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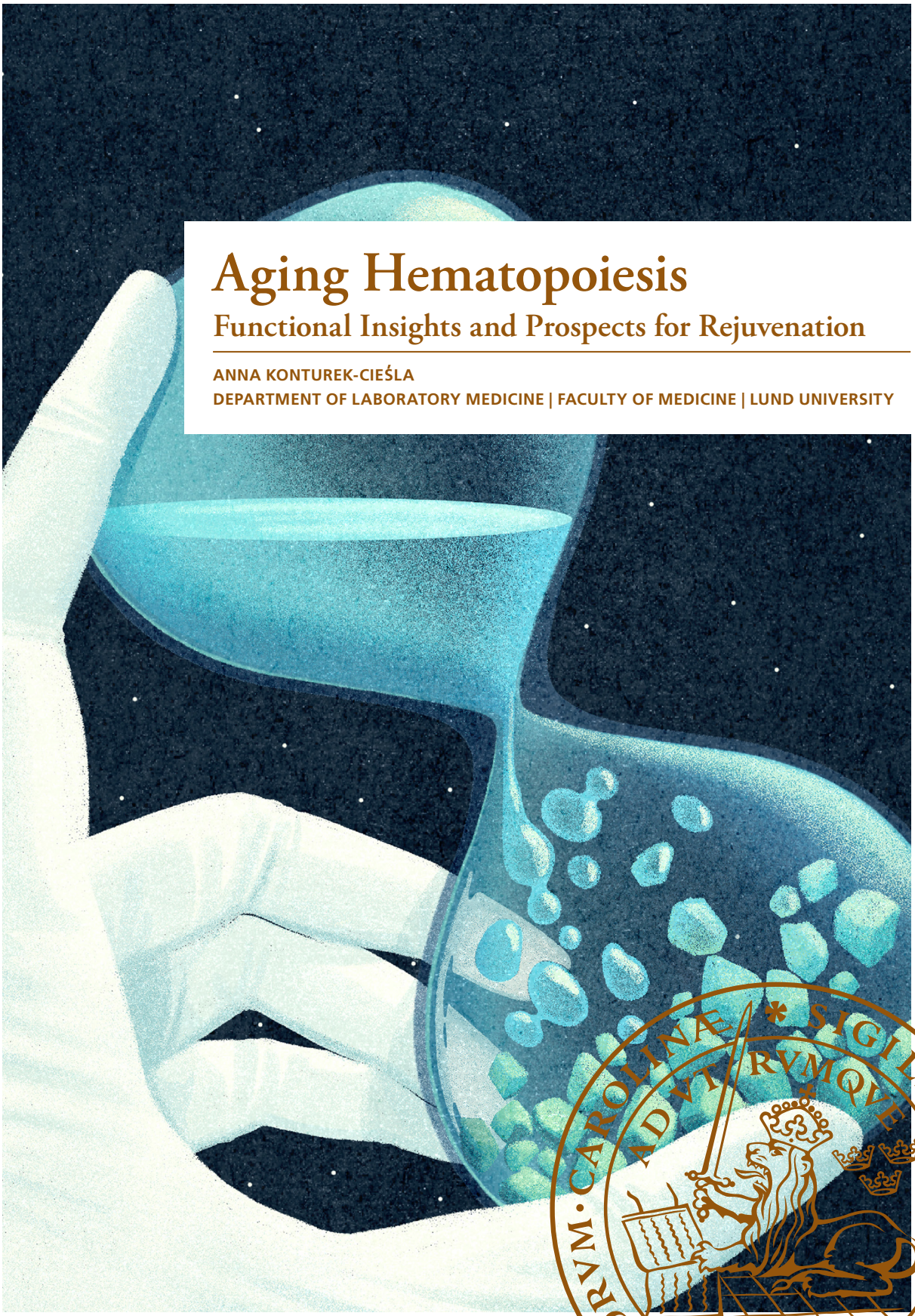

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Abstract:

Aging exerts profound effects on the hematopoietic system, leading to a loss of homeostatic control and reduced regenerative capacity. Clinical consequences of these changes include anemia, compromised immune function and an increased prevalence of myeloid disorders among the elderlies. A growing body of evidence suggests that defects at the stem and progenitor cell levels contribute to many of the hematopoietic aging phenotypes. In this thesis, I examined the function of hematopoietic stem cells (HSCs), the source of all mature blood cells, during adulthood and upon aging. By using an *in vivo* lineage tracing mouse system and high-throughput RNA profiling, I demonstrate a gradual decline in HSC multilineage differentiation capacity, with the most significant reduction in their lymphoid output. Decreased lymphopoiesis could be traced back to the primitive lymphoid progenitor subset, MPP Ly-I, revealing defects in the transition of HSC to these cells. To address age-related HSC decline, we employed a recently developed HSC culture system which allows for activation and robust self-renewal of input HSCs. Although *ex vivo* culture of aged HSCs failed to correct their *in vivo* function, we nevertheless observed that the aged HSC pool contains rare multilineage/lymphoid competent clones, which makes these promising candidates for strategies aimed at hematopoietic rejuvenation. In parallel, we investigated molecular changes associated with age-related HSC decline by re-analyzing existing transcriptomic data on HSC aging. We discovered only partial overlap in HSC aging signatures between prior studies, which prompted us to address the potential causes for such variation. We found that cell isolation procedure and sample handling both impact on the molecular profiles of the cells. In particular, incubation of cells at elevated temperature triggered a stress signature which overrides the genuine molecular profile of the cells. Notably, this stress response was age-independent, but with implications for prior interpretations of HSC aging mechanisms. Finally, given the intrinsic nature of many of the age-associated HSC phenotypes, we explored the potential for cell replacement therapy through young HSC transplantation to reinstate a more youthful hematopoietic function in older subjects. While several key parameters needed to be optimized, including non-invasive conditioning regimen prior to transplantation and HSC transplant doses, we demonstrated successful integration of young HSCs in aged recipients. Importantly, these young cells largely retained their function in the aged environment, contributing effectively to hematopoietic output, and particularly to mature lymphocytes. Hence, this data suggest that non-invasive transplantation of young HSCs holds promise as a potential strategy to alleviate some hematopoietic aging phenotypes. Collectively, this thesis work provides new insights into hematopoietic aging and set the stage for more comprehensive investigations of HSC transplantation as a potential approach for hematopoietic rejuvenation.

Key words: Hematopoietic stem cells, aging, lineage tracing, transcriptome profiling, transplantation

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Wisdom comes with winters

Oscar Wilde

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Abstract

Aging exerts profound effects on the hematopoietic system, leading to a loss of homeostatic control and reduced regenerative capacity. Clinical consequences of these changes include anemia, compromised immune function, and an increased prevalence of myeloid disorders among the elderly. A growing body of evidence suggests that defects at the stem and progenitor cell levels contribute to many of the hematopoietic aging phenotypes. In this thesis, I examined the function of hematopoietic stem cells (HSCs), the source of all mature blood cells, during adulthood and upon aging. By using an *in vivo* lineage tracing mouse system and high-throughput RNA profiling, I demonstrate a gradual decline in HSC multilineage differentiation capacity, with the most significant reduction in their lymphoid output. Decreased lymphopoiesis could be traced back to the primitive lymphoid progenitor subset, MPP Ly-I, revealing defects in HSC transition to these cells. To address age-related HSC decline, I employed a recently developed stem cell culture system which allows for activation and robust self-renewal of input HSCs. Although *ex vivo* culture of aged HSCs failed to correct their *in vivo* function, I nevertheless observed that the aged stem cell pool contains rare multilineage/lymphoid competent clones, which makes these promising candidates for strategies aimed at hematopoietic rejuvenation. In parallel, I investigated molecular changes associated with age-related HSC decline by re-analyzing existing transcriptomic data on HSC aging. I discovered minimal overlap in HSC aging signatures between prior studies, which prompted us to address the potential causes for such variation. I found that cell isolation procedures and sample handling both have impact on the molecular profiles of the cells. In particular, incubation of cells at elevated temperature can trigger a stress signature which overrides the genuine molecular profile of the cell. Notably, this stress response was age-independent but with large implications for prior interpretations of HSC aging mechanisms. Finally, given the intrinsic nature of many of the age-associated HSC phenotypes, I explored the potential for cell replacement therapy through young HSC transplantation to reinstate more youthful hematopoietic function in older subjects. While several key parameters needed to be optimized, including a non-invasive conditioning regimen prior to transplantation and HSC transplant doses, I demonstrated successful integration of young HSCs in aged recipients. Importantly, these young cells largely retained their function in the aged environment, contributing effectively to hematopoietic output, particularly to mature lymphocytes. Hence, this data suggest that non-invasive transplantation of young HSCs holds promise as a potential

strategy to alleviate some hematopoietic aging phenotypes. Collectively, this thesis work provides new insights into hematopoietic system aging and sets the stage for more comprehensive investigations of HSC transplantation as a potential approach for hematopoietic rejuvenation.

Abbreviations

ABC	Age-associated B cell
BM	Bone marrow
CLP	Common lymphoid progenitor
CRA	Competitive repopulating assay
EPCR	Endothelial protein C receptor
(LT) HSC	(Long-term) Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
GMP	Granulocyte-macrophage progenitor
IEG	Immediate early gene
IERsig	Immediate early response signature
IL	Interleukin
LMPP	Lymphoid-primed multipotent progenitor
LSK	Lineage-Sca-1+c-kit+ cell population
mAb	Monoclonal antibody
MPP	Multipotent progenitor
MPP Ly	MPP with lymphoid biased reconstitution
MkP	Megakaryocyte progenitor
PB	Peripheral blood
PVA	Polyvinyl alcohol
SAP	Saporin
TBI	Total body irradiation
vWF	von Willebrand factor

Introduction

Aging associates a progressive loss of physiological integrity. This is accompanied by changes in tissue homeostasis, which manifest with reduced renewal at steady state and impaired regeneration following injury. The mechanisms that drive this deterioration are complex, but given that cell replacement is assured by tissue-specific adult stem cells, the age-related decline in stem cell function is recognized as one underlying cause of aging[1]. Stem cells, similar to other cell types, are subject to age-associated insults, which lead to accumulation of damage (to nucleic acids, proteins, and lipids) over time. However, due to their capacity to self-renew and differentiate, stem cells can additionally propagate these harmful lesions to other cells. A failure to efficiently repair this damage or to eliminate cells carrying such lesions can consequently trigger stem cell exhaustion or malignant transformation. Therefore, understanding the mechanisms that orchestrate stem cell function during normal development and upon aging is key for addressing age-associated conditions and developing better therapeutic strategies.

In this thesis work I studied hematopoietic stem cells (HSCs), which to date represent the only adult stem cell type routinely employed in clinical applications for disease treatment. HSCs constitute a rare population of cells residing in the bone marrow (BM) and secure lifelong production of white blood cells, erythrocytes and platelets in a continuous process termed *hematopoiesis*[2]. In humans, this production translates to the astounding number of ~10^{16} cells, or a mass corresponding to 10 times the body weight during a 70-year lifespan[3,4]. The cellular components of the blood participate in various vital processes, including defense mechanisms, oxygen and nutrient transport, and hemostasis. Thus, the flexibility of HSCs to respond to organismal demands is essential to maintain homeostasis.

In our studies I explored the functional and molecular characteristics of adult HSCs and investigated changes that underlie their decline during physiological aging. I also examined the potential of HSC-based cell therapy to alleviate age-related conditions, including the progression of hematopoietic diseases. For many reasons, including the lack of *in vivo* models that would allow to study human HSCs, the murine system serves as the experimental paradigm for hematopoietic development. Therefore, in our work, I employed laboratory mice to address the objectives of this thesis.

Hematopoietic Stem Cells

Stemness encompasses two fundamental properties that enable cell to support tissue homeostasis over time. The first is *self-renewal*, which defines a cell's capacity to replicate itself and sustain its pools over extended time. The second feature is *multilineage differentiation*, indicating the ability to generate full repertoire of cell types associated with given tissue. HSCs fulfill both of these criteria. This has been formally demonstrated over 50 years ago through a series of seminal experiments involving BM transplantation into irradiated mice.

In the early 1960s, Till and McCulloch found that BM cells transplanted into lethally irradiated mice form macroscopically distinguishable colonies in recipients' spleens, which they termed spleen colony-forming unit (CFU-S)[5]. In the following studies, the same group showed that each individual CFU-S derives from a single cell and that it has the ability to form new colonies upon secondary transplantation. These colonies contained myeloerythroid cells and some of them could also generate lymphocytes[6-8]. Based on these findings, Till and McCulloch proposed that CFU-S represent primitive HSCs capable of both self-renewal and multilineage differentiation. However, later studies challenged this concept demonstrating that distinct spleen colonies are formed early after transplantation and that the CFU-S reported by Till and McCulloch derived from hematopoietic progenitors rather than *bona fide* HSCs[9].

In the mid-1980s, subsequent studies employed retroviral vectors-mediated gene transfer to enable functional examination of murine HSCs at the clonal level[10-13]. The technique exploited the property of retroviruses to insert into random genomic locations, thus creating unique, traceable integration sites in each targeted cell. By analyzing sequences flanking integration sites, several groups observed the same insertion positions in both myeloid and lymphoid cells, indicating a shared lineage origin[10-14]. Furthermore, when evaluating tagged BM cells after re-transplantation into secondary recipients, Keller et al. discovered that in some instances, identical clones could be detected in both primary and secondary mice[11]. Serial transplantation studies by Harrison et al. revealed enduring stem cell activity even after multiple rounds of transplantation, which in an extreme example equaled eight years of analyses[15,16]. Thus, it became evident that blood supporting stem cells are long-lived, with the lifespan far exceeding that of their donor. Based on this and the finding that retroviral tags persisted in mature cells long-term after transplantation, it was postulated that the HSC compartment exhibits *clonal stability*[11,16,17]. This model assumed that individual cells possess extended lifespans and are continuously recruited to the ongoing blood production. On the other hand, other studies documented clonal fluctuations over time and hence proposed an alternative model, wherein hematopoiesis is maintained by the *succession* of HSC clones with finite lifespans rather than deriving from stable, long-lasting clones[12,14]. Irrespective of the proposed model, these investigations unequivocally demonstrated the existence of

strictly defined blood stem cells in the BM. Indeed, the HSCs described displayed capacity for both self-renewal and multilineage differentiation.

