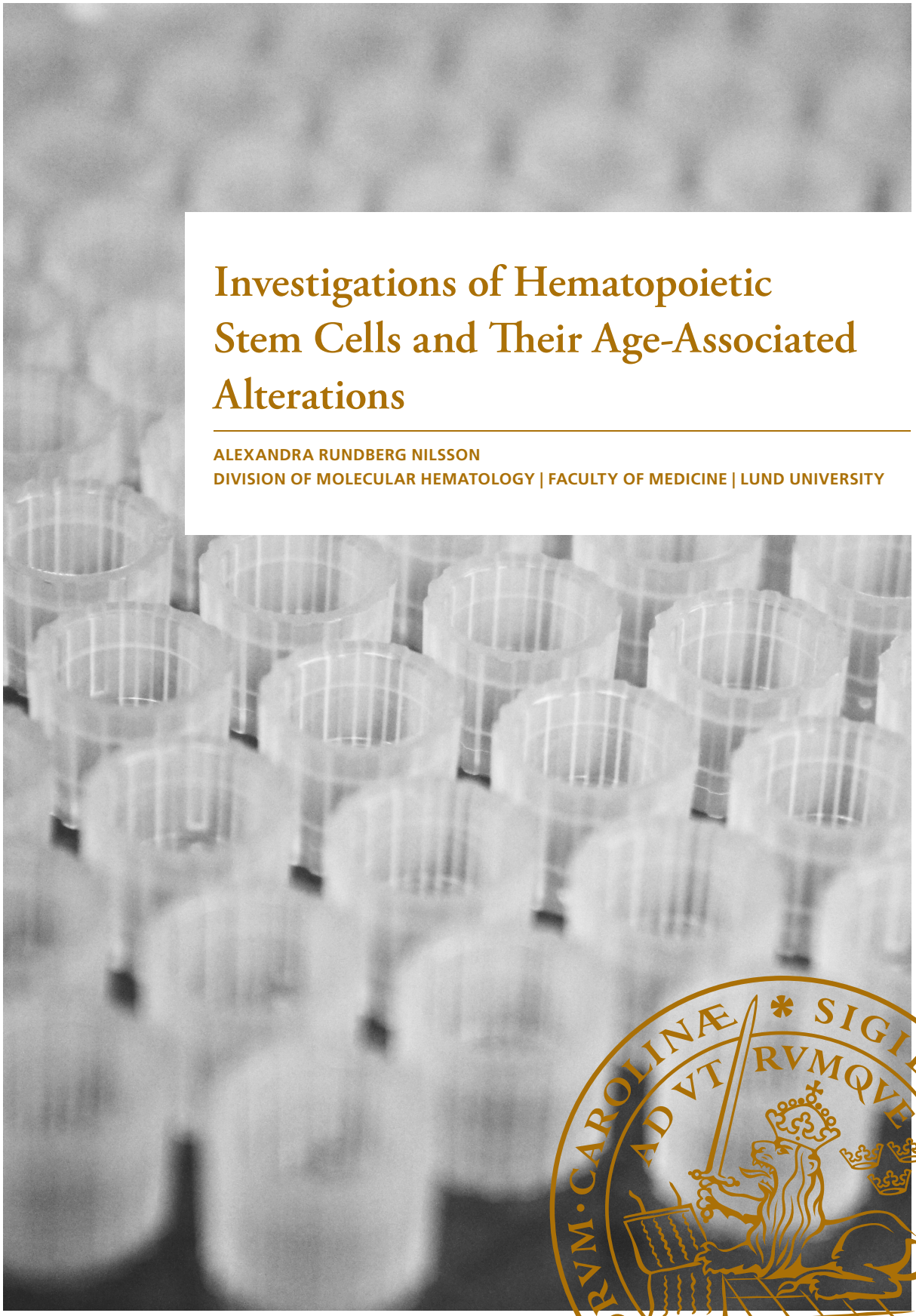

Investigations of Hematopoietic Stem Cells and Their Age-Associated Alterations

ALEXANDRA RUNDBERG NILSSON
DIVISION OF MOLECULAR HEMATOLOGY | FACULTY OF MEDICINE | LUND UNIVERSITY

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Alexandra Rundberg Nilsson

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Abstract

The hematopoietic stem cell (HSC) has and continues to be extensively investigated, and represents by far the most studied somatic stem cell. Development of various experimental technologies, including fluorescence activated cell sorting (FACS), have been crucial for research advancements within the hematopoietic field. However, utilization of flow cytometry techniques put high demands on study design, execution, and data analysis. In article I, we addressed a number of important aspects of flow cytometry-based experiments, particularly for experiments evaluating cells present at low frequencies, such as HSCs, and/or of limited sample amounts. Specifically, we highlighted the importance of different types of positive and negative controls. We also presented a 17-parameter analysis design for simultaneous investigation of numerous different mature and immature human hematopoietic cell populations, including HSCs, megakaryocyte/erythrocyte progenitors (MEPs), granulocyte/macrophage progenitors (GMPs), common lymphoid progenitors (CLPs), $CD11b^+$ myeloid cells, $CD19^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells, in one bone marrow sample. Lastly, we discussed data visualization alternatives that allow for appropriate presentation of analyzed results.

Serial transplantation is considered the gold standard approach for *in vivo* evaluation of long-term HSC capacity. Despite the extensive use of this methodology, such experiments are not conducted uniformly between laboratories. In article II, we therefore compared the two most common strategies for serial transplantation (serial transplantation of whole bone marrow [wBM] *versus* serial transplantation of purified HSCs). We revealed that donor-derived cells were not evenly distributed among separate bones within individual mice three months after transplantation – a time point that is frequently used for readout of bone marrow engraftment and/or isolation of cells for serial transplantations. We showed that such unequal distribution could impede isolation and serial transplantation of wBM with representative chimerism levels. Serial transplantation of purified HSCs would however correct for uneven chimerism levels. Furthermore, serial transplantation of wBM was associated with a relative lymphoid skewing, likely as a result of long-lived lymphoid-restricted non-HSCs. Again, serial transplantation of purified HSCs is preferential as it ensures evaluation of lineage output from the designated candidate HSC population. We also demonstrated that serial transplantation of purified HSCs enable distinction of long-term effects (occurring in secondary recipients) from short-term effects (presented in primary recipients), which may be hindered when wBM is transplanted. Therefore, we highly recommend utilization of purified HSCs for serial transplantation purposes. We also presented a proposed serial transplantation design that would maximize the chances to correctly evaluate long-term HSC competence.

Lastly in this thesis, we investigated the effects of aging on human and murine hematopoietic stem and progenitor cells (HSPCs). Such investigations are important for understanding of the underlying mechanisms that cause age-associated alterations of the hematopoietic system, including immune impairments, and increased incidences of anemia and myeloid malignancies. Murine studies indicate that at least some age-associated hematologic changes are linked to alterations occurring in the most immature hematopoietic compartments. Such studies in humans are sparse, as well as conflicting, but necessary to establish species-conserved and species-specific aging patterns, and to confirm mice as relevant model organisms for investigations of aging hematopoiesis. In article III, we revealed that humans and mice demonstrate several similar hematologic aging patterns, including increased HSC frequencies, and lymphoid differentiation impairments. Both species also exhibited prominent transcriptional lineage-skewing patterns of aged HSCs toward enrichments of megakaryocytic/erythroid signatures, while genes involved in lymphoid specification were markedly downregulated. In conclusion, these results confirmed several HSC aging similarities across the human-to-mouse species barrier, as well as demonstrated novel lineage-skewing patterns within aging HSPCs.

Key words: Hematopoietic stem cells, aging, serial transplantation, self-renewal, flow cytometry, FACS, gene expression

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Alexandra Rundberg Nilsson

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UNIVERSITY

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“Whenever I’m about to do something, I think, ‘would an idiot do that?’,
and if they would, I do not do that thing. “
- Dwight K. Schrute

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Original Papers and Manuscripts

Papers included in this thesis

Paper I

Frequency determination of rare populations by flow cytometry: a hematopoietic stem cell perspective.

Rundberg Nilsson A, Bryder D, Pronk CJ.

Cytometry A. 2013 Aug;83(8):721-7. doi: 10.1002/cyto.a.22324. Epub 2013 Jul 9. Review.

Paper II

Probing hematopoietic stem cell function using serial transplantation: Seeding characteristics and the impact of stem cell purification.

Rundberg Nilsson A, Pronk CJ, Bryder D.

Exp Hematol. 2015 Sep;43(9):812-7.e1. doi: 10.1016/j.exphem.2015.05.003. Epub 2015 May 29.

Paper III

Human and Murine Hematopoietic Stem Cell Aging Is Associated with Functional Impairments and Intrinsic Megakaryocytic/Erythroid Bias.

Rundberg Nilsson A, Soneji S, Adolfsson S, Bryder D, Pronk CJ.

PLoS One. 2016 Jul 1;11(7):e0158369. doi: 10.1371/journal.pone.0158369. eCollection 2016.

Papers not included in this thesis

Genome-wide RNAi Screen Identifies Cohesin Genes as Modifiers of Renewal and Differentiation in Human HSCs.
Galeev R, Baudet A, Kumar P, Rundberg Nilsson A, Nilsson B, Soneji S, Törngren T, Borg Å, Kvist A, Larsson J.
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Abstract

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Abbreviations

AGM	Aorta-gonad-mesonephros
APC	Antigen-presenting cell
CB	Cord blood
CFU-E	Colony-forming unit erythroid
CFU-S	Colony-forming unit in spleen
CLP	Common lymphoid progenitor
CMRP	Common myeloid repopulating progenitor
CNS	Central nervous system
DC	Dendritic cell
DDR	DNA damage response
DEG	Differentially expressed gene
d-HSC	Definitive hematopoietic stem cell
DSB	Double-strand break
E	Embryonic day
ELP	Early lymphoid progenitor
EMP	Erythro-myeloid progenitor
ErP	Erythroid progenitor
FACS	Fluorescence activated cell sorting
FMO	Fluorescence minus one
GM	Granulocyte/macrophage
GMLP	Granulocyte-monocyte-lymphoid progenitor
GMP	Granulocyte-macrophage progenitor
GO	Gene ontology
GSEA	Gene set enrichment analysis
GVHD	Graft-*versus*-host disease
GVT	Graft-*versus*-tumor
H3K4me3	Trimethylation of histone H3 at lysine 4
HCT	Hematopoietic cell transplantation
HIV	Human immunodeficiency virus
HR	Homologous recombination
HSC	Hematopoietic stem cell

HSPC	Hematopoietic stem and progenitor cell
iPSC	Induced pluripotent stem cell
IRP	Internal reference population
LMPP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharid
MEP	Megakaryocyte-erythrocyte progenitor
Mk/Er	Megakaryocyte/erythroid
MkP	Megakaryocytic progenitor
MkRP	Megakaryocyte repopulating progenitor
MLP	Myelo-lymphoid progenitor
MPP	Multipotent progenitor
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NHEJ	Non-homologous end joining
NK cell	Natural killer cell
pGM	pre-granulocyte-macrophage
preCFU-E	pre-colony-forming unit erythroid
preMegE	pre-megakaryocyte/erythrocyte progenitor
RA	Retinoic acid
RBC	Red blood cell
Rho-123	Rhodamine-123
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SL-MkP	Stem-like megakaryocyte-committed progenitor
TF	Transcription factor
TPO	Thrombopoietin
UPR^{mt}	Mitochondrial unfolded protein response
vWF	von Willebrand factor
WBC	White blood cell
wBM	Whole bone marrow
WT	Wild-type

Background

The Power of Blood

Through history, blood has been considered a mystical and powerful element, containing both healing and illness-causing powers (**Figure 1**). In many faiths and religions, blood was sacrificed to Gods as a token of respect and hope of good fortune. Blood from young individuals, especially virgins, was regarded particularly pure and was even used to bathe in or transfuse with for immortalization or rejuvenation purposes (1). Interestingly, a clinical study aimed at rejuvenating elderly individuals by infusion of plasma from younger individuals was recently initiated in the United States, although the scientific design of this particular study has been widely questioned (2). Nevertheless, the theory behind the trial stems from studies where the circulatory systems of young and aged mice were joined together (heterochronic parabiosis), demonstrating rejuvenating effects in muscle (3), heart (4) and central nervous system (CNS) (5) of the aged mice. Two additional similar human trials involving serum transfusions from young to elderly individuals were also recently initiated – one evaluating potential effects on cognitive symptoms in Alzheimer's patients, and the other on age-associated frailty (2). Results from these studies have not yet been presented.

Bloodletting (withdrawal of blood) has a long history and presumably began in Egypt about 3,000 years ago. It was widely used during the time of Hippocrates (~460-370 B.C.) when sickness was believed to arise from imbalances between the four basic humors; blood, phlegm, black bile, and yellow bile. One way to address these illnesses was through bloodletting, thus restoring the humor equilibrium. Although the original use of the bloodletting practice is largely rejected since well over 100 years, a few conditions, such as hemochromatosis (a disorder causing accumulation of iron), polycythemia vera (a disorder with overproduction of mainly erythrocytes and platelets), and postoperative care of skin grafts and re-implantation surgery (where leeches are used to stimulate blood circulation), are still treated with bloodletting-like therapies (6, 7).

