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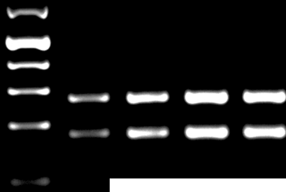

Gata2

Hematopoietic stem cell dynamics during regenerative stress

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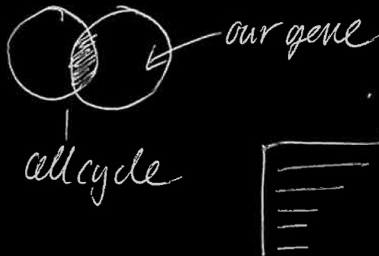

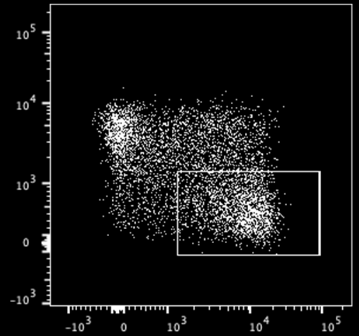

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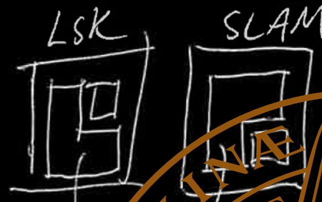

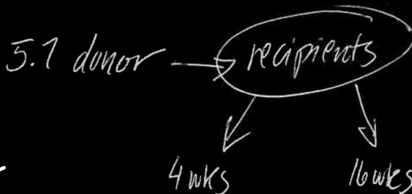

Hematopoietic stem cell dynamics during regenerative stress

Hematopoietic stem cell dynamics during regenerative stress

Anna Rydström

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 18 of January 2024 at 09.00 in Belfrage lecture hall, BMC D15, Klinikgatan 32, Lund

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Abstract: The hematopoietic system can be viewed as a hierarchical tree structure with the hematopoietic stem cell (HSC) at the apex, and the multitude of effector cells at the bottom. At homeostasis, HSCs reside in close contact with bone marrow niche cells that provide signals to keep them in a quiescent state. During conditions of severe stress, inflammatory cytokines from immune and niche cells activate the entire HSC population to proliferate. Once inflammation is cleared and the hematopoietic balance is restored, the HSCs return to quiescence.

Due to their ability to self-renew and to give rise to all cells of the hematopoietic system, HSCs are the reason why hematopoietic stem cell transplantations can replace an entire hematopoietic system and thereby provide a lifelong cure for many hematological diseases. However, proliferation of HSCs is linked to decreased reconstitution potential and must be tightly regulated during stress conditions to ensure long-term functionality. In addition, programs regulating stemness and differentiation are dysregulated in many leukemias. For these reasons it is a long-standing goal to understand the mechanisms and genes regulating the transition between activation and quiescence in HSCs.

The work in this thesis has focused on the dynamic processes of hematopoiesis during regenerative stress, and especially the changes occurring in HSCs after transplantation. We performed a comprehensive profiling of hematopoietic stem and progenitors following transplantation. We found an initial expansion of progenitors producing megakaryocytes and erythrocytes, while the HSC population is regenerating over an extended period of time. We observed a progressive recovery of HSC reconstitution potential with time and identified a set of genes with gradual expression changes that might be important for regulation of HSC activation. We further wanted to improve the identification of functional regenerating HSCs and found the cell surface protein Macrophage-1 antigen (MAC-1) to be transiently expressed after transplantation. MAC-1 expression correlated with HSC functionality and identified a molecularly distinct subpopulation that are the first to return to quiescence. Finally, we investigated how different conditioning regimens can be used to analyse properties in HSCs important for engraftment after transplantation. We compared irradiation conditioning to a conditional Gata2 knockout mouse model which allowed selective and temporally controlled depletion of HSCs. When HSCs from different genetic backgrounds were transplanted in a competitive setting to these mice, we observed that even small differences in HSC proliferation and adhesion gave fundamentally different outcomes in engraftment depending on the type of conditioning. Collectively, this work provide new insights on important features of HSC regeneration after transplantation, knowledge that can advance future investigations and clinical applications.

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Till Ella och Axel

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Abstract

The hematopoietic system can be viewed as a hierarchical tree structure with the hematopoietic stem cell (HSC) at the apex, and the multitude of effector cells at the bottom. At homeostasis, HSCs reside in close contact with bone marrow niche cells that provide signals to keep them in a quiescent state. During conditions of severe stress, inflammatory cytokines from immune and niche cells activate the entire HSC population to proliferate. Once inflammation is cleared and the hematopoietic balance is restored, the HSCs return to quiescence.

Due to their ability to self-renew and to give rise to all cells of the hematopoietic system, HSCs are the reason why hematopoietic stem cell transplantations can replace an entire hematopoietic system and thereby provide a lifelong cure for many hematological diseases. However, proliferation of HSCs is linked to decreased reconstitution potential and must be tightly regulated during stress conditions to ensure long-term functionality. In addition, programs regulating stemness and differentiation are dysregulated in many leukemias. For these reasons it is a long-standing goal to understand the mechanisms and genes regulating the transition between activation and quiescence in HSCs.

The work in this thesis has focused on the dynamic processes of hematopoiesis during regenerative stress, and especially the changes occurring in HSCs after transplantation. We performed a comprehensive profiling of hematopoietic stem and progenitors following transplantation and found an initial expansion of progenitors producing megakaryocytes and erythrocytes, while the HSC population is regenerating over an extended period of time. We observed a progressive recovery of HSC reconstitution potential with time and identified a set of genes with gradual expression changes that might be important for regulation of HSC activation. We further wanted to improve the identification of functional regenerating HSCs and found the cell surface protein Macrophage-1 antigen (MAC-1) to be transiently upregulated after transplantation. MAC-1 expression correlated with HSC functionality and identified a molecularly distinct subpopulation that is the first to return to quiescence. Finally, we investigated how different conditioning regimens can be used to analyse properties in HSCs important for engraftment after transplantation. We compared irradiation conditioning to a conditional *Gata2* knockout mouse model which allowed selective and temporally controlled depletion of HSCs. When HSCs from different genetic backgrounds were transplanted in a competitive setting to these mice, we observed that even small differences in HSC proliferation and adhesion gave fundamentally different outcomes in engraftment depending on the type of conditioning. Collectively, this work provide new insights on important features of HSC regeneration after transplantation, knowledge that can advance future investigations and clinical applications.

Populärvetenskaplig sammanfattning

Vårt blodsystem är kroppens största organ och utgör mer än 70% av alla våra celler. Den största andelen är röda blodkroppar som transporterar syre och koldioxid till och från våra celler. Utöver dem finns blodplättarna, små cellfragment som gör att blodet koagulerar vid skada, och de vita blodkropparna, som skyddar oss från infektioner. Blodcellerna förnyas konstant och trots att de fyller så olika funktioner så kan de bildas från en och samma cell - blodstamcellen. Blodstamceller är få till antalet och finns i benmärgen hos vuxna, där de flesta är vilande och overksamma och bidrar relativt lite till att producera nya blodceller. Vid behov har de emellertid en enorm potential att dela sig för att göra nya stamceller och för att mogna ut till olika blodceller, egenskaper som gör dem till den funktionella enheten i benmärgstransplantationer. De gener och mekanismer som styr hur blodstamceller fungerar är i många fall gener som även är inblandade i blodcancer. Dessa faktorer gör det extra viktigt att studera stamceller, för att förstå hur de fungerar, hur vi kan använda dem på bästa sätt i till exempel transplantationer, och för att förstå vad som går fel när en cell blir en cancercell.

Min avhandling består av tre arbeten där jag har använt mig av musen som modellsystem för att studera olika aspekter av transplantation av blodstamceller.

I ***artikel 1*** ville vi få en helhetsbild av blodbildningens olika faser efter transplantation. Vi undersökte hur olika populationer förändras över tid, hur stamcellernas funktion påverkas, och hur uttrycket av gener i olika populationer förändras och skiljer sig åt. Vi kunde se att stamcellerna i den första fasen främst bildade en specifik population progenitorer som i sin tur mognar till röda blodkroppar och blodplättar. Samtidigt så försämrades stamcellernas funktion att bilda nya stamceller. Vi kunde dessutom identifiera 130 gener som visade ett gradvis förändrat uttryck över tid, och som kan vara viktiga för hur blodstamcellerna styrs efter transplantation.

I ***artikel 2*** var målet att förbättra identifieringen av funktionella blodstamceller när de transplanterats. Detta är en förutsättning för att studera de mekanismer som styr hur stamcellerna reagerar vid olika situationer. Problemet är att vissa av de proteiner som finns på cellens yta och som används för att känna igen olika celltyper ändras efter transplantation, vilket försvårar identifieringen. Vi såg att MAC-1, ett protein som vanligtvis finns på en speciell typ av vita blodkroppar, uttrycks tillfälligt även på blodstamceller. De stamceller som har mest MAC-1 på sin yta är dessutom de mest funktionella stamcellerna.

I ***artikel 3*** undersökte vi hur olika grader av så kallad konditionering påverkar hur effektivt stamcellerna hittar till benmärgen och bidrar till blodbildning efter transplantation. Konditionering betyder att man skapar plats i benmärgen för de transplanterade stamcellerna genom att ta bort konkurrerande stamceller. Detta görs vanligtvis genom gammastrålning som inte bara dödar befintliga stamceller utan

även skadar andra celler i benmärgen. Vi etablerade en ny musmodell för konditionering, där vi kunde ta bort konkurrerande celler både före och efter transplantation utan att förstöra resten av benmärgen. Därefter visade vi att olika grader av konditionering kan användas för att utvärdera olika egenskaper som stamcellerna behöver efter att de transplanterats.

Sammanfattningsvis beskriver min avhandling hur blodstamceller förändras efter en transplantation, hur vi effektivt kan identifiera dem med hjälp av en ny markör, samt hur olika typer av konditionering av benmärgen kan användas för att analysera egenskaper som är viktiga hos stamcellerna vid en transplantation.

List of publications

Articles included in this thesis

Paper I

Rydström A, Grahn THM, Niroula A, Mansell E, van der Garde M, Pertesi M, Subramaniam A, Soneji S, Zubarev R, Enver T, Nilsson B, Miharada K, Larsson J, Karlsson S. Functional and molecular profiling of hematopoietic stem cells during regeneration. *Experimental Hematology 2023;127 :40-51.*

Paper II

Rydström A, Mansell E, Sigurdsson V, Sjöberg J, Soneji S, Miharada K, Larsson J. MAC-1 marks a quiescent and functionally superior HSC subset during regeneration. *Stem Cell Reports 2023;18(3):736-748.*

Paper III

Miharada N, **Rydström A**, Rak J, Larsson J. Uncoupling key determinants of hematopoietic stem cell engraftment through cell-specific and temporally controlled recipient conditioning. *Stem Cell Reports 2021;16(7) :1705-1717.*

Articles not included in this thesis

Warsi S, Dahl M, Smith EMK, **Rydstrom A**, Mansell E, Sigurdsson V, Sjoberg J, Soneji S, Rorby E, Siva K, Grahn THM, Liu Y, Blank U, Karlsson G, Karlsson S. Schlafen2 is a regulator of quiescence in adult murine hematopoietic stem cells. *Haematologica 2022;107(12):2884-2896.*

Liu Y, Schmiderer L, Hjort M, Lang S, Bremborg T, **Rydström A**, Schambach A, Larsson J, Karlsson S. Engineered human Diamond-Blackfan anemia disease model confirms therapeutic effects of clinically applicable lentiviral vector at single-cell resolution. *Haematologica 2023;108(11):3095-3109.*

Safaee Talkhoncheh M, Baudet A, Ek F, Subramaniam A, Kao YR, Miharada N, Karlsson C, Oburoglu L, **Rydström A**, Žemaitis K, Alattar AG, Rak J, Pietras K, Olsson R, Will B, Larsson J. Ciclopirox Ethanolamine Preserves the Immature State of Human HSCs by Mediating Intracellular Iron Content. *Blood Advances 2023.*