Since these pioneering discoveries, BM transplantation became the gold standard assay to evaluate stem cell function. However, while it was acknowledged that the cells contributing to transplantation outcome comprised both primitive cells and more differentiated progenitors, the exact identities of these cells remained largely unknown. Therefore, parallel studies were aimed at prospective isolation and characterization of functional HSCs.

Tools to study hematopoietic stem cells

Isolation methods

In the late 1960s, flow cytometry and fluorescence-activated cell sorting (FACS) emerged as a tool for rapid multiparametric analysis and cell isolation. Since then, the methodology has evolved into a front-line technique within the hematology research toolkit. Flow cytometry/FACS exploits the natural property of cells/particles to scatter light and emit fluorescence upon laser excitation. Consequently, when labeled with fluorochrome-conjugated monoclonal antibodies (mAbs), cells can be identified based on their size, granularity, and surface marker expression.

Early work led by the Weissman group demonstrated that long-term HSC (LT-HSC) activity resides within the fraction of BM cells that lack expression of markers associated with mature blood cells (Lineage markers) and are positive for stem cell antigen-1 (Sca-1) and tyrosine kinase receptor c-kit[18,19]. This population, known as Lineage- Sca-1+c-kit+ (LSK), encompasses distinct hematopoietic stem and progenitor cell (HSPC) subsets, among which HSCs are estimated to account for approximately one in thirty cells[2]. Following the initial discovery, several groups identified additional markers, including CD34[20], CD135 (Flt3)[21,22], Slam family markers[23] and CD201 (EPCR)[24] that further enrich LSK fraction for LT-HSCs, and allow to sub-fractionate early multipotent progenitor (MPP) cell subsets. Over the last two decades, various laboratories have adapted distinct combinations of these markers, thereby establishing their own phenotypic definitions of LT-HSC and MPPs. This was further complicated by inconsistent nomenclature across different groups and resulted in considerable confusion within the field. Recently, a suggestion for a consensus has been established, defining murine LT-HSCs as LSK CD150+CD48-CD135- [25].

Apart from cell surface markers, HSCs can also be enriched based on their relatively refractory behaviors in response to cytostatic drugs or retention of cell-permeable

dyes. For example, due to HSC's increased resistance to cytostatic drugs, which interfere with cell division, a number of earlier studies subjected donor mice to 5-fluorouracil (5-FU) treatment to enrich for HSCs before retroviral marking of BM cells[10-12]. Indeed, later studies confirmed that LT-HSCs are largely quiescent[26-28]. The low proliferative activity of LT-HSCs can be also traced using yet another technique that employs cell proliferation dyes. Finally, HSCs can be isolated using "side population" protocol developed by Goodell and colleagues, which relies on their capacity to efflux fluorescent DNA binding dye Hoechst 33342[29]. Notably, both protocols that utilize fluorescent dyes and "side population" approach necessitate incubation of cells at elevated temperatures, which, as we show in *Paper II*, impacts cells' molecular characteristics.

Lastly, with recent advances in transgenic and knock-in mouse models, the identification of HSCs has expanded to include the expression of stem cell-specific genes. In these systems, the regulatory elements of the gene of interest, typically promoters, are used to drive the expression of a reporter cassette, such as a fluorescent protein. The selection of a gene is based on its' expression profile, and so it dictates the specificity of the system. Ideally, the selected marker should be restricted to the most primitive HSCs, but at the same expressed at high levels to allow robust labeling. Moreover, heterozygous loss of the gene selected for targeting should not disrupt the normal function of the cell.

A number of distinct fluorescent reporter mouse strains have been established to allow identification and isolation of murine HSCs. These include the knock-in lines, such as *Fgd5*-mCherry and -ZsGreen[30], *Ctnnal1*(a-catulin)-GFP[31], and *Hoxb5*-tri-mCherry[32] and transgenic strains, like *Pdzk1ip1*-GFP[33], *Vwf*-EGFP[34] and -tdTomato[35], *Tek*-GFP[36] and *Gprc5c*-EGFP[37]. Among these, the driver genes *Vwf*, *Tek* and *Gprc5c* encoding respectively vWF, TIE2, and GPRC5C, have been previously linked to hematopoiesis. Von Willebrand factor (vWF) is a glycoprotein involved in platelet aggregation during thrombus (blood clot) formation[38]. In the transgenic system, *Vwf* expression was shown to mark not only mature platelets but also a subset of HSCs associated with a platelet-biased differentiation pattern[34,35]. TIE2, a receptor tyrosine kinase, and GPRC5C, an orphan G protein coupled receptor, have both been demonstrated to regulate HSC quiescence through the TIE2/angiopoietin[39] and GPRC5C/hyaluronic acid[40] signaling pathways, respectively. Additional mouse Cre reporter strains have been developed and are primarily employed for lineage tracing analyses, which are discussed in detail in the subsequent sections in this thesis.

While the HSC immunophenotype described above applies to most commonly used laboratory mouse strains, it should be noted that certain surface antigens are strain dependent[41]. Moreover, the expression of some markers depends on the developmental stage and can also change following stress[41]. The canonical example for the latter is transient upregulation of Sca-1 in response to interferon/poly I:polyC[42]. In this thesis (*Paper II*), we further show that the expression of intracellular

reporters can also be subject to change. Therefore, while surface markers and other cellular features can facilitate identification and prospective isolation of candidate HSCs, the functional stem cell activity is measured by other means.

Evaluation methods

Transplantation assays

The capability to effectively restore the constituents, and thus function, of a recipient's organ after transplantation is considered a property of stem cells. Although highly purified HSCs have limited ability to sustain survival upon transplantation into lethally irradiated recipient[43], they are critical for long-term hematopoietic regeneration. Therefore, transplantation continues to be the prevailing method to assess *in vivo* HSC function.

In its simplest form, transplantation involves injection of test cells into pre-conditioned recipients, followed by analysis of test cell-derived output in the peripheral blood (PB) in time-resolved manner (*direct transplantation*). As such, it provides a qualitative measure of stem cell performance. The assay can be also performed in the mirrored regimen, where control cells are transplanted into pre-conditioned test animals (*reverse transplantation*); that helps distinguish intrinsic vs. extrinsic regulators of hematopoiesis. In *competitive repopulating assay* (CRA), test cells are co-injected with a known number of competitor HSCs (usually provided as unfractionated BM) into recipients, which enables quantification of stem cell activity within the fraction of test cells relative to the competing BM[44]. Finally, *serial transplantation* is the most rigorous assay that measures HSC's ability to sustain hematopoiesis in consecutive transplantation recipients[41].

In CRA, the functional potential of test cells (T) is measured in repopulating units (RU) using the following formula:

$$\mathrm{RU_T} = \mathrm{RU_C}\,\frac{\%\ \text{test cells}}{100 - \%\ \text{test cells}}$$

where $\mathrm{RU_C}$ = value of competitor RU, defined as 1 per 10^5 BM cells

The advantage of calculating RU over quantifying raw chimerism percentage is that it accurately reflects the functional activity of test cells relative to the function of competitor cells and allows direct comparisons between different tested cell types. This is especially important, considering that virtually any parameter in the assay, including type of donor cells (purified HSCs vs. unfractionated BM), their dose, conditioning regimen, etc., can be changed. That said, while transplantation is routinely employed to assess stem cell potential, the method seems not to be

standardized across different laboratories with significant implications for conclusions on HSC performance[45].

The most common strategy to assess HSC-derived hematopoiesis following transplantation employs the murine CD45 congenic system, in which donor and competitor mice differ in their *Ptprc* alleles. The products of these alleles, also known as CD45.1 and CD45.2 antigens, can be distinguished using distinct mAbs, thereby allowing determination of donor reconstitution in PB leukocyte populations. The caveat of the system is that it precludes evaluation of chimerism in erythrocytes and platelets, which lack expression of CD45 antigen. To overcome this limitation, some groups utilize transgenic reporter mouse lines, e.g. Kusabira-Orange[46] or UBI-GFP/BL6[47], which permanently label all PB lineages and hence enable discrimination of donor and recipient cells irrespective of their *Ptprc* alleles.

Mature PB cells of distinct lineages vary considerably in their lifespans: platelets and granulocytes are short-lived and recycle within days, erythrocytes have a lifespan of approximately 120 days, while mature lymphocytes can persist for years[48]. In this context, long-term reconstitution in the myeloid lineage is the best indicator of ongoing LT-HSC activity following transplantation[49]. As T lymphocyte pools can be sustained *in vivo* through homeostatic proliferation, independent of continuous HSC input, the reconstitution in this lineage by donor-derived cells provides insights into HSC multipotency rather than ongoing stem cell activity. Thus, the *in vivo* HSC function should be evaluated based on reconstitution levels within individual PB lineages and not solely by assessing total chimerism in PB, which is a common practice.

Finally, while transplantation enables assessment of HSC function across developmental stages, until now most studies have been carried out in adult recipients. However, given the diverse environmental cues to which HSCs are exposed throughout life, evaluating stem cell function while neglecting the age-related remodeling of their niches might be suboptimal. The major limitation in adjusting donor and recipient age is differential response of young and elderly recipients to traditional conditioning regimens, which is required for effective donor cell engraftment. Alternative low-intensity conditioning approaches have been developed and as we present in this thesis (*Paper III*) are likely to broaden the applicability of transplantation assay in recipients spanning different age ranges. I describe these strategies in more detail in the following sections.

In vivo labeling assays

Proliferation assays

Most label retention assays developed to study cell proliferation adhere to a common principle, wherein initial cell labeling is progressively lost during each subsequent cell division. Thus, cells with high proliferation rates can be discriminated based on the rapid loss of the label, while non-dividing (quiescent) cells retain the label over

time. Labeling methods typically involve agents such as nucleotide analogues, intracellular dyes, or membrane protein-binding molecules, which irreversibly mark DNA or cellular proteins and are then halved into two daughter cells during mitosis. In label retention transgenic mouse models, labeling can be achieved through the inducible pulse of expression of histone-2B coupled with fluorescent protein (H2B-FP). These fusion H2B proteins become integrated into nucleosomes and then, as the cell divides, are equally distributed among its offspring.

An early assay developed to study cell proliferation relied on thymidine analogue, bromodeoxyuridine (BrdU). BrdU is administered through *in vivo* injections and integrates into the DNA of cells actively dividing during the labeling period. Therefore, by evaluating BrdU incorporation[50,51] and dilution[28,52], it is possible to determine *in vivo* HSC proliferation dynamics over time. However, because BrdU detection requires fixation of cells prior to analysis, the assay is incompatible with functional assessments of viable cells. Furthermore, BrdU was reported to have mitogenic activity, which introduces bias and can compromise reliable quantification of cell divisions[28,53]. To minimize cellular toxicity, improved methods were developed, employing protein-binding dyes, such as carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE was successfully applied to study HSC division upon transplantation into unconditioned recipients[54] and also later in the context of stimulation with cytokine receptor agonists[55]. As I show in this thesis, alternative dyes, like CellTrace labels allow for equally robust analysis of HSPC proliferation, albeit with the advantage of wider available versatility in fluorochrome combinations (*Papers III* and *IV*).

The major limitation of CFSE- and CellTrace-based techniques is that they require *ex vivo* cell labeling, which precludes investigation of proliferation dynamics at steady state. Such analyses were later made possible by the development of biotin-based labeling technique[53] and novel transgenic mouse strains. In the former assay, a modified biotin reagent was used to label surface membrane proteins, thereby providing new non-invasive approach for assessing *in vivo* HSC proliferation[53]. This method however suffers from low resolution, which ultimately makes it less applicable for long-term analyses.

Transgenic mouse lines engineered to express H2B-FP belong to tetracycline (Tet) controlled genetic systems, with an ability to switch genes off (Tet-Off) or -on (Tet-On). In both systems, H2B-FP expression is under the control of tetracycline responsive element (TRE) and a transcriptional activator, which can be reversibly induced by dosing animals with tetracycline or its derivative doxycycline (Dox)[56]. In the Tet-Off system, tetracycline-controlled transactivator (tTA) binds to TRE in the absence of Tet/Dox and dissociates upon administration of the drug, which leads to repressed expression of H2B-FP. By contrast, the Tet-On system relies on reverse tetracycline-controlled transactivator (rtTA), which stimulates TRE-regulated expression of H2B-FP only in the presence of Tet/Dox[56] (Fig. 1A). The expression of tTA or rtTA is regulated by the selected promoter/locus, such as ubiquitous

ROSA26[26,27] or more HSPC-specific loci[28] (*Paper I*). While H2B-labeling offers several advantages over previously discussed systems, with one being the non-invasive *in vivo* label induction that permits functional and molecular assessments, the system also has its limitations. One of the main concerns relates to background fluorescence present even in the non-induced setting[57], and division-independent label dilution due to H2B-FP degradation[58].