Research advancements progressively led to demystification of blood. In 1628, blood was found to circulate within the body, and in 1674, Van Leeuwenhoek showed that blood could be visualized under the microscope. Blood transfusions were performed first between animals, then between animals and humans, and finally between humans. Unfortunately, many of the first transfusion attempts between humans were accompanied by disastrous fatalities, with both donors and recipients losing their lives due to infections, extensive blood loss, and immunological incompatibilities. In 1901, the ABO blood group system was discovered, and could later be exploited to diminish rejection of donor blood after transfusion. During World War I, transfusion became a life-saving treatment for many injured soldiers. Regrettably, it was later discovered that transfused blood had occasionally transmitted fatal viruses, such as human immunodeficiency virus (HIV), and Hepatitis C, which led to a downfall and distrust for blood treatments (1). Today, however, all blood donations in member states of the World Health Organization (WHO) are screened for at least HIV-1, HIV-2, Hepatitis B, Hepatitis C, and Syphilis (8).

Following discoveries that revealed regenerative potentials of bone marrow-derived cells, transplantation of such cells to rescue and recover the hematopoietic system after cytotoxic therapies (e.g. during leukemia treatment) was considered a very promising element in different treatment strategies. In addition, hematologic rescue would allow harsher application of available treatment methods to eradicate

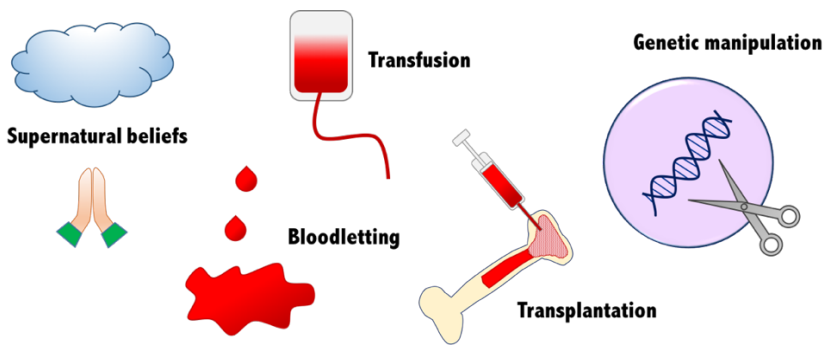

Figure 1. From religion to advanced genetic manipulations.
Blood cells have been exploited in many different ways with hope of obtaining beneficial outcomes. Based on supernatural beliefs, blood was sacrificed to Gods. Furthermore, bloodletting was during extensively periods used in ancient medicine to re-establish fluid balances. Medical advancements later paved the way for the usage of blood cells in more proper medical therapies. Transfusions and transplantations represent lifesaving treatments, and today's research developments even allow for specific manipulations of the blood cell genome. Such genetic therapies can potentially cure diseases caused by genetic mutations using the patients' own cells. Gene manipulations are also extremely valuable for basic research purposes, e.g. when functions of specific genes, or mutation-caused disorders are investigated.

diseased cells. However, due to immune system incompatibilities, similar to those observed following blood transfusions, the first patients receiving hematopoietic cell transplantations (HCTs) died. Later on, successful transplantations were performed using identical twin donors, and later even with immune system matchings of unrelated individuals (9). Much of the initial failure following transplantations in humans was caused by graft rejection or graft-*versus*-host disease (GVHD). Due to the inbred nature of laboratory animals, this effect was in general not observed in mouse experiments. In the process of overcoming the hurdles of therapeutic HCTs, progress was achieved through fine-tuning of immune compatibility matching, immunosuppressive therapy, and cancer-ablation treatments (including total body irradiation and chemotherapy). Although GVHD was a problem, it was also discovered that transplantations with accompanied GVHD had decreased likelihoods of relapse, presumably caused by a graft-*versus*-tumor (GVT) effect where donor cells attack tumor cells (10, 11). Unfortunately, HCTs are still very risky, and constitute a last resort in many treatment strategies. Despite age-associated increased risks of developing many leukemias and blood disorders where HCTs can be used in the treatment process (10), there are therefore relatively fewer elderly individuals that receive HCTs. Today, a new era of blood cell-derived transplantation therapies is emerging where gene therapy techniques possess potential to correct, introduce, or inactivate genes in patients' own cells and/or in cells engineered to serve specific functions.

The Mature Blood Cell System

The word *hematopoiesis* stems from the Greek words *haima* (αἷμα), meaning "blood", and *poiesis* (ποιεῖν), meaning "to make", and refers to the formation of blood cells. In adults, this process primarily takes place in the bone marrow, while fetal hematopoietic activity occurs within multiple sites and with variant functionality (discussed further in "**Hematopoiesis Through Ontogeny**"). The effector cells of the hematopoietic system that conduct immune reactions, promote healing, and transport essential molecules, are the mature blood cells. These can be roughly divided into three main groups: 1) ***red blood cells*** (RBCs; erythrocytes), 2) ***thrombocytes*** (platelets), and 3) **w*hite blood cells*** (WBCs; leukocytes). Erythrocytes are the most abundant cells in the human body (12). Their main function is to transport oxygen and carbon dioxide (13). Platelets represent the second most common cell type (12) and specialize in blood clotting to stop

bleeding (14). WBCs make up the immune system and include cells of the ***innate immune system,*** also referred to as the *non-specific*, or *immediate*, immune system, as well as the ***adapted immune system***, also referred to as the *acquired,* or *specific,* immune system (15, 16) (**Figure 2**). Blood cells can also be divided into ***myelo-erythroid*** (many times referred to only as "myeloid") and ***lymphoid*** lineages, based in their proposed cellular origins. Although alternative pathways have been demonstrated, a myelo-erythroid *versus* lymphoid subdivision generally places erythrocytes, platelets, granulocytes, monocytes, macrophages, and subsets of dendritic cells (DCs) within the myeloid lineage, whereas B cells, T cells, natural killer (NK) cells and other DC subsets belong to the lymphoid lineage. The myelo-erythroid lineage can be further divided into stricter ***myeloid*** *versus* ***megakaryocytic/erythroid*** lineages (**Figure 2**).

The innate immune system is the first line of immune defense that harmful agents encounter. It recognizes and responds to infections in short-term universal fashions (16). The innate immune system typically includes granulocytes, monocytes/macrophages, DCs, and NK cells (**Figure 2**). *Granulocytes* combat invading particles through release of toxic substances and phagocytosis (where foreign particles are encapsulated and digested) (16). Granulocytes also recruit more specialized immune cells to infection sites. *Monocytes* are recruited to damaged or infected tissues where they differentiate and reside as *macrophages*. Similar to granulocytes, macrophages remove dying cells and pathogens through phagocytosis (16). The macrophages are however larger than granulocytes and can therefore engulf larger particles. Macrophages are also more long-lived as they secrete digested products to protect themselves. Furthermore, macrophages are involved in antigen-presentation to T cells (16). *Dendritic cells* are also antigen-presenting cells (APCs). They constantly consume particles in their surroundings, including viruses and bacteria, which they then present on their surfaces, thus communicating to the adaptive immune system to respond (16). *NK cells* specialize in recognizing cells that have been altered, e.g. cancer and virus-infected cells (16). Contributing roles for NK cell subsets have however been implicated in both innate and adaptive immunity (17).

The adaptive immune system, mainly consisting of B and T cells, generates more specific responses than the innate immune system and gives rise to an immunological memory (16), which amongst others is the basis for vaccination as a clinical regimen. It however takes longer to evoke than the innate immune response since it must to be primed. After activation (directly by antigens or by

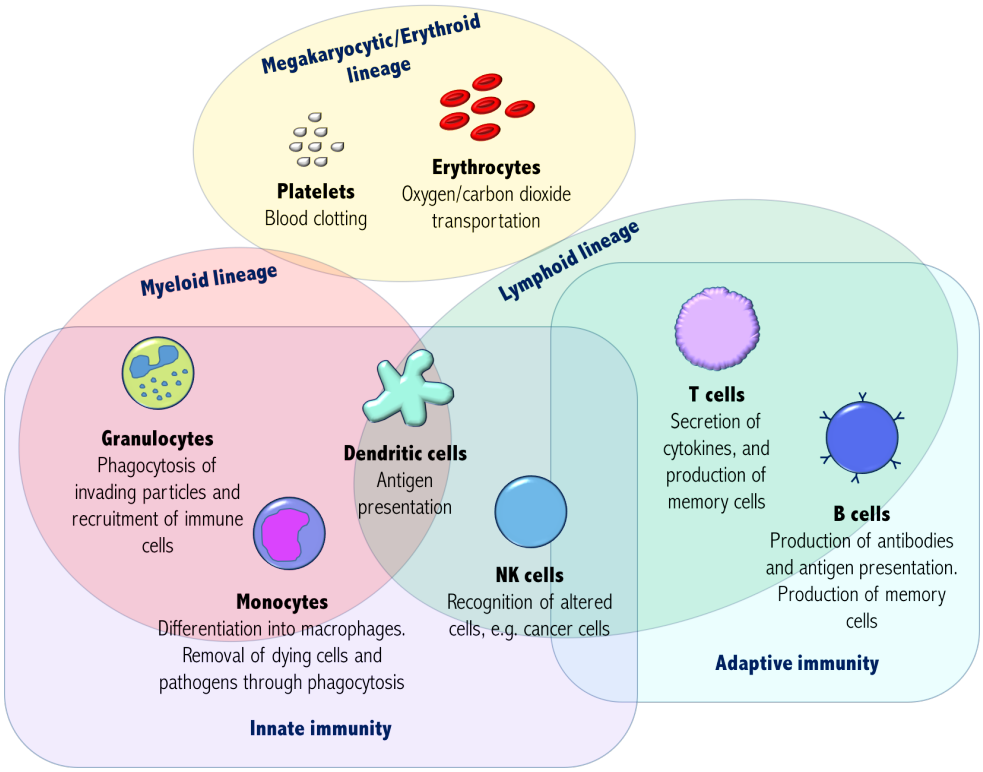

Figure 2. Mature blood cells.
Mature blood cells can be subdivided based on e.g. their cellular origins (lineages) or immune functions. Depicted in this figure are lineage subdivision into myeloid, lymphoid, and megakaryocytic/erythroid lineages, as well as immunological subdivision into innate and adaptive immune compartments.

other immune cells), *B cells* combat infections by converting into antibody-secreting plasma cells. The antibodies bind to the antigens and act by neutralizing or marking the invading object for destruction. B cells also produce memory B cells that remain in the body and that can be activated quicker upon repeated exposure (16). *T cells* consist of different subsets that together regulate the immune response by destruction of infected cells and tumor cells, production of memory T cells, and secretion of cytokines that influence behaviors of other immune cells. A subset of T cells has also been implicated in regulation of self-tolerance (16).

The Discovery of Hematopoietic Stem Cells

Although blood was a cornerstone of treatments and various religious beliefs for millennia, it was not until more modern times that the process of blood cell production was better comprehended. In 1906, Alexander Alexandrovich Maximov was the first to suggest that the mature effector blood cells were produced through differentiation from stem cells (18). Though first encountered with skepticism, his general idea holds to this day. It took almost another 50 years until researchers proposed that these hematopoietic stem cells (HSCs) reside in the bone marrow or spleen. This work was pioneered by the tragic events following the nuclear bombings during the aftermath of the World War II. Studying the survivors that died with delayed kinetics following radiation exposure contributed tremendously to insights of radiation-damaging effects. During the days/weeks following the irradiation these patients suffered many severe complications, including bone marrow failure. Based on those observations, it was discovered in the early 1950s that animals exposed to lethal doses of irradiation could survive when the bone marrow or spleen was protected, or when subsequently injected with cells from bone marrow or spleen from non-irradiated mice (19, 20). Ground-breaking work in this field was further performed by Till and McCulloch, who injected bone marrow cells from donor mice into lethally irradiated recipients, and observed that these gave rise to colonies with mixed mature lineage output in the spleen (21). Although the spleen colonies in these experiments only contained cells of erythroid, megakaryocytic, and myeloid lineages, subsequent studies revealed that the initiating cells of these colonies also harbored lymphoid potential, thereby suggesting a common progenitor for all these lineages (22). Furthermore, the number of colonies in the spleen showed a linear relationship to the injected number of nucleated bone marrow cells. This, together with the observed multi-lineage output, made the authors hypothesize that spleen colonies originated from single cells that the authors referred to as “colony-forming units in spleen” (CFU-S). Irradiation of donor bone marrow suspensions prior to injection, demonstrated a linear relationship between irradiation dose and survival of the number of CFU-S. Experiments using irradiation-induced chromosomal abnormalities later confirmed that individual colonies did originate from single cells as > 90 percent of the cells in the same colony harbored identical chromosomal abnormalities (23, 24). Collectively, these findings indicated that the

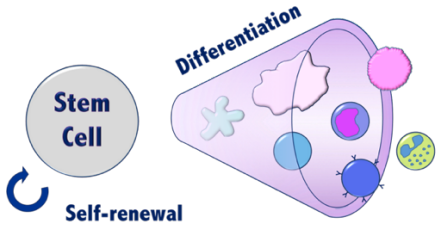

Figure 3. Key characteristics of stem cells.
Stem cells possess two key characteristic properties that enable continuous regeneration throughout a lifetime: **1)** Durable self-renewal, and **2)** Ability to produce differentiated progeny.

spleen colonies stemmed from single cells with multi-lineage potentials, potentially HSCs.

With experimental findings suggesting HSC behavior came a demand for assays capable of investigating stem cell functions. Preferentially, these assays would test the potential of proposed HSCs not only to generate differentiated progeny, but also to self-renew (produce copies of themselves) – the two key characteristics of stem cells (**Figure 3**). Serial transplantation of spleen colonies into new hosts, revealed that cells from these colonies were able to spawn new multi-lineage colonies in spleens, indicative of self-renewal (25). However, it was later suggested and proved in more thorough investigations that the majority of these colonies were produced from progenitor cells (described in the next chapter) rather than stem cells (26-29).