Silvério-Alves R, Kurochkin I, **Rydström A**, Vazquez Echegaray C, Haider J, Nicholls M, Rode C, Thelaus L, Lindgren AY, Ferreira AG, Brandão R, Larsson J, de Bruijn MFTR, Martin-Gonzalez J, Pereira CF. GATA2 mitotic bookmarking is required for definitive haematopoiesis. *Nature Communications 2023;14(1):4645.*

Abbreviations

5-FU	5-fluorouracil
AML	acute myeloid leukemia
BrdU	bromodeoxyuridine
CFSE	carboxyfluorescein
CFU	colony-forming unit
FACS	fluorescence activated cell sorting
G-CSF	granulocyte colony-stimulating factor
GVHD	graft versus host disease
H2B-FP	histone H2B-fluorescent protein
HCT	hematopoietic cell transplantation
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
IFN	interferon
JAK/STAT	Janus-activated kinase/signal transducer and activator of transcription
LT-HSC	long-term hematopoietic stem cell
KO	knockout
LSK	Lin^-, $SCA\text{-}1^+$, $cKIT^+$
MAC-1	macrophage-1 antigen
MDS	myelodysplastic syndrome
MegE	megakaryocyte/erythroid
Mk	megakaryocyte
MPP	multipotent progenitor
MSC	mesenchymal stem cell
NK	natural killer cell
PCR	polymerase chain reaction
ROS	reactive oxygen species
SS	steady state
WBM	whole bone marrow
WT	wild type

Introduction

In 1958, the first successful allogeneic bone marrow transplantation was performed on six physicists accidently exposed to lethal or near-lethal doses of irradiation (Jansen, 2005; Mathe et al., 1959). In a time where HLA-matching was not possible, this was a high-risk treatment that had previously failed due to severe graft versus host disease (GVHD). Today, hematopoietic cell transplantation (HCT) is a standard treatment with curative potential for more than 70 diseases such as inherited immunodeficiencies, relapsed leukemias and bone marrow failures. The reason why HCT works long-term is the rare and insignificant-looking hematopoietic stem cell (HSC). It harbors the ability to self-renew and to differentiate to all the mature blood cells and is thereby capable of rebuilding a whole new blood system in a transplanted recipient. Although HCT is a powerful treatment, there are still severe side-effects and limitations associated with it, such as risk of GVHD, difficulties finding matched donors, toxicity from conditioning, and susceptibility to infections due to cytopenia during the recovery phase. HSCs are the most studied adult tissue stem cells, in part due to their unique accessibility from a liquid tissue. It has become clear that genes regulating self-renewal and differentiation in HSCs often are dysregulated in cancers. Taken together, this highlights the importance of continuous research on HSCs with the aim of improving transplantation outcome, but also to understand how HSC function can be linked to malignant transformation and hematopoietic disorders.

What are stem cells and why are they important?

The definition of a stem cell is based on its dual ability to divide and give rise to new copies of itself by a process called self-renewal, and to differentiate to multiple mature cell types. In the adult organism, most tissues contain a small pool of tissue-specific stem cells harbouring the potential to differentiate to a subset or all of the mature cell types of this tissue. In some tissues with a need for high turnover of cells such as the skin and intestine, the stem cells are continuously dividing to ensure sufficient production of mature cells (Banjac et al., 2023). This is not true for the most regenerative tissue in the body, the hematopoietic system. A majority of the HSCs are quiescent during homeostasis and contribute only marginally to the production of mature blood cells, but with the ability to serve as a reservoir of

protected cells that come to use under situations of severe stress. The rate of stem cell self-renewal and differentiation is under strict regulation to ensure tissue homeostasis and function by controlling cell numbers and ratios. Stem cells have great potential in the clinic, where their proliferative and differentiation potential can be used to treat various disorders and malignancies (Kirkeby et al., 2023; Menasché et al., 2015; Sacchetti et al., 2018; Shapiro et al., 2021). Before I go more into depth on the function and regulation of HSCs, I will introduce the hematopoietic system, and some of the methods that are commonly used to study HSC biology.

What is the hematopoietic system and why is it important?

There are many fascinating aspects about our hematopoietic system. Although all mature blood cells originate from the HSC, they carry out very different and essential functions in our body. The hematopoietic system can broadly be divided into myeloid and lymphoid cells (Figure 1). The myeloid compartment can be further separated into erythrocytes, megakaryocytes/platelets (Mk), conventional dendritic cells, monocytes, and granulocytes, while the lymphoid compartment consists of T- and B-cells, natural killer (NK) cells and plasmacytoid dendritic cells.

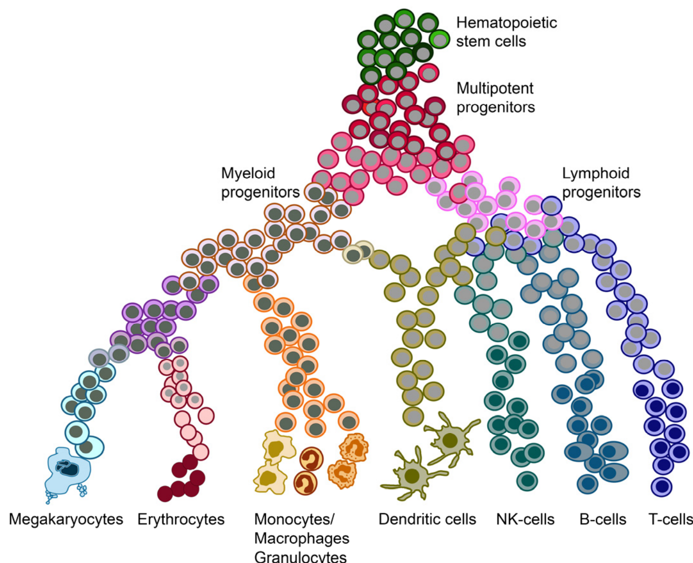

Figure 1. The hematopoietic hierarchy. Schematic representation of the hematopoietic system and the gradual differentiation from hematopoietic stem cells via progenitors to all lineages of the blood system.

The red blood cells, erythrocytes, transport oxygen from the lungs to all cells of the body, where they exchange it for carbon dioxide which is transported back to the lungs. These cells make up 70% of all cells in the body and are thus by far the most common cell type. Since the lifespan of human erythrocytes is approximately 115 days (45 days in mice) after which they are cleared by macrophages in the spleen, two million erythrocytes need to be produced every second to maintain homeostasis (Dholakia et al., 2015; Palis, 2014). With age, there is a gradual decline in erythropoiesis that likely arises from a decrease in the subpopulation of HSCs and/or multipotent progenitors (MPPs) that produces erythrocytes, often leading to mild anemia in the elderly (Busch et al., 2015; Konturek-Ciesla et al., 2023).

Platelets are the smallest constituents of our blood system and are responsible for the blood clotting in wound healing. They are anuclear cytoplasmic fragments with a predetermined lifespan of 10 days formed by shedding from megakaryocytes, where each cell gives rise to thousands of platelets (Mason et al., 2007; Trowbridge et al., 1984). A precise regulation of platelet levels in the blood is essential, since insufficient levels increases the risk of hemorrhage, and too high levels might lead to thrombosis.

The leukocyte constituents of the myeloid compartment, including monocytes, macrophages, granulocytes, and dendritic cells, make out the innate, or non-specific, immune system. Together they form the first line of defense against both unwanted microbes entering the body through recognition via pathogen-associated molecular patterns (PAMPs), as well as playing essential roles in tissue damage clearing and reacting to tumor cells through recognition of damage-associated molecular patterns (DAMPs) (Kono and Rock, 2008). Their immediate role is to neutralize the unwanted cells by releasing granules containing anti-microbial proteins and removing cells or cell debris by phagocytosis. In addition, cells of the innate immune system release large amounts of inflammatory cytokines that act as chemoattractants for the adaptive immune cells. Dendritic cells are professional antigen-presenting cells that not only phagocyte and neutralize cells, but also process the engulfed material and present it to naïve T-cells that in turn becomes activated. The adaptive immune cells belong to the lymphoid compartment, where B- and T-cells are responsible for a highly specific immune response, where each clone recognizes a unique antigen. Finally, NK cells are effector lymphocytes of the innate immune system important in controlling both infections and tumor growth. Diseases that have negative effects on the production or function of parts of the immune system often come with severe effects on protection against both infections and cancer cells (Bekker et al., 2023; Fischer et al., 2015; Kolter et al., 2022).

The hematopoietic system can be viewed as a hierarchical tree structure, with the rare and quiescent HSC at the apex and the large number of mature effector cells at the bottom. Between these extremes are various progenitors with gradually restricted potential, that are more frequent than HSCs and highly proliferative. Progenitors are essential for a swift response to various perturbations, but are also,

as will be described more in the coming sections, likely responsible for the bulk of blood cell production during homeostasis. The classical view of the hematopoietic tree has been that of clearly defined and discrete populations that differentiate in stepwise manner (Orkin and Zon, 2008). Over the past two decades this view has changed, and the current view is instead that both HSCs and progenitors are more plastic, but with intrinsic bias towards a certain fate (Loughran et al., 2020; Zhang et al., 2018). Practically, this means that a cell has potential to differentiate along several paths, but with a preference to a specific cell type.

Methods to study HSCs

Although the ultimate goal of most medical oriented research is to acquire knowledge of how the human body works and how to treat diseases and malignancies, we cannot assess this information experimentally in humans due to an international consensus of ethics (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, 2013; Greek et al., 2012; Katz, 1996). We therefore largely rely on information from experiments performed using cell lines and various animal models. Different aspects of hematopoiesis are studied mainly in zebrafish and mice, and to some extent non-human primates. The mouse is the most commonly used model, due to the relatively close similarities to the human hematopoietic system, the abundance of genetic models and the relative ease of handling and maintaining the animals. Most of the content in this thesis will be focused on experiments and knowledge from the mouse hematopoietic system.

HSC identification and isolation

A prerequisite to study the function of HSCs is to be able to identify and isolate them. The liquid nature of blood and bone marrow and the non-adherent nature of most hematopoietic cells in culture, including HSCs, make them particularly suitable to analyse using flow cytometry. This technique, based on a combination of fluidics and optics, was developed in the 1960s and has since then developed into a high throughput method that can analyse thousands of cells per second. The fluidic part forces a cell suspension into a tight stream, only allowing one cell at a time to pass. At a specific point, each cell passing through the stream is hit by one or several lasers. Lasers of different wavelengths can be used to excite combinations of fluorophores on the cell's surface. The emitted light is then passed via mirrors and filters to detectors that record information on the emitted fluorescence as well as cell size and granularity. In fluorescence activated cell sorting (FACS), this technique is modified by applying a piezoelectric force to the stream, creating cell-containing droplets that can be charged and sorted.

The identification of both immature and mature hematopoietic cell types requires the use of a combination of fluorescence-conjugated monoclonal antibodies that bind to epitopes on the cell surface. Decades of collected efforts have made it possible today to identify adult mouse HSCs with a purity of approximately 1 in 2 cells (Kiel et al., 2005). Beginning in the 1980's, Weissman and colleagues identified long-term HSCs (LT-HSCs) in the fraction of the bone marrow negative for markers of mature lineages (Lin$^-$), while expressing SCA- and 1 cKIT (Ikuta and Weissman, 1992; Spangrude et al., 1988). This population, also known as LSK, is highly enriched for the most primitive stem- and progenitor populations and can be further refined by adding CD135 (Adolfsson et al., 2001; Christensen and Weissman, 2001), CD34 (Osawa et al., 1996) and the SLAM markers CD48 and CD150 (Kiel *et al.*, 2005). HSCs are negative for CD48, CD135 and CD34, while expressing CD150, and a commonly used combination to identify adult HSCs is LSK, CD48$^-$, CD150$^+$, or LSK-SLAM. An alternative to using antibody-based recognition of a combination of surface markers is to take advantage of the high expression of efflux pumps on HSCs. These efflux pumps are used to clear the cells from harmful molecules such as the DNA-binding Hoescht dye, and HSCs can be identified as the cells with the lowest content of Hoescht, also known as side population or SP cells (Goodell et al., 1996).