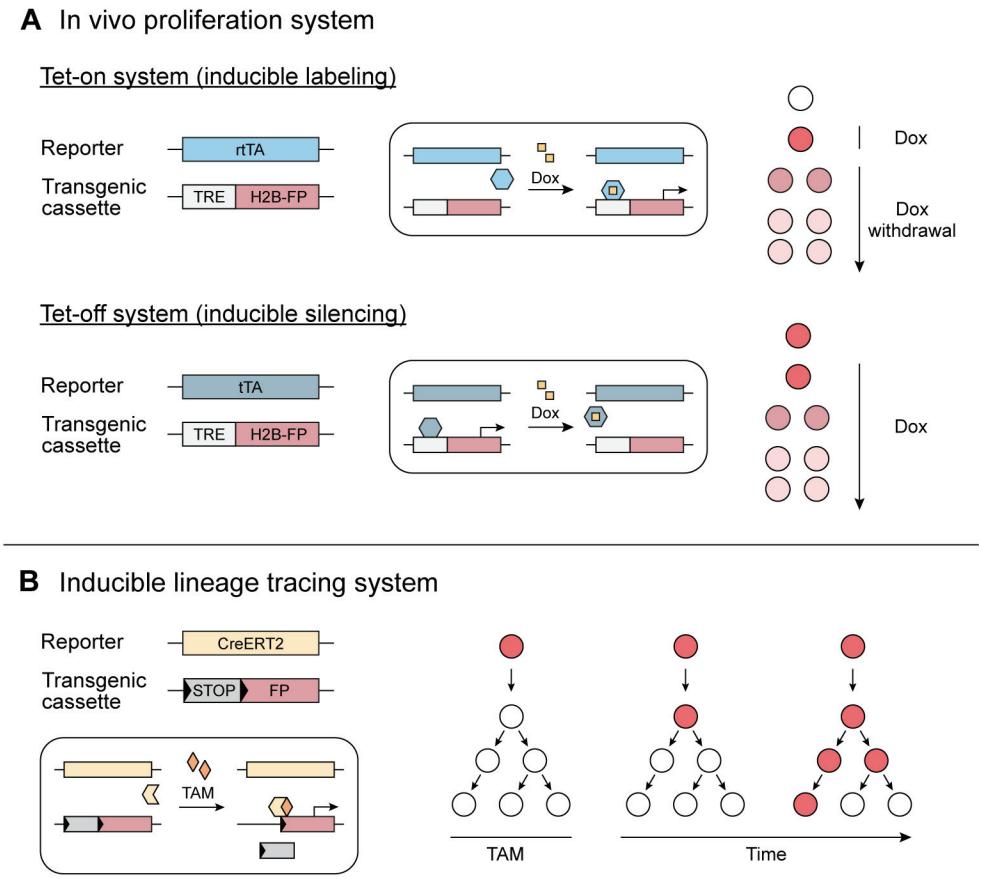

Figure 1. In vivo labeling assays. (A) Label retention systems to track cell proliferation. (B) Lineage tracing system for tracking of cellular differentiation. Dox, doxycycline; FP, fluorescent protein; TAM, tamoxifen; TRE, tetracycline responsive element.

Lineage tracing

Lineage tracing, also known as fate mapping, is the method which allows for prospective tracking of *in vivo* cellular differentiation. In this system, selected cell population of interest is irreversibly labeled with heritable marks that are passed to its progeny (Fig. 1B). Hence, by analyzing the dynamics of label propagation, lineage tracing allows to infer precursor-to-progeny relationship and the magnitudes and kinetics of ongoing differentiation events. The method has been classically used in developmental biology, but over the years it also became an essential tool in stem cell and cancer research.

One of the first genetic tracing studies tracking HSC fate transitions involved retroviral-mediated gene transfer[10-14] to create diverse genetic barcodes. In all these experiments, labeled cells were transplanted into irradiated or genetically deficient recipients, and the donor-derived progeny expressing distinct tags were analyzed over time[10-12]. These first attempts to resolve clonal-level HSC dependencies provided unprecedented insights into HSC biology, including new conceptual frameworks for their population dynamics (as discussed in previous section). More recent approaches for *ex vivo* cellular marking employ advanced DNA barcoding libraries, which offer markedly increased sensitivity in detecting individual HSC clones[59] and in some cases even allow to correlate HSC differentiation with specific molecular signatures[60,61]. The major drawback of these tools is the necessity for *ex vivo* cell labeling and transplantation into irradiated recipients, both of which are well recognized to affect cell behavior[48].

Strategies that overcome the need for *ex vivo* cell manipulation rely on transgenic mouse systems that generate heritable tags *in situ*. The most commonly applied system is based on Cre/loxP that exploits the activity of Cre enzyme to mediate site-specific recombination at the genomic regions flanked by loxP sites[56] (Fig. 1B). Importantly, these recombination events occur exclusively in cells or tissues expressing Cre enzyme, regulated by site-specific promoters. Recombination can lead to either deletion or inversion of the DNA fragment flanked by two loxP sites, which has been used for generation of fluorescent labeling[33,62,63] or DNA barcoding systems[64], respectively. In fluorescent labeling systems, loxP-flanked stop cassette (also referred to as lox-STOP-lox or LSL) is located between the promoter and a cDNA sequence that encodes selected fluorescent reporter. Upon Cre expression, this cassette is irreversibly removed, which activates permanent expression of reporter cDNA. Typically, the LSL-fluorescent reporter cassette is targeted to the ubiquitously expressed ROSA26 locus. A variety of Cre reporter mouse lines has been generated to characterize hematopoietic development[4]. However, most of these models were unsuitable for HSC lineage tracing, because of their constitutive expression (Cre recombinase could become active at any given time point), with the initial labeling not specific to HSCs. Therefore, the Cre/loxP systems were further refined to provide specific and temporal control of HSC labeling.

To achieve strict temporal control of initial labeling period, Cre recombinases were engineered by incorporating the ligand binding domain of estrogen hormone receptors (ER) resulting in formation of CreER fusion protein[65]. In the absence of the ER agonist tamoxifen (TAM or its active metabolite 4-hydroxy-tamoxifen, 4-OH TAM), the CreER fusion remains inactive in the cytoplasm. Upon 4-OH TAM binding to ER, the fusion protein translocates to the nucleus where it mediates recombination events (Fig. 1B). Over time, the ER part of the fusion transgene was further modified to increase its' sensitivity to exogenous 4-OH TAM and simultaneously reduce responsiveness to endogenous estrogens (e.g., CreERT2)[66].

Similar to the reporter mouse models (discussed in section *Isolation methods*), the specificity of HSC lineage tracing systems is conferred by stem cell-associated marker genes. Our laboratory has previously established an *Fgd5*-CreERT2-based system which allows for specific labeling of phenotypic HSC compartment[63]. Other HSC lineage tracing systems that exploit fluorescent proteins rely on expression of marker genes such as *Pdzk1ip1*[33] and *Tie2*[62]. Although all these marker genes were reportedly enriched in HSCs, the initial stem cell labeling varied between these studies. This demonstrates that the selection of Cre driver impacts the HSC labeling and further interpretations of fate mapping results.

As opposed to population-level tracing models (where the fraction of cells is labeled with indistinguishable tags), *in vivo* barcoding systems provide additional clonal resolution to the fate mapping data. In these models, individual cells are labeled with unique DNA barcodes, hence allowing dissection of their clonal outputs over time[64,67]. An exciting new approach combines *in vivo* cellular barcoding with transcriptomic profiling[68,69], which now provides a tool to unravel cell fate determinants at the single cell level.

Transcriptome profiling

In parallel with developments of *in vivo* tracing systems, the molecular assays, particularly transcriptome profiling, have advanced in leaps and bounds, providing new perspectives on hematopoietic cell identities and relationships. The available toolkit for transcriptome profiling spans from quantitative reverse transcription polymerase chain reaction (qRT-PCR) to multimodal single cell assays, which allow to address concepts that were previously considered unapproachable. For example, by combining the aforementioned *in vivo* cellular barcoding with high-throughput RNA-sequencing, it became possible to determine molecular factors that associate with particular developmental programs in individual primitive HSCs[68,69].

Among mRNA profiling techniques, qRT-PCR and microarrays are two probe-dependent assays that allow measurement of multiple genes simultaneously from bulk samples. qRT-PCR utilizes gene specific primers for the measurement of mRNA transcripts, which makes the technique highly sensitive, but at the low throughput. Microarray approaches rely on hybridization of fluorescently labeled

target cDNA to a matrix covered with thousands of synthetic oligonucleotide probes, which can measure expression levels of thousands of genes in a single experiment. The assay was extensively employed prior to advent of RNA sequencing techniques to study HSC transcriptomic profiles, also in the context of aging. However, although microarray analysis was high throughput, it suffered from the limited dynamic detection range. Moreover, the number of samples that could be evaluated simultaneously was limited to the number of available microarray chips.

In contrast to probe-dependent assays, sequencing-based approaches have been developed to interrogate the entire genomic sequences. As a result, RNA sequencing allows to analyze a broader spectrum of genes, unlike qRT-PCR approaches with pre-selected primer panels or microarray analysis with a defined number of probes. The standard RNA sequencing workflow begins by extracting RNA and enriching it for mRNA. The mRNA is then converted into a library of cDNA fragments with specific adapters attached to one or both ends. These fragments are subsequently amplified and subjected to high-throughput sequencing, yielding short reads from one (single-end sequencing) or both ends (paired-end sequencing). Next steps involve computational analysis, where the reads are aligned to a reference sequence and processed during downstream analysis steps[70]. The constantly evolving technologies, reagents, and sequencing platforms have propelled the refinement of this general method, resulting in almost 100 mRNA sequencing protocols available today[71]. Out of those, the single cell RNA sequencing (scRNA-seq) appeared as the major breakthrough, as it enables comprehensive analysis of transcriptomes in thousands of single cells. In hematopoietic research, scRNA-seq allows to dissect cellular heterogeneity within phenotypically homogeneous HSPC subsets and can help reconstructing hematopoietic differentiation trajectories. In addition, with the latest single cell multi-omics approaches, it became possible to perform integrative analyses of transcriptome with epigenome and proteome data[72].

While molecular profiling is a highly sensitive method, it is also susceptible to technical artifacts. This is probably best exemplified in a study on muscle stem cells, which showed that cell isolation procedures alter global transcriptome signatures[73,74]. Similar observations were made in studies on other cell types[75], suggesting that the response to technical variations is rather conserved among tissues and cells. The source of these technical variations is not limited to sample handling and processing, but also includes biological factors (e.g., source of RNA, genetic background), experimental design (e.g., number of samples and batches) and analytical procedures (e.g., data filtering). Therefore, to achieve reliable transcriptome profiling data, it is essential to address technical considerations related to specific RNA profiling techniques but also to optimize associated experimental workflows.

Hematopoiesis and hematopoietic stem cell fates

Most of the mature blood cell types are short-lived with a need for constant replenishment. Quantitatively, this dynamics translates to a daily production of 10^{12} new cells in human[76]. To achieve this extraordinary rate, HSPCs are organized in a hierarchical manner to allow for rapid amplification of cell numbers at each developmental stage. HSCs reside at the top of this hierarchy and cycle infrequently[26-28]. This limited mitotic activity serves to protect genome integrity, counteracting replication-related DNA errors. In addition, quiescent HSCs are metabolically inactive and therefore less likely to be exposed to toxic side-products of cellular metabolism, including free radicals and reactive oxygen species[2].

Once activated, HSCs commit to one of the several fates (Fig. 2A). During self-renewing symmetric division, HSC generates two daughter stem cells to increase HSC numbers. When symmetric division occurs in the context of differentiation, this results in enhanced mature cell output at the cost of HSC loss. Finally, HSCs can undergo asymmetric division, simultaneously perpetuating themself and generating differentiated progeny[77]. The facultative commitment to one of these fates compensates for HSCs losses due to differentiation, cell death or migration into the circulation (Fig. 2A).

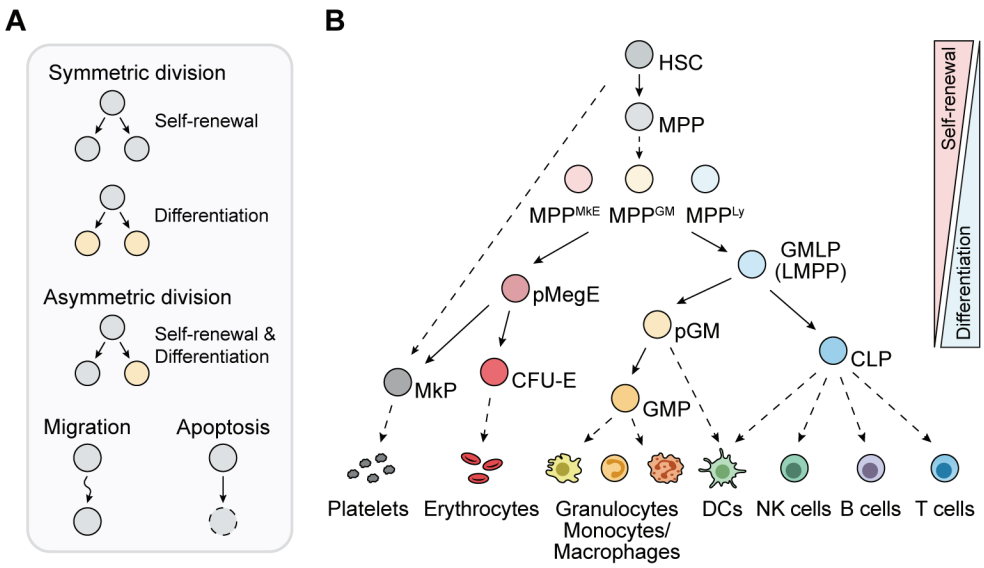

Figure 2. HSC fates. (A) Upon activation HSC can divide symmetrically to give rise to daughter HSCs. HSC differentiation involves both symmetric and asymmetric cell divisions resulting in at least one progenitor cell. HSC can also migrate into the circulation or undergo apoptosis in response to distinct stimuli. (B) The proposed model of hematopoietic development. Detailed description is provided in the main text. CFU-E, colony forming unit-erythrocyte; DCs, dendritic cells; MkP, Megakaryocyte progenitor; pMegE, pre-megakaryocyte/erythroid progenitor.

Differentiation of HSCs commences with an irreversible transition to the MPP compartment. Similar to HSCs, MPPs display multilineage potential and low proliferation rate[27,78]. However, given their inability to support long-term hematopoiesis following transplantation, MPPs have traditionally been considered to lack self-renewal potential[78]. On the other hand, lineage tracing and mathematical modeling suggest that in an unperturbed setting these cells can self-renew to a certain degree[4,62]. Regardless, dominant lineage segregation appears to occur downstream of MPPs (Fig. 2B).

Hematopoietic progenitors are indispensable to support mice survival after lethal irradiation[79] and, based on recent data, to compensate for various hematopoietic perturbations[80,81]. Hence, it appears that homeostatic control is, to a large extent, maintained at progenitor cell levels. The current knowledge on multi- and oligopotent progenitors primarily derives from transplantation and gene expression studies, which established developmental relationships among distinct cell subsets (Fig. 2B). In this model, cellular differentiation associates with progressive loss of multilineage potential, also known as *lineage commitment*, and is orchestrated by complex regulatory networks of lineage-specific genes and transcription factors. For example, cross-antagonism between transcription factors GATA1 and PU.1 has been suggested to segregate erythroid and lympho-myeloid differentiation programs respectively[82], although this view was later challenged by demonstration that both factors reinforce, rather than initiate, lineage specification[83]. Regardless, low levels of PU.1 were shown to promote lymphopoiesis, with TCF1, GATA3, and EBF1 and PAX5 further promoting T and B cell specification, respectively[84,85]. When progenitor cells present low levels of lineage-affiliated genes, they retain their alternative potentials, a phenomenon referred to as *lineage priming*. Several studies have proposed that lineage-associated programs manifest already at the level of MPPs and thus result in their biased differentiation output.