The Hematopoietic Hierarchy

During the process of hematopoiesis, HSCs generate continuously more differentiated cell types in a step-wise manner and ultimately the mature effector blood cells (**Figures 2 and 4**). Due to the short-lived nature of mature blood cells, constant replenishment is required throughout a lifetime. This is a high demand, with approximately a trillion (10^{12}) new blood cells being produced daily (30). It is estimated that an average human adult produces his own body-weight in erythrocytes, granulocytes, and platelets, during a 7-year period (31), demonstrating the enormous pressure that is put on the cells that are responsible for lifelong maintenance of hematopoiesis.

Many studies addressing the hierarchical relationships between cells of the hematopoietic system have been conducted in mice during *in vitro* (outside a living organism), and/or at non-steady state conditions. Observed potentials may therefore differ from those occurring during physiological conditions (discussed further in "**Studying HSCs**"), but may also represent non-linear or alternative differentiation pathways that are initiated in response to specific output requirements. Differences in hierarchical organization and lineage potential of phenotypically similar cell populations have also been reported between cells isolated from different developmental sources (32, 33). Continuously increasing research point to that hematopoietic cells are primed for differentiation into specific mature blood cell types at very early stages in the differentiation hierarchy (33-42). As a consequence, proposed HSPC subsets are not homogenous populations consisting of single cells with equal differentiation abilities. These elements complicate establishment of a simple hematopoietic differentiation cascade outlay. The hematopoietic organization described below is therefore a generalized outlay of adult hematopoiesis (depicted in **Figure 4**).

During the differentiation route from HSCs to mature cells, intermediate cell types arise with increasingly restricted potentials (**Figure 4**). These progenitor cells are crucial for expansion of the number of cells that can be produced from each HSC. A hierarchical organization of the blood cell system also minimize proliferation-induced damages in HSCs as proliferative pressure can be put on downstream cells (43). The very first steps of HSC differentiation result in progressive loss of self-renewal capacity, but continued full multi-lineage potential (43). These intermediate steps are referred to as intermediate HSCs (IT-HSCs) (44), short-term HSCs (ST-HSCs) (45), or multipotent progenitors (MPPs) (46, 47), dependent on the level of self-renewal competence and reconstitution ability that the cells retain (48). Several studies suggest that the first branching point in lineage differentiation potential arise at the MPP stage between the megakaryocytic/erythroid (Mk/Er) lineage and the myeloid/lymphoid lineage (**Figure 4**), as cells harboring both myeloid and lymphoid potential, but very limited Mk/Er potential have been identified (37, 49, 50). Different names/types of bipotent myelo-lymphoid progenitors have been suggested, such as lymphoid-primed multipotent progenitors (LMPPs) (32, 37, 50), granulocyte-monocyte-lymphoid progenitors (GMLPs) (51, 52), and myelo-lymphoid progenitors (MLPs) (32). Hints of these cells' lineage potentials are revealed in their gene expression patterns, where key myeloid (such as PU.1 and C/EBP-α) and lymphoid (such as PAX5 and GATA-3) transcription factors (TFs) are expressed, while expression of erythroid TFs (such

as GATA-1), are lacking (32). These myelo-lymphoid progenitor populations that are identified in different studies are suggested to represent different steps in the differentiation cascade, contain several more specific cell types, and/or be the result of assay-specific potentials/interpretations. A recent study based on transcriptional profiles of individual murine steady state HSPCs suggests that three trajectories (towards Mk/Er, granulocyte/macrophage, and lymphoid, respectively) arise already at the ST-HSC level (53).

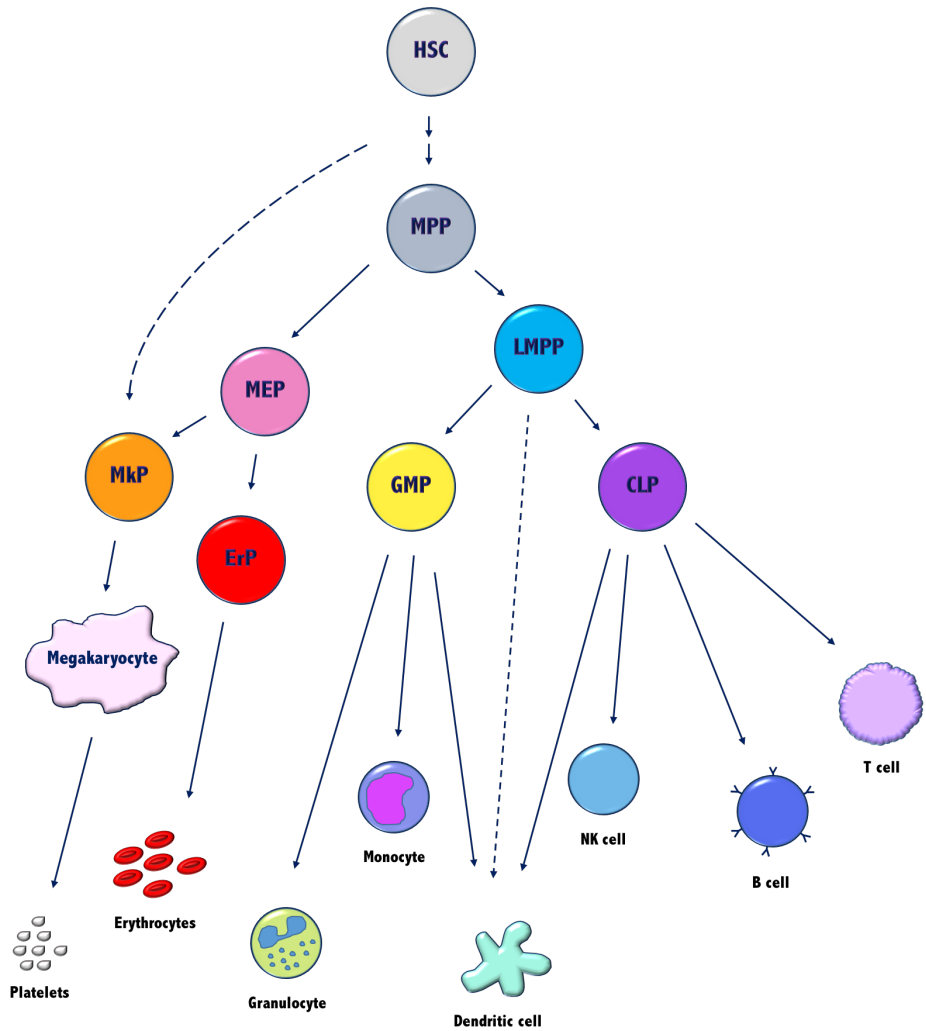

Figure 4. Simplified schematic of the hematopoietic hierarchy.

HSC = hematopoietic stem cell, MPP = multipotent progenitor, LMPP = lymphoid-primed multipotent progenitor, MEP = megakaryocyte/erythrocyte progenitor, GMP = granulocyte/macrophage progenitor, CLP = common lymphoid progenitor, MkP = megakaryocytic progenitor, ErP = erythroid progenitor.

During the course of differentiation, mouse multipotent progenitors first lose their Mk/Er potential, and then their granulocyte/macrophage potential (49). Studies of human umbilical cord blood (CB) confirm that granulocyte potential is lost very early in the differentiation cascade also in the human setting (54). Downstream of bipotent myelo-lymphoid progenitor cells, myeloid-restricted granulocyte-macrophage progenitors (GMPs) (55), with potential to generate granulocytes, monocytes, and myeloid DCs (35, 56), have been identified (**Figure 4**). Recent single cell transcriptome analysis suggests that early myeloid progenitor populations consist of several subgroups that are already primed for differentiation fates, indicating that commitment to mature myeloid differentiation fate is established at earlier stages in the hierarchy (34). *In vitro* functional evaluations confirm that the GMP compartment is heterogeneous with single cells exhibiting distinct differentiation potentials (35).

Downstream of bipotent myelo-lymphoid progenitor cells, the common lymphoid progenitor (CLP), which gives rise to B cells, T cells, NK cells, and DCs, has also been discovered (57-59) (**Figure 4**). Some studies question a restricted lymphoid potential of CLPs since $Mac1^+$ (a marker commonly used to detect monocytes and thereby myeloid lineage) cells are produced from CLPs. Other studies on murine CLPs have however shown that the produced $Mac1^+$ cells concomitantly express CD11c, indicating that these cells are DCs (58), and thus agree with a restricted lymphoid potential. At least a proportion of CLPs can give rise to all of the four main lymphoid lineages on a clonal level (58). There are however also alternative differentiation pathways for lymphoid lineages, which is demonstrated by the identification of separate differentiation trajectories from LMPPs into the T cell linage that pass through as well as outside of the CLP stage (60).

Megakaryocytic and erythroid lineages have been largely overlooked in many *in vivo* (within a living organism) studies. Underlying this is their absence of the pan-hematopoietic CD45 (Ly5) marker, that is widely used to distinguish between donor, competition, and host contributions in competitive transplantation experiments. Several recent studies have however shed lights on these pathways, and shown that particularly the megakaryocytic lineage is primed very high up in the developmental hierarchy, even as early as within the LT-HSC compartment (36) (discussed more in "**HSC Heterogeneity**"). Mk/Er progenitors (MEPs) as well as Mk-restricted progenitors have been shown to arise from MPPs (33), but also directly from ST-HSCs (37), and LT-HSCs (38), thereby bypassing the MPP stage (**Figure 4**). Mk-committed progenitors within the phenotypically defined

HSC pool (38, 61) can be generated within one cell division from LT-HSCs, but do not possess serial transplantation capacities (38). Such stem-like Mk-committed progenitors (SL-MkPs) are quiescent during steady state, but can be quickly recruited under acute stress conditions and produce platelets with rapid kinetics (61), demonstrating that different trajectories within the hematopoietic hierarchy may represent differentially regulated pathways that are activated upon distinct requirements.

Studying HSCs

Early studies had no opportunity to isolate pure HSCs and relied solely on evaluation of very heterogeneous populations of cells. Although these cells could be individually marked, there was little possibility to trace back and isolate enriched populations exhibiting similar behaviors. Development of isolation techniques, such as ***fluorescence activated cell sorting (FACS)***, have contributed tremendously to advancements in HSC research. FACS methodology allows for isolation of single cells on the basis of distinct cell-surface markers, and is today widely used for HSC analysis and purification purposes. Application of index sorting practices (where marker distributions of individually sorted cells are saved), enable further backtracking after single cell evaluations. Flow cytometry-based applications of very infrequent populations of cells that are defined by multiple markers, such as HSCs, require particularly careful consideration of sample preparation, acquisition, as well as analysis strategies, and are discussed in **Paper I**. Neglecting these factors may limit or distort information extracted from such analyses.

Extensive investigations of murine HSCs have resulted in that these can be isolated to higher purity with phenotypical markers than human HSCs (**Figure 5**). Marker combinations enriching for both human and murine HSCs usually include exclusion of cells expressing mature surface lineage markers (lin^-). Murine HSCs can be further enriched by inclusion of Sca-1 and c-Kit markers ($L^-S^+K^+$, or simply LSK). This combination enriches mouse HSCs to a purity of about 1 in 30 (43). Additional markers, such as $CD34^-Flt3^-$, or $CD48^-CD150^+$, can enrich additionally for mouse HSCs with long-term repopulation activity to about one in every five cells (62) (**Figure 5**). Most phenotypical markers used for mouse HSC isolation cannot be used as enrichment markers of human HSCs. The functional ability of

Figure 5. Markers used for HSC isolation.
Representation of phenotypical markers routinely used for FACS-isolation of mouse and human HSCs. Conventionally used murine markers allow for a higher purity of HSCs (about 1 in 5 cells) compared human markers, which can only enrich HSCs to a purity of about 1 in 10. Alternative markers represent markers that can be added to or replace the commonly used markers. For replacement strategies, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (mouse) and Lin^-CD34^+ (human) are typically still included.

immature hematopoietic cells to efflux Rhodamine-123 (Rho-123) and Hoechst 33342 (40, 63-65) is however shared, and can be utilized to enrich for primitive hematopoietic cells in both species. Purification of human HSCs usually rely on the CD34 antigen, which is expressed on human HSPCs (66). It should be mentioned that some $CD34^-$ human HSCs have also been isolated from CB (67-69), where they were suggested to be developmentally higher up in the hierarchy, i.e. more immature (68) than $CD34^+$ CB HSCs. However, studies of human bone marrow HSCs show that $CD34^+$ HSCs can generate $CD34^-$ HSCs that then in turn can produce new $CD34^+$ cells (70), indicating perhaps more dynamic relationships. Nevertheless, calculations suggest that > 99 % of human HSCs are likely included in the $CD34^+$ population (71). Further markers used for isolation of human HSCs include $CD38^-$ (71), $CD45RA^-$ (72), and $CD90^+$ (73). Additional inclusion of $CD49f^+$, thereby using a $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ surface marker phenotype, provides enrichment of long-term repopulating human HSCs to about 1 in every 10 cells (74).