It is important to be aware that the expression of cell surface markers may vary depending both on the developmental stage and the physiological state of the HSCs. During fetal development, the HSC pool expands, and cells are dividing extensively, in contrast to the quiescent adult HSCs. The HSCs that reside in the fetal liver express MAC-1, a marker typically expressed on mature macrophages and NK-cells (Morrison et al., 1995). As investigated in Paper II, various kinds of stress influence the expression of cell surface markers on HSCs, and both SCA-1 and MAC-1 are upregulated as a response to stress, while the expression of cKIT is transiently lost (Pietras et al., 2014; Randall and Weissman, 1997; Snapper et al., 1991). Alternative strategies to account for these changes can be to exclude these variable markers and identify markers stable to stress (Rabe et al., 2020), or to use the side population mentioned above. In aged mice the phenotypic HSC pool expands, while the overall functional potential declines. Although there is evidence of subsets of HSCs with high fitness also in old mice (Mansell et al., 2021), no marker combination has yet been found to prospectively identify these cells efficiently.

Methods to evaluate HSC function and traits

When studying HSC biology, some of the key features to be measured include differentiation and self-renewal potential, homing and engraftment ability, proliferative and metabolic status, as well as variations in gene and protein expression. There are numerous ways to assess these different aspects of HSCs, and below I will introduce a few of the most commonly used methods.

In vitro assays

The colony-forming unit (CFU) assay is a commonly used method to assess hematopoietic progenitor frequency, proliferation potential and differentiation capacity. Here, individual cells are grown in a semi-solid medium containing cytokines and growth factors that support differentiation of myeloid, erythroid and megakaryocyte cells. Cells are plated at a concentration to allow for the growth of individual colonies that are typically classified based on morphological and phenotypic criteria (Wognum, 2015). More recently, antibody-based analyses by microscopy or flow cytometry were developed, to allow for faster and more objective classification (Pamphilon et al., 2013; Radulovic et al., 2019; Thompson et al., 2023). To assess lymphoid potential and allow differentiation of lymphoid B- and T-cells *in vitro*, co-culture with OP9 and OP9-DL1 stromal cells is required (Nakano et al., 1994; Schmitt and Zúñiga-Pflücker, 2002).

The long-term culture-initiating cell (LTC-IC) assay allows for the identification and quantification of more primitive progenitors and stem cells. Here, hematopoietic cells are cultured on a stromal cell layer for more than 4 weeks in culture media supporting both self-renewal and differentiation. During this time, committed progenitors become terminally differentiated, while the more primitive cells are maintained. After 4 weeks, the remaining cells are assayed for their potential to generate CFUs (Sutherland et al., 1990; Wognum, 2015).

A longstanding goal is the possibility to expand HSCs *in vitro* while maintaining their stem cell function. This can be useful in experimental settings to assess self-renewal potential, but most importantly, it harbours great clinical potential, expansion of cord blood HSPCs to increase availability of donors for HCT, and expansion of corrected HSCs in gene therapy to name a few. Recent progress have been made to transiently expand both mouse and human HSCs *in vitro* (Fares et al., 2014; Sakurai et al., 2023; Wilkinson et al., 2019), and clinical trials are ongoing using expanded cord blood in transplantations (Saiyin et al., 2023). There are still considerations when evaluating expanded cultures since current protocols also expand non-HSCs, resulting in large heterogeneity (Wilkinson *et al.*, 2019; Zhang and Lodish, 2005). To resolve this, phenotypic profiling combined with functional evaluation of expanded cultures have identified SCA1, cKIT, and EPCR to be stably expressed (Che et al., 2022; Zhang and Lodish, 2005), CD48 and CD244 to be a stably absent (Koide et al., 2022; Noda et al., 2008), while TIE2 is downregulated upon culture (Zhang and Lodish, 2005).

In vitro assays are relatively easy to perform, informative regarding differentiation potential, and to some extent also to evaluate self-renewal. However, the environment created in a culture dish, with defined culture media, growth factors, stromal cells, adhesion factors, controlled temperature and CO_2 levels, is still not sufficient to reflect the complex environment in a living organism. For this reason, we still rely on *in vivo* models for precise assessment of HSC function.

Transplantation

To fully evaluate the potential of HSCs to reconstitute mature multilineage output and to self-renew, transplantation remains the gold standard method. Typically, in a so-called competitive transplantation, test donor cells are injected together with a reference population of competitor cells into preconditioned recipients, where conditioning is necessary to create niche space and to remove competition (Figure 2).

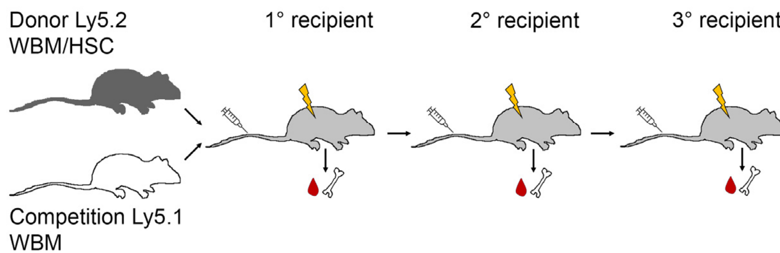

Figure 2. HSC transplantation in mice. Donor HSCs are transplanted together with competitor cells to recipients pre-conditioned by irradiation. The congenic Ly5.1 and Ly5.2 mouse strains are used to distinguish donor cells from competitor and recipient cells. Engraftment is evaluated by measuring donor chimerism in peripheral blood and bone marrow. Long-term reconstitution potential is evaluated by serial transplantation to secondary and tertiary recipients.

The kinetics of the output of mature donor cells of myeloid and lymphoid cells is measured in peripheral blood. At the end of the experiment, the donor chimerism in the bone marrow is assessed. Depending on what aspects of HSCs you are evaluating, a number of different transplantation approaches can be employed. If the aim is a qualitative measurement of functional HSCs, competing a set number of donor whole bone marrow (WBM) cells against a set number of competitor WBM cells is a common procedure (Szilvassy et al., 1990). When purified HSCs are transplanted in competition with either HSCs or WBM, the outcome allows for a more precise evaluation of HSC function (Rundberg Nilsson et al., 2015). A quantitative assessment of HSCs can be achieved by using the limiting dilution assay. Here, a series of dilutions of donor cells are transplanted together with a set number of support cells. The number of mice negative for donor cells is then used to estimate the HSC frequency using Poisson statistics (Szilvassy *et al.*, 1990). Finally, to evaluate the long-term reconstitution potential of HSCs, serial transplantations of engrafted cells from primary to secondary and tertiary recipients are considered the most stringent assay (Dykstra et al., 2007; Purton and Scadden, 2007).

To distinguish between donor and recipient cells, the congenic mouse strains Black6 Ly5.1 and C57/Black6 Ly5.2, that differ only in their *Ptprc* alleles are commonly used. This locus contains around 300 genes, one of them coding for the CD45

antigen that can be identified using antibodies specific for the Ly5.1 and Ly5.2 variants of CD45. If competing transplanted cells from these strains against each other in a preconditioned recipient, it is important to be aware of the slight advantage of the Ly5.2 HSCs. The possible mechanisms behind this difference and how conditioning affects the outcome was further investigated in Paper III. To avoid this difference in fitness, the Scadden lab developed the congenic CD45.1^{STEM} mouse strain that differs only with one amino acid compared to the Ly5.2 (Mercier et al., 2016). Since the CD45 antigen is not expressed on platelets and erythrocytes, donor contribution to these subsets cannot be discriminated using the Ly5.1 and Ly5.2 strains. To track and monitor platelets and erythrocytes in transplanted animals, donor cells from transgenic reporter mouse strains such as UBI-GFP/BL6 and Kusabira-Orange that express fluorescent proteins in all hematopoietic lineages can be used (Schaefer et al., 2001; Yamamoto et al., 2013).

Reconstitution of hematopoiesis following transplantation is a multi-step process that measures the combined output of many qualities of HSCs, such as homing to the bone marrow, lodging in the niche and the generation of hematopoietic output, altogether referred to as engraftment. A specific type of transplantation can be employed to specifically assess the homing ability of HSCs. Here, a large number of donor cells are transplanted to unconditioned or conditioned recipients, and then retrieved from the bone marrow within 15 hours for analysis by flow cytometry (Heazlewood et al., 2014; Szilvassy et al., 2001). If two cell types are compared, they can be fluorescently labelled with separate dyes before transplantation, to allow for direct comparison in the same recipient. The homing assay is challenging since it requires transplantation of many cells to re-isolate a sufficient number of cells for statistically meaningful analysis. We demonstrate in Paper III that the *Gata2* mouse model provides an alternative model with high sensitivity to measure small differences in homing qualities.

In the experimental setting, transplantation is typically preceded by irradiation conditioning to create niche space and to eliminate competition. This is also relevant from a clinical perspective, where irradiation can have the dual role of creating space for the incoming graft as well as part of the treatment in killing malignant cells. When removal of malignant cells is not a necessity, alternative conditioning regimens that can allow for efficient engraftment by a more selective depletion of resident HSCs, while leaving niche cells and mature hematopoietic cells intact, might be advantageous. Experimental approaches of mild pre-conditioning include antibody regimens (Czechowicz et al., 2007; Palchaudhuri et al., 2016), mobilization (Omer-Javed et al., 2022), or using genetically modified mice with impaired HSC function as recipients (Migliaccio et al., 1999; Wang et al., 2009).

Gene expression analysis

The development of the polymerase chain reaction (PCR) in the 1980s (Saiki et al., 1985), and the sequencing of the human genome in the early 2000, are two

milestones on the way to today's current high throughput single cell RNA sequencing. Before this, gene expression was typically measured by probe-based techniques like quantitative reverse transcription-PCR (qRT-PCR) and microarrays. The common denominator for these techniques is the initial conversion of mRNA to cDNA. The DNA molecule is more resistant to degradation than RNA and enables subsequent DNA amplification. In qRT-PCR, a set of pre-selected genes, including house-keeping reference genes, are amplified using specific primers. Although this technique is sensitive and can quantify lowly expressed genes, it is limiting in the number of genes that can be measured. High throughput platforms such as Fluidigm is based on the same technique and can measure expression of 96 genes in 96 single cells but has limitations to accurately quantify genes with low and high expression at the same time.

RNA sequencing is a high throughput technique that measures the entire transcriptome in single cells or in a pool of bulk cells. The sequencing protocols include steps of cDNA synthesis, amplification, and barcoding, albeit in different orders depending on the technique. Compared to probe-based techniques, RNA sequencing is an unbiased approach that can be used in an explorative manner to identify differences between cells, to determine cell identity, and, in the case of single cell sequencing, also provide information on the heterogeneity of a population. More recently, techniques applying RNA sequencing to histological tissue samples, so called spatial transcriptomics, are used to provide insight to the localisation of cellular subsets (Stark et al., 2019). Gene expression is also regulated epigenetically by modulating accessibility of the DNA, thereby allowing, or preventing transcription to occur. Several applications of next generation sequencing have been developed to measure epigenetic modifications. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is an antibody pull-down method identifying regions of the genome that interacts with specific proteins, such as transcription factors or histones carrying specific modifications (Park, 2009). A broader picture of chromatin accessibility can be obtained by the assay for transposase-accessible chromatin using sequencing (ATAC-seq). Here, a hyperactive transposase with sequencing adapters that inserts into nucleosome-free regions is used to determine sites where DNA is open and accessible for transcription (Grandi et al., 2022).

Genetic mouse models

The wealth of information coming from transcriptomic analysis has helped to draw a more comprehensive picture of HSC identity and heterogeneity. Yet, this picture is merely descriptive and does not provide mechanistic evidence of the function of genes. One of the most powerful techniques to study gene function is to delete parts of, or the entire sequence of a gene, thereby creating a knockout (KO). Today, KO in cell lines or animal models can be created efficiency and with high accuracy thanks to the development of the CRISPR/Cas9 technology. The Cas9 protein has

endonuclease activity and can induce double strand breaks in the DNA molecule. The specificity is mediated by a guide RNA, directing the Cas9 to a specific site in the genome. The repair of the DNA breaks can induce inactivation of a gene, or insertion of a new genetic element (Knott and Doudna, 2018). Another way of modifying gene expression is to introduce sequence specific double stranded RNA molecules such as small interfering RNA (siRNA) or short hairpin RNA (shRNA). These molecules bind to specific mRNAs which induce their degradation, resulting in reduced expression of a specific gene (Hannon and Rossi, 2004; Rao et al., 2009).