Multipotent progenitors with known lineage biases include MPP^{MkE}, MPP^{GM} and MPP^{Ly} and can be separated based on the expression of CD48, CD150 and Flt3 markers[25]. MPP^{MkE} corresponds to a previously identified MPP2 population that possesses megakaryocyte/erythroid potential[86]. MPP^{GM} and MPP^{Ly} differ in expression of Flt3 at the cell surface and have been suggested to display biased myeloid and lymphoid differentiation potentials, respectively[25]. These subsets have been associated with previously identified granulocyte-monocyte-lymphoid progenitor (GMLP) or lymphoid-primed multipotent progenitor (LMPP) populations[82,87,88]. The next step in lymphoid specification involves common lymphoid progenitors (CLPs)[89], which give rise to B, T and NK cells, while retaining limited potential for myeloid differentiation[90,91]. Importantly, while B cell specification continues in the BM, the next stages of T lymphopoiesis take place in the thymus. Myeloid development associates with generation of pre-granulocyte/macrophage progenitors (pGM) and granulocyte/macrophage progenitors (GMPs)[92]. The latter subset decomposes into monocyte progenitors and

several intermediate subsets of neutrophil precursors[93-97]. Finally, megakaryocyte potential segregates already at the earliest stage of hematopoietic hierarchy. While this developmental path is initiated at the level of MPP/MPP^{MkE} progenitors[86], more recent studies suggested that it can also derive directly from the HSC compartment (Fig. 2B) [35,46,98,99].

Megakaryocyte restriction is not the only fate associated with a fraction of HSCs. Previous large-scale single HSC transplantation studies reveled the existence of myeloid- and lymphoid-biased HSCs[100-103]. Notably, these subsets were reported to display varying responses to cytokines, such as interleukin (IL)-7[103] or transforming growth factor-β (TGF-β)[100]. More recent *in situ* barcoding studies identified multipotent, differentiation-inactive ("childless")[68,69] and myelo-erythroid restricted[69] HSCs, whose fates were shown to associate with distinct molecular signatures. In another barcoding study, leveraging HSC labeling followed by serial transplantations, distinct "low-" and "high-output" HSC clones were identified. These clones presented different capacities for long-term multilineage reconstitution, which could be linked to distinct transcriptomic profiles[60]. In all, these findings suggest that HSC fates are, at least in part, inscribed transcriptionally and can be resolved by multimodal single cell profiling.

Apart from dissecting hierarchical order among HSCs and their progenies, significant effort was also made to reveal the kinetics of cellular differentiation. By using HSC-specific lineage tracing mouse models, we and others have demonstrated fundamental differences in the emergence of HSC-derived mature populations[33,63,104]. In particular, the generation of platelets outpaces other lineages, followed by myeloid and erythroid cell production. Conversely, HSC differentiation to B and T lymphocytes occurs with a marked delay compared to other cell types[33,63,104]. This differentiation dynamics is in stark contrast to HSC-derived hematopoiesis following transplantation, where all mature blood lineages deriving from HSCs emerge at earlier time points[63].

While state-of-the-art assays and newer single-cell technologies have substantially expanded our knowledge on hematopoietic development, they all associate with built-in limitations. Perhaps the most striking example comes from high-throughput single-cell RNA sequencing (scRNA-seq) studies that proposed the concept of transcriptional priming, wherein the expression of lineage-associated genes in multipotent HSPCs drives their lineage commitment. As a consequence, hematopoietic differentiation would involve unipotent progenitors, without obligatory transition through developmental stages characterized by mixed lineage potential[96,105]. This interpretation can be challenged, as gene expression patterns might not be the only determinant of cell potential. Thus, complementary functional and molecular approaches are critical to fully resolve the population-level complexity among HSPCs.

Hematopoietic system aging

Aging associates with numerous changes impacting hematopoiesis and hematopoietic tissue function. This is reflected by a reduced capacity of old hematopoietic system to sustain homeostasis and effectively respond to organismal demands in response to injury or stress. Clinically, these effects manifest in the increased prevalence of anemias, susceptibility to infectious diseases and poor responses to vaccination frequently observed among elderlies. Moreover, aging strongly correlates with blood malignancies, including myeloproliferative disorders or leukemias. The widespread effects of aging on the hematopoietic system encompass alterations in mature cell compartments, remodeling of hematopoietic tissues, and systemic changes in circulating factors. Because these elements are interdependent, they collectively contribute to reduced regenerative potential of the senile hematopoietic system.

While age-related changes affect all mature cell compartments, the most notable alterations are observed within the adaptive immune system, particularly T and B cell pools. For instance, T cell aging associates with population shifts from naïve to memory T cell phenotypes. This change is largely attributed to age-associated involution of the thymus, which initiates already at the puberty[106]. As the production of new naïve T cells gradually declines with age, existing T cells undergo compensatory expansion through homeostatic proliferation to sustain T cell pools in the periphery. Notably, both antigen-driven clonal expansions and homeostatic proliferation lead to the acquisition of memory-like phenotypes, which result in a reduced diversity of available T cell receptor repertoires and compromised responses to new antigens[107]. Furthermore, aging promotes the accumulation of T cells with proinflammatory properties, contributing to chronic inflammation and immune dysfunction in older individuals[107]. Age-related involution of the thymus results from decreased numbers of thymic epithelial cells, which support its structure, and from expansion of adipocytes, whose byproducts may be toxic to developing thymocytes[108]. However, this process is also influenced by reduced influx, expansion, and maturation of T cell progenitors. For instance, our laboratory has previously demonstrated that blocking early lymphopoiesis in the BM results in thymic atrophy already in young mice[88]. Therefore, in addition to established interventions such as pharmacological treatments with growth hormones and cytokines to restore thymic mass[108], strategies aimed at enhancing BM lymphopoiesis are essential for improving thymic output and enhancing T cell-dependent immunity in the elderly.

Akin to changes observed in the T cell compartments, aging also impacts the diversity and function of mature B cell subsets. This is exemplified by an increase in numbers of antigen-experienced long-lived B cells in aged individuals. Aged naïve B cells exhibit impaired class switch recombination, which impacts the quality of newly produced antibodies. Connected to this is the observation that aged B cells

tend to produce autoreactive antibodies[109]. Overall, these alterations contribute to reduced diversity of peripheral B cell repertoire and compromised antibody production[108,110]. Furthermore, aging promotes accumulation of age-associated B cells (ABCs)[111,112], which possess unique transcriptional, phenotypic, and activation characteristics, distinct from other mature B cell subsets[113]. These ABCs have been shown to inhibit early B lymphopoiesis by producing pro-inflammatory cytokine – tumor necrosis factor-alpha (TNF-α)[114]. Intriguingly, Keren et al. reported that elimination of mature B cells in old mice, either through antibody-mediated depletion[115] or by employing B-cell deficient mouse models[116], alleviates age-associated reduction in B lymphopoiesis. Thus, it was suggested that declines in B cell production during aging are at least in part an adaptive homeostatic response to the accumulating ABCs[116]. Moreover, increasing evidence suggests that ABCs are causatively linked to a broad spectrum of autoimmune and autoinflammatory diseases, not the least due to their contribution to the chronic inflammatory milieu[113].

As emphasized in previous sections, aging and age-related changes in adaptive immunity entail systemic increase in the levels of pro-inflammatory molecules. Indeed, low-grade chronic inflammation that develops with age, commonly referred to as *inflammaging,* is characterized by elevated serum levels of cytokines and mediators, including IL-6, C-reactive protein, TNF, fibrinogen, IL-1β and other molecules[106,110]. The candidate sources of age-associated inflammation encompass the aforementioned activated T cells and ABCs, but also distinct subsets of myeloid cells, adipocytes and cells that exhibit the senescence-associated secretory phenotype. Chronic inflammation contributes to diminished antigen-specific responses, reduced vaccination efficacy, but also impairs tissue integrity and affects the function of other hematopoietic cells[106,110].

While age-related changes in mature cell composition, thymic involution and low-grade chronic inflammation undoubtedly contribute to gradual decline in cell-based immunity, it is well established that impaired lymphopoiesis also results from cellular and molecular alterations within the most primitive HSPC compartments. Accordingly, BM CLPs and early B cell progenitors decrease in numbers[117-119] and the remaining lymphoid precursors exhibit compromised function upon aging[118,119]. The impaired lymphoid differentiation was further associated with alterations within LMPP/GMLP fraction[120]. It was demonstrated that aged LMPPs downregulate expression of lymphoid-associated transcripts, possess decreased differentiation capacity[120] and have higher proliferation kinetics compared to their young counterparts[27,120]. Apart from changes at the level of lymphoid progenitor cells, aging is also accompanied by decreased levels of erythroid progenitors, as well as increased levels of megakaryocyte progenitors and GMPs[117,121-123]. Mounting evidence suggests that these alterations at least in part originate from alterations within the HSC compartment.

Hematopoietic stem cell aging

Initial insights into the biology of HSC aging derived from transplantation studies, comparing the function of BM or purified HSCs from young and aged donor mice in lethally irradiated recipients. These studies revealed pronounced changes in the functionality of stem cells upon aging and pointed toward intrinsic alterations as the underlying cause of age-related hematopoietic decline.

When evaluating reconstitution potential of unfractionated BM from young and aged mice upon serial transplantation, early studies concluded that HSC function is not affected by age[124,125]. However, with the advent of flow cytometry and identification of surface markers that enrich for stem cell activity, subsequent studies revealed that highly purified aged HSCs are less effective in multilineage reconstitution compared to their young counterparts[117,126,127]. This was shown to coincide with an increase in HSC pool size, a finding that holds true regardless of the marker combination or isolation protocol used[101,117,128-130]. Notably, while aged HSCs outnumber their young counterparts by several folds, this expansion varies significantly among individual mice[127]. Increased numbers of aged HSCs were also observed upon their transplantation into young recipients, indicating that age-related HSC expansion is an intrinsic and transplantable property of these cells[117]. Hence, when considering both decreased reconstitution potential and numerical expansion of HSCs during aging, this demonstrates severely compromised function per individual cell. Apart from their reduced reconstitution potential, aged HSCs display striking defects in their lymphoid differentiation[117,126,127,130]. Consequently, the reduced lymphocyte production alters the composition of mature blood cells in favor of myeloid lineage - a phenomenon frequently referred to as a *myeloid bias*.

HSC heterogeneity and the dynamics of HSC pool

The HSC compartment could be viewed as a uniform population of cells with equal functional activities. Thus, any changes that arise during development and/or aging would affect each clone in a similar manner. However, it is now well recognized that despite their immunophenotypic similarity, HSCs are functionally heterogeneous[48]. Early transplantation studies demonstrated that individual HSCs differ in their lifespans, magnitudes of differentiation and the type of mature blood cells they produced (lineage bias)[102,103]. While the identification of such clones was at that time purely retrospective, later studies discovered the markers that helped enrich for certain cellular behaviors. For example, the expression of marker protein CD150 has been shown to discriminate between HSC clones with myeloid- and balanced/lymphoid-biased differentiation preferences[126,131]. HSCs expressing higher levels of CD150 tend to produce a myeloid-biased output[126,131]. Additionally, such cells display increased expression of myeloid-associated transcripts, suggesting that lineage bias is inherently primed by differential gene expression[126]. Similar lineage bias was found for HSCs displaying distinct capacity for Hoechst

dye efflux (described previously side population)[100]. Notably, the identified myeloid- and lymphoid-biased subsets appeared to respond differently to TGF-β. Thus, it was postulated that extrinsic factors, such as TGF-β, could be involved in regulation of the HSC population dynamics[100]. An alternative strategy to separate HSCs with distinct lineage potentials employed the combination of CD41 and CD150 surface markers[132]. The comparison between CD41-positive and CD41-negative HSCs revealed enrichment for myeloid differentiation potential within the former fraction[132]. Finally, by using reported mouse models, the Jacobsen laboratory discovered a subpopulation of *Vwf*-expressing HSCs that present increased propensity for platelet differentiation[34,35]. It is however noteworthy that while oligolineage/platelet-restricted reconstitution associated with the expression of *Vwf*, a proportion of *Vwf*+ HSCs with multilineage reconstitution potential could be also observed[35].

Given the heterogeneity of the HSC compartment in young age and altered reconstitution patterns of aged HSCs, it is intuitive to propose the existence of a causative link between aging and changes in the composition of the HSC pool. The experimental evidence for heterogeneity of aged HSC compartment derives from studies employing transplantation of limited numbers/single HSCs. Similar to young mice, individual aged HSCs were shown to differ in the magnitude by which they support blood formation and in their preferential differentiation trajectories[100,121,126,127,133,134]. Most studies revealed age-related accumulation of clones with myeloid-biased output at the expense of lymphoid-competent HSCs[100,126,127,132-134]. Importantly, these cells could be prospectively isolated using the aforementioned markers CD150[126], CD41[132] or dye efflux ability[100]. Additional evidence exists for age-associated increase in platelet-biased HSCs[121] and accumulation of myeloid-restricted progenitors within the phenotypic HSC compartment[135].

By employing genetic barcoding and transplantation approaches, we and others have demonstrated that both young and aged HSC clones actively contribute to hematopoiesis[134,136]. In line with previous data, our detailed analysis further revealed increased frequency of myeloid-biased clones as a consequence of aging. While the frequency of clones with B cell and/or erythroid potentials did not change with age, a striking decrease was observed for aged HSC clones harboring T cell potential. Together with the observed overall reduction in differentiation ability of aged HSCs, this entails that aging not only drives changes in the clonal composition of the HSC compartment, but also affects the function of individual cells[134].

Regulation of hematopoietic stem cell aging

Intrinsic regulation

HSC aging associates with changes at every level of gene expression control described by the central dogma of molecular biology. A number of previous studies identified age-related alterations in HSCs that affect their genome, transcriptome, and proteome[137]. Notably, these changes impact established hallmarks of aging, indicating again that stem cells, similar to other somatic cells, are subject to a common set of age-associated insults.

