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UNDERSTANDING THE HETEROGENEITY OF THE HEMATOPOIETIC STEM CELLS

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Cover illustration: Scanning Electron Microscope (SEM) of circulating human blood cells, National Cancer Institute. Bruce Wetzel, and Harry Schaefer, created in February 1982. Visible in the picture: Erythrocytes, lymphocytes, a monocyte, a neutrophil, and platelets.
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UNDERSTANDING THE HETEROGENEITY OF THE HEMATOPOIETIC STEM CELLS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my beloved parents,

Canım aileme,

What we know is a drop, what we don't know is an ocean.

Isaac Newton

ABSTRACT

The hematopoietic system is replenished and maintained throughout life by rare hematopoietic stem cells (HSCs) that reside in the bone marrow (BM) of adult mammals. Over the last 20 years, the advancement in the field lead to the acknowledgement of the heterogeneity within the HSC compartment unraveling the presence of HSC subsets with certain mature blood lineage preferences so called lineage-biased (Li-bi) HSCs. Studying the heterogeneity and lineage bias within the HSC compartment is crucial not only to understand the functional and molecular mechanisms behind this lineage skewing but can also shed light on the emergence of hematological malignancies subsequently paving the way to find new therapeutic targets, better treatment options and more selective alternatives of BM transplantation.

Recent developments have taken advantage of immunophenotypic markers for prospective isolation of cells. The cell surface markers can be used to enrich for HSCs but cannot purify. Current markers cannot resolve heterogeneity within the HSC compartment, highlighting the importance of continuing efforts on identifying new cell surface markers that enrich Li-bi HSC subtypes. In **paper I**, we demonstrate that CD49b cell surface marker subfractionates the most primitive HSC compartment into two; $CD49b^{-}$ HSCs with myeloid bias, high self-renewal potential and the most quiescent state, and $CD49b^{+}$ HSCs with lymphoid bias, lowered self-renewal potential and more proliferative state. Furthermore, we show that both subsets have similar transcriptome profiles but distinct epigenetic landscapes highlighting that the lineage-bias is regulated via epigenetic mechanisms. In **paper III**, we show that using the additional cell surface marker CD229, the remaining heterogeneity within the $CD49b^{+}$ HSCs can be resolved into two functional subsets as $CD49b^{+}CD229^{-}$ and $CD49b^{+}CD229^{+}$. The $CD49b^{+}CD229^{-}$ fraction shows long-term and stable reconstitution and the $CD49b^{+}CD229^{+}$ fraction enriches for multipotent progenitor cells having short term activity.

Hematopoietic aging is associated with myeloid skewing, delayed, and reduced immune response and higher incidences of myeloid malignancies. The composition of HSC compartment changes with a shift toward an increased proportion of myeloid biased HSCs in elderly both in human and mice. However, the molecular mechanisms behind this phenomenon are not completely understood. **In paper II**, we show that the $CD49b^{-}$ HSC maintains its myeloid bias in the peripheral blood of the young, young adult and old age groups whereas the $CD49b^{+}$ HSC shifts from lymphoid bias in young and young adult to lineage-balance (no bias) in aged mice. In addition, we demonstrate that both subsets are equally active in young and have similar chromatin landscapes with different levels of accessible regions in old mice.

The B cell lineage priming occurs downstream of HSCs starting at the branching point of multipotent progenitors in the hematopoietic hierarchy. The B cell development is highly regulated by transcriptional factors. In **paper IV**, we show that combined loss of transcription factors FOXO1 and FOXO3 prevents the B cell development by blocking it at the BLP stage.

Moreover, we demonstrate that FOXO3 plays a crucial role in regulating the B cell lineage priming higher up in the hematopoietic hierarchy already as early as the LMPP level.

Collectively, this thesis identifies cell surface markers that resolves the functional heterogeneity of the HSCs, gives insights into how the lineage bias is regulated during aging, and unravels the effect of transcription factors in B cell development.

LIST OF SCIENTIFIC PAPERS

I. *Ece Somuncular*, Julia Hauenstein, Prajakta Khalkar, Anne-Sofie Johansson, Özge Dumral, Nicolai S. Frengen, Charlotte Gustafsson, Giuseppe Mocci, Tsu-Yi Su, Hugo Brouwer, Christine L. Trautmann, Michael Vanlandewijck, Stuart H. Orkin, Robert Månsson, and Sidinh Luc. **CD49b identifies functionally and epigenetically distinct subsets of lineage-biased hematopoietic stem cells.** *Stem Cell Reports, 2022, Vol. 17, 1546-1560*

II. *Ece Somuncular*, Julia Hauenstein, Tsu-Yi Su, Özge Dumral, Charlotte Gustafsson, Efthymios Tzortzis, Aurora Forlani, Anne-Sofie Johansson, Robert Månsson and Sidinh Luc. **Aging is associated with functional and molecular changes in distinct hematopoietic stem cell subsets.** *Manuscript*

III. *Ece Somuncular*, Tsu-Yi Su, Özge Dumral, Anne-Sofie Johansson and Sidinh Luc. **The combination of CD49b and CD229 reveals a subset of multipotent cells with short-term activity within the hematopoietic stem cell compartment.** *Manuscript*

IV. Lucia Peña-Pérez, Shabnam Kharazi, Nicolai Frengen, Aleksandra Krstic, Thibault Bouderlique, Julia Hauenstein, Minghui He, *Ece Somuncular*, Xiaoze Li Wang, Carin Dahlberg, Charlotte Gustafsson, Ann-Sofie Johansson, Julian Walfridsson, Nadir Kadri, Petter Woll, Marcin Kierczak, Hong Qian, Lisa Westerberg, Sidinh Luc and Robert Månsson. **FOXO Dictates Initiation of B Cell Development and Myeloid Restriction in Common Lymphoid Progenitors.** *Frontiers in Immunology, 2022, Vol 13:880668*

CONTENTS

LIST OF ABBREVIATIONS

AGM	Aorta-gonad-mesonephros
ALP	All lymphocyte progenitor
Bala	Lineage-balanced
BCR	B cell receptor
BLP	B cell biased lymphocyte progenitor
BM	Bone marrow
CD	Cluster of differentiation
CFU-E	Colony forming unit-erythroid
CFU-S	Colony forming unit-spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
Csf1r	Colony stimulating factor 1 receptor
DAR	Differentially accessible region
DC	Dendritic cell
DEG	Differentially expressed gene
dHSC	Definitive hematopoietic stem cell
Dntt	DNA nucleotidylexotransferase
E	Embryonic day
EBF1	Early B cell factor-1
ELP	Early lymphoid progenitor
ETP	Early thymic progenitor
FBM	Fetal bone marrow
FL	Fetal liver
FoB	Follicular B cells
FOXO	Forkhead box O
FS	Fetal spleen
GC	Germinal center
GMP	Granulocyte-macrophage progenitor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell

IgH	Ig heavy chain
IgL	Ig light chain
IL7Rα	Interleukin receptor 7 alpha
immB	Immature B cells
IT	Intermediate-term
Itgα2	Integrin alpha2
LMPP	Lymphoid-primed multipotent progenitor
LT	Long-term
Ly-bi	Lymphoid-biased
LY6D	Lymphocyte antigen 6 family
MEP	Megakaryocyte-erythroid progenitor
MHC	Major histocompatibility complex
MkRP	Megakaryocyte repopulating progenitor
MPP	Multipotent progenitor
My-bi	Myeloid-biased
MZ	Marginal zone
NK	Natural killer cell
PAX5	Paired box 5
PB	Peripheral blood
PCA	Principal component analysis
Pl-bi	Platelet-biased
Pre-B	Pre-B cell
Pre-GM	pre-granulocyte-macrophage progenitor
Pre-MegE	pre-megakaryocyte-erythroid progenitor
Pro-B	Pro-B cell
Rag	Recombination activating gene
SP	Side population
ST	Short-term
Tcf3	Transcription factor 3
TF	Transcription factor
UMAP	Uniform manifold approximation and projection

V(D)J	Variable (V), diversity (D), joining (J)
vWF	Von Willebrand factor
WBC	White blood cell
YA	Young adult
YS	Yolk sac

1 INTRODUCTION

1.1 THE MATURE BLOOD SYSTEM

The blood is composed of various types of cellular components with distinct functions. It takes part in innate and adaptive immune responses, gas transportation, and hemostasis. Red blood cells (erythrocytes) carry oxygen, nutrients and hormones into other tissues whilst removing carbon dioxide and waste from them and can circulate up to 120 days (Liggett and Sankaran, 2020). Platelets (thrombocytes) which originate from megakaryocytes play role in blood clotting and healing the scarred tissue and have only nine days of lifespan in the peripheral blood (Rieger and Schroeder, 2012). White blood cells (WBCs, leukocytes) take part in innate and acquired immune responses. Myeloid cells (monocytes/macrophages, granulocytes) have a short life cycle in circulation (4-11 days) and are part of the nonspecific innate immune responses that act as a first line of defense against bacterial, fungal, and viral infections caused by pathogens (Dahl, 2009; Janssen et al., 2016). Lymphocytes (B cells and T cells) are the members of the adaptive immune system with relatively long lifetime (several months up to years) that regulate the immune response and long-term protection against an ongoing infection (Borghans and Ribeiro, 2017; Colucci, 2009; Corfe and Paige, 2009; Khodadadi et al., 2019; Schwarz and Bhandoola, 2009). Dendritic cells (DCs) act as the linkers of the primary and secondary immunity. In addition to their ability to induce the innate response, DCs can regulate the form of T cell-mediated secondary response and control autoimmunity (Mellman, 2013).