Genetically modified mice provide important tools to study the function of individual genes in the context of an organism. In conventional knockout mice, a specific region is deleted entirely from the genome in all cells. If a gene is essential in multiple tissues, or during different times of development, the use of conditional KO mouse models can be more informative. Here, the Cre-lox system provides a versatile tool, where a genetic locus flanked by loxP sites can be excised by expression of the Cre recombinase (Sauer, 1998; Sauer and Henderson, 1989). To achieve tissue specific or inducible excision, the Cre recombinase can be expressed under the control of specific promoters. The Vav-Cre model allows for deletion of a gene specifically in endothelial and hematopoietic cells, starting around embryonic day 10.5 (E10.5) (Georgiades et al., 2002; Stadtfeld and Graf, 2005). Several models of temporally controlled expression of Cre have been developed, including Mx-Cre, Er-Cre, and Tet-on and Tet-off. In the Mx-Cre model, the viral mimetic poly(IC) drives Cre expression from an interferon-responsive promoter mainly in hematopoietic cells (Kühn et al., 1995). In the Er-Cre model, the Cre recombinase is fused to an estrogen receptor and retained in the cytoplasm until tamoxifen administration allows for the translocation to the nucleus (Ventura et al., 2007). The Tet-on and Tet-off models express an inactive reverse tetracycline-controlled transactivator (rtTA), or an active tetracyclin-controlled transactivator (tTA), respectively. When rtTA binds to the tetracyclin derivative doxycycline (Dox), it allows for binding to a tetracyclin responsive element (TRE), thereby driving Cre expression (Tet-on). The tTA can bind to TRE and drive expression of Cre in the absence of Dox, while the addition of Dox inactivates the tTA (Tet-off) (Gossen and Bujard, 1992; Gossen et al., 1995; Kistner et al., 1996). When using these systems, it is important to be aware of their limitations. In the case of the Mx-Cre model it is known that administration of poly(IC) induces proliferation in HSCs (Essers et al., 2009), and that experimental procedures that elicit an interferon response, such as transplantation, can induce spontaneous expression of Cre (Velasco-Hernandez et al., 2016). Moreover, expression of Cre is not limited to hematopoietic cells, but also occurs in other tissues such as the bone marrow stromal compartment and the liver. Similar to poly(IC), tamoxifen has proliferative but also apoptotic effects on different HSPC populations (Sánchez-Aguilera et al., 2014), and Tet-models have demonstrated leaky expression from the TRE (Challen and Goodell, 2008; Morcos et al., 2020). Taken together, although these mouse models provide useful tools to study the function of individual genes, or to trace specific

population of cells, it is essential to include proper controls to account for the side effects described above.

In addition to models created to study gene function, reporter mouse strains are used to identify cells with a specific gene expression. Several HSC- specific reporter mice have been created, including *Fgd5*-mCherry and -ZsGreen, *Pdzk1ip1*-GFP and *Tek*-GFP (Gazit et al., 2014; Ito et al., 2016; Sawai et al., 2016). The same genes have also been used as inducible lineage tracers of HSC-generated progeny (Busch *et al.*, 2015; Sawai *et al.*, 2016; Sawen et al., 2018). In these mice, a fraction of the HSC pool is irreversibly induced to express a fluorescent marker that is passed on to all progeny upon proliferation and differentiation. The label propagation provides information on the identity and amount of cell types produced, and the kinetics of the process.

The hematopoietic stem cell

A brief history of the discovery of HSCs

The definition of a hematopoietic stem cell is based on its potential to self-renew and to produce mature blood cells of all lineages. Therefore, to demonstrate the existence of such a cell, those are criteria that need to be experimentally assessed. In the 1960s, before the common use of FACS, PCR and genetically engineered mouse models, Till and McCulloch identified multilineage colonies forming on the spleen of irradiated mice transplanted with bone marrow cells (Till and Mc, 1961). When inducing unique chromosomal aberrations by irradiation, they demonstrated that each spleen colony contained the same aberration, and therefore came from one clone (Becker et al., 1963). Furthermore, cells from these colonies could be transplanted and give rise to new colonies (Siminovitch et al., 1963), thereby demonstrating both self-renewal and multipotent potential. Twenty years after these discoveries, three separate groups used the random integration of retrovirus into the genome as a more efficient way of introducing unique labels and demonstrated multilineage differentiation of serially transplanted bone marrow (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). With refined labelling and isolation techniques to obtain purified HSCs, Osawa et al. were the first to demonstrate self-renewal and multilineage potential within one HSC by transplantation of single cells (Osawa *et al.*, 1996).

Ontogeny of the hematopoietic system

During fetal development, hematopoiesis occurs in two waves. In the first wave, primitive erythrocytes, macrophages, and megakaryocytes emerge from blood

islands in the extraembryonic yolk sac around E7.5 in the mouse embryo (Silver and Palis, 1997). The primitive erythrocytes differ from the adult, as they maintain their nucleus and express a fetal-specific form of hemoglobin (Barker, 1968). In the second, definitive hematopoietic wave initiated at E10.5, HSPCs emerge from the ventral side of the dorsal aorta within the aorta-gonads-mesonephros region (AGM), in a process called endothelial-to-hematopoietic transition (Medvinsky and Dzierzak, 1996). Around this time, HSCs can be detected also in the yolk sac and placenta, but whether these are sites of HSC emergence remains debated (Medvinsky et al., 2011). As they are formed, the HSCs migrate via the circulatory system to the fetal liver where they undergo extensive proliferation to expand the HSC pool during E12-E16 (Ema and Nakauchi, 2000; Müller et al., 1994). Finally, prior to birth the HSCs migrate to the bone marrow where they gradually take on the characteristics of adult HSCs (Bowie et al., 2007).

HSCs during homeostasis

Quiescence and dormancy

During homeostasis, HSCs reside in the bone marrow niche where 70% are quiescent (Passegue et al., 2005; Wilson et al., 2008). Mathematical models based on label dilution kinetics of bromodeoxyuridine (BrdU), histone H2B-fluorescent protein (H2B-FP), and carboxyfluorescein (CFSE) have suggested the co-existence of two HSC populations, one active population dividing every 9-36 days, and one more dormant population dividing every 56-145 days (Foudi et al., 2009; Takizawa et al., 2011; Wilson *et al.*, 2008). Moreover, label retention in HSCs correlates both with the current cell cycle state and the reconstitution potential (Foudi *et al.*, 2009; Qiu et al., 2014; Säwén et al., 2016; Takizawa *et al.*, 2011; Wilson *et al.*, 2008). However, these label retention assays all have technical limitations. BrdU incorporates in the DNA as a cell divides, and since HSCs are quiescent there is a risk of leaving the most quiescent cells unlabeled. In addition, BrdU also induces proliferation in HSCs (Takizawa *et al.*, 2011; Wilson *et al.*, 2008), a confounding trait in a molecule used to assay proliferation. H2B-FP expression was induced with different variants of the Tet-on system described above. This system has shown to be leaky and induces low level of labeling in the absence of Doxycyclin. Moreover, low level degradation of the labeled protein dilutes the signal independently of cell divisions (Morcos *et al.*, 2020; Waghmare et al., 2008). To trace CFSE label dilution, cells are labeled *ex vivo* followed by transplantation. *Ex vivo* handling of HSCs can induce proliferation or apoptosis, which might affect the result of the experiment. In addition, when homeostatic conditions are investigated, it also involves transplantation to unconditioned recipients, a method that results in very low engraftment and thereby a risk of large variability. Irrespective of these shortcomings, there is a consensus that there are both active and quiescent HSCs

existing side by side, and that their divisional history is correlated to reconstitution potential.

Contribution to hematopoiesis by HSCs

For a long time, the dogma was that HSCs had a major contributing role in hematopoiesis, both under homeostatic and stress conditions. In part, this was a consequence of the assays used to define HSCs and the technical limitations of these assays. The definition of a tissue stem cell is its ability to self-renew long term and to have multilineage potential, and the only way to demonstrate this was traditionally by transplantation. The notion that HSCs are the main source of steady state hematopoiesis was questioned a decade ago, due to the development of new *in vivo* barcoding and lineage tracing models. Work from independent groups demonstrated how HSCs in an unperturbed homeostatic setting only displayed marginal contribution to the production of mature blood cells (Busch *et al.*, 2015; Sun et al., 2014). Instead, a large pool of progenitors specified at an early age, were responsible for the bulk of hematopoiesis. At one given point, many progenitors were demonstrated to be active during a short period of time, resulting in a highly diverse, polyclonal output of mature blood cells. These same progenitors, when transplanted, are unable to give rise to long term hematopoiesis. In contrast, transplanted HSCs give rise to oligoclonal hematopoiesis with fewer long-lived HSCs producing large clones, highlighting the vast differences between steady state and stress hematopoiesis. In a follow-up study employing the same *in vivo* barcoding model, Camargo's group did, however, detect substantial contribution specifically to megakaryocytes from HSCs (Rodriguez-Fraticelli et al., 2018). These findings have since been questioned, using other lineage tracing models (Chapple et al., 2018; Sawai *et al.*, 2016) or a multi-fluorescent clonal tracking model (Yu et al., 2016), where a substantial hematopoietic output from HSCs at steady state was demonstrated. These conflicting results might come from an initially low labelling frequency of the HSC pool that may represent a more dormant subpopulation, thereby underestimating the level of HSC differentiation in the earlier studies (Busch *et al.*, 2015). Similarly, if a barcoded HSC divides and the outcome is two differentiating daughter cells, that clone can no longer be traced back to an HSC, again leading to an underestimation of HSC contribution (Sun *et al.*, 2014). However, the large clonal complexity observed by Sun et al., that when summed up over time would exceed the number of stem cells, gives support to the idea of progenitors being a major contributor to steady state hematopoiesis. From the other perspective, the lineage tracing models used to claim an active contribution are also labelling progenitors to some extent (Chapple *et al.*, 2018; Sawai *et al.*, 2016), and given the expansion potential of progenitors, this could have confounding effects at the level of label propagation.

HSC heterogeneity

Heterogeneity of the HSC pool has been demonstrated both molecularly and functionally (Challen et al., 2010; Gekas and Graf, 2013; Loughran *et al.*, 2020; Sanjuan-Pla et al., 2013). Single cell transcriptome analyses have shown that HSCs exist in a continuum of transcriptional states, with gradual differences rather than distinct subpopulations (Cabezas-Wallscheid et al., 2017; Pietras et al., 2015). Single cell transplantations and barcoding have demonstrated functional heterogeneity, both in terms of lineage bias and expansion potential (Carrelha et al., 2018; Dykstra *et al.*, 2007; Morita et al., 2010; Rodriguez-Fraticelli et al., 2020; Rodriguez-Fraticelli *et al.*, 2018). There is evidence of both stable inherited traits (Carrelha *et al.*, 2018; Yu *et al.*, 2016), as well as traits changing over time (Morita *et al.*, 2010). Interestingly, the existence of progenitors displaying self-renewing lineage restricted output *in vivo*, while still maintaining multipotent potential when cultured *in vitro*, sheds new light on the definition of HSCs (Carrelha *et al.*, 2018; Morita *et al.*, 2010; Yamamoto *et al.*, 2013). Can you define such a cell as an HSC, even if it does not effectuate its full potential? Similarly, how do you define a cell displaying multilineage and self-renewal properties during homeostasis, as proposed by Sun et al., but without potential to reconstitute upon transplantation?

Extrinsic regulation of HSCs

The word "niche" is described in the Oxford Languages dictionary as "a comfortable or suitable place". In the world of stem cells, the niche is the location and composition of cells that creates a safe harbour for stem cell maintenance and function. The adult HSCs reside in the bone marrow niche, where they receive signals from the surrounding cells that limit them from entering cell cycle. Historically, two distinct niches have been proposed: the endosteal niche lining the trabecular bone, and the perivascular niche, together containing endothelial cells, mesenchymal stem cells (MSC), megakaryocytes, macrophages, osteoblasts, osteoclasts, sympathetic nerves, and non-myelinating Schwann cells (Figure 3A).