FACS-purification allows for *in vivo* and *in vitro* functional evaluations of candidate HSC populations, as well as snap-shot investigations of cellular states. Competitive primary and serial transplantations of HSCs that are isolated based on the above-described markers are extensively used in HSC research and represent gold standard assays for evaluation of *in vivo* HSC competence. Serial transplantations are utilized in investigations where long-term competence and self-renewal capacity of HSCs are assessed, while primary transplantations are used for evaluations of more short-term behaviors. Serial transplantations force cells to undergo self-renewal and lineage differentiation. Only true HSCs are believed to possess capacity to endure multi-lineage maintenance of hematopoiesis through serial transplantations, and long-term HSC capacity can thus be studied in

an accelerated and/or challenged setting. Various progenitor and/or mature blood cell populations have however also shown relatively long-lived potentials. Despite the extensive use, serial transplantation experiments are not conducted uniformly between studies. In **Paper II**, we therefore evaluated and discussed advantages and pitfalls of different serial transplantation approaches, and found that serial transplantation study design could severely impact transplantation outcomes and interpretations of HSC behavior. Additionally, inconsistency in transplantation strategy complicate comparisons of results from different laboratories. Based on the findings in our study, we suggested a preferential experimental outlay that would maximize the chances to accurately assess long-term HSC competence.

Although transplantations are extremely useful for evaluations of *in vivo* biological performances they do not reflect physiological conditions. To avoid improper interpretations of non-steady state behaviors as steady state performances, recent experimental techniques allow for *in situ* (local; in its original position) labeling of HSC populations, thus reflecting conditions more similar to normal physiological conditions. The use of such techniques has demonstrated several key differences in HSC behavior between "stressed" and steady state hematopoiesis, although conflicting results between different steady state studies have been reported (75-78). Though these methods are much less invasive, they still require slight deviation from steady state, such as doxycycline or tamoxifen administration, which may influence on cell performance (79-81). Although these models enable evaluation of more unperturbed hematopoiesis, it is possible that HSC performances during steady state conditions of laboratory animals differ from normal human HSC behaviors, as pathogens, environment, and lifestyle conditions likely repeatedly challenge human hematopoiesis throughout life.

Advancements in single cell techniques, including sequencing (82), epigenetic evaluation (83, 84), and cell fate tracing (85, 86), allow for discrimination of regulatory patterns within populations that have previously been overshadowed by heterogeneity. Such distinct purity is many times not required during actual clinical therapeutic applications. They could however be necessary to uncover underlying regulatory mechanisms, as well as for the development and evaluation of new therapies. Combinations of different experimental methodologies, together with the ever-increasing bioinformatics tools and programs developed, will likely contribute extensively to new advancements within the hematopoietic field.

Humans *Versus* Mice

Ethical and technical constraints hinder application of numerous experimental techniques on human cells. Consequently, most basic research is conducted using model organisms with human similarities. One of the most important advantages with model organisms is their permissiveness for complex genetic manipulations. Such manipulations can be utilized to study e.g. specific gene functions in developmental- and tissue-specific contexts. Mice are commonly used in hematopoietic research, and are highly comparable to humans regarding genetics, anatomical organization, and physiology (71, 87). Differences between humans and mice, including size, lifespan, environmental exposure, and biological variation, result in both advantages and disadvantages for experimental research (**Table 1**).

Hematologic research is often conducted with inbred mice showing very limited biological variance (71, 88). As a consequence, common traits are relatively easily and quickly discovered when using low quantities of mice. Inbred mice do however not capture the biological variation that is observed among humans. Moreover, the relatively sterile conditions that laboratory mice are housed and bred under do not reflect the normal exposure to pathogens that humans encounter, which constitutes another constraint. The shorter lifespans (71) (around 3-4 years) and quick reproduction (88) of mice serve as enormous advantages for many

Table 1.
Examples of benefits and pitfalls connected to the usage of mice as surrogate organisms for human biology.

	Benefits	Pitfalls
Variance	• *Common traits are discovered faster and more easily* • *Reduced quantity*	• *Does not capture human biological variance*
Lifespan	• *Accelerated aging* • *Multiple generations in a relatively short time* • *Quick reproduction*	• *Distinct metabolic rates and lifetime turnover of cells*
Genetics	• *Allows for genetic modification that are unfeasible in humans*	• *All murine genes do not have or are not fully comparable to human counterparts*
Housing	• *Small → easy handling and transportation* • *Relatively cheap*	• *Distinct environmental exposure to microorganisms*

biological questions. Despite their short lifespans, mice develop aging phenotypes, which is greatly advantageous in aging research. However, human cells exhibit several-fold lower metabolic rates than mouse cells (87, 88). Human cells also have shorter telomeres (discussed further in "**Protective and Causative HSC Aging Mechanisms**"), and are thus more sensitive to replication-induced senescence (87). As human cells have higher lifetime turnover than mouse cells, the shorter telomere length may be a protective mechanism to avoid accumulation of replication-induced damage on proliferating cells (87, 88), and could play a more influential role in human than murine HSC aging.

Some differences between humans and mice can be overcome by generation of transgenic mouse models where human genes are introduced directly into the mouse genome (such as introduction of human-specific receptors that allow for infection with viruses that are normally not supported in mice) (89). When mouse models still fail to capture human cell behaviors, xenograft models, which allow for transplantation of cells between species, can be utilized. So-called *humanized mouse models* are engineered to allow for human reconstitution (90, 91), and are frequently used in experimental settings where *in vivo* behaviors of human HSCs are investigated. The bone marrow milieu in these animals differ however vastly from that in humans, and hematopoiesis is severely skewed and does not allow for long-term output of all mature lineages (90). Despite all constraints with humanized mouse models, they do allow for *in vivo* evaluation of some otherwise unmanageable experimental procedures on human cells.

In conclusion, utilization of mice as model organisms in experimental research is highly advantageous. Complex genetic alterations are achievable and evaluated using mice in ways that are impossible in humans (87). Not all findings are however translatable to the human system, and species-conserved patterns should therefore be addressed before utilizing mouse models. It should be noted though that incorrect interpretation of mouse experiments has been highlighted as a contributing cause of mouse-to-human irreproducibility (92), which may indicate that mice and humans are more similar than what is suggested in some translational research.

Hematopoietic Stem Cell Heterogeneity

Mouse single cell experiments have shown extensive heterogeneity within the HSC compartment with regards to e.g. lineage potential, self-renewal capacity, and cycling status (**Figure 6**). Such investigations have also revealed that robust self-renewal potential is not necessarily linked to balanced multi-lineage output. Durable serial reconstitution capacity of adult HSCs is however highly linked to slow proliferation kinetics (75, 93) (**Figure 6**). Several markers, including $Hoxb5^{+}$ (94), $CD150^{high}$ (94, 95), reactive oxygen species $(ROS)^{low}$ (96), $CD41^{+}$ (39) , $Sca\text{-}1^{high}$ (75, 97), $c\text{-}Kit^{low}$ (75, 98), $Tie2^{+}$ (99), $CD229^{-/low}$ (42) and $CD61^{+}$ (100), have all shown enrichments of cells with more robust reconstitution potentials within the proposed HSC compartment. Enrichments of HSCs with high reconstitution abilities using ROS^{low} (96) and $Tie2^{+}$ (99) phenotypes are both associated with mitochondrial functionality – ROS^{low} for obvious reasons as ROS are metabolic by-products produced in the mitochondria during energy metabolism (discussed further in "**Protective and Causative HSC Aging Mechanisms**"), but also $Tie2^{+}$ HSCs since these cells represent a subgroup within candidate HSCs with higher levels of mitochondria clearance (mitophagy) (99).

Numerous studies have also observed differences in the ability of single mouse HSCs to produce distinct mature lineages (**Figure 6**). Lineage-bias within the phenotypical HSC population has been proposed to arise when HSCs differentiate and commit to lineage fates at so early stages that extensive self-renewal potentials are still present and HSC-defining phenotypical markers have not yet been altered (101). Markers that are used to enrich for HSCs with high reconstitution abilities often also enrich for cells that show myeloid-biased (39-41), or balanced (41, 99) lineage output, while lymphoid-biased HSCs (41, 42) have lower reconstitution potentials (**Figure 6**). These findings have spawned a theory of that lymphoid-biased HSCs are differentiated progeny of LT-HSCs. It has also been suggested that lineage-biases within candidate HSCs arise when lineage committed progenitors return to stem cell states (102). HSC subtypes with distinct lineage preferences can also be subdivided into α, β, γ, and δ cells. α and β HSCs have robust serial transplantation abilities, and present with myeloid-biased and balanced lineage outputs, respectively. γ and δ cells, only reconstitute primary, but not secondary, recipients and are lymphoid-biased (41). It should be noted that hematopoiesis per se is myeloid-biased as extensively higher myeloid than

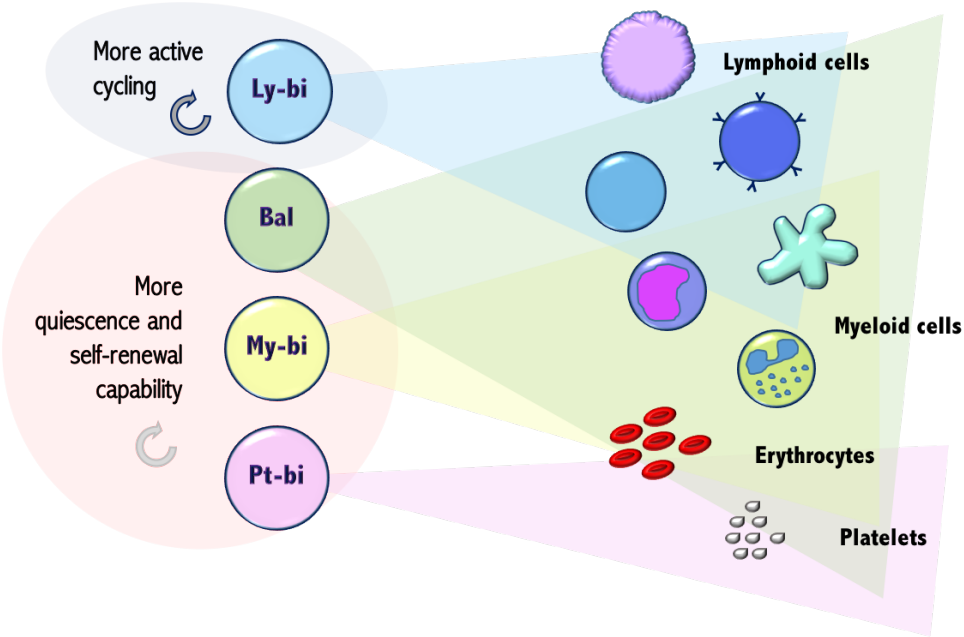

Figure 6. Mouse HSC heterogeneity.
HSCs are heterogeneous with regards to e.g. lineage potential, and proliferation status. Ly-bi = Lymphoid-biased HSCs, predominantly generating lymphoid progeny. Bal = Balanced HSCs, producing balanced outputs of myeloid and lymphoid cells. My-bi = Myeloid-biased HSCs, mainly generating myeloid progeny. Pt-bi = Platelet-biased HSCs, primed to generate cells of the megakaryocytic lineage.

lymphoid progeny is generated (78), which is presumably linked the short-lived nature and higher replenishment demand of myeloid cells.

With emergence of new models allowing for evaluation of donor-derived platelets in competitive transplantation settings came discoveries of platelet-biased cells within the HSC compartment (**Figure 6**). These include platelet-committed progenitors (38, 61), but also platelet-biased von Willebrand factor (vWF)-positive LT-HSCs (36) that can be activated upon acute platelet needs. High expression of c-Kit is also correlate to platelet-bias within the HSCs compartment. These c-Kit^{high} cells show increased platelet production at early time points after transplantation, while c-Kit^{low} HSCs are responsible for the majority of platelet production at later time points (98), indicating that c-Kit^{high} HSCs are activated during acute platelet needs.

Hematopoiesis Through Ontogeny

Hematopoiesis changes extensively during the course of an individual's lifetime. During embryonic development, hematopoietic activity is present in several different regions and with varying output. Although adult hematopoiesis is mainly restricted to one region, the bone marrow, essential alterations still occur with increasing age.

Emergence of hematopoiesis

Embryonic hematopoiesis is roughly divided into two waves. Two models explaining the emerging hematopoiesis and the two waves have been hypothesized. In the first model, the two hematopoietic waves are developed separately and independently of each other. In the second model, cells developed during the second wave originate from cells that were developed during the primitive wave (103). The first wave of hematopoiesis, also referred to as ***primitive***, or *transient*, ***hematopoiesis***, is transitory and occurs during embryonic

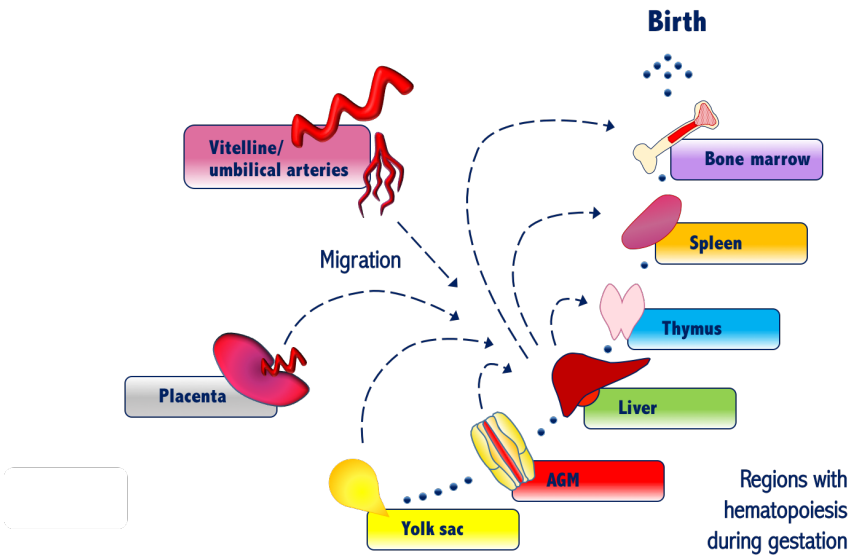

Figure 7. Sites with hematopoietic activity during development.
Hematopoietic activity first occurs in the yolk sac during very early development, after which production of hematopoietic cells can be found in the placenta, vitelline/umbilical arteries, and in the AGM region. Hematopoietic cells then seed the fetal liver, in which a massive expansion of HSCs occurs. From the fetal liver, HSCs migrate to the thymus, spleen, and ultimately the bone marrow, which is the major hematopoietic organ during adult life.

days 7-11 (E7-11) in mice (104). Transient hematopoiesis emerges from the extra-embryonic yolk sac at E7.0 (105, 106), where primitive forms of nucleated RBCs (104) and macrophages (106) are generated. At E8.25, erythro-myeloid progenitors (EMPs) emerge (106, 107). These then migrate to the liver (**Figure 7**), where they generate enucleated RBCs and fetal monocytes (106, 108).