The blood is highly regenerative due to relatively short lifespan of the mature blood cells, producing nearly one trillion (10^{12}) cells day-to-day in the adult human bone marrow (BM) (Doulatov et al., 2012). Hematopoiesis is the process of continuous formation, maintenance, and turnover of blood cells throughout embryonic development and adulthood where hematopoietic stem cells (HSCs) are the precursors of this hematopoiesis (Orkin and Zon, 2008).

1.2 HEMATOPOIETIC STEM AND PROGENITOR CELLS

The current understanding of hematopoietic stem cell biology and its application was founded in the 20th century. In 1945, Ray Owen discovered that dizygotic twin cattle (two separate eggs fertilized with two different sperm) hosted the red blood cells of both twins (Owen, 1945). Later in 1952-53, transplantation of mono- and dizygotic skin grafts of the cattle twins and hematopoietic cell infusions in embryonic and neonatal mice shed light into the acquired immune tolerance and chimerism which paved the way to understand and apply the transplantation technique in stem cell research (Billingham et al., 1952, 1953).

In 1945, the explosion of the atomic bomb and the subsequent radiation in Hiroshima and Nagasaki shattered the lives of Japanese citizens, causing massive death toll. Following

weeks of the tragedy, survivors of the explosion began to show signs of hair loss, low blood cell count, internal bleeding, and subsequently death. Whilst investigating the cause of the mortality of this the second wave, scientists discovered that the hematopoietic failure due to radiation was the reason. It was later noticed that hematopoietic failure can be prevented using lead as a spleen shield (Jacobson et al., 1949, 1950) and can even be recovered by intraperitoneal or intravenous injection of spleen and bone marrow cells post-radiation (Jacobson et al., 1951; Lorenz and Congdon, 1954; Lorenz et al., 1952). Despite differing in major histocompability complex (MHC) classes, successful engraftment of allogeneic donor BM cells in lethally irradiated mice followed by no further rejection of skin transplant substantiated the chimerism tolerance (Main and Prehn, 1955) which was later confirmed by chromosomal markers (Ford et al., 1956; Nowell et al., 1956). Since this knowledge, irradiation has been widely used as a useful application for clearing the hematopoietic niche in engraftment experiments.

1.2.1 In The Search for Hematopoietic Stem Cells

Till and McCulloch conducted a series of ground-breaking studies laying the foundation of hematopoietic stem cell biology that started as attempts to understand radiation sensitivity. In 1961, they discovered that intravenous BM cell injection into lethally irradiated mice resulted in colony-forming unit-spleen (CFU-S) that contained myeloid cells, erythrocytes, and megakaryocytes. Additional cells were detected but could not be identified due to their undifferentiated state. The mice that did not get a protective BM dose died from hematopoietic failure within the first two weeks post-radiation. Of the ones that survived, the number of cells injected was directly correlated to the number of colonies observed indicating that a single BM precursor can give rise to a heterogeneous pool of cells. (Till and McCulloch, 1961). To decipher the origin of the colonies, the sublethally radiated recipient mice were injected with BM cells followed by immediate exposure to sublethal radiation while eliminating the indigenous BM cells in the recipient and thus allowing the donor cells to be individually marked with chromosomal aberrations. They hypothesized that after 10 days of engraftment, if the spleen colony carry the same chromosomal abnormality, this would evidently show that the cells originated from a single BM cell. This method verified that a single blood-forming cell is capable of giving rise to different types of blood cells (Becker et al., 1963).

Further studies demonstrated that when the engrafted cells from individual spleen colonies were secondary transplanted into lethally radiated mice, the primary cells could repopulate the secondary recipients, deducing that spleen colonies were able to self-renew (Siminovitch et al., 1963) It was later on shown that repeated serial transplantations diminish the self-renewal ability in these cells. Surprisingly, the colonies were found to be capable of giving rise to myeloerythroid progeny but also the lymphoid progeny (Siminovitch et al., 1963; Wu et al., 1967). Later studies conducted in the end of 20th century illuminated that day 8-12 spleen colonies that rescue the myeloablated animals are mainly composed of committed hematopoietic progenitors, hence could not sustain the hematopoietic system long-term and

have multilineage potential (Jones et al., 1990; Nakorn et al., 2002; Ploemacher and Brons, 1988).

Through these studies, hallmark characteristics of HSCs were established: multipotent differentiation ability with generation of different blood lineages and ability to give rise to cells in re-transplantation with self-renewal ability. Due to extensive self-renewal potential and multipotency, HSCs are capable of long-term repopulation and maintenance of the entire hematopoietic system.

1.2.2 Prospective Isolation of Hematopoietic Stem and Progenitor Cells

Hematopoietic stem cells are found at a frequency of one in 10,000-20,000 BM cells (Spangrude et al., 1988; Szilvassy et al., 1990). Due to the rare frequency of HSCs, one of the main challenges of the field has been to prospectively isolate and further characterize the HSCs for subsequent clinical applications (Ng and Alexander, 2017).

Attempts at isolating HSCs revealed several physical properties: resistance to 5-fluorauracil (Hodgson and Bradley, 1979) and gamma-radiation (Ploemacher et al., 1992), separation based on counterflow density (Jones et al., 1990) and light density (Visser et al., 1984), ability to efflux Hoechst 33342 intracellular dye (Side population, SP) (Goodell et al., 1996) and Rhodamine 123 mitochondrial dye (Uchida et al., 1994; Zijlmans et al., 1995). Advances in flow cytometry and fluorescence activated cell sorting (FACS) systems rapidly facilitated the prospective isolation of hematopoietic stem and progenitor cells (HSPCs), performed by marking the antigens on the cell surface (Ng and Alexander, 2017; Spangrude et al., 1988). Since then, these techniques play essential roles in identification and functional characterization of HSPCs and mature blood lineages in the research field.

Hematopoietic stem and progenitor cells can be identified by the lack of mature lineage cell surface markers. Combination of these differentiated lineage markers include B cells (B220, CD19), T cells (CD4 and CD8), granulocytes (Gr-1), monocytes (CD11b or Mac-1) and erythrocytes (Ter-119) and are excluded when isolating HSPCs (Spangrude et al., 1988). Remarkably, HSCs express low levels of Thy-1 (CD90.1) antigen, T cell specific marker and hence are isolated as Thy-1^{lo} (Muller-Sieburg et al., 1986). HSPCs express stem cell antigen-1 positive (Sca-1^{+} or Ly-6A/E^{+}) (Morrison and Weissman, 1994; Spangrude et al., 1988; Uchida et al., 1994), protooncogene c-Kit positive ($CD117^{+}$ or c-kit^{+}, steel factor receptor) (Ikuta and Weissman, 1992) cell surface markers and are present within the Lin^{-}Sca-1^{+}c-kit^{+} (LSK) compartment. Additional cell surface markers that enrich for HSPCs include FMS-like tyrosine kinase (Flt-3, Flk2 and CD135) (Adolfsson et al., 2001, 2005; Christensen and Weissman, 2001; Månsson et al., 2007; Yang et al., 2005), CD34 (Osawa et al., 1996), CD48 (Signaling lymphocyte activation molecule family 2, Slamf2), CD150 (Slamf1), CD244 (Slamf4) (Kiel et al., 2005; Kim et al., 2006) and CD201 (Endothelial protein C receptor, EPCR) (Balazs et al., 2006; Kent et al., 2009; Rabe et al., 2020; Wilson et al., 2015), endothelial cell-selective

adhesion molecule (ESAM) (Ooi et al., 2009; Sudo et al., 2012; Yokota et al., 2008) and CD41 (Miyawaki et al., 2015).

Investigations focusing on the functionality of HSPC populations are conducted based on the usage of different marker combinations; therefore, there is an added complexity to reach an agreement on which antigens purify the most primitive HSCs. Commonly used HSC markers are LSK Flt-3$^-$CD34$^-$CD48$^-$CD150$^+$ (Wilkinson et al., 2020) and HSCs can additionally be separated by CD201$^+$, CD244$^-$, Rhodamine 123$^-$ (Rho$^-$) and Hoechst 33342$^-$ (SP) tip. Using reporter mice, HSCs are found to be enriched within populations that express certain genes such as Fgd5$^+$ (Gazit et al., 2014), von Willebrand factor (vWF$^+$) (Sanjuan-Pla et al., 2013), Homeobox B5$^+$ (Hoxb5, Hox-2.1) (Chen et al., 2016) α-catulin$^+$ (Ctnnal1) (Acar et al., 2015), ecotropic viral integration site 1$^+$ (Evi1$^+$, histo-lysine N-methlytransferase MECOM) (Kataoka et al., 2011) and Krt7$^+$ (Tajima et al., 2017).