Sympathetic nerve cells confer a circadian regulation of CXCL12 expression and thereby a daily, small-scale HSC mobilization, while the non-myelinating Schwann cells regulate TGF-beta activation (Katayama et al., 2006; Méndez-Ferrer et al., 2008; Yamazaki et al., 2011). The main cellular component of the endosteal niche is the osteoblast, a bone-forming cell that expresses N-cadherin, angiopoietin, and osteopontin. Osteoblasts were initially proposed to provide essential adhesion with HSCs via homophilic N-cadherin binding (Hosokawa et al., 2010a; Hosokawa et al., 2010b; Xie et al., 2009; Zhang et al., 2003). By conditional KO of N-cadherin in HSCs it was later demonstrated to be redundant for HSC function, both during homeostasis, stress and for homing to the bone marrow (Kiel et al., 2009; Kiel et al., 2007). Instead, the bone, and the bone-forming progenitors are suggested to play an important role by promoting the formation or maintenance of the perivascular niche (Chan et al., 2009; Sacchetti et al., 2007; Zhou et al., 2010).

With the improvement of specific HSC surface markers (Kiel *et al.*, 2005), it became possible to identify where in the bone marrow HSCs localise and the cells found in their proximity. It became clear that the majority of steady state HSCs reside adjacent to sinusoids, in close proximity to endothelial cells and mesenchymal stromal cells (Kiel *et al.*, 2005). Conditional deletion of *Scf* or *Cxcl12* in

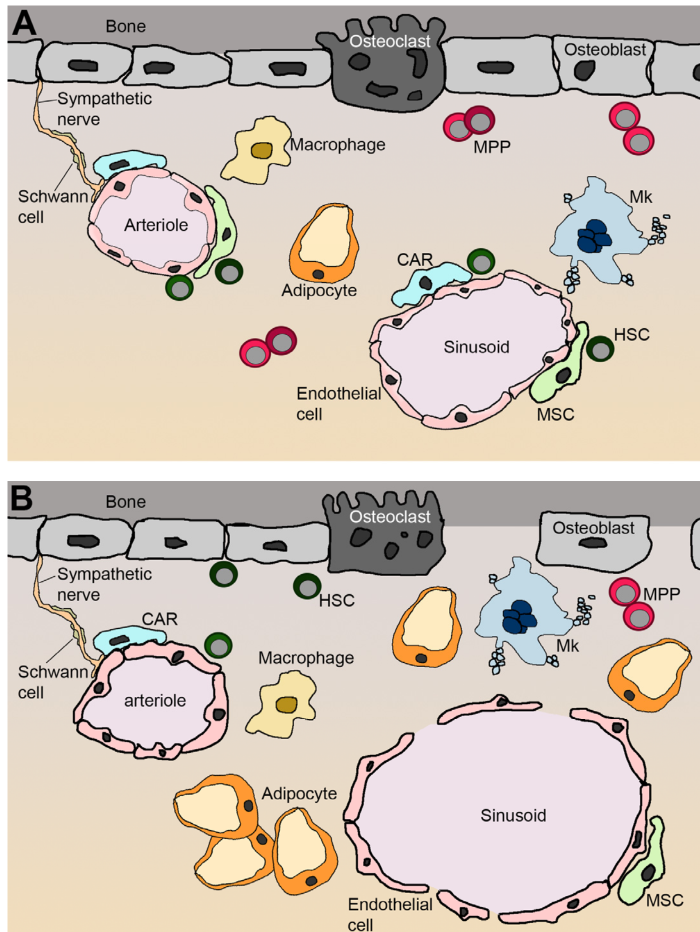

Figure 3. The bone marrow niche during homeostasis and stress. (A) HSCs reside in close proximity to sinusoids and arterioles during homeostasis, where a variety of niche cells support HSC maintenance and quiescence. (B) Stress induced by irradiation and chemotherapy leads to changes in niche composition and structure. HSCs adapt to these changes by shifting their location closer to the arterioles and bone.

endothelial and LeptinR^{+} mesenchymal stromal cells, but not Nestin^{+} stromal cells or osteoblasts, identified these signals and their cellular source to be essential for HSC maintenance (Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). Although the majority of bone marrow HSCs are located close to sinusoids,

the more rare arterioles in connection with NG2$^+$ stromal cells, are suggested to be essential for maintaining quiescent HSCs (Asada et al., 2017; Kunisaki et al., 2013). In further support of this, NTN1 expression in the arteriolar niche was recently found to be a positive regulator of HSC quiescence via binding to the NEO1 receptor (Renders et al., 2021). Interestingly, the composition of the niche changes with age, with the sinusoidal niche being better preserved and thereby supporting quiescent HSCs in the aged bone marrow (Renders *et al.*, 2021; Saçma et al., 2019). Recent work mapping the bone marrow microenvironment by single-cell and spatial transcriptomic analysis have identified new cellular subsets, added detailed information on cellular localisation, and mapped changes induced by stress or malignant transformation (Baccin et al., 2020; Baryawno et al., 2019; Severe et al., 2019; Tikhonova et al., 2019).

Intrinsic regulation of HSCs

While the niche provides extracellular cues essential for HSC regulation, cell-intrinsic regulators such as transcription factors and metabolites are the key players that propagate the external signals to control and execute the fate of HSCs.

Quiescent HSCs are characterized by their low metabolic rate and low protein synthesis. While metabolically active cells mostly produce ATP by oxidative phosphorylation, HSCs rely on the less energy efficient pathways of glycolysis and fatty acid oxidation. This is regulated in part by expressing high levels of HIF1a, a master regulator of metabolism. HIF1a mediates the activation of PDK, an enzyme which shunts pyruvate to lactate conversion instead of entering the tricarboxylic acid (TCA) cycle (Simsek et al., 2010; Takubo et al., 2013). For HSCs, the choice of using glycolysis is not only based on energy demands, but also a way to avoid accumulation of reactive oxygen species (ROS), a byproduct of oxidative phosphorylation that can lead to oxidative stress and inhibition of self-renewal (Ito et al., 2004). The transcription factor Meis-1 regulates Hif-1a and restricts ROS-levels in HSCs (Simsek *et al.*, 2010; Unnisa et al., 2012).

mTOR is another major regulator of cellular metabolism which promotes proliferation and protein synthesis (Thoreen et al., 2012). For HSCs it is critical to have a strict regulation of mTOR, since increased signaling through mTOR leads to HSC activation and exhaustion, while inhibition leads to increased HSC quiescence, bone marrow failure and pancytopenia (Chen et al., 2008; Guo et al., 2013; Wu et al., 2021). The PML-PPAR-delta pathway for fatty acid oxidation is used by HSCs to regulate asymmetric and symmetric divisions (Ito et al., 2012; Ito *et al.*, 2016). Taken together, metabolism in HSCs is not merely a passive consequence or requirement of proliferation, but an active regulator of quiescence and activation (Morganti et al., 2022; Takubo *et al.*, 2013).

MYC proteins are a family of transcription factors that regulate cell cycle and growth, and that are commonly dysregulated in many cancers. In HSCs MYC has

been shown to control the balance between self-renewal and differentiation, partly by regulating the expression of adhesion molecules (Laurenti et al., 2008; Wilson et al., 2004). Interestingly, MYC levels are mostly regulated by the rate of protein degradation. Since HSCs have limited capacity to degrade proteins they need to restrict protein synthesis to maintain low levels of protein misfolding. Increased protein production leads to proteasome overload, disrupted proteostasis and increased MYC levels due to insufficient degradation (Hidalgo San Jose et al., 2020).

The pioneer transcription factor GATA2 is required during embryogenesis in the generation of definitive HSCs, where it regulates the expression of other HSPC transcription factors (de Pater et al., 2013; Silvério-Alves et al., 2023), and conventional *Gata2* KO mice die at E10 due to severe anemia (Ling et al., 2004; Rodrigues et al., 2005). GATA2 is also essential for the maintenance of adult HSCs, and induced deletion in hematopoietic cells leads to depletion of the LSK compartment (Menendez-Gonzalez et al., 2019). Furthermore, *Gata2* mutations are associated with development of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Bruzzese et al., 2020; Hahn et al., 2011; Luesink et al., 2012). In Paper III we used an inducible *Gata2* KO mouse model to achieve depletion of HSPCs, and demonstrated how these mice could be used as recipients with a time-controlled conditioning regimen.

Additional transcription factors essential for HSC regulation are HLF, MECOM, and the HOX family to mention a few (Germeshausen et al., 2018; Komorowska et al., 2017; Magnusson et al., 2007). They ensure a proper balance between quiescence/proliferation and self-renewal/differentiation essential for sustaining hematopoiesis. When this balance is perturbed, either by overexpression or loss of function, it can lead to leukemia or bone marrow failure (Bindels et al., 2012; Germeshausen *et al.*, 2018; Golub et al., 1999; Inaba et al., 1992; Thorsteinsdottir et al., 2001; Voit et al., 2023).

Epigenetic regulators add another layer of complexity to gene expression. They act as structural or chemical modifiers of the DNA or the histone proteins which packs the DNA molecule. DNA methylation commonly causes repression of gene expression, while acetylation of histones often leads to transcriptional activation. A well-described epigenetic regulator is DNMT3a which is essential for HSC differentiation. DNMT3a is a methyltransferase, and mutations in this gene results in both hyper- and hypomethylation that can lead to the development of clonal hematopoiesis, AML, and MDS (Challen 2011, Yan 2011, Bowman 2018). IKZF2 acts both as a transcription factor and an epigenetic regulator. By altering chromatin accessibility, it regulates self-renewal and differentiation preferentially in leukemic stem cells (Park 2019). Other examples of epigenetic modifiers are BMI1 which acts by restricting protein synthesis to maintain proteostasis and thereby maintaining quiescence (Burgess 2022), and CTCF that by regulating 3D chromatin structures promotes the transition from quiescence to activation in HSCs (Takayama 2021).

HSCs during stress

Besides the continuous process of steady state hematopoiesis, the hematopoietic system is constantly "on call" to respond to various kinds of stressors such as infection, blood loss, mobilisation, as well as genotoxic and chemotoxic insults causing tissue damage. A common denominator for these situations of stress is the inflammatory response they elicit. Inflammation is mediated by the release of cytokines such as interferon alpha and gamma (IFNa, IFNg), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor alpha (TNFa) and interleukins (IL), from cells of the immune system as well as cells in the niche (Zhang et al., 2012). These pro-inflammatory cytokines can transduce their signals via the Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), or nuclear factor-kappa B (NFkB) pathways (Baker et al., 2007; Hemmati et al., 2019; Liu et al., 2017; Ramalingam et al., 2020), thereby initiating proliferation in HSCs (Baldridge et al., 2010; Essers *et al.*, 2009; Lévesque et al., 2003; Sato et al., 2009; Yamashita and Passegue, 2019). The precise mechanism of G-CSF is still not clear, but it disrupts part of the niche and reduces CXCL-12 levels, leading to HSC mobilization (Lévesque *et al.*, 2003; Petit et al., 2002; Tay et al., 2017). Depending on the type of stress and the need connected to that stress, different cells in the hematopoietic hierarchy become activated. Depletion of specific lineages such as B-cells or granulocytes directly induces proliferation in restricted progenitor populations, and indirectly by cellular feedback, also multipotent progenitors, while HSCs remain quiescent (Fanti et al., 2023; Säwén *et al.*, 2016). Whether depletion of erythrocytes by serial bleeding induces proliferation in HSCs is debated (Fanti *et al.*, 2023; Walter et al., 2015). Infection, mobilisation, irradiation, and chemotherapy on the other hand, induce proliferation of HSCs (Baldridge *et al.*, 2010; Busch *et al.*, 2015; Chapple *et al.*, 2018; Sawai *et al.*, 2016; Säwén *et al.*, 2016; Walter *et al.*, 2015; Wilson *et al.*, 2008).