The primitive hematopoietic wave is followed by a second wave of hematopoiesis, which is termed ***definitive hematopoiesis.*** This wave is characterized by the presence of definitive HSCs (d-HSCs), capable of sustaining durable hematopoiesis following transplantation. D-HSCs mature from pre-HSCs, which can develop into cells capable of long-term reconstitution in NK-deficient Rag2$\gamma c^{-/-}$ mice but not in wild-type (WT) mice if cultured with thrombopoietin (TPO) and stromal cells prior to transplantation (109, 110). The fetal liver does not contain *de novo* production of d-HSCs but is nevertheless a site for massive expansion of d-HSCs (109). D-HSCs can be found in the fetal liver at around E11.5 (111), after which the extensive expansion immediately occurs. As very few d-HSCs are found in the fetus before liver seeding, it is believed that the rapid expansion of d-HSCs in the liver is initiated primarily by maturation of seeded pre-HSCs (109). Prior to liver seeding, some d-HSC have however been observed in several areas, including the yolk sac (109, 111), vitelline/umbilical arteries (112), placenta (111), and the aorta-gonad-mesonephros (AGM) region (109, 111, 112). From the fetal liver, hematopoietic cells then migrate to the spleen, thymus, and ultimately the bone marrow, where they reside throughout adult life (110, 113) (**Figure 7**).

It is still under debate where the d-HSCs that seed the bone marrow and ultimately give rise to adult hematopoiesis originate, or if several sources exist in parallel. Lack of source-specific factors (103), as well as the early onset of circulation, beginning at E8.25 (114), complicate investigations of d-HSC origin. Fate mapping experiments suggest that the yolk sac is contributing to at least a fraction of adult hematopoiesis (115, 116), but does not rule out parallel sources. Several other studies support the notion of *de novo* d-HSC production in the AGM region (117-119).

Adult hematopoiesis

During postnatal, ***adult hematopoiesis***, blood cell production is mainly maintained within the bone marrow. At around 2-3 weeks after birth, mouse HSCs switch to adult behaviors. Compared to fetal HSCs, adult HSCs are more quiescent and demonstrate lower reconstitution potentials upon transplantation. Fetal and adult HSCs also show differences in lineage output and in response to external stimuli (120-123). The fetal-to-adult HSC switch occurs concomitant with decreased expression of the transcriptional regulator Sox17, which is required for development and maintenance of fetal, but not adult HSCs (124). Increased expression of the transcription factor C/EBP-α has also been implicated in the switch, where it controls HSC cycling/quiescence (125). Other regulators involved in the developmental switch include Bmi-1 (126), Ezh2 (127), and the Lin28-let-7-Hmga2 pathway (128-130).

Several mature blood cell subsets have shown to primarily or even restrictively arise from fetal hematopoietic sources, and cannot be generated from adult HSCs. Such cells include several tissue-resident macrophages, including microglia (in brain), Langerhans cells (in epidermis), and Kupffer cells (in liver) (131), as well as B-1a cells (132), and certain T cells (128). The majority of the tissue-resident macrophages originates from the yolk sac (108). As fetal and adult HSCs comprise very different functionalities, careful consideration should be applied when selecting cellular source for experimental studies. This applies not least to human HSCs, where CB-derived HSCs are often selected over bone marrow-derived HSCs due to their high availability and superior engraftment potential in humanized mouse models.

Aging hematopoiesis

Aging typically presents with tissue function impairments, and diminished capacity to maintain homeostasis (133). Hematopoietic aging further associates with e.g. adaptive immune response impairments (134, 135), clonal hematopoiesis (136, 137), and increased incidence of several hematological complications, including anemia (138) and myeloid malignancies (139). At least some age-associated mouse hematopoietic phenotypes have been linked to alterations within the primitive HSC compartment. Aged mouse HSCs are also increased in frequency (140, 141), have impaired reconstitution capacities (141), and show

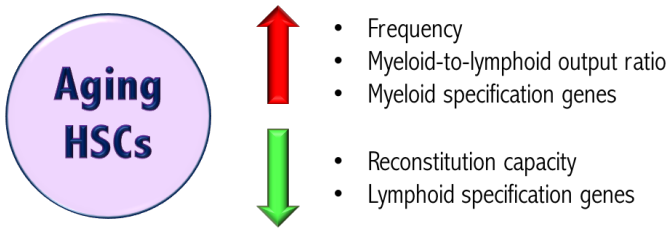

Figure 8. Shared characteristics of human and mouse HSC aging.
Aging of HSCs associates with increased HSC frequencies, but impaired reconstitution capacities. Furthermore, aged HSCs present with myeloid-biased output behaviors, that are presumably linked to increased expression of myeloid-specific genes and downregulated expression of lymphoid-specific genes.

increased myeloid-to-lymphoid output ratio (primarily caused by reduced lymphoid output) (141-143). Although strain-specific, increased mouse HSC frequencies correlate with increased HSC numbers (140) (**Figure 10**). It is plausible to assume that the increased frequency of HSCs at least partially represents compensatory means aimed at rescuing impaired functionalities. Moreover, transcriptional studies have revealed that myeloid specification genes are upregulated in aged mouse HSCs, while genes associated with the lymphoid lineage are downregulated (141). Genes involved in DNA repair, chromatin organization, cell cycle, and DNA replication also show differential expression patterns between young and aged mouse HSCs (144, 145) (**Figure 8**). Aging of human HSCs is much less studied but has reported some similar phenotypes, including increased HSC frequencies, increased myeloid-to-lymphoid output, and altered transcriptional expression patterns of lineage-associated genes (146) (**Figure 8**). Conflicting results have however also been reported (147). **Paper III** in this thesis aimed to clarify age-associated changes of human HSPCs, and compared these to alterations in mouse HSPCs to identify species-conserved age-related patterns.

Protective and Causative HSC Aging Mechanisms

The long-lived nature of HSCs makes them plausible targets for transformations that can drive aging phenotypes and malignant initiation. Several elements have been suggested to contribute to HSCs aging, including accumulation of destructive cellular products and damage, mitochondrial dysfunction, epigenetic alterations, proliferative exhaustion, and environmental influences. HSCs however harbor

several mechanisms that protect against damage accumulation. Proposed mechanisms of the former and latter are discussed below.

Protective mechanisms

Most adult HSCs reside in a non-dividing ***quiescent*** cell cycle state (140, 148) and are only occasionally recruited into cell cycle. The quiescent nature protects HSCs against cell cycle-induced damage, such as cell cycle stress, DNA replication errors, and senescence (**Figure 9**). However, quiescent HSCs rely on the more error-prone non-homologous end joining (NHEJ) mechanism for reparation of DNA double-strand breaks (DSBs) (149, 150), as the less error-prone homologous repair (HR) machinery requires cell cycling. HSCs with high proliferation history are more likely to undergo differentiation (27) and lose long-term reconstitution capacity (75, 93) compared to HSCs that have undergone fewer cell divisions. Stress-induced exit of HSCs from quiescent states is linked to increased metabolic activity, ROS production, and generation of DSBs (151).

To further minimize ROS-induced damage, HSCs rely on anaerobic ***glycolysis*** rather than oxidative phosphorylation for mitochondrial energy production (152). Glycolysis generates less ATP, but also less ROS (**Figure 9**). Furthermore, human mammary stem-like cells ***separate mitochondria asymmetrically*** during proliferation so that differentiating daughter cells receive higher proportions of old mitochondria than daughter cells that maintain stem cell characteristics (153) – a feature that is potentially also shared by other stem cells, including HSCs. Additional protection from oxidative stress is provided by ***low oxygen concentrations*** in the bone marrow environment where HSCs reside (154, 155) (**Figure 9**).

Telomeres are repetitive non-coding sequences at the ends of linear chromosomes, which serve as protective elements during replication. Due to the inability of DNA polymerases to replicate linear chromosomes to the very ends, DNA chromosomes shorten during each cell division. Telomeres hinder attrition of the cellular genome (**Figure 9**), and prevent against interpretation of chromosome ends as DSBs. When telomeres reach critical lengths, apoptosis or senescence is induced. Extension of telomeres through activation of the telomere-building enzyme telomerase is a known hallmark of cancer cells, and required for indefinite proliferation. Some stem cells, including HSCs, also demonstrate slight telomerase

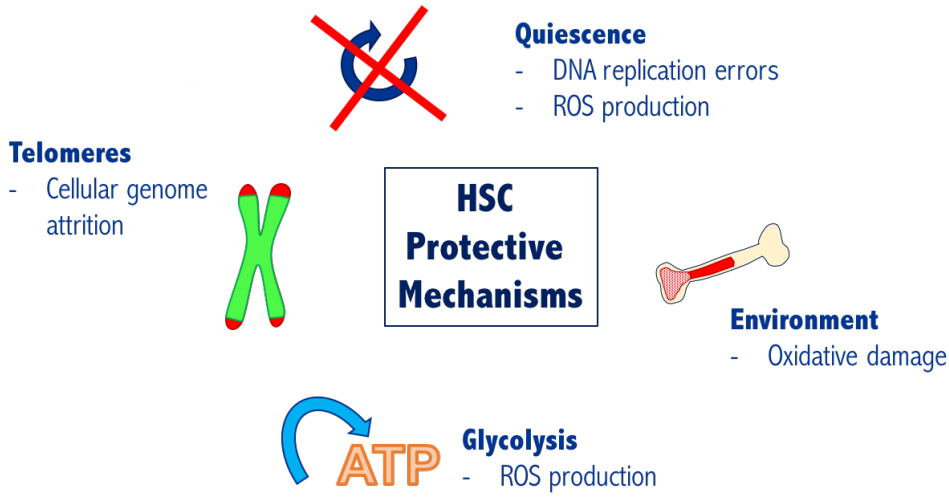

Figure 9. HSC protective mechanisms and features they protect against.
HSCs harbor several protective mechanisms that prevent accumulation of damage. Telomeres protect against attrition of the cellular genome, while the quiescent nature of HSCs protects against cell cycle-induced damages, including replication errors and generation of ROS. HSCs also rely on glycolysis for energy production, which results in lower ROS production compared to oxidative phosphorylation. HSCs are additionally protected against oxidative stress by the low oxygen environment in the bone marrow that the HSCs reside in.

activity (156), which may be a means to reduce telomere shortening in long-lived proliferating cells.

Clonality and aging

Several studies have demonstrated transcriptional alterations during aging of HSCs. As mentioned earlier, these changes include upregulation of genes involved in myeloid specification, while lymphoid-specification genes are downregulated on a population basis (141, 146) (**Figure 10**). Such findings suggest that the age-associated increased myeloid-to-lymphoid output ratio is linked to changes occurring in the HSC compartment. Two hypotheses have been suggested to explain transcriptional alterations in aging HSCs. The first hypothesis is based on ***clonal evolution***, where alterations in gene expression patterns occur within individual HSCs. In the second theory, called ***clonal composition shift***, the composition of functionally different HSC clones is altered through relative expansion/reduction of different subsets. Both of these suggestions would lead to overall transcriptional changes. It is likely that both mechanisms contribute to the aging phenotype. In support of age-associated changes in HSC clonal composition are findings showing that the frequencies of myeloid-biased (39) and platelet-

biased (157) HSC clones increase with age, while lymphoid-biased clones decrease (158) (**Figure 10**). Aged HSCs not only demonstrate overall impairments in repopulation activity, but also on per cell bases when HSCs are separated into myeloid-biased and lymphoid-biased HSCs (159), indicating that also clonal evolution contributes to HSC aging (**Figure 10**).

Myeloid-biased HSCs show superior reconstitution potentials compared to lymphoid-biased HSCs upon transplantation (39-42). Therefore, it would be plausible to assume that clonal selection over time would lead to clonal composition alterations in the HSC pool in favor of myeloid-biased clones. Acquired mutations may however also provide favorable behaviors and trigger expansion or increased survival of individual HSC clones. Age-associated accumulation of specific somatic mutations in human peripheral blood cells indicate that aging is associated with clonal hematopoiesis (160, 161), where decreasing numbers of clones are responsible for hematopoiesis. A study of a 115-year old woman suggested that her hematopoiesis was sustained by only two clones (162). Around 80 % of the mutations found in clonal hematopoiesis are in genes connected to epigenetic regulation or RNA splicing (160), suggesting that such alterations are involved in expansion advantages. Of these, mutations in the epigenetic regulators DNMT3, TET2, and ASXL1 are most common (161). Although clonal hematopoiesis is linked to increased risks of developing hematologic malignancies, the majority of individuals with clonal hematopoiesis never acquire any of these disorders (161).