1.3 THE HEMATOPOIETIC HIERARCHY IN ADULT BONE MARROW

Throughout life, HSCs undergo a series of divisions to maintain the HSC pool and to replenish the mature blood lineages. Restoration of the hematopoietic system requires a progressive and stepwise lineage commitment of HSCs to fully differentiated blood lineages. Even though we may just have begun to decipher the complexity of hematopoietic hierarchy and most likely have oversimplistic models, understanding the molecular and cellular aspects of lineage commitment is crucial for normal and malignant hematopoiesis (Cheng et al., 2020; Jacobsen and Nerlov, 2019; Orkin, 2000; Orkin and Zon, 2008; Reya et al., 2001).

Since the late 1980s, multicolor FACS technology with readily available antibodies has been extensively used to isolate and characterize HSPC populations. Starting with the identification of long-term (LT) reconstituting HSCs and multipotent progenitors (MPPs) (Muller-Sieburg et al., 1986; Spangrude et al., 1988), a new era in hematology emerged with a focus to unravel the function and lineage commitment of the bone marrow cells. Henceforth, hematopoietic hierarchy has been intensively studied and continuously revised.

1.3.1 The Classical Model

The classical model proposes that the hematopoietic hierarchy has a tree-like structure with stepwise differentiation of lineage commitment in which HSCs reside at the top of the hierarchy (Bryder et al., 2006). In this model, HSC pool is grouped into two fractions: LT-HSCs (LSK Flt-3$^-$CD34$^-$) (Morrison and Weissman, 1994; Osawa et al., 1996) and short-term (ST) HSCs (LSK Flt-3$^-$CD34$^+$) (Yang et al., 2005). The progressive differentiation of LT-HSCs to ST-HSCs is followed by oligopotent MPPs (LSK Flt-3$^+$CD34$^+$) (Adolfsson et al., 2005). While both ST-HSCs and MPPs have transient and declining repopulating ability (less than 4 weeks) with no self-renewal potential, LT-HSCs have stable and long-term repopulating ability (more than 16 weeks), can self-renew and are secondary transplantable.

Lineage commitment begins at the MPP stage with the bifurcation into lymphoid and myeloid lineages. MPPs give rise to both a common lymphoid progenitor (CLP) (Kondo et al., 1997) and a common myeloid progenitor (CMP) (Akashi et al., 2000; Nakorn et al., 2002). Identified with immunophenotypic markers Thy-1.1^{-}Sca-1^{low}c-kit^{low}IL7Rα^{+}Flt-3^{+}, CLPs only generate lymphoid lineage (B, T, NK, and dendritic cells). On the other hand, CMPs (Lin^{-}Sca-1^{-}c-Kit^{+} (LK) IL7Rα^{-}CD34^{+}FcγRII/III^{low}) have myeloid, erythrocyte and megakaryocyte potentials (Kondo et al., 1997). CMP branches into oligopotent granulocyte-macrophage progenitor (GMP; Lin^{-}Sca-1^{-}c-Kit^{+}IL7Rα^{-}CD34^{-}FcγRII/III^{high}) and megakaryocyte-erythroid progenitor (MEP; Lin^{-}Sca-1^{-}c- Kit^{+}IL7Rα^{-}CD34^{-}FcγRII/III^{low}) (Akashi et al., 2000; Arinobu et al., 2007; Rieger et al., 2009). Granulocyte (consisting of basophil, eosinophil, and neutrophil), monocyte (containing macrophage and monocyte derived DC), and DC lineages are derived from GMP, whereas MEP gives rise to megakaryocytes that produces platelets and to erythroblasts that differentiate into erythrocyte lineage (Cheng et al., 2020; Orkin and Zon, 2008).

In the classical model, lymphoid lineages consist of B, T, NK and dendritic cells, and myeloid lineage contains monocytes, granulocytes and platelets and erythrocytes. In the revised hematopoietic hierarchy models, platelet and erythrocyte lineages are identified to be in a separate branch than monocyte and granulocyte lineages (Jacobsen and Nerlov, 2019).

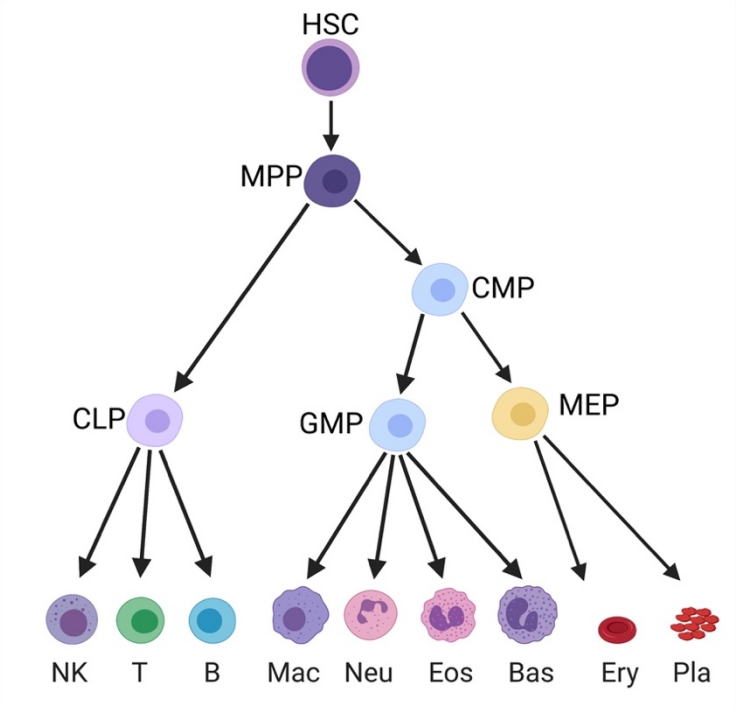

Figure 1. The classical model of hematopoietic hierarchy.

HSC: Hematopoietic stem cell, MPP: Multipotent progenitor, CMP: Common myeloid progenitor, GMP: Granulocyte-macrophage progenitor, MEP: Megakaryocyte-erythroid progenitor, CLP: Common lymphoid progenitor, NK: Natural killer cell, B: B cell, T: T cell, Mac: Macrophage, Neu: Neutrophil, Eos: Eosinophil, Bas: Basophil, Ery: Erythrocyte, Pla: Platelet. Adopted from Jacobsen and Nerlov, 2019.

1.3.2 The Revised Model

The classical hierarchy suggests that the initial bifurcation at MPP level strictly restricts myeloerythropoiesis and lymphopoiesis with intermediate progenitors (CMP bifurcated into MEP and GMP, and CLP). However, prospective isolation of these progenitors followed by transplantation and *in vitro* differentiation assays revealed the heterogeneity within oligopotent progenitors of both branches (Jacobsen and Nerlov, 2019).

As colony forming unit assays exhibited formation of colonies with oligo-, multi-, bi- and unipotent lineage potentials in early myeloid lineage commitment, heterogeneity within CMP fraction was further dissected using cell surface markers. Taking advantage of CD41 and CD9 markers, megakaryocyte progenitors (MkP) were identified (Nakorn et al., 2003). Within GMP, CMP and MEP fractions, heterogeneous endoglin (CD105) and CD150 expressions suggested that these populations can be further subfractionated into four subtypes: Progenitors with erythrocyte potential: colony forming unit-erythroid (CFU-E; Lin^{-}c-$Kit^{+}Sca\text{-}1^{-}CD41^{-}Fc\gamma R^{-}CD150^{-}CD105^{+}$Ter-$119^{-}$) and Pre-CFU-E ($Lin^{-}$c-$Kit^{+}Sca\text{-}1^{-}CD41^{-}Fc\gamma R^{-}CD150^{+}CD105^{+}$); myeloid potential: pre-granulocyte-macrophage progenitor (pre-GM, Lin^{-}c-$Kit^{+}Sca\text{-}1^{-}CD41^{-}Fc\gamma R^{-}CD150^{-}CD105^{-}$); and megakaryocyte and erythrocyte potential: pre-megakaryocyte-erythroid progenitor (pre-MegE; Lin^{-}c-$Kit^{+}Sca\text{-}1^{-}CD41^{-}Fc\gamma R^{-}CD150^{+}CD105^{-}$). Megakaryocyte progenitor population was also immunophenotypically identified as Lin^{-}c-$Kit^{+}Sca\text{-}1^{-}$ $CD150^{+}CD41^{+}$ (Pronk et al., 2007).