In response to these activation signals, HSCs change a number of features compared to the steady state situation. There is a downregulation of adhesion molecules leading to mobilisation from the niche, an increased metabolic activity, especially of oxidative phosphorylation, resulting in proliferation (Christodoulou et al., 2020; Morganti *et al.*, 2022; Venezia et al., 2004) (Figure 4, left). While proliferative activity can be readily measured by BrdU uptake, expression of cell cycle proteins in combination with DNA content, or label retention, these assays do not provide information on the outcome of proliferation. Here, the HSC has three options: (1) expansion by symmetric self-renewal division, differentiation by asymmetric division (2), or symmetric commitment division (3) (Figure 4, right). In the case of transplantation of purified HSCs, the congenic Ly5.1/Ly5.2 strains are readily used to measure donor contribution both to the stem cell pool and mature progeny. Employing lineage tracing models have demonstrated how 5-fluorouracil (5-FU)

efficiently induces both self-renewal and differentiation in HSCs (Busch *et al.*, 2015; Chapple *et al.*, 2018; Sawai *et al.*, 2016). While the viral mimetic poly(IC) induces differentiation of HSCs (Sawai *et al.*, 2016), bacterially induced sepsis induced robust proliferation of HSCs without differentiation (Fanti *et al.*, 2023). The increase in proliferation induced by sepsis was, however, counteracted by a similar increase in apoptosis, hence resulting in an unchanged HSC pool size.

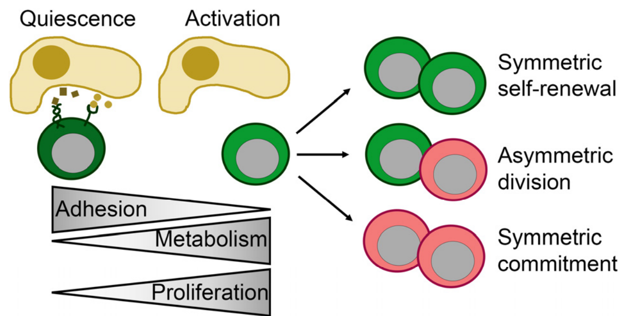

Figure 4. HSC activation during stress. HSCs respond to stress by mobilization from the niche, increasing their metabolism and initiating proliferation. The outcome of proliferation can be HSC expansion by self-renewal, or differentiation by asymmetric or symmetric commitment divisions.

The signals on a systemic level that regulates the decision whether HSCs expand or differentiate are still poorly understood. On the cellular level, there is evidence for regulation of fatty acid oxidation as well as asymmetric partitioning of mitochondria and lysosomes driving this decision (Ito *et al.*, 2012; Ito *et al.*, 2016; Katajisto et al., 2015; Loeffler et al., 2019).

In addition to the effects on the hematopoietic system, the stress induced by irradiation and chemotherapy also affects the cells of the bone marrow niche (Figure 3B). These regimens trigger an increase in adipogenesis, while osteoblasts, osteoclasts, mesenchymal stem cells, and endothelial cells are reduced in numbers and function (Cao et al., 2011; Domen et al., 1998; Dominici et al., 2009; Quach et al., 2015), which is likely part of the reason for HSC egress and activation under these circumstances. Both chemotherapy and irradiation have been shown to cause specific disruption of sinusoidal niche cells (Hooper et al., 2009; Kunisaki *et al.*, 2013; Saçma *et al.*, 2019), and HSCs seem to adapt by shifting their location closer to the arterioles or endosteum (Hooper *et al.*, 2009; Xie *et al.*, 2009). Pleiotrophin (PTN) is a growth factor mainly secreted by stromal cell during steady state. Following irradiation, the expression of PTN is increased in the bone marrow with endothelial cells as the main source (Himburg et al., 2018), demonstrating how the niche is adapting to stress. Another adaptation to irradiation is the secretion of cholinergic signals from nerve cells activate endosteal MSCs to regulate HSC quiescence (Fielding et al., 2022). Recently, the identification of subsets of

macrophages and MSCs important for hematopoietic reconstitution after transplantation provide insight in possibilities to improve HSC engraftment (Abbuehl et al., 2017; Kaur et al., 2018; Severe *et al.*, 2019).

Once inflammation is cleared, HSCs return to quiescence (Pietras *et al.*, 2015; Säwén *et al.*, 2016; Venezia *et al.*, 2004; Walter *et al.*, 2015; Wilson *et al.*, 2008). Depending on the severity of the stress, and the proliferative pressure on the HSC, the return to homeostasis can take days or months (Säwén *et al.*, 2016; Venezia *et al.*, 2004; Walter *et al.*, 2015). If HSCs are exposed to extensive, repeated, or chronic inflammation (Bogeska et al., 2022; Essers *et al.*, 2009; Ganuza et al., 2019; Kovtonyuk et al., 2022), or if genes regulating quiescence such as *Hes1*, *FoxO*, *Socs2, Hlf* and *Irf2* are dysregulated (Komorowska *et al.*, 2017; Ma et al., 2020; Sato *et al.*, 2009; Tothova et al., 2007; Vitali et al., 2015), this can lead to stem cell exhaustion, bone marrow failure, and clonal hematopoiesis. The impaired function can be caused by increased DNA damage, dysfunctional mitochondria, ROS-induced oxidative stress (Hinge et al., 2020; Ito *et al.*, 2004; Walter *et al.*, 2015). A deeper understanding of how these mechanisms can be harnessed to preserve HSC function is of great importance.

Aims of the thesis

HSCs reside in the bone marrow, where the surrounding niche provides essential signals to keep them in a quiescent state. They serve as a long-term reservoir throughout life but with an impressive capacity to rebuild the entire hematopoietic system if needed. The regulation of quiescence and activation is essential, both to ensure a balanced hematopoiesis output, but also to avoid exhaustion and the risk of malignant transformation. With this work I aimed to characterize the changes that HSCs undergo during conditions of severe stress and the effect of different conditioning regimens on the mechanisms of engraftment. A deeper knowledge of the processes regulating HSC activation and engraftment will aid in the understanding of both normal and malignant hematopoiesis, and hopefully contribute to improve treatments of hematopoietic disorders and malignancies. Specifically, the overarching aims of the work summarised in this thesis were:

Paper I

- To characterise the population dynamics and molecular regulation of HSPC regeneration after transplantation.
- To identify genes displaying gradual expression changes during regeneration and that may act as regulators of HSC activation.

Paper II

- To evaluate if the expression of the cell surface marker MAC-1 is affected by regenerative stress after transplantation.
- To investigate if there is a correlation between MAC-1 expression and reconstitution potential in regenerating HSCs.
- To map the molecular features of HSC MAC-1 subsets.

Paper III

- To establish a genetic conditioning model with selective and temporally controlled depletion of resident HSCs by conditional deletion of *Gata2.*
- To use this model to assess different functional aspects important for HSC homing and engraftment after transplantation.

Summary of results

Paper I

In this paper we aimed to perform a comprehensive profiling of hematopoietic stem and progenitor populations during the entire regenerative phase following transplantation after irradiation. To this end, we transplanted a large cohort of mice with a high dose of HSPCs. The mice were then sacrificed at 8 different timepoints for phenotypic analysis of blood and bone marrow, as well transcriptomic analysis of bone marrow HSPCs. We hypothesized that a gradual change in stem cell functionality during regeneration would be governed by gradual increases or decreases in critical genes regulating stem cell function.

The initial phase of regeneration was characterized by the production of myeloid cells both in blood and bone marrow. In particular, the megakaryocyte/erythroid progenitor HPC-2 population expanded, while the more primitive MPP and HSC populations were reduced. This gradually reverted, and by 13 weeks the balance between the HSPC populations was similar to that seen in steady state mice. Functional assessment of stem cell function was performed by secondary transplantation of cells from the different phases of regeneration. Compared to steady state HSCs, the HSCs from all the regenerating phases had significantly reduced engraftment capacity but showed a clear trend of increasing reconstitution with time after initial transplantation. The transcriptomic analysis demonstrated that HPC-1, HPC-2, and HSCs from all phases maintained their typical population signatures, while at the same time showing altered expression of genes controlling cell cycle, MYC and mTOR signalling. Changes in genes regulating fatty acid metabolism and glycolysis were specific to HSCs. By fitting the transcriptomic data from HSCs to a decay rate model we found a set of genes displaying a gradual increase or decrease in expression during regeneration. These genes overlapped significantly with known gene sets associated with HSC function, as well as genes that are differentially expressed in HSCs depending on their divisional history.

Paper II

In this paper we aimed to investigate if the surface antigen MAC-1 was upregulated on HSCs as a response to regenerative stress after transplantation, and if subpopulations of HSCs expressing different levels of MAC-1 displayed functional differences. In addition, we wanted to know if the expression of MAC-1 was required for such functional variation.

We observed a transient increase in MAC-1 expression during the early recovery phase after transplantation. Moreover, the expression of MAC-1 showed a positive correlation with HSC functionality as assessed in secondary transplantation assays, thereby allowing for a more efficient identification and isolation of long-term HSCs during regeneration. To identify the molecular differences between these functionally distinct subpopulations of HSC based on MAC-1 expression, we performed RNA sequencing. We observed differences in expression of genes regulating lineage priming, adhesion, stemness, metabolism and cell cycle, all processes important in the regulation of HSC function. Interestingly, we found a significant overlap between genes upregulated in the MAC-1 positive HSCs and genes known to be expressed in HSCs with low divisional history. In support of this observation, we found that HSCs expressing MAC-1 were more quiescent than MAC-1 negative HSCs, which contrasts with previous reports.

Next, we tested if the expression of MAC-1 had any role in regulating HSC function. To assess this, we used a conventional knockout mouse model of the CD11b subunit, that leads to a complete lack of MAC-1 expression in homozygous mice. We observed no difference in the frequencies of HSPCs or the cell-cycle state of HSCs, between wild type (WT) and KO littermates at steady state. To examine the role of MAC-1 during regenerative stress, we performed a competitive serial transplantation with WT or KO cells against equal numbers of competitor cells. Analysis of reconstitution in both primary and secondary recipients revealed no major differences in levels of engraftment or lineage output, suggesting that the expression of MAC-1 is dispensable for the regenerative capacity of HSCs.

Finally, we addressed whether the upregulation of MAC-1 in regenerating HSCs and the associated changes in cell cycle and function could be observed under other conditions of stress. To this end, we exposed mice to 5-FU, a chemotherapeutic agent causing myeloablation and activation of HSCs. Similar to what we observed after transplantation, we observed a transient upregulation of MAC-1 following 5-FU treatment, and that HSCs expressing high levels of MAC-1were more quiescent than MAC-1 negative HSCs. When assessed functionally by transplantation, the MAC-1 positive HSCs displayed superior reconstitution.

To conclude, MAC-1 is a useful marker to identify and isolate a functionally superior subpopulation of HSCs during regeneration, and thereby provides a promising tool to gain further knowledge of HSC regulation during proliferative stress.

Paper III

The gold standard assay for characterization of multipotency and self-renewal in HSCs is transplantation. Traditionally, a conditioning regimen such as irradiation is employed to create nice space and to remove the overwhelming competition of resident HSCs. In this paper we compared irradiation conditioning to a genetic

mouse model where selective depletion of resident HSPCs can be induced in a time-controlled manner by conditional deletion of *Gata2*.

When inducing deletion of *Gata2*, we achieved efficient depletion of HSCs, demonstrated by lack of engraftment when transplanting WBM from these mice in a competitive setting. This prompted us to test if *Gata2* deletion could serve as a pre-conditioning regimen by using the KO mice as recipients for transplantation. While control recipients with intact HSPC populations showed very low levels of transplanted donor cells, the *Gata2* deficient recipients displayed engraftment levels similar to irradiation conditioned recipients, although with slower reconstitution kinetics.

Next, we investigated if our model could be a permissive recipient even prior to HSC depletion, and thus whether donor HSPCs could engraft in an unperturbed environment in full competition with resident cells. We transplanted a large dose of HSPCs and selectively depleted recipient HSCs 4 weeks later by *Gata2* deletion. As expected, barely any donor cells were detected in the blood prior to recipient HSC deletion. Interestingly, however, upon deletion of *Gata2,* donor reconstitution increased substantially over time, while only low levels were observed in control recipients. This suggests that *Gata2* deletion can be used as a conditioning regimen both before and after transplantation allowing for efficient engraftment in a minimally perturbed environment.