The notion that the strict maintenance of genomic integrity is essential for proper HSC function had been supported by previous studies demonstrating that HSCs derived from mice deficient in DNA damage repair develop premature stem cell defects, including impaired self-renewal[138-140]. Because phenotypes observed in these mice recapitulate to some extent physiological HSC aging, it was suggested that the accumulation of DNA lesions might be causally linked to stem cell aging. Indeed, aged HSCs harbor increased amounts of γH2AX foci[140,141], which has historically been interpreted as a sign of accumulating DNA double-strand breaks (DSBs). On the other hand, γH2AX also marks stalled/collapsed replication forks, and thus later work suggested that replication stress, rather than accumulation of DSBs, drives HSC aging[141]. Regardless, later work proposed that HSCs indeed accumulate DNA lesions over time; this can be however effectively repaired once the cells start to proliferate[142]. Notably, another study arrived at an opposite conclusion, instead proposing that stress-induced HSC proliferation leads to increased accrual of DNA damage[143].

A vast number of studies have focused on delineating molecular changes that associate with hematopoietic aging by comparing transcriptomes of young and aged HSCs[117,121,128,141,144-150]. These studies have revealed a plethora of distinct mechanisms with little overlap between individual reports. Among these changes were alterations in expression of genes involved in multilineage differentiation. For instance, Rossi et al. demonstrated upregulated expression of myeloid-associated genes in aged HSCs, while young stem cells were enriched for transcripts involved in lymphoid specification[117]. This study was the first to provide evidence that at least some of the aging phenotypes, including myeloid skewing, might be evident when looking at the transcriptomic signature. Notably, altered gene expression profiles found in aged HSCs persisted after transplantation, indicating that the core aging signature is stable even after transplantation-dependent stress[149]. HSC aging was also proposed to associate with upregulated expression of genes involved in megakaryocyte specification[121]. Another study concluded that a change in cell cycle dynamics is implicated in HSC aging phenotypes, including regulation of self-renewal[145]. Besides, the cycling status of aged HSCs has been under debate, as distinct studies reported lower[129], higher[54,145,147] and similar[27,130] frequencies of quiescent cells among aged HSCs as compared to their younger counterpart. Some

studies also pointed to an altered inflammatory response as a main contributor to age-associated hematopoietic decline[128,146]. This is also debatable as evidenced by two reports from the same laboratory reporting paradoxically opposite signature enrichment, with aged HSCs displaying either upregulated[128] or downregulated[148] expression patterns of genes involved in inflammatory and stress responses. Regardless, both studies documented increased expression of gene encoding the adhesion molecule P-selectin, which was later confirmed by an independent group[151]. It should be noted that a number of studies provided evidence for additional pathways and molecular processes involved in HSC aging, including alterations in mitochondria function[152], deregulated autophagy and metabolism[153] or replication stress[141].

A recent meta-analysis of existing transcriptome profiling datasets proposed a core aging signature and pointed to membrane-associated transcripts as the most consistently deregulated genes in HSC aging[151]. This study further implied increased transcriptional heterogeneity of the aged HSCs and identified clones that possessed "young-like" molecular signature. At the same time, the authors highlighted common study-to-study variations, although they did not provide an explanation for this discrepancy[151]. Our analyses presented in this thesis indicate that the group of transcripts that differed most among these datasets constitute immediate early genes (IEGs).

IEGs encompass a set of genes that are rapidly activated in response to a wide range of cellular stimuli. They are involved in regulation of cellular response to stress, known as *immediate early response* and play important function in biological processes such as immune activation, cell growth and differentiation. IEGs, as opposed to their secondary response genes, do not require *de novo* synthesis of factors critical for gene expression. Therefore, their activation is rapid and not halted by protein synthesis inhibitors. Importantly, their expression is transient, typically peaking at 30-60 min after stimulation and followed by upregulation of genes involved in secondary responses[154]. The canonical examples of IEGs include *Fos*, *Jun* and *Egr1*, with some of them previously implicated in the regulation of HSC function[155,156].

Distinct transcriptional signatures of young and aged HSCs can be attributed to changes in epigenetic regulation. In support to this notion, we previously observed that reprogrammed aged hematopoietic progenitors were functionally and transcriptionally equivalent to young HSPCs[134]. More direct analyses of the epigenomic landscape in young and aged HSCs revealed age-associated changes with respect to DNA methylation, histone modification and chromatin architecture. In line with this, epigenetic modifiers are important for maintenance of HSC self-renewal and differentiation[157]. This is further supported by the observation that individuals who develop blood disorders or disease-associated conditions, such as clonal hematopoiesis, frequently harbor mutations in genes encoding epigenetic regulators. Previous reports documented locus-specific differences in DNA

methylation profiles that associate with HSC aging[148,158]. One of the most consistently observed epigenetic alterations upon aging is hypermethylation (deposition of repressive marks) at genetic loci of Polycomb Repressive Complex 2, which in itself is responsible for establishing repressive histone marks[148,158,159]. Similar to DNA methylation patterns, aging affects the landscape of histone modifications. One notable example is acetylation of histone 4 (H4K16ac, an activating mark), which not only is reduced, but also exhibits more dispersed expression over the nucleus as compared to young HSCs[160]. This characteristic, referred to as loss of epigenetic polarity, results from age-related increase in Rho GTPase Cdc42 activity, which regulates H4K16ac levels and polarity. Targeting Cdc42 with the small molecule CASIN was shown to ameliorate some of the HSC aging phenotypes[160] and this treatment was more recently proposed as longevity promoting strategy[161].

Extrinsic regulation

HSCs occupy specific BM environments, referred to as *niches*, which comprise a network of hematopoietic and non-hematopoietic cells, the latter encompassing endothelial cells, mesenchymal stromal cells, osteoblasts, adipocytes, and others. The interactions between HSCs and distinct BM niche components ensure proper hematopoietic homeostasis by regulating stem cell quiescence, self-renewal, proliferation, and differentiation. During aging, the BM microenvironment undergoes substantial remodeling, which is manifested by the accumulation of adipocytes, degeneration of the vascular system, altered innervation and bone loss[162]. Apart from changes in the BM structure and cellular composition, aging associates with increased local levels of pro-inflammatory factors, including for instance IL-1β, IL-6, TGF-β and Ccl5[162].

Previous studies have unequivocally established that several HSC aging phenotypes result from intrinsic changes within HSC compartment[117,163]. However, increasing evidence suggests that age-related alterations in the BM microenvironment might also contribute to HSC aging and the skewed production of blood cells. For instance, studies have linked elevated levels of IL-1β in aged mice to enhanced myelopoiesis[164]. Increased myeloid output has also been linked to rises in concentration of Ccl5[165] or reductions in insulin-like growth factor-1 (IGF-1)[166] and osteopontin[167]. Additionally, age-related decline in erythropoiesis and HSC platelet bias were attributed to elevation in IL-6 and TGF-β, respectively[122]. Consequently, targeting these factors with mAbs proved successful in restoring some of the aging phenotypes. In the context of rejuvenating interventions, conflicting reports exist on the beneficial effects of young bloodborne factors and/or BM niche on the function of aged HSCs. Some studies indicate that a youthful environment can enhance the function of aged HSCs[165,167], while others refute this idea[117,168,169]. Adding complexity to the matter, most transplantation studies involve irradiation-based

conditioning, a procedure known to induce the production of inflammatory cytokines likely to influence the outcome of reconstitution[170].

Age-associated disorders and disease conditions

Clinical manifestations of age-related hematopoietic decline include anemia, decreased immunity, or distinct forms of blood cell expansion, including clonal hematopoiesis, myelodysplastic syndrome, and leukemia. Among these, anemia is the most common, affecting up to more than 20% of elderly individuals[171]. The causes of anemia in the elderly are multifactorial and often include malnutrition and coexisting diseases, such as bone marrow failure, non-hematopoietic neoplasms, chronic infections and inflammaging[171]. As previously discussed, inflammaging can lead to the development of various immune alterations, collectively referred to as *immunosenescence*. Immunosenescence leads to compromised function of immune system in older individuals, making them more vulnerable to infectious diseases and less responsive to vaccination. Additionally, it restricts the effectiveness of immunotherapies used against autoimmune syndromes and cancer[172].

Numerous prior studies have shown that some individuals carry genetic mutations in their blood cells in the complete absence of any overt manifestations of disease. It was further shown that such mutations occur in less than 5% of individuals younger than 50 years but become more prevalent with time, reaching 10-20% of people above 70 years[173]. This associates with a clonal dominance of the mutated HSCs. Hence, it became evident that this condition, known as clonal hematopoiesis (CH) occurs normally during life and strongly associates with advanced age. If the mutations occur in genes that are not leukemia-drivers, this state is defined as Age-Related Clonal Hematopoiesis (ARCH). On the other hand, when CH mutations occur in genes associated with hematological malignancies with variant allele frequency of at least 2%, this is referred to as clonal hematopoiesis of indeterminate potential (CHIP). Although CHIP is not a disease in itself, it increases the risk of leukemic transformation, and associates with higher morbidity and mortality from atherosclerotic cardiovascular diseases and ischemic stroke[174].

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid neoplasms characterized by abnormal growth of aberrant HSCs. MDS manifests in cytopenia, BM dysplasia, and ineffective hematopoiesis, leading to increased risk of acute leukemias. The most frequently observed mutations in MDS are found in splicing-related genes (e.g., *Sf3b1*, *Srsf2*, *Zrsr2* or *U2af1*) and epigenetic regulators (e.g., *Dnmt3a*, *Tet2* or *Asxl1*)[175]. MDS can further progress to acute myeloid leukemias. Among elderly, there is a high incidence and mortality from acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL), which differ in their dynamics, lineage-skewing, and prognosis. Although both malignancies

strongly associate with advanced age, they vary in disease progression and originate from distinct cell subsets, with AML stemming from more primitive blood cells and CLL sourced from a B cell committed population. Notably, for most of these hematopoietic diseases, the only curative regimen is BM transplantation.

Hematopoietic stem cell-based therapies

Since the initial demonstration that HSPC transplantation can provide therapeutic effects in lethally irradiated recipients[176], the procedure has become widely used in the clinic to treat various hematological disorders. As a treatment modality, HSPC transplantation enables replacement of patients' malignant cells with healthy donor cells, which regenerate the entire hematopoietic system via the self-renewal and differentiation of donor HSCs. Worldwide, the number of HSPC transplantations is on the rise, being employed to treat various blood and immune disorders, including described above leukemias, myelodysplastic syndromes, hemoglobinopathies and immunodeficiencies[177]. HSPC transplantation is also applied for other diseases, mainly cancer, where the cytostatic treatment used to eradicate malignant cells leads to collateral destruction of hematopoiesis. HSPC transplantation can be divided into *autologous*, where the recipient is transplanted with their own HSPCs or *allogeneic*, in which case the recipient receives HSPCs from another healthy individual.

Transplantation also provides a powerful research tool to study various facets of hematopoiesis. Experimentally, successful transplantation typically requires conditioning of recipient mice to enable donor cell engraftment. Standard regimens involve administration of cytotoxic chemotherapy or total body irradiation (TBI). Such treatment effectively eliminates host cells and provides space in the BM for the transplanted cells. In some settings, conditioning also serves to reduce responses from the host immune system and consequently the risk of graft rejection. This is also relevant in experimental studies using the CD45 congenic mouse system, as immunogenicity of specific CD45 antigens was found to limit donor cell engraftment in unconditioned (as we show in *Paper IV*) or minimally conditioned recipients[178]. Yet, the unspecific nature of conventional strategies leads to many deleterious short- and long-term side effects, including multi-organ damage, blood deficiencies and increased risk of infection. Moreover, radiation induces increased production of pro-inflammatory cytokines through bystander effect, which negatively impacts tissue integrity and regeneration[179]. All these factors are likely to influence the fate of transplanted cells, and indeed previous studies demonstrated distinct differentiation patterns of donor HSCs when transplanted into irradiated and unconditioned recipients[180].

The use of mAbs instead of chemotherapy and/or TBI for transplantation conditioning was launched by Czechowicz and colleagues who applied anti-CD117

treatment to block critical signal that promotes survival of HSPCs[181]. However, because the strategy worked only in an immunocompromised setting, the consecutive studies used anti-CD117 in combination with low dose TBI[182] or anti-CD47 mAb[183] to condition immune intact hosts. Regardless, the anti-CD117 mAb-based treatment is currently under phase 1/2 clinical trial as a conditioning regimen for patients with severe combined immunodeficiency (NCT02963064). An alternative strategy to targeting CD117 is based on CD45 (pan-leukocyte) antigen[184]. In this approach, anti-CD45 mAb is conjugated with ribosome-inactivating protein saporin (SAP) to create immunotoxin, which upon internalization into the cytosol halts protein synthesis leading to death of targeted cells (Fig. 3). CD45-SAP was reported to efficiently deplete endogenous HSPCs, allowing for high-level donor cell engraftment in wild type recipients and experimental murine models of Fanconi Anemia[184], immunodeficiency[185] and hemophilia A[186].

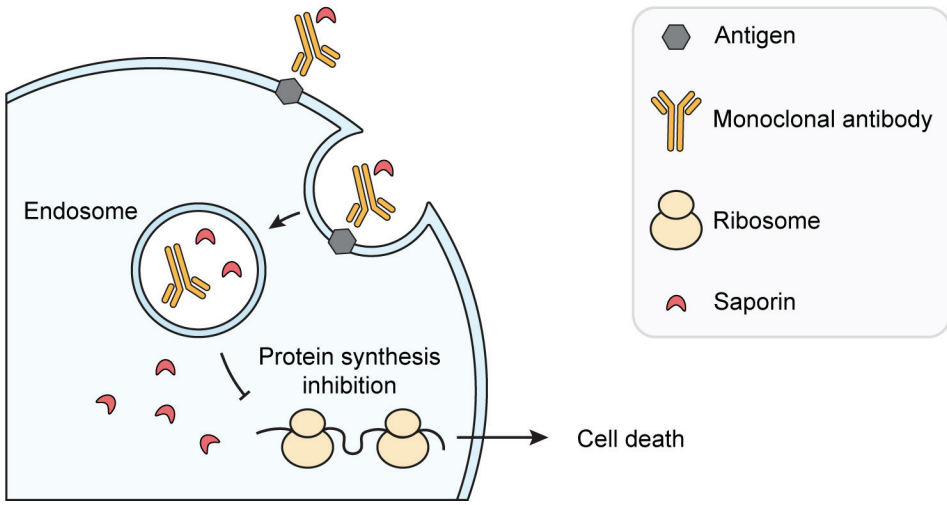

Figure 3. Mechanism of immunotoxin-based cell depletion.