In the classical model, the segregation of the lymphoid branch was suggested to occur at the CLP stage, generating B, T and NK cells with no apparent myeloerythroid lineage. This view was first challenged using recombination-activating gene 1 (Rag-1) reporter mouse model proposing the presence of an early lymphoid progenitor (ELP; LSK $CD27^{+}$) present within LSK compartment. Capable of giving rise to all lymphoid lineages, ELP was suggested to give rise to the CLP population with mainly B cell capacity (Igarashi et al., 2002). Further investigations on T cell progeny demonstrated the presence of a circulating early thymic progenitor (Early thymic progenitor, ETP; LSK $CD25^{-}$) that does not derive from a CLP (Allman et al., 2003; Schwarz and Bhandoola, 2004). Moreover, the LSK compartment was shown to contain fraction of cells that can generate both myeloid and lymphoid lineages but are devoid of megakaryocyte and erythrocyte potential, identified as lymphoid-primed multipotent progenitor (LMPP; $LSKFlt\text{-}3^{hi}$) (Adolfsson et al., 2005; Lai and Kondo, 2006; Månsson et al., 2007; Yoshida et al., 2006).

In addition to characterization of the hematopoietic hierarchy downstream of MPPs, the top part of the hierarchy has also been subjected to exploration of additional subtypes. Using H2B reporter mouse model and BrdU label retention approach, MPPs were subfractionated into four distinct populations based on their quiescence and longevity from highest to lowest: MPP1/ST-HSC (LSK $CD48^{-}CD150^{+}Flt\text{-}3^{-}CD34^{+}$); MPP2 (LSK $CD48^{+}CD150^{+}Flt\text{-}3^{-}CD34^{+}$); MPP3 (LSK $CD48^{+}CD150^{-}Flt\text{-}3^{-}CD34^{+}$); MPP4 (LSK $CD48^{+}CD150^{-}Flt\text{-}3^{+}CD34^{+}$) (Wilson et al., 2008). Further investigation of the MPP subsets revealed their distinct lineage biases:

myeloid biased progenitors MPP2 with MegE and MPP3 with myeloid preferences and lymphoid biased progenitor MPP4 (overlapping with LMPP) (Cabezas-Wallscheid et al., 2014; Pietras et al., 2015). Identification of these new progenitor populations has resulted in the proposal of a revised roadmap of hematopoietic hierarchy.

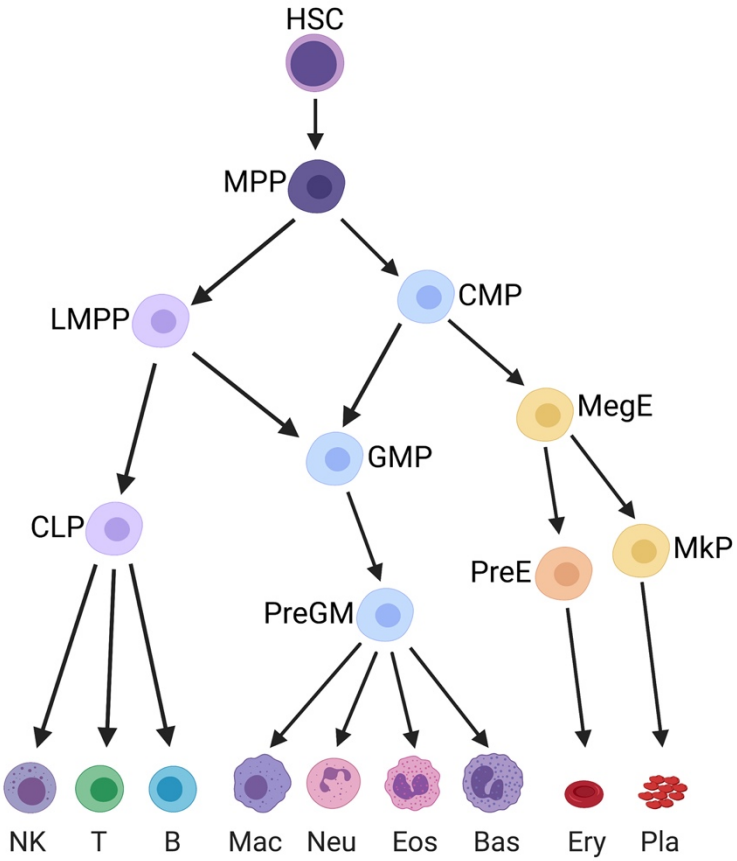

Figure 2. The revised model of hematopoietic hierarchy.

HSC: Hematopoietic stem cell, MPP: Multipotent progenitor, CMP: Common myeloid progenitor, LMPP: Lymphomyeloid-primed progenitor, GMP: Granulocyte-macrophage progenitor, CLP: Common lymphoid progenitor, PreGM: Pre-Granulocyte-monocyte, PreE: Pre-erythrocyte, MkP: Megakaryocyte progenitor, , NK: Natural killer cell, B: B cell, T: T cell, Mac: Macrophage, Neu: Neutrophil, Eos: Eosinophil, Bas: Basophil, Ery: Erythrocyte, Pla: Platelet. Adopted from Jacobsen and Nerlov, 2019.

1.4 HETEROGENEITY WITHIN ADULT HEMATOPOIETIC STEM CELL COMPARTMENT

In the classical view, the HSC compartment resides at the apex of the hematopoietic hierarchy and is composed of homogeneous cells that are all equally capable of giving rise to all mature blood lineages. Advances in single cell lineage tracing and transplantation paved the way to understanding the HSC behavior at a clonal level and revealed the existence of heterogeneity within the HSC pool.

Prospective isolation of the HSC populations at a bulk cell level made it possible to subfractionate the HSC pool based on longevity and self-renewal ability. As the population-level investigations can only demonstrate the averaged output of HSC behavior, it remains important to decipher self-renewal and lineage commitment of HSCs at a single cell level. Pioneered in early 2000, these investigations revealed that HSCs are not functionally homogeneous but rather heterogeneous, differ in their longevity and self-renewal capacity, and have distinct intrinsic lineage preferences (Dykstra et al., 2007; Müller-Sieburg et al., 2002).

1.4.1 Classification of Hematopoietic Stem Cells Based on Longevity

In transplantation experiments, successful replenishment of the blood system after the ablation of endogenous BM cells relies on the transplanted HSPC population. While earlier studies indicate that injected progenitor cells with limited reconstitution abilities can rescue the myeloablated mouse (Siminovitch et al., 1964; Till and McCulloch, 1961), long-term maintenance of the hematopoietic system ultimately depends on the ability of HSCs to sustain HSC number and its differentiation to progenitor cells and to mature blood lineages. Growing number of studies have demonstrated that HSCs differ in their ability to sustain long-term hematopoiesis, underlining that the primitive HSCs are the most durable ones (Eaves, 2015; Ema et al., 2014).

The longevity of HSCs is assessed by their stable myeloid output in the peripheral blood (PB) (Adolfsson et al., 2001; Morrison and Weissman, 1994) due to extremely short half-life of circulating myeloid cells (approximately 1 day) (Athens et al., 1961; Dancey et al., 1976; Doherty et al., 1988). HSCs are classified as LT-HSCs only if they can stably repopulate all the blood lineages (myeloid, B and T cells) for at least 4-6 months post-transplantation and can sustain stable multilineage output in the secondary transplantation (Adolfsson et al., 2001; Dykstra et al., 2006; Ema et al., 2005; Kiel et al., 2005; Morita et al., 2006a; Morrison and Weissman, 1994; Morrison et al., 1997; Osawa et al., 1996). On the other hand, ST-HSCs have multilineage potential that lasts less than 4 months and do not reconstitute in the secondary hosts (Christensen and Weissman, 2001; Yang et al., 2005).

Using the single cell transplantation approach, two studies acknowledged the intermediate-term (IT) HSC even though there is no consensus on its lifespan in neither one of

the investigations. Important to note here is that these studies analyzed two phenotypically different IT-HSC populations that might have led to divergent results (Benveniste et al., 2010; Yamamoto et al., 2013). Benveniste *et al.* demonstrated that IT-HSC engrafts the recipients up to 8 months with stable lymphoid output but had declining erythrocyte and myeloid repopulation patterns that became undetectable after 4 months (Benveniste et al., 2010). On the contrary, Yamamoto *et al.* described IT-HSCs as a population that has stable multilineage reconstitution in primary hosts up to 6 months. When secondary transplanted, the lymphoid lineage was maintained while platelet and erythrocyte lineages declined.

1.4.2 Lineage-Biased Hematopoietic Stem Cells

1.4.2.1 Myeloid and Lymphoid Biased Hematopoietic Stem Cells

Understanding the behavior of a single HSC *in vivo* has been pioneered by the Sieburg group. HSC clones were generated by using long-term culture initiating cell (LTC-IC, limiting dilution) assay, in which unfractionated BM cells are co-cultured with S17 stromal cell line. Subsequently, colonies derived from a single initiating HSC are transplanted into sub-lethally irradiated $W^{41}W^{41}$ mice (Cho and Müller-Sieburg, 2000; Müller-Sieburg et al., 2002). Observations of myeloid- and B-lineage output in primary transplantations revealed the presence of HSC subtypes with distinct lineage dominance defined as lineage-biased (Li-bi) HSCs comprising of 30% of the HSC pool. Lymphoid (L)-to-myeloid (M) ratio analysis of unmanipulated mice in the PB set the base for lineage-balanced (Bala) HSCs with a mean of 6.0±2.0. In transplanted mice, donor-derived L/M ratio demonstrated lineage skewing towards myeloid and lymphoid hence categorized as myeloid- (My-bi) and lymphoid- (Ly-bi) biased HSCs, respectively. To rule out that the lineage skewing is a result of initial co-culture conditions, secondary transplantation was performed. To their surprise, the cells that reconstituted in the secondary and tertiary recipients followed similar lineage dominance patterns as in the primary underlining that 1) biased HSCs have extensive self-renewal ability, 2) lineage skewing is not a result of lineage commitment and 3) lineage-bias is preprogrammed (Müller-Sieburg et al., 2002).