Poly(IC) injections employed to induce the deletion of *Gata2* induce an interferon alpha response. Since interferon alpha is known to induce proliferation in HSCs, we wanted to exclude the possibility that the inflammatory effect of poly(IC) itself had a major impact on the results. To this end, we used an estrogen responsive genetic model where tamoxifen is used to induce *Gata2* deletion. Similar to our previous observations, when inducing depletion of recipient HSCs 4 weeks after transplantation, we observed robust reconstitution of donor cells specifically in *Gata2* deleted mice.

Finally, with this model at hand, we explored how different conditioning regimens can affect the outcome of a transplantation, and how we can take advantage of these differences to assess functional aspects of HSCs. We took advantage of the congenic strains Ly5.1 and Ly5.2, where HSCs from Ly5.2 have a slight advantage in competitive transplantations to irradiated recipients. We therefore transplanted equal numbers of WBM from Ly5.1 and Ly5.2 into either lethally irradiated recipients, *Gata2* pre-induced recipients, or *Gata2* recipients where deletion was induced 4 weeks post transplantation. As expected, donor engraftment was very similar between Ly5.1 and Ly5.2 in irradiated recipients, with Ly5.2 cells demonstrating an advantage in the BM. In contrast, the Ly5.1 cells were in advantage in the pre-induced *Gata2* recipients. This advantage was accentuated in post-induced *Gata2* recipients, with barely detectable levels of Ly5.2 cells in both blood and BM. Since both homing and proliferation are qualities of HSCs that

influence their reconstitution, we sought to test if the Ly5.1 and Ly5.2 strains differed in these aspects. We found, using independent assays, that Ly5.1 HPSCs homed more efficiently than Ly5.2 HSPCs, while Ly5.2 were slightly more proliferative. Our *Gata2* model is thus able to uncouple these properties and detect alterations in homing capacity not seen in conventional irradiated recipients. In conclusion, we provide a temporally controlled model of HSC depletion suitable to assess homing properties of HSCs.

Discussion

During the past 70 years the use of HSCs in transplantations have revolutionised treatments of many hematological disorders, and this progress goes hand in hand with the increased knowledge in HSCs biology. Since the first experimental evidence of the existence of HSCs in the 60s, it is now possible to identify and isolate HSCs at high purity for both functional and molecular analysis. The papers included in this thesis add pieces of information on the impact of regenerative stress on HSCs, and how we can study different functional aspects of HSC homing and engraftment.

The role of HSCs in steady state versus stress hematopoiesis

Chemotherapy and irradiation followed by transplantation puts enormous pressure on the hematopoietic system to replenish the lost and damaged cells. This pressure feeds back to HSCs that in turn rapidly adapt by entering cell cycle. In contrast to steady state hematopoiesis, which is mainly driven by progenitors, HSCs contribute significantly to hematopoiesis during severe stress, and after transplantation this results in a switch from polyclonal to oligoclonal hematopoiesis (Busch *et al.*, 2015; Sun *et al.*, 2014). Although the self-renewal capacity of HSCs is crucial for long-term engraftment, the proliferation potential of downstream progenitors is essential for a rapid recovery. The important role of progenitors also in a transplantation setting is clearly seen in Paper I, where a rapid expansion of HPC-2 megakaryocyte/erythroid (MegE) biased progenitors is the immediate output of the transplanted HSPCs. This population seems to be particularly important in stress hematopoiesis since it is only marginal in size during steady state. The reason for their rapid expansion after transplantation might be two-fold: First, the efficient ablation of megakaryocytes and megakaryocyte progenitors by irradiation, in combination with the short lifespan of mature platelets, creates an acute need for platelet production. Second, lineage tracing has demonstrated relatively fast label progression from HSCs to HPC-2 and Mk progenitors, demonstrating the potential of HSCs to rapidly differentiate along this path (Sawen *et al.*, 2018). Importantly, similar to what has previously been demonstrated during steady state, regenerating HPC-2 do not possess long-term self-renewal properties when transplanted.

Interestingly, we did not observe any major changes in frequency or number of the HPC-1 population, the cells suggested by Camargo and Rodewald (Busch *et al.*, 2015; Sun *et al.*, 2014) to be the main contributors of steady state hematopoiesis, suggesting that this population is highly regulated to meet long-term demands, and is less dynamic in acute hematopoiesis, compared to HPC-2 and HSCs. In addition, although lineage tracing demonstrated the fastest label progression from HSCs to MPPs during steady state (Sawen *et al.*, 2018), we observed a slow recovery of the MPP population after transplantation.

It is possible that HSCs continuously give rise to progenitors also during steady state conditions, and that this occurs through symmetric commitment divisions. If a barcoding system is used to trace hematopoietic output, symmetric commitment divisions leave no HSCs behind to be detected. Lineage tracing using different reporter systems are not limiting in this aspect and have demonstrated an active flow from the HSC compartment to progenitors and mature cells (Busch *et al.*, 2015; Sawai *et al.*, 2016; Sawen *et al.*, 2018), a flow that seems to diminish with age (Konturek-Ciesla *et al.*, 2023). Depending on the reporter system employed, the flow rate of label propagation that is a measurement of differentiation output is different, reflecting the difficulties with using specific reporters and ways of inducing the label (as discussed in the introduction). If the results suggesting significant HSC contribution to steady state hematopoiesis would hold true, it implies that the organism would rely on HSCs to maintain long-term stable hematopoiesis. One argument against this is work demonstrating how normal hematopoiesis is still maintained when 90% of the HSC pool is depleted (Schoedel et al., 2016), while the effect is much more adverse when both stem and progenitors are depleted, as seen in our *Gata2* model in Paper III. In addition, the large number of unique mature clones produced over an extended period of time exceeds the complexity of the HSC pool (Busch *et al.*, 2015; Sun *et al.*, 2014). On the other hand, we and others have observed donor output in peripheral blood when transplanting large numbers of HSCs to unconditioned recipients, (unpublished data), (Lu et al., 2019; Shimoto et al., 2017). If transplantation to unconditioned recipients can be considered similar to steady state hematopoiesis, these findings provide evidence for HSC contribution to homeostatic hematopoiesis. Yet, in this settings, it is possible that the handling and processing of cells prior to transplantation activates the HSCs, thereby overestimating their role of HSCs in steady state hematopoiesis.

Another aspect to consider is how accurate our mouse models recapitulate steady state hematopoiesis in humans. Experimental animals are kept in a controlled environment, protected from pathogens. Since inflammatory signals are known to activate stem cells, the lack of infection and inflammation in our mouse models represents an artificially clean setting leading abnormally low stress. Naturally, humans are exposed daily to various bacteria and virus, thereby creating a higher demand on the immune system. Steady state hematopoiesis in humans could

therefore be more dependent on the activity of HSCs. Indeed, tracing naturally occurring somatic mutations in HSPCs and peripheral blood demonstrates how human HSPCs actively contribute to granulocytes and B-cells (Lee-Six et al., 2018). Interestingly, there is evidence for differences in post-transplantation hematopoiesis between humans and mice. Taking advantage of the random integration of lentivirus in patients receiving gene therapy, Biasco et al. performed clonal tracking of 15 different blood cell lineages over a 4-year period. During the first 6 months they observed a first hematopoietic wave with lower clonal diversity, likely produced by progenitors, followed by a second long-lasting wave with higher clonal diversity sustained by HSCs (Biasco et al., 2016).

In summary, steady state hematopoiesis is propagated mainly by multipotent HPC-1 progenitors with a low contribution from the HSC compartment. In contrast, transplanted HSCs exit quiescence to initiate an immediate output of rapidly proliferating HPC-2 MegE biased progenitors, while the HPC-1 population remains relatively stable during regeneration.

Functional decline as a consequence of transplantation

The definition of long-term HSCs is based on their ability of multilineage reconstitution in serial transplantations. In both Papers I and II we observe a decline in reconstitution in secondary recipients. An overall lower number of functional HSCs during regeneration was demonstrated by Passegué and colleagues, who observed impaired reconstitution potential during early regeneration when assessing HSC activity by limiting dilution transplantations with unfractionated bone marrow (Pietras *et al.*, 2015). This is in a way expected and can partly be explained by the decline in functionality that is known to accompany divisional history (Qiu *et al.*, 2014; Wilson *et al.*, 2008). Interestingly, the engraftment levels partly recovered with time after the initial transplantation, suggesting that divisional history is not the only determining factor. There are several possible explanations for the decline in reconstitution potential:

1) The most potent HSCs may alter their marker profile during acute stress and could fall outside the conventional LSK SLAM gate.

2) The acute need to replenish the blood system during early regeneration instructs HSCs to differentiate, and a fraction of the cells defined phenotypically as HSCs is primed to differentiate and lacks the ability to self-renew, thereby “contaminating” and diluting the population.

3) Parts of, or all HSCs could temporarily exist in a reversible state of proliferation that would impair their homing and engraftment potential.

In Paper I we used MAC-1 as a negative marker in the lineage cocktail, why this subpopulation was most likely excluded from our analyses. This could partly

explain the lower engraftment levels seen during the early regenerative phase. Yet, we do know, from transplanting MAC-1 low and negative cells, that these populations contain HSCs, albeit with lower purity. In Paper II, by excluding MAC-1 from the lineage cocktail we were able to capture essentially all HSCs within the LSK SLAM gate, since cells outside of this gate generated very low levels of engraftment when transplanted.

It is more complicated to address the question of non-HSCs "contaminating" the regenerating HSC population, since many markers alter their expression as a response to an inflammatory environment (Pietras *et al.*, 2015; Randall and Weissman, 1997). HSCs downregulate expression of cKIT, and both HSCs and progenitors upregulate the expression of SCA-1. Of note, MAC-1 is upregulated specifically in HSCs and MPPs and not in more restricted progenitor populations (unpublished data). In Paper II, as an alternative approach, we replaced cKIT and SCA-1 with EPCR, thereby defining HSCs as Lin-, $CD150^{+}$, $CD48^{-}$, $EPCR^{+}$, and still observed a functional difference correlated to MAC-1 expression, excluding the possibility of SCA-1 expressing progenitors diluting the HSC population.

The transcriptomic analysis in Paper I revealed molecular overlap between early regenerating HSCs and HPC-2, as well as an upregulation of cell cycle genes. Since the analysis was done on bulk cells, we cannot say if these traits reflect the entire stem cell population, or only a subpopulation, but it might indicate that a fraction of these cells are primed to differentiate. However, the identification of MAC-1 as a marker of a functionally superior subset of HSCs during early regeneration supports the notion of HSC heterogeneity. By adding MAC-1 to the standard LSK SLAM phenotyping of HSCs we were able to identify a small subset of HSCs with distinct molecular and functional features. $MAC\text{-}1^{+}$ HSCs were not only superior in long-term engraftment, but also more quiescent and with higher expression of genes promoting stemness. Since all HSCs are activated and induced to proliferate when transplanted to irradiated recipients (Takizawa *et al.*, 2011; Wilson *et al.*, 2008), MAC-1 thereby identifies the first HSCs to return to quiescence.

In conclusion, our findings in Paper I and II give support to the existence of subpopulations of HSCs during the process of regeneration, and that the functional decline is a combination of divisional history, cell cycle status and priming to differentiation.

Regulation of activation and quiescence in HSCs

To find novel regulators of HSC activation, we reasoned that genes regulating HSC activation would display a gradual increase or decrease in expression with time after stress insult. The decay rate model we employed for this purpose identified a set of genes that overlapped significantly with published gene sets linked to HSC function,

some of which had previously described roles in HSC regulation. One shortcoming of this experiment is the lack of molecular data during the first three weeks after transplantation. This was due to technical limitations, since it proved challenging to sample a sufficient number of cells during this time. Results from Dong et al. confirmed the difficulty in retrieving transplanted cells at early timepoints, even without relying on cell surface markers for identification. They did demonstrate however, that the majority of transplanted HSCs downregulate HSC signature genes and upregulate the expression of genes related to myeloid and MegE lineage restriction, while a small subset increased the expression of genes regulating quiescence (Dong et al., 2020). This raises the question if these subsets of HSCs that evolve in response to stress arise from stochastic events, or if they represent inherently different cells that thus have responded in different ways to the same stress stimuli.