Further compositional changes in aging mouse HSCs include decreased frequency of cells with polar distribution of laminar proteins. Loss of such polarity is linked to increased activity and scattered distribution of Cdc42 (a small RhoGTPase known to regulate cell polarity, including actin and tubulin distribution). Pharmacological inhibition of Cdc42 in aged HSCs increases the frequency of polarized cells and ameliorates several age-related phenotypes, including the impaired reconstitution capacity and lineage skewing (163) (**Figure 10**).

Finally, it has also been suggested that the compositional distribution of aging HSCs is altered with regards to the frequency of HSC that are actively cycling. Whether or not the HSC cycling state is altered during aging is still under debate however, as lower (143, 146, 164), higher (165), and more similar (75, 140) frequencies of quiescence in aged HSCs have all been reported.

Genetics and epigenetics

Accumulation of ***genomic damage*** has been suggested to drive HSC aging. Indeed, mouse models with defective DNA repair mechanisms present with several HSC aging-like phenotypes, including decreased self-renewal, impaired reconstitution ability, and increased apoptosis, although typically not with increased myeloid-to-lymphoid output ratio (166). These conditions often ultimately result in LT-HSC exhaustion (151, 167). DNA damage is usually measured indirectly by the presence of phosphorylated histone H2AX (γ-H2AX) foci, which are phosphorylated at DSBs during recruitment of repair proteins. Observations based on such foci have suggested increased DNA damage in aging HSCs (167, 168) (**Figure 10**), thus providing a link between aging and DNA damage accumulation. Phosphorylation of H2AX can however also occur during replication stress, which was found in a study by Flach *et al*. to be the main cause of age-associated accumulation of γ-H2AX foci (169). Another study suggested that although DSBs increase in aging HSCs, they are robustly repaired when the cells enter cell cycle (170), which is in contrast to studies showing that stress-induced HSC cell cycle entry results in accumulation of DNA damage (151, 171).

Rejuvenation of aged HSCs by passage through induced pluripotent stem cell (iPSC) states has demonstrated that changes in DNA sequence per se are dispensable for normal murine HSC aging (172). In this study, old mouse HSCs were reprogrammed into iPSCs from which new mice were subsequently generated. HSCs generated from the iPSCs did not exhibit aging phenotypes (172), indicating that epigenetic components rather than changes in DNA sequence drive HSC aging. These experiments do however not rule out that DNA damage responses (DDRs) induced by accumulated DNA damage are involved in HSC aging. DNA damage checkpoints evoked by DNA damage can result in cell cycle arrest, senescence and apoptosis (173), which may all contribute to the aging HSC phenotype. In support of this are findings showing that several age-induced HSC features, including myeloid/lymphoid output skewing and reduced reconstitution potential, are ameliorated upon deletion of the DDR-associated protein Periodic circadian clock 2 (Per2) (174) that normally increases in aging HSCs (preferentially in lymphoid-biased HSCs).

The term ***epigenetics*** describes patterns of gene expression or phenotype that are inheritable independently of DNA sequence, and include specific modifications to the DNA itself, including DNA methylation or hydroxymethylation, and to

histones, such as methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation (175, 176). Changes in the epigenetic landscape have been suggested to influence age-associated alterations of self-renewal and differentiation (53, 144, 177). The expression of chromatin modifiers is altered in the aged HSC compartment (141, 145) (**Figure 10**), and includes decreased expression of the chromatin organizer Special AT-rich sequence-binding protein 1 (Satb1) (178). Overexpression of Satb1 in aged HSCs improves *in vitro* lymphoid generation (178), demonstrating involvement of Satb1 in the aging HSC phenotype. Chromatin modifiers are also linked to regulation of DDR, telomere maintenance, and cellular metabolism (175), thus connecting epigenetics to several suggested aging mechanisms. Changes in the epigenetic landscape of aging HSCs further include increased DNA methylation at several lymphoid and erythroid loci (179) – a marking that is typically associated with gene repression. Moreover, the activating histone modification trimethylation of histone H3 at lysine 4 (H3K4me3) is increased at genes associated with HSC self-renewal (144). It has been suggested that old HSCs promote self-renewal over differentiation in a process that involves reduced TGF-β signaling (144). Single-cell RNA sequencing studies of young and aged HSCs have demonstrated that relatively fewer aged cells are engaged in differentiation trajectories (53), agreeing with reduced differentiation potentials. Together, these results clearly allocate a role for epigenetics in the aging HSC phenotype. The previously-mentioned successful re-setting of the aged HSC epigenetic landscape demonstrate however that these changes can be reversed (172). It should be noted that epigenetic profiling of young and aged HSCs has due to technical constraints only been conducted on pools of HSCs, where contribution of compositional change is not assessed. Furthermore, although the iPSC-induced epigenetic re-setting of aged HSC caused rejuvenation (172), it is possible that functions associated with the iPSC state or elsewhere during the developmental progression contribute to HSC rejuvenation and may not be fully recapitulated solely by direct conversion of old-to-young epigenetic landscapes.

Telomere attrition, as mentioned previously, ultimately results in senescence or apoptosis, and is yet another suggested mechanism for HSC aging (**Figure 10**). Telomere deficiency-mediated syndromes, such as dyskeratosis congenita, present with premature aging-like phenotypes (180), which supports influence of telomeres on aging. Moreover, telomerase-deficient mouse HSCs show impairments in repopulation capacity, self-renewal, and proliferation, as well as exhibit increased apoptosis (167). Telomerase activation does however not abolish

serial transplantation exhaustion of HSCs (181), which contradicts contribution of telomere shortening to normal mouse HSC aging. In further support of a dispensable role for telomere shortening in normal mouse HSC aging, is the observation that laboratory mice have several-fold longer telomeres than humans (182), but still present with similar age-associated HSC impairments. Telomere attrition-induced responses may however play more influential roles for human than for mouse HSC aging.

Mitochondria and ROS

In the "***mitochondrial theory of aging***", aging phenotypes are driven by ROS-induced cellular damage (183). Mitochondrial genome is, unlike the nuclear genome, not protected by histones, and the polymerase responsible for replication of the mitochondrial DNA (mtDNA) is more error-prone than nuclear DNA-replicating polymerase (184). This has led to a theory of a vicious cycle where ROS-induced mitochondrial damage causes defective mitochondria that in turn drive further production of ROS. Given the close proximity of ROS and mitochondria, and an age-related increase in ROS levels (**Figure 10**), this is a plausible theory. However, as mutations of mtDNA have shown to cause little to no increase in ROS production, this theory has been questioned (185). Furthermore, although mouse models with defective mtDNA repair machinery accumulate mtDNA mutations and present with aging-like hematopoietic phenotypes, these show distinct mechanisms compared to normal aging (186). The amount of mutations in these models is also considerable higher than in the normal aging setting (187).

It was recently demonstrated that aged murine HSCs contain higher levels of ROS compared to young HSCs (**Figure 10**), which is caused by both increased production of ROS and diminished anti-oxidant pathways (143). As mentioned previously, lower levels of ROS in HSCs associate with increased reconstitution capacities (96), thus suggesting a link between age-associated increase in ROS and the reduced HSC function. It should be mentioned however that though ROS can unequivocally be harmful, it is also involved in cell signaling and stem cell fate regulation (188, 189). Further indications of ROS involvement in aging are provided by studies showing that loss of the signaling transduction protein SH2B (Lnk) in flies protects against oxidative stress, and results in lifespan extension (190). Interestingly, Lnk-defective aged mouse HSCs also display improved

functions, including higher reconstitution potentials and more balanced lineage output patterns (191) (**Figure 10**).

Alterations of mitochondrial metabolism have frequently been implicated in studies where increased longevity is achieved. These studies typically involve factors of the mitochondrial respiration machinery, stress response machinery, and/or antioxidant factors. However, the majority of these studies are performed in yeast, worms, or flies (192, 193), and results have been very conflicting (193-198). To complicate matters further, tissue-, timing-, and dose-dependent effects have also been observed (193, 199, 200). Surprisingly, several studies have observed lifespan extensions upon mitochondrial stress, which is presumably caused by activation of the mitochondrial unfolded protein response (UPR^{mt}), and/or altered insulin signaling (199, 201). Mouse lifespan is generally not affected by antioxidant alterations (202, 203), although positive effects have occasionally been reported (204). Circulating serum levels of insulin-growth factor-1 (IGF-1) decrease in aging humans (205), which is suggested to influence aging phenotypes. Contradictory however, reduction of IGF-1 in mice increases the maximum lifespan (206). Furthermore, reduction of IGF-1 levels induced by prolonged fasting of aged mice improve HSC behaviors with regard to reconstitution potential and lineage skewing (207). The mammalian target of Rapamycin (mTOR) is a nutrient-sensing protein that is activated by insulin signaling (208) and is upregulated with age. Inhibition of this pathway in mice ameliorates aging HSC phenotypes, including the effects on HSC numbers, reconstitution capacity, and myeloid skewing (209) (**Figure 10**).

Possibly also connected to mitochondrial function and aging are sirtuins, which are a class of nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent proteins with deacetylase or ADP-ribosyl transferase activity. Sirtuins are found in the nucleus, cytoplasm, and mitochondria. Both NAD^{+} and sirtuin activities decrease with age (**Figure 10**), indicating a potential link to HSC aging. Sirtuin activation has been implicated in organismal longevity, although a lot of controversy has been reported here also (210). Nevertheless, upregulation of SIRT3 in old mouse HSCs decreases ROS levels and improves regeneration capacity (211) (**Figure 10**). SIRT1 (212) and SIRT7 (213) have also shown involvements in aging-like phenotypes of HSCs, although SIRT1 has shown conflicting results (214).

Taken together, factors involved in mitochondrial and ROS activities appear to be very complexly regulated, difficult to interpret, and perhaps not species-conserved.

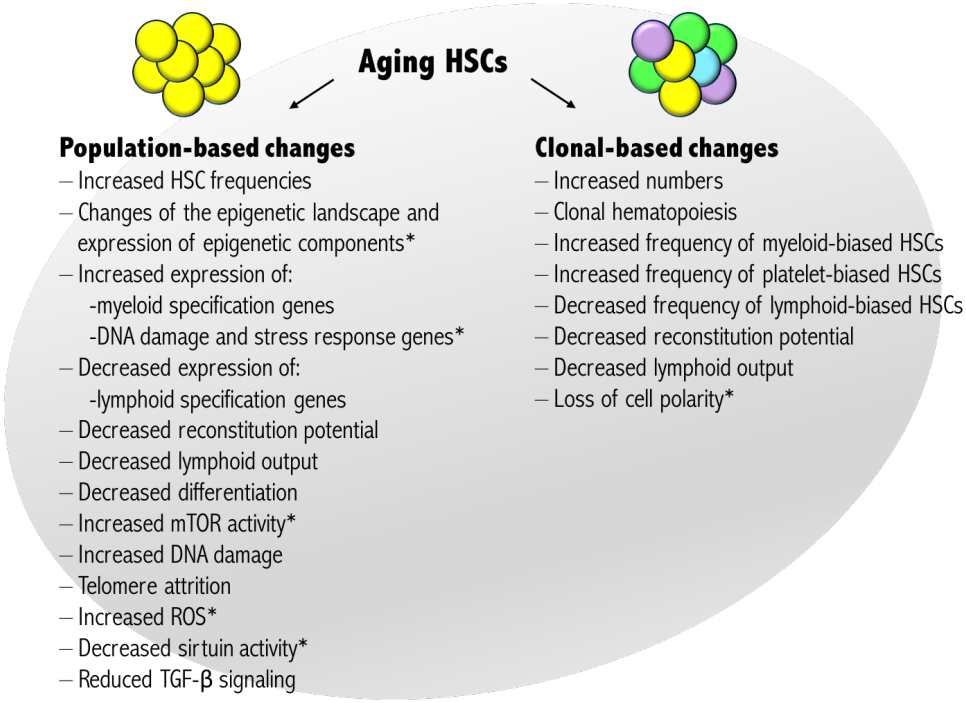

Figure 10. Population-based and clonal-based alterations of aging HSCs.
Numerous changes have been identified in the aging HSC compartment. Clonal studies have revealed that many of these are impacted by alterations in the clonal composition of HSCs. Additional studies are required to uncover compositional contribution to several HSC aging effects that have not yet been addressed on a per cell level. Such distinction is necessary for the understanding of underlying factors that drive HSC aging. *Rejuvenated/improved functions of aged HSCs when counteracted.

On this note, it is worth mentioning that interventions aimed at altering metabolic activity is predicted to show little to no effect in non-obese human populations as human cells are much more metabolically stable than the cells in the animals where rejuvenation effects were observed (215).

Niche

It was early suggested that HSCs reside in specific environments that support HSC functions (29). These locations are termed HSC niches and are still debated as technical limitations hinder simultaneous visualization and functional assessment of HSCs in the bone marrow environment. Two different niches are generally proposed – the *endosteal niche* that lines trabecular bones and mainly supports quiescence, and the *perivascular niche* of the sinusoidal blood vessels that

regulates proliferation and differentiation of HSCs (216). Both cells of hematopoietic origin, such as megakaryocytes (217-220), monocytes/macrophages (221), regulatory T cells (Tregs) (222), and osteoclasts (223), as well as of non-hematopoietic origin, including osteoblasts (224), endothelial cells (225), stromal cells (226), and adipocytes (227, 228), have been implicated as niche cells important for HSC regulation. Although these cells have shown to be influential on HSC behavior, less is known about their alterations and potential involvements in HSC aging (229).