To understand the mechanism of lineage-bias and the lineage-skewing in downstream progenitors, the Sieburg group further investigated the potential of Li-bi HSCs to give rise to donor-derived myeloid and lymphoid precursors *in vitro* co-cultures formed by BM cells isolated from primary recipients. Both Bala and Ly-bi HSCs generated B-cell precursors whereas My-bi HSCs had 3-fold reduction in their capacity as compared to Bala HSCs. In addition, My-bi HSCs had reduced ability to form T-cell precursors. On the other hand, both My- and Bala HSCs could efficiently produce myeloid precursors with lowered generation capacity in Ly-bi HSCs. Even though a reduced number of thymic-progenitor cells were generated from My-bi HSCs, when transplanted, the formed T-cell precursors could efficiently give rise to mature T cells as the control BM. These results indicated that despite lineage skewing being present, Li-bi HSCs can still generate all the lineages, albeit less efficiently.

Mechanistically, the donor derived lymphoid cells from My-bi HSCs as compared to Bala HSCs showed no difference in terms of cytokine production and receptor expression except for interleukin-7 receptor alpha (IL7Rα). Diminished representation of IL7Rα expressing donor-derived cells in the BM and spleen of mice with My-bi HSC also accounted for their lack of response to IL-7 in culture. These results overall suggest that My-bi HSCs are preprogrammed potentially at the epigenetic level for reduced lymphopoiesis (Muller-Sieburg et al., 2004).

A detailed comparison of various transplantation settings in which 97 individual HSCs from *in vitro* and *in vivo* limiting dilution assays and based on purification (Lin^-Rho^-SP cells) were assessed for their kinetics in the PB. This demonstrated that although the approaches are different, there are only a limited number of HSC behaviors observed. These results suggested that HSC behavior is not stochastic but rather predetermined with limited number of reconstitution patterns. Of those 16 patterns detected, Lin^-Rho^-SP HSCs only covered a subset of HSC patterns indicating that phenotypic enrichment is a powerful tool to identify certain HSC subtypes (Sieburg et al., 2006).

Lineage tracing of HSCs at a clonal level gained momentum after the single cell transplantation approach. The Eaves group investigated the behavioral patterns of single HSCs ($CD45^{mid}Lin^-Rho^-SP$) using three different settings in serial transplantations: 1) Isolation of single HSCs from fresh BM followed by transplantation, 2) transplantation of single HSCs expanded in a 4-day culture and 3) transplantation of single HSCs expanded in the 10-day culture. A comprehensive analysis of long-term lineage repopulation categories of individual HSCs was conducted based on myeloid-to-lymphoid (M/L) ratio (calculated by GM:(B+T); G: Granulocyte, M: Myeloid, B: B cell, T: T cell) and four types of long-term cells (≥4 months, α, β, γ, δ) were identified. Of note, the freshly purified HSCs and 4-day expanded HSCs had similar frequencies of α, β, γ and δ cells *in vivo*, but 10-day cultured HSCs demonstrated only β and γ patterns and lost their durability suggesting that in vitro conditions might cause a shift or exhaustion in the lineage output of HSCs. Among the patterns observed, only α and β cells were highly durable throughout the serial transplantation, while γ and δ cells did not reconstitute in the secondary recipients even though >1% myeloid repopulation was sustained in primary recipients with γ cells (but not in recipients with δ cells) (Dykstra et al., 2007).

The HSC is classically defined with its multipotency (ability to give rise to all mature blood lineages) and extensive self-renewal potential (ability to generate itself, secondary transplantable). β cell followed the criteria of HSC definition, having multilineage capacity and sustained high level of reconstitution. Interestingly, the α cell gave rise to mostly myeloid cells with low level of reconstitution in primary transplantation, but it could extensively repopulate in the secondary and tertiary mice showing its latent potential for hematopoiesis and quiescence. While 50% of the α and β cells sustained the lineage preferences throughout serial transplantations, the other half changed their pattern. Categorically, α cell could give rise to β or occasionally to γ cells and β cell could generate γ and/or δ cells revealing the hierarchy

among the cell types. (Dykstra et al., 2007). According to the lineage bias categorization, α cell can be classified as My-bi, β cell as Bala, γ/δ cells as Ly-bi HSCs.

It is important to note that the HSC categorization in both Sieburg (M-bi, Bala and Ly-bi HSCs) and Eaves (α, β, γ/δ cells) groups was based on similar approaches but consisted of different calculations. Sieburg group's analysis considered only donor-derived WBCs in calculation, whereas recipient cells were also taken into account by the Eaves group. Direct comparison of both studies using Morita et al. data (Morita et al., 2010) illustrated that both approaches classify similar HSC subsets albeit the categorization of the Eaves group was more restrictive (Ema et al., 2014).

1.4.2.2 Markers to Identify Myeloid and Lymphoid Biased Hematopoietic Stem Cells

Phenotypic enrichment of LT-HSC paved the way to investigate various markers for better purification of Li-bi HSC subsets. One of those markers is CD150 in which $CD150^{+}$ is known to enrich for LT-HSCs. On the other hand, $CD150^{-}$ fraction as it is suggested to have limited self-renewal ability. In addition, My-bi HSCs are present within the $CD150^{+}$ and Ly-bi HSCs are present within the $CD150^{-}$ fraction (Kent et al., 2009; Kiel et al., 2005). Nakauchi's research group identified Li-bi HSCs within the CD150 fraction of $LSKCD34^{-}$ cells by performing single cell transplantation. Enrichment of Li-bi HSCs is represented as follows: My-bi HSCs in $CD150^{hi}$, Bala HSCs in $CD150^{int}$ and Ly-bi HSCs in $CD150^{-}$ fractions from highest to lowest self-renewal abilities. Albeit at a lower level, $CD150^{int}$ and $CD150^{-}$ subsets could reconstitute secondary transplanted mice in which lymphoid dominant/restricted output progressively increased, respectively. Within the $CD150^{hi}$ fraction, HSCs with low level of myeloid-restricted repopulation as well as latent HSCs that engrafted ≥3 months post-transplantation were detected (Morita et al., 2010).

Enrichment of Li-bi HSCs is also shown within the SP^{LSK} population (stained with Hoechst 33342). When transplanted both bulk and at a clonal level, the lower SP^{LSK} demonstrated myeloid bias whereas the upper SP^{LSK} was lymphoid biased in both PB and BM progenitors. Their lineage preference was enhanced when both populations were competitively co-transplanted suggesting that it is possible to enrich for My-bi HSCs within the lower SP^{LSK} and Ly-bi HSCs within the upper SP^{LSK} where both populations in a mixed environment would prefer to give rise to lineages that they are intrinsically inclined to. BrdU labeling and Ki-67 staining indicated that My-bi HSCs are more quiescent whereas Ly-bi HSCs are more proliferative. Assessment of the overlap between the SP populations and CD150 cell surface marker in single cell transplantations revealed that CD150 does not necessarily add onto resolving the heterogeneity when SP is used (Challen et al., 2010). Although SP is a very good marker to isolate for LT-HSC and has been extensively used, Hoechst staining is subjected to several factors such as overstaining and consequential shifting of SP cells to non-SP population that make the staining somewhat unreliable (Morita et al., 2006b). Additional markers such as CD41 (Gekas and Graf, 2013), CD86 (Shimazu et al., 2012) and CD229 (Oguro et al., 2013)

also enrich for Li-bi HSCs within LSKCD48^{-}CD150^{+} HSCs: CD41^{+}, CD229^{-}, and CD86^{-} for My-bi and CD41^{-}, CD229^{+}, and CD86^{+} for Ly-bi HSCs.

1.4.2.3 Platelet Biased Hematopoietic Stem Cells

Aforementioned studies explored the lineage bias based on the detection of donor- and recipient-derived cells using CD45 (Ly5) congenic system. Since only leukocytes express CD45 but not platelets and erythrocytes, the investigations lacked the contribution of these two lineages. The generation of reporter mouse models expressing fluorescent proteins either as pan- or under the genes targeting platelet and erythroid lineages made it possible to investigate the presence of additional Li-bi HSCs and their hierarchical order.