HSC activation, a one-lane or multi-lane path?

As mentioned earlier, the outcome of proliferation in response to different kinds of stress can be different, but many cellular pathways regulating metabolism and proliferation are initially common in HSCs (Fanti *et al.*, 2023; Giladi et al., 2018; Mann et al., 2018; Venezia *et al.*, 2004). In contrast, a recent publication by Fast et al. compared the acute response in HSCs to G-CSF, prostaglandins, and poly(IC), and showed unique transcriptional responses to each of these stimuli (Fast et al., 2021). This raises the question on how specific the stress response is in HSCs to various stimuli, but also if the activation to a specific stimulus would always follow the same path in all HSCs.

When we designed the experimental layout for Paper I, we made a simplified hypothesis that HSC activation by transplantation would follow the same path up until the point where they make the decision if they should differentiate or not. This path would be the same, irrespective of when they become activated during the regenerative process, therefore the same states of activation would exist throughout regeneration but at different ratios (Figure 5, model A). Alternatively, the same stimulus could induce multiple paths of activation, possibly pre-determined by primed HSC subpopulations (Figure 5, model B).

The experimental setups in Papers I and II, where we perform both transplantations and RNA-sequencing on bulk populations, prevents us from making any strong claims regarding intrinsically different HSCs and how individual HSCs respond to activating cues. However, in support of model A, we observed an overlap of the differentially expressed genes identified with the decay rate model in Paper I, and genes that were differentially expressed between the 4-week MAC-1 subpopulations in Paper II. 12 out of 117 genes with decreased expression over time also had lower expression specifically in MAC-1^{+} compared to MAC-1^{-} HSCs, while 2 of the 13 genes with increased expression over time had higher expression in MAC-1^{+}

compared to MAC-1$^-$ HSCs. The conclusion is that some of the genes with a gradual change in expression can also be found in distinct subpopulations at one specific time point, in agreement with model A.

In favour of model B, there is a well-documented existence of HSC subpopulations with epigenetically inherited traits (Dykstra *et al.*, 2007; Yu *et al.*, 2016), and myeloid-biased and lymphoid biased HSC subpopulations respond differently to TGF-beta and IFN-gamma signalling molecules induced by irradiation (Challen *et al.*, 2010; Matatall et al., 2014). Of note, in contrast to our observation where MAC-1$^+$ HSCs are more quiescent and express higher levels of lymphoid genes, the lymphoid biased HSCs identified by Challen et al. were more proliferative compared to the myeloid biased (Challen *et al.*, 2010).

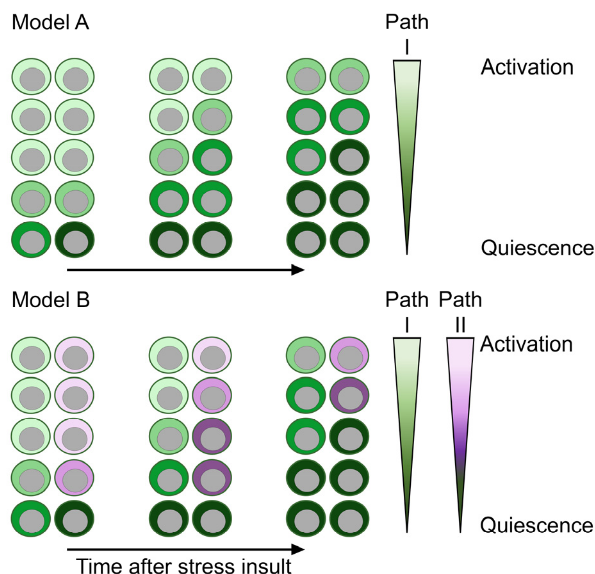

Figure 5. Models of unique or parallel paths of HSC activation. HSC activation by one stimulus induces the same unique path in all HSCs (model A). The same stimulus can induce multiple paths of activation that exist in parallel (model B).

The highly inflammatory environment following irradiation activates the entire pool of HSCs to proliferate (Wilson *et al.*, 2008), and it is essential for the maintenance of LT-HSC function to return to quiescence. Many inflammatory cytokines signals through JAK/STAT pathway, that in turn induces expression of *Socs2*, a transcription factor that functions as negative feedback (Baker *et al.*, 2007; Posselt et al., 2011). Our observation in Paper II of an increased expression of *Hlf* and *Socs2* specifically in the more quiescent MAC-1$^+$ HSC subpopulation, suggest that they might be key transcription factors regulating the transition from activation to quiescence. Both *Hlf* and *Socs2* are redundant for steady state hematopoiesis, while

KO of either of these genes compromises the ability for HSCs to return to quiescence after activation, eventually leading to HSC exhaustion (Komorowska *et al.*, 2017; Vitali *et al.*, 2015).

Driving forces of HSC activation

One interesting aspect of HSC activation after transplantation is whether the main regulator of HSC proliferation is the need to replenish the blood system and the stem cell pool, or if the main driver is the inflammatory environment. Since irradiation-induced inflammatory cytokines emerge simultaneously, and probably as a consequence of tissue damage and death of hematopoietic and niche cells, it is difficult to address which of the two is the main regulatory factor of HSC proliferation. Interestingly, the number of active clones and the longevity of each clone is dependent on the dose of transplanted HSCs (Brewer et al., 2016). A large donor dose induces differentiation of short-lived myeloid clones not seen to the same extent when transplanting limited number of HSCs. This might be a way for the organism to spare a fraction of the HSCs long term, while another fraction is allowed to rapidly differentiate to replenish the blood system. It also suggests that the regulation of HSC activation is fine-tuned by feedback signals propelled by the hematopoietic need, and not only driven by the initial burst of inflammatory cytokines.

We are currently investigating this by transplanting a wide range of donor HSC doses together with WBM support and thereafter determining both mature hematopoietic output as well as recovery of the stem cell pool size in primary and secondary recipients. We speculate that if the hematopoietic need is the main regulatory factor, the HSC pool size would fully recover, irrespective of the initial transplanted dose. Consequently, a smaller dose of transplanted donor HSCs would need to proliferate more to reach this pool size. Unpublished data from this study revealed that the total phenotypic HSC pool fully recovered, but that the contribution from the donor HSCs varied, suggesting that HSC self-renewal is regulated by the hematopoietic need. We did observe an inverse correlation between the transplanted dose and the average fold expansion of donor HSCs, suggesting that the lower dose have a higher proliferative history. Unexpectedly, this difference in proliferative history was not reflected in their differentiation capacity when transplanted to secondary recipients, while the reconstitution of the HSC pool corresponded to the initial input dose (unpublished data). These results partly contradict observations made by Weissman and Rossi, who observed a positive correlation between long term reconstitution and initial transplant dose (Beerman et al., 2013; Pawliuk et al., 1996). Our preliminary results suggest that the need for hematopoietic recovery is the dominant regulator of HSC activation, but that the proliferative stress they experience upon transplantation is enough to impair their fitness, in particular their ability to differentiate. To further strengthen this

hypothesis, it would be interesting to use our *Gata2* model as preconditioned recipients to investigate if a less inflammatory bone marrow environment would affect this outcome.

The niche in steady state and after transplantation

Niche space versus outnumbering competition

Engraftment and efficient reconstitution are multi-step processes that requires many different qualities in HSCs. First, they need to find their way to the BM via long range soluble signals, known as homing. This is followed by a closer interaction with specific niche cells known as lodging. Once the HSCs are residing in the bone marrow niche, to contribute to reconstitution they need to divide to produce mature hematopoietic cells and contribute to reconstitution. Transplantation is normally preceded by irradiation conditioning which efficiently kills the recipient's hematopoietic cells, thereby creating a need for the incoming donor cells to become activated and participate in the replenishment of the hematopoietic system. The classic view of the BM niche has been that it is the limiting factor for the HSC pool size. Substantial engraftment of transplanted HSCs would not be possible unless the niche is cleared from resident cells. In contrast, with the *Gata2* model we demonstrate that engraftment can occur even without prior conditioning. When transplanting HSPCs to non-conditioned recipients and depleting the recipient HSPCs after 4 weeks by *Gata2* deletion, we observed a robust output of mature cells from the donor HSCs. This means that the transplanted cells were capable of engrafting in the BM niche but outnumbered by the recipient cells, therefore their hematopoietic output was below detection until the competition was removed. Our results suggest that niche space is not the only limiting factor for engraftment and is supported by previous work done by Shimoto et al., who demonstrated how transplantation of very large doses of HSCs both increases the total HSC pool size, as well as allowing for detection of blood output from transplanted cells (Shimoto *et al.*, 2017).

Prospects of conditioning

It is clear, however, that niche availability plays a significant role in engraftment of HSCs. Recent findings have demonstrated how the use of mobilizing agents such as G-CSF and Plerixafor can be used not only to collect HSPCs by apheresis, but also as a means of mild conditioning that allows for efficient engraftment of transplanted bone marrow cells (Canarutto et al., 2023; Guderyon et al., 2020; Omer-Javed *et al.*, 2022). Further development of mild forms of conditioning such as lower dose of

radiation, mobilization, or the use of antibodies that specifically clears HSPCs (Czechowicz *et al.*, 2007; Dahl et al., 2021; Palchaudhuri *et al.*, 2016), is particularly interesting in the case of transplantations of patients with hematological disorders that does not require malignant cells to be killed by irradiation or chemotherapy. To accompany such advancements, it becomes important to better understand the different steps in engraftment under homeostatic conditions. For this purpose, the *Gata2* mice would be a good model. It allows for depletion of recipient HSCs both before and after transplantation and provides a sensitive readout of subtle differences in homing/lodging that may go undetected in transplantations into irradiated recipients. In a similar manner, the use of toxin-conjugated antibodies can deplete specific cell populations *in vivo* and can be used as alternative conditioning regimens. The CD45-SAP immunotoxin regimen specifically targets the CD45.2 isoform while sparing CD45.1 and could possibly be used to specifically deplete host CD45.2 HSCs while sparing already engrafted CD45.1 donor HSCs also after transplantation (Palchaudhuri *et al.*, 2016).

The effects caused by irradiation and chemotherapy on the niche microenvironment plays an important role in HSC regeneration following transplantation. The selective ablation of the sinusoidal niche is compensated for by re-localisation of HSCs to the endosteal and arteriolar niches and demonstrates the importance and capability of both niche and hematopoietic cells to adapt to stress (Fielding *et al.*, 2022; Himburg *et al.*, 2018; Hooper *et al.*, 2009; Saçma *et al.*, 2019; Xie *et al.*, 2009). In this context, less is known whether subtypes of HSCs react and adapt in a similar manner, and how differences between HSCs and leukemic stem and progenitor cells influence transplantation outcome in this regard. In Paper III we demonstrated that even small differences in HSC proliferation and adhesion can read out differently depending on the conditioning. It would be interesting to investigate how HSC subtypes differ in their homing and engrafting properties depending on the conditioning, and if such differences could be used to improve and adapt transplantation regimens.

Closing remarks

It is difficult to predict if or how individual findings in basic research will impact the research community, clinical advancements and, in the end, our society. My hope is of course that the years of fun, but hard work will be one stepping stone for future research in regenerative medicine. Currently, personalised and precision medicine is becoming an alternative for a growing number of diseases, and gene therapy is one example where there is hope for cures of currently incurable diseases. Here, a better understanding of factors regulating homing and engraftment after mild conditioning can provide better outcomes after transplantation, and we hope that our *Gata2* model can add information in this aspect. The long-term risks of proliferative stress inflicted on transplanted HSCs highlight the importance to regulate the

regenerative process with extreme precision. The work in Paper I and II have improved current protocols to identify regenerating HSC and added to the knowledge of genes and processes regulating activation and quiescence. These findings can be important for future improvements of transplantations, *ex vivo* expansion of HSCs, and help in understanding mechanisms of malignant transformation.

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