Several aging phenotypes are indisputable intrinsically imprinted as demonstrated by recapitulation of aging characteristics upon transplantation of aged HSCs to young hosts (141, 230). However, slight myeloid skewing is also observed upon transplantation of young bone marrow cells to aged recipients, indicating that also extrinsic factors contribute to HSC aging (230). The myeloid skewing inflicted by the environmental niche has been linked to increased levels of the inflammatory cytokine RANTES in the aged bone marrow (not caused by increased expression within LSK cells), which impair T cell potential (231). Megakaryocytes are found in close proximity to HSCs in the bone marrow (217-219), thus suggesting a direct involvement of megakaryocytes in HSC regulation. Megakaryocytes regulate HSC quiescence and expansion in a process where TGF-β (218) and CXCL4 (219) are influential. As myeloid-biased and lymphoid-biased HSCs exhibit distinct responses to TGF-β (40), age-associated reduction in TGF-β signaling in the HSC pool (144) (**Figure 10**) may be caused by altered megakaryocyte-HSC interactions. Altered stimulation of TGF-β signaling may be influential on clonal composition changes in the HSC compartment, but it is also possible that the reduced overall TGF-β signaling is a consequence, rather than a causation, of compositional changes. Environmental involvement in HSC aging is further suggested by age-related functional defects of bone marrow stromal cells (232, 233). Stromal cells expressing the P450 retinoid-inactivating enzyme 26B1 (CYP26) are important for regulation of self-renewal in human $CD34^{+}CD38^{-}$ hematopoietic cells by inhibiting retinoic acid (RA) signaling (which otherwise induce differentiation) (234). Changes of stromal cells could thus be linked to alterations in self-renewal capacity in aging HSCs. Moreover, the age-associated increased adipogenesis (235) could potentially also influence HSC functionality as adipocyte-conditioned medium has shown to increase myeloid and decrease lymphoid output from bone marrow cells in cell cultures (228). In conclusion,

several potential involvements of the bone marrow niche to HSC aging have been suggested, although direct influences are yet to be demonstrated.

Inflamm-aging

Aging associates with a chronic, low-grade inflammation, referred to as immunosenescence or "inflamm-aging", concomitant with increased circulation of pro-inflammatory cytokines. Included in the immunosenescence concept are also age-associated immune response impairments and increased autoimmune susceptibility (236). There is thus indisputably at least a correlative link between inflammation and aging. HSPCs respond directly to infection, in which ST-HSCs and MPPs produce numerous amounts of inflammatory cytokines, including TNF-α, IL-6 and IL-1β (143, 237). Several studies suggest initial proliferative effects of HSCs upon different inflammation provocations, such as lipopolysaccharide (LPS) stimulation (238), or interferon signaling (151, 239, 240). The increased proliferation is then followed by reduced self-renewal ability upon long-term exposure. Aging and inflammation-induced HSC phenotypes also contain other similar features, including myeloid-biased output productions (238, 241). Together, these findings are indicative of a potential inflammatory contribution to HSC aging. Inflammation-induced effects could possibly compose much higher contributions to normal human aging phenotypes than what is captured in the relative sterile steady state housing environments of laboratory mice.

Senescence

Senescence is a state of irreversible growth arrest that is accompanied by metabolic alterations and secretion of damaging factors referred to as senescence-associated secretory phenotype (SASP). Senescence can be trigged through many different stressors, such as telomere attrition, ROS, and interferon exposure. These stressors evoke different pathways and definite markers for senescence-induction are therefore lacking. Nevertheless, frequent markers used for indication of senescence include $p16^{Ink4a}$, and expression of senescence-associated β-galactosidase (242). Senescent cells accumulate with age in several tissues (143, 243). Clearance of senescent cells ameliorates age-associated degeneration (244, 245), suggesting an impact of senescent cells on aging. Treatment of mice with ABT236 (an inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL), removes

senescent cells and mitigates age-related reconstitution impairment and lineage skewing of HSCs (245). Clearance of $p16^{Ink4a}$-expressing cells in mice also extends lifespans (244), but hematopoietic effects were not evaluated in this study. $p16^{Ink4a}$ expression has been reported in old LT-HSC, and $p16^{Ink4}$ KO mice show increased frequencies of HSCs (246), suggesting a role for $p16^{Ink4}$ in HSC aging. Such findings were however challenged in subsequent investigations, which found no expression of $p16^{Ink4a}$ in either young nor aged HSCs (247), indicating that potential beneficial effects on HSCs from removal of senescent cells are likely not caused by removal of $p16^{Ink4}$-positive HSCs.

Aging summary

The global average lifespan in humans is continuously increasing (248), and as a consequence so are age-related pathologies. Improved knowledge of age-associated alterations will most definitely contribute to further development of treatments involved in promoting continuously increasing health spans. Several mechanisms have been suggested to drive aging phenotypes in HSCs. Many of these pathways also interact, thus complicating distinction between causal and correlative effects. Aging-accompanied functional alterations of HSCs are largely intrinsically determined, as transplantation of aged HSCs to young recipients recapitulates aging phenotypes (141). Extrinsic components have however also been identified. It is important to note that "normal" aging mechanisms may differ from aging-like phenotypes. Furthermore, separating aging-*inducing* mechanisms from aging-*induced* mechanisms is challenging, but required to understand the complicated system of interacting factors that drive and execute aging phenotypes. Nevertheless, successful rejuvenation attempts, including epigenetic re-setting, re-polarization of tubular protein distribution, and removal of senescent cells, indicate that aging phenotypes are not eternally fixed, but can be reversed, and bring hope to development of not only protective, but also restorative therapies.

Discussion of Articles

Article I

Frequency determination of rare populations by flow cytometry: a hematopoietic stem cell perspective.
Rundberg Nilsson A, Bryder D, Pronk CJ.
Cytometry A. 2013 Aug;83(8):721-7. doi: 10.1002/cyto.a.22324. Epub 2013 Jul 9. Review.

Aim: To evaluate crucial aspects that can influence analysis and interpretation of flow cytometry experiments when studying infrequent cell populations, such as HSCs.

Flow cytometry-based experimental methods are frequently used in HSC research. Today's instrumental advancements and fluorochrome availability allow for increasing numbers of markers being separated. Such advancements are accompanied by increased demands for sample preparation and analysis. We therefore in this report set out to highlight some important aspects in connection to this, particularly for analysis of very infrequent cell populations, such as HSCs. We also present a new 17-parameter combination for simultaneous evaluation of an extensive number of mature and immature hematopoietic cell populations, including HSCs, MEPs, GMPs, CLPs, $CD11b^+$ myeloid cells, $CD19^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells, in one human bone marrow sample.

First, we discuss the utilization of controls, including biological, gate-setting, and compensation controls. We presented internal reference populations (IRPs), that are based on gate-setting according to preexisting knowledge of expression levels in other populations, as a complement to fluorescence-minus-one (FMO) controls. Although differences in autofluorescence and unspecific binding may differ between populations, IRPs are very valuable for capturing of general unspecific binding. Such controls may also spare precious sample when used as replacements for FMOs, particularly if many markers are used. Sparing of valuable samples can

also be achieved by the usage of compensation beads when correcting for spectral overlap. This is of particular advantage when the antibody only binds to a minor fraction of the cells. Although compensation of signals using stained sample cells is sometimes necessary, compensation beads are many times better alternatives.

Multicolor flow cytometry allows for identification of populations that require multiple markers. Increasing the number of different fluorochromes in a sample however also increases spectral overlap and unspecific binding, which may impair the potential to separate signals. Moreover, when analyzing the data of a flow cytometry experiment, it is important to remove unwanted events before analyzing the expression levels of the intended populations. This "pre-gating" allows for exclusion of e.g. dead, clumped, and undesired cells. Undesired cells can preferentially be identified by antibodies conjugated to the same fluorophore, a so called "dump channel", which minimizes the number of different fluorophores used. Analysis of flow cytometry data would preferentially also include at least one positive marker for each analyzed population to separate the intended cells from other negative events. Acquiring of a sufficient number of events is also crucial to distinguish true positive signals from false positive events and to establish proper estimates of frequency levels.

Finally, we discussed how flow cytometry data can be presented to allow for clear insights of the interpretation of the analyzed data. We discuss choice of plot graph, biexponential display, visualization of controls to demonstrate positive *versus* negative signals, and sample comparison alternatives. Contour plots are frequently used and give good overviews of intensity distributions of signals. Dot plots are however more useful when presenting plots/gates with few events, especially when containing scattered outliers whose significance can otherwise be difficult to interpret. Furthermore, visualization of events lying on negative axes by exponential display is key to ensure inclusion of all intended events. When presenting and comparing data, consideration should also be taken to what type of comparison that is most appropriate, whether it is absolute numbers or frequencies, and in the latter case, within what population that is compared.

Article II

Probing hematopoietic stem cell function using serial transplantation: Seeding characteristics and the impact of stem cell purification.

Rundberg Nilsson A, Pronk CJ, Bryder D.

Exp Hematol. 2015 Sep;43(9):812-7.e1. doi: 10.1016/j.exphem.2015.05.003. Epub 2015 May 29.

Aim: To evaluate crucial aspects in study design of serial transplantation experiments that may affect interpretation of HSC self-renewal capacity and lineage output.

Serial transplantation of HSCs is the main method used for evaluation of long-term *in vivo* self-renewal ability. Experimental study design however differs between laboratories, and may severely hamper both comparisons of different studies and interpretation of results. To address this matter, we in this study discuss and demonstrate how different experimental parameters, specifically competitive serial transplantation of whole bone marrow (wBM) or FACS-purified HSCs, can affect result interpretation. Based on these results we then propose the outlay of an experimental design that maximizes the possibility to correctly evaluate long-term self-renewal capacity of HSCs. We also highlight the importance of keeping biological replicates separated, as stochastically acquired outlier effects could otherwise be transferred to whole groups of animals.

Most often, wBM isolated from competitively transplanted recipients is used for serial transplantation purposes. Due to the high cellularity in the bone marrow of bones relative to what is required for survival of lethally irradiated recipients, bone marrow is typically isolated from only one or a few bones. By competitively transplanting mice and evaluating donor contribution levels in individual bones of the hind limbs three months after transplantation, we could show that HSC donor chimerism levels fluctuated distinctly among separate bones of individual recipients. This finding indicated that very limited HSC recirculation occurs within the first three months after transplantation, and that chimerism levels obtained by analysis of only a few bones may therefore poorly represent the overall chimerism. Unevenly distributed donor-HSCs may in turn also hamper evaluation of long-term HSC ability if wBM from a limited number of bones is serially transplanted. Re-purification and subsequent transplantation of donor-derived HSCs would

however correct for bone distribution differences, although not for differences in clonal distributions, which is why isolation of cells for serial transplantation from multiple bones is always encouraged.

Another caveat with the usage wBM as a source for serial transplantations is the potential presence of long-lived progenitor and/or mature cells that hinder correct evaluation of HSC output. To approach this concern, we compared long-term mature B, T, and myeloid cell output levels in recipients transplanted with wBM to those transplanted with purified HSCs. Recipients transplanted with wBM exhibited a relative T-lymphoid bias among donor-derived cells, potentially caused by the presence of long-lived lymphoid cells. This finding suggests that non-HSCs can contribute to long-term mature output in transplantation experiments, and lineage output may thus not be reflective of that from the serially transplanted HSCs.

Competitive serial transplantation of re-purified HSCs permit discrimination of short-term effects, seen in primary transplantation, and long-term effects, observed in serial transplantations, while serial transplantation of wBM may transfer short-term effects into the long-term evaluation, thereby obscuring distinction between these effects. In these cases, wBM transplantations could e.g. aggravate interpretations of conditions in which HSCs initially present with increased proliferation followed by exhaustion. The increased number of HSCs transplanted from primary recipients could then mask per cell HSC impairments and be interpreted as exhibiting similar or even superior long-term performances. Unless long-term outcomes are normalized to those observed in the short-term evaluation, comparisons of mice serially transplanted with wBM may not reflect long-term HSC behaviors. Moreover, occasions where e.g. the relative frequency of HSCs in the bone marrow is decreased due expansion of non-HSC populations would lead to fewer numbers of HSCs being transplanted in wBM samples. A lower donor chimerism may then wrongfully be interpreted as impaired HSC function. Transplantations of purified HSCs would avoid these issues and allow for direct comparisons of per cell HSC performances in serially transplanted hosts.

Based on the findings and speculations in this study we therefore suggest that serial transplantation of purified HSCs, compared to of wBM, is advantageous for evaluations of long-term HSC behavior. However, in contrast to transplantation of purified HSCs, transplantation of wBM does not exclude HSCs residing within other phenotypical marker combinations than those used for HSC isolation. At

times where this is the case, determination of HSC frequencies would have to be established through other methods, and evaluations of HSCs cannot be based on phenotypical markers of HSCs elsewhere. Therefore, we believe that this argument typically does not outplay the beneficial consequences of serially transplanting purified HSCs.