Investigation based on the *Kusabira-Orange* (KuO) mouse model in which KuO protein expression is detected in all the blood lineages including platelet and erythrocytes (Hamanaka et al., 2013) revealed the presence of long-term repopulating cells with platelet unilineage output within LSKCD34^{-}CD150^{+}, in addition to the unilineage myeloid lineage previously detected. Analysis at a clonal level revealed that these cells have limited capacity for self-renewal in secondary transplantations and have therefore been suggested to rather be megakaryocyte repopulating progenitors (MkRP) differentiated from LT-HSCs. This observation was based on paired daughter cell assay, where single HSC is allowed to divide in the culture once and each divided cell is transplanted separately (Yamamoto et al., 2013).

Von Willebrand factor (Vwf) is one of the platelet specific proteins involved in hemostasis. It is found in platelets and endothelial cells (Denis et al., 1998) and in a fraction of phenotypic HSCs (Kent et al., 2009; Månsson et al., 2007). Jacobsen & Nerlov groups generated a *Vwf*-eGFP reporter mouse model in which *Vwf* expression was restricted to endothelial cells, platelets, and megakaryocyte progenitors. Expression of *Vwf* subfractionated phenotypically defined LT-HSCs (LSKCD34^{-}CD48^{-}CD150^{+}) into two fractions as vWF-eGFP^{-} (~40%) and vWF-eGFP^{+} (~60%). Through serial transplantations with limited number of cells (10, 5 and 1), vWF^{+} HSCs showed stable long-term reconstitution with platelet- or platelet/myeloid-bias whereas vWF^{-} HSCs demonstrated lymphoid bias. Both fractions sustained their lineage bias in secondary transplantation and while vWF^{+} HSCs could generate both vWF^{-} and vWF^{+} HSCs, vWF^{-} HSCs could only give rise to vWF^{-} HSCs suggesting a hierarchical order (Sanjuan-Pla et al., 2013).

Further investigation by crossing the *Vwf-tdTomato* strain with a *Gata-1 eGFP* mouse model allowed the tracking of both donor-derived platelets and erythrocytes in serial transplantations of single vWF^{-} and vWF^{+} HSCs. Unravelling the presence of platelet-restricted (P-rest) HSCs, in addition to the platelet-biased (P-bi) HSCs identified earlier, the P-rest HSCs preferentially gave rise to and sustained only the platelet lineage and its progenitors *in vivo* but could give rise to all other lineages *in vitro* (Carrelha et al., 2018). Important to note is that P-bi HSCs identified in Carrelha *et. al* (Carrelha et al., 2018) were LT-HSCs that repopulated the platelet lineage stably as compared to MkRP shown to be derived from LT-

HSCs in Yamamoto *et al.* (Yamamoto et al., 2013) as in the latter research where vWF was not used to distinguish the HSC fractions.

1.5 HEMATOPOIESIS THROUGH ONTOGENY

1.5.1 Embryonic Hematopoiesis

Throughout embryonic development two waves of hematopoiesis occur: primitive and definitive hematopoiesis. The first wave of hematopoiesis is primitive, ensuring the survival of the mammalian embryo via embryonic oxygenation. The second wave, also known as definitive hematopoiesis, originates and maintains the adult hematopoietic system throughout life (Mikkola and Orkin, 2006).

Primitive hematopoiesis is initiated post-gastrulation with the migration of a committed mesodermal precursor cell to the yolk sac (YS). Transient nucleated erythrocytes are the first primitive cells to emerge in the YS at embryonic day (E) 7-7.5 (Silver and Palis, 1997) in mice. The fusion of allantois with the chorion to form the placenta establishes the circulation at E7.5-E8.25. Following that at E9, when the embryo is enveloped in the YS connected with vitelline and umbilical vessels to the placenta, erythromyeloid progenitors begin to colonize in the fetal liver (FL) (Medvinsky et al., 2011).

The definitive hematopoiesis is a process where all the mature blood lineages (erythrocytes, myeloid cells, and lymphocytes) are generated and sustained throughout life by definitive HSCs (dHSCs). It starts with detectable dHSCs in vitelline and umbilical vessels and the emergence of dHSCs in aorta-gonad-mesonephros (AGM) region of the embryo at E10.5, and thereafter dHSCs are also present in the placenta, YS, and FL. Thymus is colonized at E11, followed by the fetal spleen (FS) at E12.5 and finally the fetal BM (FBM) at E15 (Dzierzak and Speck, 2008; Gekas et al., 2005). Seeded by the circulating hematopoietic cells that originate from AGM, FS initiates macrophage differentiation (Bertrand et al., 2006). Importantly, FL remains as the main hematopoietic site for definitive hematopoiesis until birth (Bertrand et al., 2005).

1.5.2 Adult Hematopoiesis

The hematopoietic system undergoes a massive shift within four weeks after birth in mice (neonatal stage). The transition of fetal-like HSCs to adult-like HSCs occurs during this period. During the first three weeks of postpartum, fetal HSCs remain highly proliferative, expanding the hematopoietic system enormously. At the last week of neonatal stage (between 3 to 4 weeks postpartum) cycling fetal HSCs transition into quiescent adult-like HSCs (Beaudin et al., 2016; Bowie et al., 2006). Although earlier studies suggested an abrupt switch from fetal to adult HSC (Bowie et al., 2006, 2007), later studies indicated that this transition is gradual and already starts prenatally at the transcriptional and epigenetic levels (Li and Magee, 2021; Li et al.,

2020). From then onwards, adult hematopoiesis sustains the hematopoietic system throughout the adult lifetime of mammals.

1.5.3 Aging Hematopoiesis

Cellular aging is a dynamic biological process occurring over time due to the accumulation of impairments and alterations at the molecular and cellular levels resulting in functional deterioration of the cells. Hallmarks of an aging hematopoietic system are reduced innate and adaptive immune responses, declined regenerative ability upon stress, increased frequencies of myeloid-associated malignancies, and increased susceptibility to autoimmune diseases (Guidi and Geiger, 2017). As hematopoiesis is maintained by HSCs throughout life, aging-related modifications of the hematopoietic system is suggested to be conveyed by the aged HSCs which are regulated by extrinsic and intrinsic factors. Overall, old HSCs have reduced self-renewal capacity, responsiveness to environmental cues, engraftment capacity and homing ability (Dykstra et al., 2011; Kovtonyuk et al., 2016; Mejia-Ramirez and Florian, 2020).

Aged mice demonstrate an increase in the size of the phenotypic HSC pool and myeloid skewing with reduced capacity of lymphopoiesis (Dykstra et al., 2011; Rossi et al., 2005; Sudo et al., 2000). The recognition of Li-bi HSCs has shed light on the change in blood composition during aging, indicating that the frequency and the number of My-bi HSCs (Beerman et al., 2010; Challen et al., 2010; Dykstra et al., 2011) as well as Pl-bi HSCs (Grover et al., 2016) expand in old mice with a decrease in Ly-bi HSC frequency, hence contributing to myeloid skewing. Based on this, the Sieburg group suggested the clonal diversity model in which alterations during aging occur on the size and composition of HSC population rather than in an individual cell (Muller-Sieburg and Sieburg, 2006). Importantly, as the contribution of an individual HSC to aging phenotype of hematopoiesis cannot be excluded, both scenarios are likely to occur.

The aging is regulated both intrinsically and extrinsically. In the BM niche, it is suggested that the endosteal niche aids quiescence and self-renewal whereas the vascular niche creates an environment that promotes differentiation, proliferation, and migration of HSCs. Throughout aging, the amount and type of cellular components, cytokines and growth factors released from the nearby niche and immune cells also change. Exposure to these “aging signals” alters HSC properties leading to an increase in the frequency of the HSC pool and impairments in HSC function (Matteini et al., 2021; Mercier et al., 2012; Wagner et al., 2008). Additionally, cell intrinsic regulation plays a major role in the behavioral output of aged HSCs. Transplantation of aged and young adult (YA) HSCs into myeloablated YA mice demonstrated that as compared to YA HSCs, the old HSCs engraft poorly even in YA environment, suggesting the rejuvenation of old HSCs in YA environment is not possible and therefore aging related HSC functions are regulated intrinsically (Dykstra et al., 2011). Overall, understanding the mechanism of aging is an important part of HSC research with implications of new and/or better treatment options to hematological malignancies such as myeloid-related leukemia and myelodysplastic syndromes which are often seen in elderly (Rossi et al., 2008).

1.6 B CELL LYMPHOPOIESIS IN ADULT HEMATOPOIEISIS

B lymphocytes constitute an integral part of the adaptive immune system, playing a role in antibody production, secondary immune responses, and immunological memory (LeBien and Tedder, 2008). The recognition of B cells started in the late 1940s with the discovery of gammaglobulin protein in blood serum as the origin of the antibodies and that plasma cells are responsible for producing them (Fagraeus, 1947; Good and Zak, 1956). Following investigations on antibody production (Burnet, 1976; Jerne, 1955; Nossal and Lederberg, 1958) in the late 1960s, the discovery and delineation of B cells together with T cells took place in both pre-clinical and clinical studies (Coombs et al., 1969; Fröland et al., 1971; Preud'homme and Seligmann, 1972). From then onwards, different subtypes of B cells, their ontogeny, functions, and molecular programming have been extensively characterized (Laidlaw and Cyster, 2021; LeBien and Tedder, 2008; Nutt and Kee, 2007).