The obvious advantage of unconditioned transplantation is safety of the procedure and its minimal influence on the microenvironment. This is particularly relevant for studies on tissue homeostasis, cellular interactions within the native niche and the systemic and microenvironmental factors regulating cell function. However, the attempts to achieve high level, sustained myeloid chimerism in non-irradiated recipients have for the most part been disappointing[181,187]. This has led to the concept that at steady state there is limited availability of "spaces" in the BM, which restricts engraftment of donor cells[188]. Attempts to address this attractive hypothesis included experimental settings in which recipients were subjected to repetitive transplantation with donor cells[189,190]. The rationale for such approach was based on

the fact that HSCs and/or progenitors circulate under physiological conditions[191,192], and thus the steady-state egress of HSCs should leave vacant spaces in the BM at any given time. Indeed, evidence exists for improved reconstitution outcomes in recipients receiving daily injections with donor cells, as compared to single bolus transplantation[189]. Alternative approaches rely on the use of mobilizing agents, such as G-CSF or plerixafor (AMD3100), which enforce HSPC migration from the BM by altering their interaction with BM microenvironment[193-195]. These strategies have also proven successful for more effective donor engraftment[196-198]. Regardless of the conditioning protocol, transplantation outcomes highly depend on the dose of transplanted cells. Indeed, escalating doses of donor cells contribute to higher reconstitution outcomes even in non-irradiated recipients[181,199]. However, the scarcity of HSCs in the BM imposes the major limitation for such approach.

Ex vivo HSC expansion has been the long-standing goal in regenerative medicine, not the least due to its potential to increase the number of cells available for transplantation. However, despite efforts, HSCs have long been difficult to grow *ex vivo.* The most common approach to amplify stem cell numbers rely on their *in vitro* stimulation with cytokines and growth factors, such as stem cell factor (SCF) and thrombopoietin (TPO) and IL-11[200]. The culture media is further supplemented with fetal bovine serum to support cell growth. Such culture conditions allow the maintenance of HSCs *ex vivo* but provide rather modest support for their self-renewal[200]. Interestingly, it was recently suggested that *ex vivo* culture may induce proteotoxic stress leading to reduced activity of cultured HSCs. Consequently, by supplementing media with a small molecule that restores proteostasis, Kruta et al. documented improved *in vivo* function of cultured HSCs. The treatment however did not promote stem cell self-renewal[201]. An alternative recent approach employs a synthetic polymer polyvinyl alcohol (PVA) instead of serum, in combination with SCF, TPO and insulin[202]. Such PVA-based culture system allows for expansion of purified murine HSCs up to 899-fold during a 4-week culture period[202]. Further studies from Wilkinson and co-workers suggested that the PVA-based media can also enrich for HSPCs from the BM without the need for HSC purification prior to culture[203]. Most importantly, the PVA-based culture mediated HSC expansion that allowed for successful engraftment into nonconditioned recipients. With recent developments of an equivalent protocol for human HSCs expansion[204], these newer culture systems hold promise for improving safety and efficacy of transplantation procedure in the future.

Altogether, in this thesis, I tackled some of the fundamental conundrums related to hematopoietic stem cell aging. Specifically, I determined the perturbed differentiation trajectories of aged HSCs at the single cell level, revealing an early differentiation block underlying dysfunctional lymphopoiesis (*Paper I*). I have further understood possible confounding factors resulting in variability of transcriptomic profiles reported for senile HSCs (*Paper II*). Finally, I have in collaborative efforts described an *ex vivo* expansion system of HSCs (*Paper IV*) and

employed it together with mild, mAb-dependent conditioning regimen in an approach to overcome age-related hematopoietic dysfunction at steady state and in a disease context (*Paper III*).

Aims of the thesis

The overall goal of this thesis work was to gain better understanding of the physiology of HSC aging and to explore stem cell transplantation as a means to reinstate healthy hematopoiesis in the elderly. To accomplish this goal, we performed a set of experiments addressing project-specific aims that are outlined below.

In **Paper I**, I sought to identify key features of native HSC-derived hematopoiesis during chronological aging. The second objective of this project was to establish a reference molecular landscape of early hematopoiesis.

In **Paper II**, I aimed to define how cell isolation procedures impact the molecular profiles of primary HSCs and whether these responses depend on age.

Paper III was designed to explore the prospects for hematopoietic rejuvenation through a cell replacement approach. Specifically, I aimed to establish a non-invasive transplantation protocol for efficient integration of young cells into aged hosts and to further examine the function of young HSCs within an aged environment.

Paper IV was aimed to detail previously the reported PVA-based culture system and to examine the self-renewal and differentiation of HSCs in *in vitro* cultures. By addressing several key concerns related to culture conditions, we created a robust foundation for effective expansion of HSCs *ex vivo*, which was extensively exploited in *Paper I* and *Paper III* in this thesis.

Summary of included papers

Paper I

Aging associates with compromised hematopoietic function. Previous studies have linked these changes to defects within aged HSCs, highlighting their myeloid-biased differentiation patterns and decreased lymphoid potential. However, many of these investigations relied on the transplantation assay, which we now know does not faithfully reflect stem cell function under physiological conditions[48]. In parallel to transplantation-based studies, other research efforts employed transcriptome profiling to uncover molecular mechanisms involved in HSC aging. However, although these approaches provided important insights into the transcriptional states of aged HSCs, they often fell short of directly revealing information about cellular differentiation and its time-resolved dimension. Therefore, an important next step was to devise a method that would allow for the analysis of ongoing differentiation from aged HSCs at the single cell resolution. We reasoned that by combining lineage tracing with single cell transcriptomics, we could overcome some of the major limitations associated with employing each method independently. This also held the potential to provide important new insights into hematopoietic alterations during aging.

By using an HSC-specific Fgd5-based lineage tracing system, we first established that aged HSCs continue to support steady-state hematopoiesis in the native setting. We further showed that, similar as at the young age, the differentiation of aged HSCs into mature blood cells follows distinct kinetics depending on cell lineage. However, when analyzing the overall multilineage output, we observed a gradual decline in HSC activity during aging. The reduction in HSC differentiation was particularly pronounced for mature B and T cells and less evident for the platelet lineage.

To resolve age-related differentiation defects during early hematopoiesis, we next decided to employ combined fate and state mapping by single cell profiling. To this end, we first established a reference transcriptomic landscape of murine HSPCs. By combining single cell transcriptome and epitope profiling[205], we demonstrated that hematopoietic differentiation proceeds through obligatory intermediate cell stages, that are characterized by well-defined immunophenotypes. In addition, our analysis led us to uncover previously uncharacterized populations, including MPP Ly-I and MPP Ly-II. By using *in vivo* proliferation, transplantation, and mAb-based cell depletion assays, we further showed that MPP Ly-I and MPP Ly-II are immediate

progeny of HSCs and possess robust lymphoid differentiation potential. We further showed that these populations are hierarchically related, with MPP Ly-I representing a more primitive developmental stage than MPP Ly-II cells.

We next investigated the mechanisms responsible for decreased HSC contribution to mature blood cells in aged mice. For this, we projected HSC-derived cells onto the reference HSPC landscape, which allowed us to quantify changes in HSC differentiation routes during aging. We found that aged HSCs exhibit a pronounced differentiation block into all downstream progenitors, which was strikingly evident for MPP Ly-I and MPP Ly-II subsets. Based on these findings, we concluded that age-related reductions in lymphopoiesis results, at least in part, from a diminished capacity of aged HSCs to generate early lymphoid-committed progenitors, including MPP Ly cells. This hypothesis was further strengthened by our observation on reduced frequencies of MPP Ly-I and MPP Ly-II cells in aged mice.

Finally, we explored the potential of *ex vivo* HSC expansion system[202] to alter the function of aged HSCs. We first established that the PVA-based culture system effectively supports the maintenance and expansion of aged HSCs, akin to young cells. Given this finding, we reasoned that enforced activation of aged HSCs might improve their *in vivo* function. However, contrary to our expectations, the competitive transplantation experiments revealed that most *ex vivo* expanded aged HSCs fail to support long-term multilineage hematopoiesis. Interestingly, by increasing the input number of aged HSCs to *ex vivo* cultures, we reported markedly improved reconstitution and in particular, lymphocyte production. This suggests that while the pool of aged HSCs is highly heterogeneous, it retains rare clones capable of efficient lymphopoiesis.

Collectively, this study provided new insights into HSC contribution to native hematopoiesis during aging. We identified reduced multilineage differentiation as a key feature of aging HSCs. Our study also revealed age-related reductions in early lymphoid-committed progenitors, which likely contribute to age-related lymphoid deficiencies. Finally, we demonstrated the potential of a PVA-based culture system to sustain aged HSCs *ex vivo*, which offers an exciting possibility to manipulate and enhance their *in vivo* function.

Paper II

Differential gene expression acts as a molecular blueprint that dictates unique characteristics and functions of cells. In line with this, numerous studies have conducted genome-wide assessments of young and aged HSCs, aiming to uncover the underlying causes of age-related decline in HSC function. However, despite continuous efforts, a comprehensive understanding of this process is still lacking. This is largely due to discrepancies among prior studies reporting multiple distinct

mechanisms possibly involved in stem cell aging. Although the reasons for these discrepancies are unknown, we speculated that one potential explanation might be the difference in cell isolation procedure and/or downstream sample processing. This is further underscored by the fact that different laboratories employ slightly different HSC isolation protocols. These include for instance labeling of cells with target-specific probes or the use of dye uptake/exclusion technique, both of which require incubation of cells at elevated temperatures prior to cell sorting. However, little is known about how such treatments impact HSC transcriptomes.

In *Paper II*, we set out to characterize how experimental procedures may influence the molecular profiles of primary HSPCs and whether these responses are age-dependent. We initially focused on comparing gene expression patterns in young and aged HSCs from our two previously published datasets[149,150]. Because these analyses were performed as three separate batches, this allowed us to assess potential impact of different experimental handling. We found that transcripts upregulated in batch 1 comprised many IEGs, that strongly associated to TNF-α signaling pathway. This was paralleled by downregulation of genes involved in cell cycle regulation. Based on these up- and downregulated genes, we derived the immediate early response (IER) and cell cycle signatures, respectively. By performing time course gene expression analysis, we further showed that the kinetics and magnitude of IER signature (IERsig) induction is comparable for young and aged HSCs. Therefore, we concluded that while IERsig can be rapidly activated in primary HSCs, its' induction appears to be independent of age.

Using gene set enrichment analysis, we next assessed IER and cell cycle gene expression profiles in 13 datasets on murine HSC aging. To benchmark our analysis, we also included aging and young HSC signature sets derived from previous meta-analysis study[151]. We found that in all investigated datasets, young and aged HSCs displayed their respective young and aging signatures. Cell cycle signature was predominantly enriched in young HSCs. By contrast, the activation of IERsig was inconsistent across studies; some studies demonstrated its induction in aged HSCs, others observed it in young HSCs, and finally some studies revealed no association of the signature with age. These findings highlight irregular activation of IERsig across various studies, which has broad implications for interpretation of HSC aging mechanisms.

Induction of IEGs has been previously suggested as a technical artifact resulting from tissue dissociation and exposure of cells to elevated temperatures in the fields outside hematopoiesis[73-75]. To evaluate whether the same holds true in primary HSPCs, we employed a reporter mouse line for Nr4a1, a prominent IER gene, and subjected the cells to heat stress. Indeed, we observed marked upregulation of IERsig, which notably could be mitigated by the transcriptional inhibitor triptolide (TP) in a dose-dependent manner. Our subsequent scRNA-seq experiment largely validated these findings. Differential gene expression analysis further revealed

strong association of genes induced at 37°C to TNF-α signaling pathway, which was accompanied by a decrease in expression of genes involved in cell cycle pathways. Importantly, we found that TP had no noticeable impact on the molecular profiles of cells that were kept on ice. Finally, we showed that the induction of IERsig has no functional consequences on the long-term HSC activity.

In summary, our study identified a stress-associated transcriptional signature in primary HSPCs that is rapidly induced during cell isolation procedure but is uncoupled from aging. Through this work, we aim to raise awareness about it and its potential to confound interpretations of gene expression in primary HSPCs.

Paper III

As discussed in *Paper I* in this thesis, many age-associated changes in the hematopoietic system directly result from cell-intrinsic alterations occurring in HSCs. In parallel investigation in the frame of *Paper III*, we aimed to determine whether extrinsic factors also contribute to this process and to what extent age-related changes in the BM microenvironment alter HSC function. This objective was partly based on previous reports, showing that systemic and/or BM remodeling drives age-associated hematopoietic phenotypes[162]. Furthermore, we intended to explore whether cell replacement approaches, such as transplantation of young HSCs, could potentially improve hematopoietic outputs in the elderly. Traditional TBI- and/or chemotherapy-based transplantation is however poorly tolerated by older individuals. In addition, it is now increasingly evident that standard conditioning regiments alter the dynamics of transplanted cells[170], which makes the comprehensive analysis difficult. Therefore, we took the view that alternative non-invasive transplantation methods are needed to study the impact of recipient age on the function of transplanted young HSCs.

To begin addressing our research questions, we employed a previously established CD45-SAP-based conditioning regimen as a non-toxic alternative to TBI[184]. We first demonstrated that this conditioning resembles native HSC behavior better than TBI-based transplantation. At the same time, we found that CD45-SAP, much like irradiation, induces significant proliferation and decreases the ability of primary transplanted HSCs to competitively reconstitute secondary hosts.

When applied to aged mice, CD45-SAP effectively eliminated host HSCs, while sparing more mature hematopoietic cells. However, in contrast to young mice, the treatment failed to support the engraftment of freshly isolated young HSPCs. Through extensive optimization of the protocol, we showed that the transplantation barrier in aged hosts can be overcome by injecting escalating doses of HSCs and combining two mechanistically different non-invasive conditioning techniques - CD45-SAP- and mobilization-based regimens.

Systematic examination of young HSCs' performance in aged hosts revealed their function. In particular, we found comparable *in vivo* proliferation dynamics of young HSCs in CD45-SAP conditioned young and aged mice. Furthermore, we showed that transient exposure of young HSCs to aged microenvironment did not permanently damage their ability to repopulate secondary hosts. However, perhaps the most important finding from our long-term analyses was that young HSCs that successfully engrafted in aged recipients displayed superior reconstituting activity compared to the host HSCs. This was reflected by more efficient generation of B and T lymphocytes from young HSCs.