Another limitation with serial transplantation of re-purified HSCs is that these possess competitive disadvantages compared to freshly isolated competitor cells, which may result in very low chimerism levels. To avoid this, we propose that the numbers of serially transplanted HSC are increased compared to in the primary transplantation and/or that previously-transplanted bone marrow is used as competition. Setting up separate experiments for evaluation of short-term and long-term HSC behaviors, where primary recipients that are to be used for serial transplantations are transplanted with wBM would allow for re-isolation of a high quantity of HSCs as well as maximizing the proliferative pressure on HSCs in the primary setting. Bone marrow, from which HSCs are then purified from is subsequently isolated from multiple bones, and competitively transplanted into secondary recipients where per cell evaluations of HSC behaviors are conducted.

Collectively, the findings presented in this study demonstrate major concerns associated with serial transplantation of wBM when evaluating long-term HSC behavior. In comparison, serial transplantation of purified HSCs often offers more robust interpretation of long-term HSC self-renewal and lineage output capacity.

Article III

Human and Murine Hematopoietic Stem Cell Aging Is Associated with Functional Impairments and Intrinsic Megakaryocytic/Erythroid Bias.

Rundberg Nilsson A, Soneji S, Adolfsson S, Bryder D, Pronk CJ.
PLoS One. 2016 Jul 1;11(7):e0158369. doi: 10.1371/journal.pone.0158369. eCollection 2016.

Aim: To evaluate and compare age-associated changes in human and mouse HSPCs.

Aging is accompanied by numerous hematologic impairments, including compromised adaptive immune responses, and increased incidences of anemia and myeloid leukemias. In mice, such age-associated alterations have been linked to changes within immature cells at the top of the hematopoietic hierarchy. Similar investigations in humans are sparse and somewhat conflicting. We therefore set out to evaluate age-related changes within the primitive HSPC compartment in humans. As humans present with high biological variance, we based or interpretations on a large number of human samples, which allowed us to thoroughly address previously conflicting results in human studies. Simultaneously, we performed similar investigations in mice. In this study, we revealed several inter-species conserved patterns between humans and mice, as well as presented novel age-associated findings.

We first evaluated age-associated frequency alterations of HSCs, MEPs, GMPs, and CLPs in human bone marrow, as well as of HSCs, pre-megakaryocyte/erythrocyte progenitors (preMegEs), MkPs, pre-colony-forming unit erythroid (preCFU-Es), CFU-Es, pGM/GMPs, and CLPs in mice. Both humans and mice revealed increasing frequencies of HSCs with age, which we interpreted as a conserved compensatory mechanism aimed at rescuing reduced per cell competence. Consistent with age-associated impairments in adaptive immune functions, both species also revealed reduced levels of CLPs in the aged bone marrow. Moreover, aged human bone marrow presented with increased frequencies of MEPs, which may represent means aimed at counteracting the reduced erythrocyte and platelet output that is observed in aged humans. In contrast, the Mk/Er bipotent progenitor preMegE in mice was not significantly altered with age. This may demonstrate differences in hematological aging phenotypes between humans and mice and/or that the Er/Mk progenitors are not

fully comparable. In support of the former are findings showing that human aging is associated with decreased counts of both erythrocytes and platelets, while mouse aging show decreased counts of erythrocytes but increased numbers of platelets. Accordingly, downstream of preMegEs, aged mouse bone marrow presented with increased frequencies of MkPs and decreased frequencies of erythroid progenitors. Although aging of murine bone marrow was associated with a slight increase in pGM/GMP frequencies, aging of human bone marrow showed decreasing frequencies of GMPs. At least part of this may be related to the inclusion of MLPs within the investigated human GMP gate. Nevertheless, early human lineage skewing appears to mainly consist of increased priming to the Mk/Er lineage, and decreased priming to the lymphoid lineage. When we evaluated functional *in vitro* potential of aged human HSCs, we could confirm decreased proliferative potential, as well as a trend toward reduced B cell capacity. We also showed that CB HSCs were vastly superior to bone marrow HSCs in both these aspects.

We next used young and aged human bone marrow HSCs to identify differentially expressed genes (DEGs) that subsequent gene ontology (GO) analyses and establishment of age-associated HSC signatures used in gene set enrichment analysis (GSEA) were developed from. GO analyses revealed that genes upregulated in aged HSCs were enriched for biological themes involving platelet activation, and blood coagulation, further suggesting a platelet-bias in aged human HSCs. Furthermore, aged HSCs were enriched for DNA and chromatin modification themes, indicating also age-associated alterations in the epigenetic landscape. Young human HSCs were enriched for several themes associated with lymphocyte activation and differentiation, as well as immune system development, consistent with the age-associated decrease in CLP frequency and lymphoid output impairment.

Myeloid-skewing has previously been reported in human HSCs. However, as these analyses were based on subjectively chosen genes without distinction between the Mk/Er and granulocyte/monocyte lineage, and because our frequency analyses hinted towards a Mk/Er rather than a granulocyte/monocyte bias, we decided to approach a potential lineage skewing in a more unbiased manner. Recently published studies suggesting an increase of platelet-biased HSCs with age in mice prompted us to simultaneously evaluate this in mice. To establish unbiased lineage-associated signatures we identified DEGs between young human MEPs, GMPs, and CLPs, as well as between young mouse preMegEs, pGM/GMPs and

CLPs, or young mouse MkPs, preCFU-Es, pGM/GMPs and CLPs. When applying these gene sets to enrichment analyses between young and aged HSCs, we could demonstrate that aged human HSCs were enriched for MEP and GMP signatures, while young human HSCs were enriched for the CLP gene set. The three-progenitor-group-established mouse gene sets only revealed significant enrichment of preMegE-associated genes to aged HSCs, demonstrating that a strong Mk/Er skewing was also present in aged mouse HSCs. GSEA based on the four-progenitor-group comparison revealed that both MkP- and preCFU-E-affiliated genes were enriched in aged mouse HSCs. In this analysis, significant enrichment was also seen between CLP and pGM/GMP signatures to young HSCs.

Collectively, these analyses suggest that a strong skewing towards Mk/Er is present in both human and mouse aged HSCs, while the young HSC pool is associated with an enrichment of lymphoid-associated genes. Furthermore, the combined analysis of human and mouse HSCs hints for inter-species conserved lineage skewing patterns.

Populärvetenskaplig sammanfattning

Hematopoes (blodbildning) är en process som ständigt pågår under hela livet. Under denna process bildas mogna blodceller som bl a bygger upp immunsystemet och transporterar syre till kroppens vävnader. Eftersom dessa celler har begränsade livslängder måste de ersättas kontinuerligt. Varje dag produceras därför ca en biljon nya blodceller, vilket medför att en genomsnittperson producerar sin egen vikt i bara blodceller ungefär vart sjunde år. För att en sådan produktion ska kunna fortgå under en hel livstid krävs celler som har två särskilda förmågor: 1) att kunna skapa kopior av sig själva (självförnyelse) så att cellerna inte tar slut, och 2) att kunna differentiera (mogna) ut till blodsystemets alla olika celler för att ersätta förlorade celler. De celler som har dessa förmågor finns i benmärgen och kallas för hematopoetiska stamceller (HSC:s). HSC:s utgör endast en väldigt liten del av alla celler som finns i benmärgen (~0,001–0,01 %). För att kunna rena upp HSC:s behövs därför väldigt sofistikerade metoder. När HSC:s differentierar ut till mogna blodceller sker detta genom en rad mellansteg och celldelningar som är viktiga bl a för att man ska kunna få ut så många celler som möjligt från varje stamcell. Cellerna som utgör dessa mellansteg kallas för progenitorsceller.

En metod som ofta användas för att analysera och rena upp HSC:s är flödescytometri. För att kunna hitta stamcellerna använder man sig av antikroppar som binder till särskilda proteiner på cellernas yta. Genom att använda rätt kombination av antikroppar kan man identifiera stamcellerna baserat på vilka proteiner man vet ska finnas och inte finnas på deras yta. För att kunna se vilka antikroppar som har bundit till olika celler är antikropparna kopplade till fluoroforer. Fluoroforer är molekyler som kan fånga upp ljus och sedan sända ut (emittera) ljus med olika våglängder som är specifika för olika sorters fluoroforer. Genom att koppla olika fluoroforer till olika antikroppar kan man med hjälp av flödescytometern särskilja vilka antikroppar som har bundit till en viss cell baserat på de ljus de emitterar ut och man kan även mekaniskt sortera ut de celler man vill åt. Flödescytometri är ett mycket viktigt redskap inom blodcellsforskningen och har bidragit till enorma forskningsframsteg. Det används även inom kliniken för att t.ex. diagnostisera och klassificera blodcancrar. Flödescytometri är dock en

väldig känslig metod som ställer stora krav på både cellmaterial och användare. Inte minst när man är intresserad av celler som utgör en så väldigt liten andel av ett prov som vid studerandet av HSC:s, är kraven på förberedande, utförande och analys, särskilt viktiga för att resultatet ska bli korrekt. I **artikel I** har vi belyst några sådana viktiga inslag, såsom val av antikroppar och fluoroforer, nödvändiga kontroller, samt visualisering av resultaten. I denna artikel presenterar vi även en väldigt komplex antikroppskombination för humana (mänskliga) celler som gör att man kan analysera många olika omogna (HSC:s och progenitorsceller) och mogna blodcellspopulationer i ett och samma benmärgsprov. Detta är viktig för att kunna få ut så mycket information som möjligt från ett prov då mängden humant benmärgsprov man har tillgång till ofta är väldigt liten.

Då etiska och experimentella begränsningar hindrar många undersökningar av humana celler används ofta modellorganismer med egenskaper som liknar människans. Möss är väldigt vanliga modellorganismer vid blodcellsstudier då människors och möss blodsystem är väldigt lika. När man undersöker HSC:s inom forskning transplanterar man ofta HSC:s som man har renat upp från en mus till en annan. När man är intresserad av hur de beter sig på lång sikt serietransplanterar man HSC:s, vilket innebär att HSC:s först transplanteras till en mus, återisoleras från denna mus en tid efter, och sedan transplanteras vidare till en ny mus. På så sätt tvingar man stamcellerna att gång på gång bygga upp det hematopoetiska systemet. Tanken är att bara äkta HSC:s ska klara av att bygga upp det hematopoetiska systemet upprepade gånger, och på så sätt kan man studera den långvariga effekten av HSC:s i en accelererad modell utan att behöva åldra mössen under längre tider. Vi upptäckte dock att trots att detta är en väldigt utbredd metod utförs inte serietransplantationer enhetligt mellan olika laboratorium. I **artikel II** jämförde vi därför de två vanligaste strategierna (att serietransplantera hel benmärg och att serietransplantera renade HSC:s) för att undersöka om de olika metoderna medförde några särskilda för- och/eller nackdelar. Vi kom fram till att den vanligaste strategin, serietransplantation av hel benmärg, medförde stora nackdelar jämfört med serietransplantation av renade HSC:s i de allra flesta fall, vilket kritiskt kunde påverka både analys och resultat av experimentet. Här spelade faktorer såsom skillnader i HSC-frekvens mellan jämförda grupper, effekter av celler som inte var HSC:s, samt skillnader i distribution av HSC:s mellan benmärg i olika ben efter transplantation, stor roll. Med dessa resultat som grund föreslog vi sedan en utförlig serietransplantationsstrategi som maximerar chanserna att korrekt bedöma HSC:s långvariga kapacitet. Resultaten i denna studie ifrågasätter resultat från många studier som använt serietransplantation av hel benmärg som

metod och illustrerar även möjliga orsaker till att olika studier har visat olika resultat.

Möss och människor uppvisar även många liknande förändring inom blodsystemet vid åldrande. T.ex. så får äldre individer sämre immunförsvar och har större risk för att utveckla anemi (blodbrist) och olika blodcancrar. Möss och människor har dock även många olikheter, såsom storlek, livslängd, metabolism, och omgivningsfaktorer. Trots likheterna mellan möss och människor skulle det därför också kunna vara olika mekanismer som styr blodceller och blodcellsåldrandet. Det är viktigt att reda ut likheter och skillnader mellan möss och människor för att kunna säkerställa musen som en passande modellorganism att studera blodceller i, och för att kunna veta när musen inte är tillräcklig för att vi ska kunna förstå hur blodcellerna regleras i människor. Studier i möss har visat att vissa av de förändringar som sker i blodsystemet vid åldrande är kopplade till förändringar som sker i HSC:s. Liknande studier på humana celler är begränsade och delvis inkonsekventa. I **artikel III** studerade vi därför åldersrelaterade förändringar hos hematopoetiska stam- och progenitorsceller hos både möss och människor. Vi identifierade flera gemensamma förändringar, samt presenterade nya, tidigare oidentifierade förändringar. Likheterna mellan möss och människor tyder på att åtminstone vissa delar av HSC-åldrandet styrs av artbevarade mekanismer. Resultaten tyder också på att flera av de förändringar man ser hos mogna blodceller vid åldrande startar i HSC:s, men också att HSC:s försöker kompensera för åldersrelaterade försämringar.

Sammanfattningsvis belyser resultaten i denna avhandlings artiklar aspekter som är kritiska vid användandet av några av de mest utbredda metoder inom HSC-forskning. Beaktande av dessa parametrar är absolut nödvändiga för korrekt bedömning av undersökningsresultat. Vidare så har HSC:s undersökts och kartlagts ur ett åldrandeperspektiv hos både möss och människor. Sådan kunskap kan vara essentiell för utvecklande av behandlingsalternativ som förhindrar, begränsar, och/eller förbättrar åldringsrelaterade sjukdomar och tillstånd.

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