1.6.1 Becoming B Cells in the Bone Marrow: Early B Cell Fate

Terminally differentiated B cells are generated from the progenitors of the lymphoid branch in the hematopoietic hierarchy. The first hematopoietic lineage restriction occurs after MPP, when the MPPs give rise to LMPP expressing genes specific to both myeloid (*Csf1r*) and lymphoid (*Dntt, Rag1, Rag2,* sterile immunoglobulin transcripts) lineages and are devoid of megakaryocyte and erythrocyte differentiation potential (Adolfsson et al., 2001, 2005; Lai and Kondo, 2006; Månsson et al., 2007). LMPPs generate CLPs which can be subfractionated using lymphocyte antigen 6 family member D (LY6D) into $LY6D^-$ CLP (All lymphocyte progenitor, ALP) and $LY6D^+$ CLP (B cell biased lymphocyte progenitor; BLP) subpopulations. ALP holds the capacity to give rise to all lymphoid lineages (B, T, plasmocytoid DC, and NK cells). Conversely, BLP loses its potential to give rise to NK cells and have a drastically reduced capacity to differentiate into T cells; only retaining B-cell lineage commitment (Inlay et al., 2009; Mansson et al., 2010)

The early stage of B cell development is regulated by distinct transcription factors (TFs) in the bone marrow. The three pillars of B cell identity and function are Pax5, EBF, and E2A TFs, driving the differentiation from BLP to pro-B stage. B cell identity is determined by the transcriptional activation of EBF1 by E2A preceded by PAX5, therefore creating a EBF1-PAX5 positive feedback loop which creates exclusive B lineage commitment (Cobaleda et al., 2007; Nutt and Kee, 2007). Mediated by Rag1/2 recombinase proteins, BLP cells undergo two-step V(D)J recombination in the *Ig heavy chain* (*IgH*) gene. The first recombination (D_H-J_H) upregulates CD19 expression and generates pro-B cells, which is then followed by the second rearrangement (V_H-D_HJ_H) that allows the formation of the $CD25^+$ pre-B cells (Fuxa et al., 2004; Rolink et al., 1994; Rumfelt et al., 2006). In early pre-B stage (large Pre-B stage, extensive cycling), *Vpre-B and λ5 surrogate light chain* genes are rearranged allowing the formation of pre-B cell receptor (pre-BCR). This is followed by either *Igκ* or *Igλ light chain* (*IgL*) gene expressions forming mature BCR in late pre-B stage (small Pre-B stage, resting), leading to generation of immature B cells (immB) that express IgM on the cell surface (Akkaya and

Pierce, 2019; Hardy and Hayakawa, 2001; Hardy et al., 1991, 2007; Müschen et al., 2002; Poulaki and Giannouli, 2021; Rothenberg, 2014). Migrating from BM to the periphery, immB cells terminally differentiate into marginal zone (MZ) B cells (Cerutti et al., 2013) and follicular B cells (FoB) in spleen and lymph nodes (Pillai and Cariappa, 2009).

1.6.2 Getting Acquainted with The Targets: Terminal B Cell Differentiation

B cell commitment and maturation are highly regulated processes occurring in a sequential manner throughout B cell development. Before egressing from the BM, pre-B cells go through several checkpoints where only the cells that have low affinity to self-antigens are allowed to further differentiate. This is essential for two reasons: 1) to avoid formation of autoreactive B cells that target self-antigens and 2) to allow the cells to recognize auto-antigens. Once this is successfully completed, pre-B cells generate immB cells that leave the BM and egress into the periphery as transitional B cells (trB) (Almqvist and Mårtensson, 2012; Mårtensson et al., 2010).

Transitional B cells relocate to secondary lymphoid organs (spleen and lymph nodes) where they differentiate into FoB and MZ B cells. Once encountering an immune response, FoB cells migrate to germinal centers (GC) and terminally develop into either long-lived plasma cells that produce antibodies or long-lived memory B cells that recognize the re-encountered pathogen from the initial infection and triggers a secondary immune response. While FoB cells generate long lived cells which initiates high-affinity secondary humoral response, MZ B cells generate short lived plasmablasts with low-affinity response (Laidlaw and Cyster, 2021; LeBien and Tedder, 2008; Palm and Henry, 2019; Pillai and Cariappa, 2009).

1.6.3 Mechanistic Insight: Transcriptional Priming of Early B Cell Lineage

Early B cell fate is transcriptionally highly controlled in each stage from lineage priming to activation of lineage specific genes that forms the B cell identity. Starting at the LMPP stage, upregulation of Flt-3 in LMPPs results in a diminished potential of the megakaryocyte and erythrocyte lineages to retain their myeloid and lymphoid capacity (Månsson et al., 2007). In LMPPs and its downstream progenitors, the decision making of early B cell fate is dependent on the inactivation of myeloid- and upregulation of B cell specific gene programs which are mediated and regulated by TFs. Some of those TFs that play important role in B cell priming and commitment are Ikaros, PU.1, Pax5, EBF1, FoxO1, and E2A proteins.

1.6.3.1 Ikaros

Ikaros (encoded by *Ikzf1*) zinc finger protein is a TF that binds to lymphocyte specific *Dntt* and *Cd3d* genes (Georgopoulos et al., 1992; Lo et al., 1991) and is therefore responsible for both B and T lineage priming. Ikaros plays a crucial role in early lymphopoiesis, possibly at the ALP stage and/or earlier in the hematopoiesis, since Ikaros null mice (*Ikzf1*$^{-/-}$) show complete loss of B and NK cells and defects in T cell subsets (Allman et al., 2003; Wang et al., 1996). It was shown later that the LMPP population is missing due to lack of Flt-3 expression in Ikaros null

mice (Yoshida et al., 2006). Using a mouse model with conditional inactivation of Ikaros in pro-B cells (mb1-Cre x Ikzf1$^{fl/fl}$), Schwickert et al. revealed that B cell development is blocked at the pro-B stage the impaired pre-BCR upon activation (Schwickert et al., 2014). Lastly, *Ikzf1*$^{-/-}$ pro-B cells were not restricted to the B lineage, but also could commit to the myeloid lineage, underlining that Ikaros is essential for successful repression of opposing myeloid genes (Reynaud et al., 2008; Sellars et al., 2011).

1.6.3.2 PU.1

Transcription factor PU.1 (Spi-1 proto-oncogene, encoded by *Spi1*), is one of the early determinants of myeloid-lymphoid lineage fate during hematopoiesis. High PU.1 expression marks myeloid lineage priming while its gradual decrease favors B cell differentiation. The regulatory network of lymphoid priming is suggested to occur as followed: Ikaros promotes Gfi1 (Growth factor independent 1, encoded by *Gfi1*) transcriptional repressor which then dislodges PU.1 from the transcriptional regulatory circuit and therefore hindering myeloid lineage priming at the MPP stage (Spooner et al., 2009). Further analyses of PU.1 function in lymphoid progenitors was performed by using a PU.1-deficient mouse models (Spi1^{gfp}, Spi1/Mx-Cre) that detect LMPP, and CLP populations (crossed with Rag1/Cre) indicated that PU.1 is essential for Flt-3 expression in LMPP and CLP but is dispensable at the pro-B cell stage onwards (Pang et al., 2018).

1.6.3.3 E2A

E2A (Transcription factor 3, TCF3, encoded *Tcf3*) is one of the E-protein (E-box-binding TF) family members along with HeLa E-box binding protein (HEB) and E2-2. Through differential splicing, the *Tcf3* gene encodes E12 and E47 proteins which makes up the E2A protein. E2A is vital for many stages of hematopoiesis including B and T lymphopoiesis (Engel and Murre, 2001). In E2A-deficient HSCs, downregulation of self-renewal genes (p21, P27 and Gfi-1) initiated the cell cycle and therefore increased the proliferation (Semerad et al., 2009). E2A protein restricts the proliferation not only in HSCs but also in MPP and LMPPs and is therefore essential for HSPC maintenance (Dias et al., 2008). In the early B-lineage fate, E2A-deficient mice show complete absence of BLPs with only T cell differentiation potential in ALP subset indicating its essential role in ALP-to-BLP transition during B cell commitment (Inlay et al., 2009). Further analysis indicated that HEB also plays a concerted role in this transition with E2A, shown by using a E2A$^{f/+}$ and HEB-deficient mouse model with BLP arrest (Welinder et al., 2011). In addition to initiating the early B-lineage commitment by inducing EBF1 transcription and promoting transcriptional cascade with EBF1, PAX5 and FOXO1, E2A proteins together with EBF1 are found to be important for *IgH* and *Igκ* rearrangements in pro- and pre-B cells (Greenbaum et al., 2004; Lin and Grosschedl, 1995; Singh et al., 2005).

1.6.3.4 FOXO Family

Forkhead box O (FOXO) TFs are a subfamily of the Forkhead family of proteins (19 subsets) identified by conserved DNA-binding motif called “forkhead box (FOX)”. The FOXO subfamily is composed of four members which are FOXO1, FOXO3, FOXO4, and FOXO6.