Finally, we explored CD45-SAP-based transplantation as a means to counteract hematological disease progression in experimental murine model of myelodysplastic syndrome. We discovered that transplantation of healthy donor HSPCs into asymptomatic recipients resulted in decreased disease incidence and most notably, prevented the development of acute leukemias.

Collectively, our study provided new insights into HSC behavior upon transplantation into differentially conditioned hosts. We further demonstrated that CD45-SAP-based transplantation offers viable prospects to establish young HSC-derived hematopoiesis in aged subjects and to mitigate the progression of age-associated diseases.

Paper IV

HSCs are vital components of various cellular and gene therapy approaches, but their scarcity continues to restrict a broadened clinical application of these therapies. Despite numerous prior efforts to effectively expand HSCs *in vitro*, the outcomes have been unsatisfactory. A recent study proposed an alternative cell culture platform based on PVA, which enables the maintenance and *ex vivo* expansion of transplantable HSCs up to 899-fold[202]. Indeed, we confirmed the robustness of the PVA-based culture in *Papers I* and *III*, demonstrating the potential of *ex vivo* expanded cells to restore healthier hematopoiesis in aged individuals. However, at the beginning of this thesis work, detailed characteristics of the PVA-based culture system was lacking. Particularly, there was no information about the input HSC requirements, cellular outputs, culture reproducibility, and *in vivo* performance of expanded cells. Therefore, we conducted a study, where we extensively examined the self-renewal and differentiation of HSCs in *in vitro* culture.

In the first set of experiments, we determined phenotypic requirements of input cells for effective amplification of HSC activity. By transplanting *ex vivo* expanded candidate HSCs, defined as LSK CD150+CD48- (LSK SLAM), we observed a notable enrichment of long-term repopulating activity within the $EPCR^{high}$ fraction. Additionally, we demonstrated that the progeny generated *in vitro* from LSK SLAM

EPCR^{high} cells were capable of rescuing mice from lethal irradiation. This indicates that *ex vivo* culture not only supports HSC self-renewal, but also promotes the generation of more differentiated hematopoietic progenitors. Together, this established our input HSCs as LSK SLAM EPCR^{high}.

Our next analyses aimed at characterizing the cells generated *ex vivo* following 2-3 weeks of culture. By phenotypic and molecular profiling, we showed that in addition to expanded candidate HSCs, the cultures contained a large number of more differentiated progenitors. When evaluating functional performance of cultured cells, this revealed the long-term HSC activity to be restricted to LSK SLAM EPCR^{high} population. Quantitatively, these LSK SLAM EPCR^{high} cells constituted only a minor fraction of the total cultures (0.6% and 0.1% of 2- and 3-week cultures, respectively). Nevertheless, given the massive overall amplification of cell numbers, the net expansion of these cells reached up to 1,200-fold.

We also quantified HSC activity in *ex vivo* expansion cultures. By employing the CRA we compared functional activities of *ex vivo* expanded cells to unmanipulated HSCs. This revealed respective 400- and 70-fold increases in peripheral blood and bone marrow HSC chimerism from *ex vivo* expanded cells. By cellular barcoding, we further showed substantial heterogeneity in *ex vivo* expansion potential of individual input HSCs. This variation was however reduced when HSC activity was determined after expansion, suggesting that the progeny of the HSC clones that expand is more uniform than when assessing HSC activity of freshly isolated HSC clones.

Finally, we assessed the activity of *ex vivo* expanded cells in the setting of unconditioned transplantation. In agreement with previous studies[202,203], we found that amplification of HSC numbers by *ex vivo* culture allows for long-term multilineage reconstitution in unmanipulated, immunologically-matched hosts. Unexpectedly, we also observed that under such conditions, the fraction of expanded HSCs rapidly returned to quiescence after transplantation.

Collectively, our study provided insights into HSC self-renewal and differentiation in *in vitro* culture. We also presented several key considerations for *ex vivo* HSC expansion and the functional assessment of such cells *in vivo*.

Discussion

Since the beginning of the century, life expectancy has increased by more than six years, and current estimations predict that the average lifespan will continue to rise[206,207]. However, despite our lives getting longer, a similar rate of improvement is not observed for the healthspan[208]. In fact, elderly individuals frequently experience multiple age-associated conditions, which collectively extend periods of poor health and increased vulnerability to disease.

The process of aging does not spare the hematopoietic system. This is evident through the existence of conditions such as anemia, propensity for infections, increased incidence of pre-malignant states (including clonal hematopoiesis), and severe blood disorders that are frequently observed in elderly. Much like in other tissues, the decline of hematopoietic system with age has been associated with changes within its corresponding stem cells. Nevertheless, despite numerous studies detailing distinct mechanisms of HSC aging, several outstanding questions remain. How does chronological aging impact HSC activity under physiological conditions? What intrinsic and extrinsic mechanisms account for reduced function of aged HSCs? And finally, is it possible to revitalize aged hematopoietic system? The work presented in this thesis represents a step towards addressing these questions.

Adult hematopoiesis is initiated in the BM by LSK SLAM EPCR^{high} cells and proceeds through intermediate progenitor cell stages

Previous studies suggested that LT-HSC activity can be enriched using CD201/EPCR surface marker[24,209]. In line with this, in our work we have established that LSK SLAM EPCR^{high} cells have the potential for long-term multilineage reconstitution. This applied to both HSCs isolated from the BM and those subjected to *ex vivo* expansion. We further showed that LSK SLAM EPCR^{high} cells generate offspring capable of rescuing mice from lethal irradiation and have superior potential over their EPCR^{-} counterparts from *ex vivo* culture. By *in vivo* proliferation analyses, we documented a positive correlation between EPCR levels, proliferative status, and LT-HSC activity (*Papers III, IV* and unpublished data). Finally, when investigating their molecular profiles, LSK SLAM EPCR$^{+/high}$ cells displayed high expression of known HSC-associated transcripts, including *Fgd5*[30]. Combined, our studies demonstrated that adult murine LSK SLAM EPCR^{high} cells by all means represent functional LT-HSCs.

Despite their uniform immunophenotype, HSCs are functionally heterogeneous. This diversity has been previously reported for stem cell lineage outputs/biases, lifespans, and self-renewal potential[48]. In our study, we similarly observed differential behavior of individual HSC clones. In particular, our reconstitution analysis in mice transplanted with limiting number of HSCs (10 cells per mouse) revealed different levels of multilineage output (*Paper I*) with similar observations for aliquots of HSCs (10 cells per well) expanded *ex vivo* prior to transplantation (*Paper IV*). We also found that individual HSCs substantially differ in their self-renewal potential, with positive correlation between self-renewal and hematopoietic output. Interestingly, when assessing HSC clonal behavior following *ex vivo* culture, we observed less pronounced differences in hematopoietic output, suggesting that the progeny of HSCs that robustly self-renewed in culture is functionally highly similar. In a broader sense, this also refers to the concept of HSC-fate stability, where HSC fate patterns are conserved among cells derived from the same clone[210]. HSC fate stability has been suggested in previous transplantation and barcoding studies[60,101,102], and we now show that the HSC self-renewal culture system[202] provides an alternative platform to investigate this concept.

While it is well-established that HSC differentiation involves a gradual acquisition of lineage-restricted characteristics, the precise developmental pathways originating from LT-HSC and the hierarchical relationships among HSPCs are actively debated. One reason for this is the identification of HSC subsets with distinct lineage-fate biases, documented in previous HSC transplantation studies[34,100-102]. Based on transplantation and transcriptomes profiling data, other studies further suggested that the phenotypic HSC compartment contains lineage-restricted progenitors[46,105]. However, both transplantation and transcriptome profiling techniques present their own sets of limitations that restrain detailed analysis of the ongoing HSC-derived hematopoiesis. In *Paper I*, we set out to overcome these challenges by combining *in vivo* lineage tracing with single cell molecular profiling. With this approach, we demonstrated that cellular differentiation is initiated from the most primitive HSC and progresses through obligatory intermediate progenitor cell stages. Our epitope profiling further showed that downstream progenitor cells associate with distinct immunophenotypes and hence the cells with different developmental potentials are, for the most part, prospectively isolatable.

Simultaneous measurements of gene expression and surface protein abundance also allowed us to identify previously uncharacterized cell subsets, including MPP Ly-I and MPP Ly-II. Importantly, cell clusters representing these populations were distinguishable only by integration of transcriptomic and surface marker profiles, which attests the potential for multimodal analysis to uncover cellular heterogeneity, that can otherwise be missed by single modality analysis (unpublished observation). By detailed molecular and phenotypic analyses, we found that MPP Ly-I and MPP Ly-II cells represent two fractions of the MPP Ly compartment (previously referred to as MPP4[25]) that can be separated based on their EPCR and CD48

expressions. Furthermore, our functional data revealed that both populations derive from LT-HSCs and are hierarchically related. By transplantation, we showed that while MPP Ly-I and MPP Ly-II display modest myeloid differentiation potential, above all, they are characterized by strong lymphoid differentiation capacity in sub-lethally irradiated recipients. Because transplantation into irradiated recipients can enforce cellular behaviors that are less likely to occur in the native context[48], we also evaluated MPP Ly-I cell function using a milder, CD45-SAP-based conditioning and compared it to HSC performance in the same settings (unpublished data). In line with our previous observations, MPP Ly-I cells generated robust lymphoid output with only marginal contribution to myeloid lineage in this setting. We further observed that the production of mature lymphocytes was faster for MPP Ly-I than from HSCs. Finally, similar as in irradiated recipients, we did not document MPP Ly-I self-renewal in CD45-SAP-conditioned mice (unpublished data).

Collectively, our findings agree with the hierarchical structure of hematopoietic development, with LT-HSCs residing at its apex. Hematopoietic differentiation associates with significant amplification of cell numbers and generation of cells with heterogenous lineage potentials. At the same time, our data do not preclude the possibility that parallel differentiation routes from HSPCs exist, and future studies using barcoding technology, multiome integration and *ex vivo* HSC culture are likely to provide further insights into HSPC lineage commitment and differentiation.

Age-related hematopoietic decline is primarily driven by cell-intrinsic changes within aging HSCs

Based on transplantation and transcriptome profiling data, multiple prior studies reported reduced HSC function upon aging[211]. However, as pointed earlier in this thesis, these methodologies have their own caveats, which can limit comprehensive analysis of the ongoing hematopoietic differentiation at the individual cell level. Our data demonstrated that the HSC contribution to steady-state hematopoiesis gradually declines with age and is particularly pronounced for effector lymphoid lineages. While previous studies suggested that age-related decline in lymphopoiesis originates from alteration in early lymphoid progenitors[117-120], our work demonstrated that this extends to even more primitive cells, particularly MPP Ly-I. The reduced lymphoid output from aged HSCs was also evident following their *ex vivo* expansion, indicating that intrinsic factors other than activation status contribute to functional decline of aged HSCs. Intriguingly, lack of stable, high-level lymphoid reconstitution from *ex vivo* expanded aged HSCs could be overridden by increasing the starting number of input cells to the cultures. In line with our previous barcoding study[134], this provides evidence that the aged HSC compartment is heterogeneous and contains rare clones that are capable of multilineage/lymphoid differentiation. Furthermore, the fact that these clones can be effectively maintained *ex vivo* offers an exciting opportunity to explore features

of these 'fit' clones with the objective to develop strategies aimed at reinstating health in the elderlies.

To identify the molecular underpinnings of HSC aging, several studies comparatively evaluated the transcriptomics of young and aged HSCs. These analyses revealed a multitude of distinct mechanisms, including aberrant expression of genes involved in multilineage differentiation[117], elevated inflammatory and stress response[128,146], and altered regulation of cell cycle[145], among others. While the reasons for the discrepancies in reported mechanisms remain unknown, we suggested that one potential explanation is technical variability in transcriptome profiling procedures. Indeed, we showed that the exposure of primary HSPCs to temperature stress leads to a rapid induction of IER signature with simultaneous downregulation of cell cycle-related genes. Critically, the response is independent of age and has profound consequences for global transcriptome profiles. We propose that this may inadvertently lead to misinterpretations regarding molecular dynamics of the aging process. For instance, because many IEGs are constituents of the TNF-α signaling pathway, their activation during sample preparation and/or processing can confound accurate assessment of genuine inflammatory responses. As we show in *Paper II*, induction of IER signature can also further preclude reliable evaluation of cell cycle status. Finally, given that the average length of IEGs is notably shorter than that of other genes[154], their overexpression can trigger a widespread shift in the transcript size distribution. In this context, the recently reported connection between mRNA length-related transcriptome imbalance and aging is noteworthy[212]. While we reported our findings in the context of hematopoietic aging, their applicability likely extends to other published datasets, including those from areas beyond hematopoiesis[73-75]. This underscores the broad relevance of such considerations when conducting transcriptome profiling analyses.

In addition to the existing data, our own observations help us speculate about the potential mechanisms involved in HSC aging. *Stem cell exhaustion* and *loss of proteostasis* represent two previously reported hallmarks of aging[1]. In the context of HSC biology, tight regulation of protein homeostasis is essential for HSC function[213]. Consequently, even modest alterations in protein synthesis or quality can impair HSC self-renewal and differentiation[213,214]. During aging, HSCs are subject to various intracellular insults, including DNA mutations, and elevated levels of reactive oxygen species related to mitochondria dysfunction, which can collectively limit proteome integrity. Correspondingly, recent findings show that aged HSCs express increased levels of heat shock factor 1, which regulates proteostasis[201]. This perhaps suggests that aged HSCs are under proteotoxic stress and might engage distinct pathways to restore homeostasis. Our unpublished observation indicates that aged HSCs display a tendency for reduced protein synthesis when compared to their young counterparts. Notably, the rate of mRNA translation in aged HSCs varied significantly between animals, indicating that aging process is heterogeneous and differently affects individual subjects. Future studies

will be critical to understand how alterations in proteostasis contribute to HSC aging.

While in *Papers I* and *II* we primarily focused on cell-intrinsic origins of HSC aging, in *Paper III* we explored whether age-related microenvironment changes contribute to this process. For this, we established a non-invasive transplantation protocol which facilitates integration of young HSCs into aged recipients, while preserving non-hematopoietic/niche components. In line with previous observations[163], our initial analyses revealed that aging environment hinders effective engraftment of donor HSPCs. This may result from an active immune response and/or altered transplantation tolerance leading to graft rejection. However, limited donor-derived reconstitution observed in aged recipients co-injected with CD45-SAP and immunotoxins targeting mature B and T lymphocytes would argue against this. Instead, our findings suggest that aged subjects require higher doses of transplanted cells and presence of an available HSC niche in the BM to surmount the transplantation barrier.

It has been proposed that systemic and BM microenvironment changes during aging result in compromised HSC function[215-217]. In our work, we showed that young HSCs retain their proliferative capacity and could successfully repopulate secondary recipients after short-term pre-conditioning in aged hosts. Thus, this indicates that aging microenvironment *per se* it not toxic to the transplanted cells. Another observation was that CD45-SAP-mediated HSC depletion was not complete in aged mice, retaining a fraction of cells in the BM. While this could be viewed as a limitation of the methods, we in our analyses took advantage of it to directly compare the function of remaining aged HSCs to the transplanted young cells. This comparison revealed that engrafted young HSCs exhibited superior per cell multilineage differentiation as compared to aged host HSCs. This was particularly pronounced for B and T cell lineages, wherein the corresponding progenitors and naïve cells predominantly originated from the young donor HSCs. When interrogating mature B and T cell subsets, we found that the aging environment did not promote generation of ABCs from donor cells. At the same time, we noted a slight increase in donor-derived memory $CD4^+$ and $CD8^+$ T cells as compared to young transplantation recipients (unpublished observation). Taken together, this perhaps suggests that while systemic factors and age-related organ remodeling might not impede *de novo* lymphopoiesis from young HSCs, the influence of age on mature lymphoid subsets is more cell type- and context-dependent.

HSC is the bona fide unit of hematopoietic regeneration

While HSPC transplantation offers a curative treatment for various diseases, its broad therapeutic application is hindered by several clinical challenges. The main concern is a need for balancing safety of transplantation procedure while increasing chances of graft success. On one hand, cytoreductive conditioning eradicates malignant cells and provides space in the host BM for HSC engraftment, but at the

same time it associates with detrimental short- and long-term complications. On the other hand, while enhancing the rate of HSC engraftment through infusion of large HSC doses could potentially obviate the necessity for harsh cytotoxic conditioning, the scarcity of HSCs and the challenge in securing immune-matched donors remain a significant hurdle. In this context, recent advancements in low-intensity conditioning protocols[181,184] and novel HSC expansion culture systems[202,204] that I employed in my work present promising avenues for advancing the safety and efficacy of HSPC transplantation.

From a safety perspective, the transplantation procedure devoid of preconditioning regimen is the most preferable option. Earlier studies demonstrated that transplantation of purified HSCs into unconditioned hosts yields hematopoietic reconstitution, albeit with relatively modest chimerism levels[181,187]. In a more recent study, Shimoto et al. reported a notably elevated level of donor-derived chimerism in unconditioned mice upon transplantation of massive numbers (up to 2.5 x 10^5) of purified HSCs[199]. However, the fact that these experiments required isolation of donor cells from almost 300 mice per each recipient poses considerable experimental and ethical constraints. By contrast, *ex vivo* HSC expansion using the PVA-based culture system[202] provides a more feasible approach for amplifying HSC numbers. In line with this, we found that *ex vivo* expanded cells derived from 100 initial HSCs effectively supported long-term multilineage hematopoiesis even in unconditioned, immune-matched recipients. Using this system, we also re-examined the concept of niche recycling, wherein steady-state egress of host HSCs from their niches creates vacant spaces for engrafted donor cells[189]. By comparing the reconstitution outcomes from 500-expanded HSCs transplanted either as a single bolus or through five consecutive injections, we found similar chimerism levels regardless of the injection scheme. This suggests that steady-state niche recycling alone is insufficient to enhance the contribution from *ex vivo* expanded HSCs. Nevertheless, the cumulative chimerism levels from 500 expanded HSCs were markedly higher than those observed in recipients of 100 cultured HSCs, indicating that escalating doses of transplanted HSCs improve hematopoietic reconstitution.

Transplantation following low-intensity conditioning greatly enhances HSC engraftment with relatively low toxicity. In our studies, we successfully applied CD45-SAP-based transplantation, and found that this approach recapitulates some aspects of native HSC-derived hematopoiesis. We found that HSC differentiation into lymphoid and myeloid lineages in CD45-SAP-conditioned recipients followed similar kinetics as that observed under the steady-state. On the other hand, CD45-SAP treatment, much like TBI, induced proliferation of donor HSCs and markedly decreased their competitive abilities in secondary hosts. In a previous study, Lu and co-workers further demonstrated that antibody-based conditioning alters HSC differentiation patterns at the clonal level[180]. Collectively, these observations demonstrate that while antibody/immunotoxin treatment is well

tolerated and less invasive than standard TBI, it nonetheless alters the behavior of transplanted HSCs.

In addition to its clinical application in treating various disorders, HSC-based cell therapy has also been harnessed to mitigate age-associated phenotypes. For instance, repeated cycles of mobilization and transplantation of young HSCs into aged mice not only led to robust donor-derived hematopoiesis but were also shown to promote increased health- and lifespan[197]. Although in *Paper III* we did not assess the overall survival of aged mice following young HSC transplantation, we did observe that these transplanted cells efficiently generated adaptive immune cells, likely contributing to improved health. Alternative approaches for hematopoietic rejuvenation involve treating aged HSCs *ex vivo* with small molecules such as Cdc42 inhibitor CASIN[160] or subjecting aged individuals to systemic treatment with compounds like rapamycin[218] or nicotinamide ribonucleoside[219]. Given the ability of the PVA-based culture system to sustain multilineage/lymphoid-competent aged HSC clones (*Paper I*), we speculate that this approach offers a new framework for exploring other compounds for their anti-aging properties, with the ultimate goal of improving hematopoiesis in aged subjects. With these and future efforts we envision a narrowing of the healthspan-lifespan gap.

Popular scientific summary

Aging is a natural process that leads to decreased physiological fitness. For that reason, aging is one of the main risk factors for developing variety of diseases, spanning from neurological, through cardiovascular to related to immunity. Perhaps the most illustrative example is recent Covid-19 pandemic, which severely affected the entire population, but took the greatest toll among the elderly. Besides their increased susceptibility to infections, elderly people poorly respond to vaccinations and often suffer from various blood and immune conditions, including anemia or leukemias. While many factors contribute to this, it is believed that age-related decrease in the function of blood stem cells is one of the major reasons for reduced regeneration of the blood system. Therefore, understanding how aging impacts blood stem cells is essential for developing better and more informed therapeutic strategies.

The blood system comprises distinct cell types, including red blood cells, platelets, and leukocytes. Many of these cells are short lived, with a need for constant replenishment. This is achieved through the process of hematopoiesis. Hematopoiesis is initiated by a rare population of stem cells that resides in the bone marrow. They are unique because they can *self-renew*, which means that they generate copies of themselves. In addition, blood stem cells produce all different mature blood cell types due to their capacity for *multilineage differentiation*. These two properties make blood stem cells the candidate-of-choice for therapeutic interventions. Indeed, they are used in clinical applications relying on bone marrow transplantation. However, during aging the function of blood stem cells declines. We showed that they lose their capacity for multilineage differentiation, which is most pronounced for generation of new lymphocytes. By combining functional and molecular experimentation, we uncovered that senile stem cells have a reduced ability to differentiate to primitive subsets of lymphoid precursor cells. In agreement with that, we further showed that the frequency of these populations gradually decreases during chronological aging. To try to re-boot the function of old blood stem cells, we expanded them in the novel stem cell *in vitro* culture system. With this approach, we found that although the majority of cells function poorly, there exist rare individuals with high multilineage and lymphoid differentiation potential. The fact that such cells can be effectively maintained *in vitro* provides new opportunity to explore their properties with the aim to enhance blood development in older individuals.

To understand the underlying molecular causes for stem cell aging, many previous studies applied RNA profiling that provides information on molecular factors involved in the regulation of cell behavior. By comparing the genetic landscape of young and aged stem cells, these studies proposed multiple distinct mechanisms for age-related stem cell decline. These include aberrant regulation of genes involved in lymphoid differentiation, inflammation or replication stress. It is however not known why these studies reported such distinct mechanisms. We showed that one potential reason for this discrepancy is the artificially induced stress signature that can be evoked by different sample preparation procedures. We demonstrated that the induction of such a response is independent of cell age but has profound implications for interpretation of the aging process. With this study we want to raise awareness of the influence of sample preparation protocol on the readout of genetic profiling.

Given the reduced function of the blood system in the elderly, we explored whether or not it is possible to reinstate healthier blood differentiation to aged hosts by transplanting them with young blood stem cells. Transplantation is a procedure that involves conditioning - a treatment of recipients with chemotherapy and/or irradiation necessary to enable successful engraftment of donor cells. Despite its broad therapeutic potential, transplantation associates with severe side effects, that restrict its use for older patients. To overcome limitations of traditional chemotherapy or irradiation, we applied newer conditioning approaches based on the use of antibodies that were weaponized with a toxin. We found that such treatment was well tolerated in both young and aged recipients, making it a non-invasive alternative for traditional transplantation protocols. Our initial experiments revealed that advanced recipient age restricts effective engraftment of donor stem cells. This could be however overcome by transplanting escalating numbers of stem cells, which we achieved by *ex vivo* stem cell expansion. Our further analyses showed that young stem cells that had engrafted in aged recipients retained their function in the aged microenvironment and could effectively produce all blood cells, including lymphocytes. We finally showed that the non-invasive transplantation has the potential to hinder the progression of age-associated blood disorders.

Overall, this thesis work provided new insights into blood stem cell aging and highlights the potential of bone marrow transplantation to improve blood system fitness in elderlies.

Populärvetenskaplig sammanfattning

Åldrande är en naturlig process som leder till försämrad fysiologisk funktion och är en av de främsta riskfaktorerna för att utveckla en rad olika sjukdomar, som sträcker sig från neurologiska och kardiovaskulära åkommor till immunsjukdomar. Ett tydligt exempel är den senaste tidens COVID-19-pandemi där äldre visat en ökad risk att utveckla svår COVID-19. Förutom äldres ökade infektionskänslighet uppvisar äldre människor också ett dåligt immunsvar i samband med vaccination. Äldre lider även ofta av olika blod- och immunåkommor, såsom anemi eller leukemi.

Blodsystemet består av olika celltyper, inklusive röda blodkroppar (erytrocyter), blodplättar (trombocyter), och vita blodkroppar (leukocyter). Många av dessa celler är kortlivade, med ett behov av konstant påfyllning. Detta uppnås genom att nya blodceller hela tiden produceras i en process som kallas för hematopoes. Hematopoesen påbörjas i stamceller som finns i benmärgen. Dessa är sällsynta och unika i sin förmåga att förnya sig själva (s.k. självförnyelse), vilket innebär att de bildar kopior av sig själva vilket är nödvändigt för hematopoesen inte ska upphöra. Blodstamceller kan också producera alla olika mogna blodcellstyper. Dessa två egenskaper är anledningen till att ett nytt blodsystem kan byggas upp efter en benmärgstransplantation. Dessvärre avtar blodstamcellernas funktion vid åldrande. Man tror att åldersrelaterad försämring av blodstamcellsfunktionen är en huvudorsak till äldres försämrade blodsystem. Det är därför viktigt att förstå hur åldrande påverkar blodstamceller för att kunna utveckla bättre behandlingar mot åldersrelaterade sjukdomar.

I vår forskning visade vi att åldrande leder till att blodstamceller får en gradvis försämrad förmåga att bilda olika blodcellstyper, och särskilt för en typ av immunceller som kallas lymfocyter. Vi upptäckte dessutom att även om majoriteten av stamcellerna fungerar dåligt, finns det ett fåtal celler kvar som fungerar bra. Dessa celler kan odlas och expanderas i cellkulturer, vilket ökar möjligheten att studera dem för att få kunskap som kan användas för att förbättra blodbildning hos äldre individer.

För att förstå de underliggande molekylära orsakerna till stamcellers åldrande har forskare i tidigare studier jämfört de gener som uttrycks hos unga och gamla stamceller. Sådana studier har kommit fram till olika resultat, och bland annat föreslagit att gener som styr lymfoid cellutveckling, inflammation, eller stress som

uppkommer när celler delar sig, har förändrats i gamla blodstamceller. Våra studier visade att en möjlig orsak till skillnaderna i resultat är stressen som kan uppkomma när celler hanteras innan analys, vilket är oberoende av åldrande. Vi vill med dessa upptäckter öka medvetenheten om att hantering av cellprover drastiskt kan påverka resultaten från genuttrycksanalyser.

Med tanke på den försämrade funktionen hos äldres blodsystem undersökte vi om det är möjligt att återställa blodcellsbildningen genom att transplantera unga blodstamceller till gamla individer. Transplantation involverar ofta så kallad konditionering, där mottagare behandlas med kemoterapi och/eller bestrålas för göra sig av med oönskade celler och göra plats för de donatorceller som ska transplanteras. Trots sin stora potential är transplantationsproceduren dessvärre kopplad till allvarliga biverkningar, vilket begränsar möjligheten för transplantation till äldre patienter. För att överkomma sådana begränsningar tillämpade vi en ny konditioneringsmetod. Denna metod använder sig av antikroppar som är kopplade till ett toxin som dödar de celler antikroppen binder till. Vi upptäckte att behandlingen tolereras väl av både unga och äldre mottagare. Detta gör metoden till ett icke-invasivt konditioneringsalternativ till traditionell konditionering vid transplantation. I vår forskning visade vi även att åldrande är förknippat med sämre mottaglighet av transplanterade donatorstamceller. Detta kan överkommas genom att transplantera ett större antal stamceller, vilket vi uppnådde genom att expandera blodstamceller i cellkulturer innan transplantation. De unga stamceller som transplanterades till gamla mottagare behöll sin goda funktion och kunde effektivt producera alla blodcellstyper, inklusive lymfocyter. Slutligen visade vi även att denna icke-invasiva transplantationsprocedur har potential att hindra utveckling av åldersrelaterade blodsjukdomar.

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