Members of the FOXO subfamily are important for the regulation of the phosphotidlyinositol-3-kinase/AKT (PI3K/AKT) pathway and are therefore involved in vital cellular processes such as survival, growth, differentiation, cell cycle arrest and cell death (Greer and Brunet, 2005). Among the four members, *FoxO1*, *FoxO3*, *FoxO4* are expressed in the hematopoietic system. Deletion of all three (*Foxo1/3/4*$^{F/F}$*Mx1-Cre*) from the hematopoietic system resulted in a drastic decrease in the number of phenotypic LT-HSCs (LSKFlt-3^{-}CD34^{-}) and increase in cell cycle, proliferation, and apoptosis. In addition, FOXO1,3,4-deficient BM cells lost their LT repopulation ability (Tothova et al., 2007) similar to Foxo3$^{-/-}$ HSCs in serial transplantations (LSKCD34^{-}) (Miyamoto et al., 2007). Therefore, FOXO3 is important for HSC maintenance, quiescence, and self-renewal.

Early B cell development relies on FOXO1 TF and its interaction with other TFs. FOXO1-deficient mice (*Foxo1*$^{f/f}$*Vav1*^{iCre}, herein *Foxo1*$^{-/-}$) demonstrated a block at the BLP stage accompanied with a complete developmental arrest in the pro-B stage. The lack of *Ebf1* expression in *Foxo1*$^{-/-}$ mice indicated that transitioning from BLP to the pro-B stage requires synergistic collaboration of FOXO1 and EBF1 (Mansson et al., 2012). Further studies using conditional deletion of FOXO1 (*Foxo1*$^{f/f}$*mb1*^{Cre} for pro-B and *Foxo1*$^{f/f}$*CD19Cre* for pre-B cells) downstream of BLP have revealed that FOXO1 is vital for 1) activating gene expressions of *Il7-Rα* in pro-B cells, and *Rag1* and *Rag2* in pre-B cells, 2) regulation of *κ- light chain* gene rearrangement in pre–B cells. FOXO1-deficient pre-B cells also lacked pre-BCR components λ5 and VpreB1 underlining that in the absence of FOXO1 TF, pre-BCR cannot be formed. These functions could not be compensated in the presence of FOXO3 exhibiting that FOXO1 TF is indispensable for B-lineage commitment at the pre-B stage (Dengler et al., 2008).

The impact of FOXO3 in B cell development is less nuanced. While *Foxo3*$^{-/-}$ mice show reduced number of the pre-B cells and circulating B cells in the periphery and the BM, FOXO3 deficiency does not affect splenic B cells (Hinman et al., 2009) as FOXO1 absence does (Dengler et al., 2008), suggesting that FOXO1 and FOXO3 regulate their targets in early B-lineage commitment via discrete mechanisms.

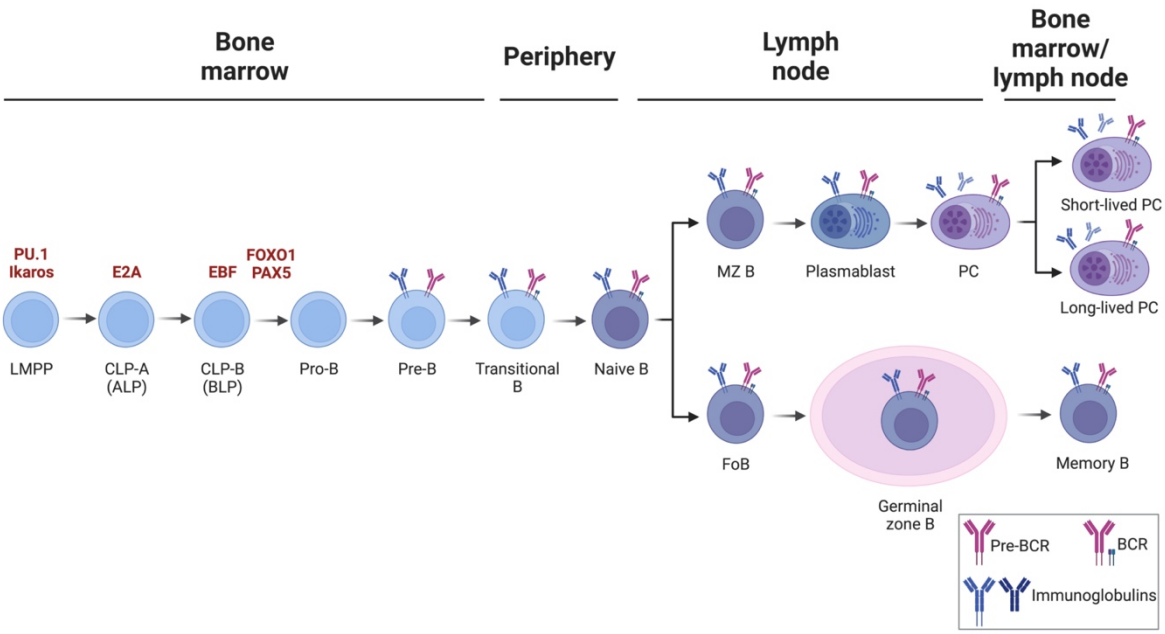

Figure 3. B cell commitment and terminal differentiation in different niches.

LMPP: Lymphoid primed multipotent progenitor, ALP: All lymphocyte progenitor, BLP: B cell biased lymphocyte progenitor, FoB: Follicular B cell, MZ: Marginal zone B cell, PC: Plasmacytoid cell, BCR: B cell receptor.

2 RESEARCH AIMS

The overall aim of the thesis is to understand and resolve the heterogeneity of the hematopoietic stem cells by functional and molecular characterization during aging.

Paper I: To investigate the functional and molecular differences of distinct HSC subsets subfractionated with CD49b.

Paper II: To dissect the functional and molecular differences of CD49b HSC subsets during aging.

Paper III: To study the functional differences between distinct HSC subsets subfractionated with CD229 and CD49b.

Paper IV: To understand the roles of FOXO transcription factors in B cell development.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Recent developments in the field of hematopoiesis revealed the presence of lineage-biased HSCs starting in early 2000s. Since then, there has been some improvements in identifying cell surface markers to enrich the HSC subtypes, but their behavioral and molecular differences remain incompletely understood. Therefore, we investigated a cell surface marker, CD49b, and explored the functional and molecular aspects of the subsets.

3.1.1 Results

In this study, we further subfractionated the previously identified and most quiescent subset of HSCs, that was enriched for My-bi HSCs (LSKCD48$^-$CD34$^-$CD150^{hi}, henceforth CD150^{hi}) (Morita et al., 2010), by using cell surface markers to investigate whether additional markers could help resolve the heterogeneity within this subset. Among the several markers tested, which have shown to be important for isolation of HSPC fraction (CD41, CD224, Flt-3, Tie-2, CD201, CD61, Esam, CD86, CD9, CD229 and CD49b), only CD41, CD229 and CD49b within CD150^{hi} exhibited phenotypic population that has bimodal distribution as negative and positive fractions (Paper I, Fig. S1A). Of these three markers, CD49b have inconsistently been shown to be a marker for ST-HSCs (Wagers and Weissman, 2006), IT-HSCs (Benveniste et al., 2010) and primed HSCs with LT reconstitution ability (Zhao et al., 2019). Hence there has not been a consensus on what type of HSPCs CD49b marks for. As our phenotypic analyses demonstrated that CD150 was heterogeneously expressed within CD49b fractions, the absence of the CD150 marker in the previous studies (Benveniste et al., 2010; Wagers and Weissman, 2006; Zhao et al., 2019) might have led to divergent results (Paper I, Fig. S1B-S1C). Moreover, as the combinations of three markers CD41, CD229 and CD49b within CD150^{hi} HSCs did not demonstrate any difference in cell cycle and *in vivo* cell proliferation analyses indicating that CD49b can be sufficient for resolving the heterogeneity; we decided to use CD49b as a candidate marker (Paper I, Fig. S1D-S1H).

Assessment of *in vitro* differentiation potential of LSKCD34$^-$CD48$^-$CD150^{hi}CD49b$^-$ (henceforth CD49b$^-$) and LSKCD34$^-$CD48$^-$CD150^{hi}CD49b$^+$ (henceforth CD49b$^+$) showed that both subsets could give rise to mainly myeloid cells but also B cells (Paper I, Fig. 1A-1B and S1I) and were also capable of efficiently producing megakaryocytes and erythrocytes (Paper I, Fig. 1C-1D) suggesting that both CD49b$^-$ and CD49b$^+$ cells can generate lymphoid and myeloid lineages *in vitro*. Although not significant, a higher total cloning frequency observed in the CD49b$^+$ subset (Paper I, Fig. 1B), prompted us to analyze cell cycle and proliferation status of the subsets. The CD49b$^-$ subset was found to be significantly more quiescent and less proliferative as compared to the CD49b$^+$ subset (Paper I, Fig. 2A-2D). In agreement with this, tracking the cell division kinetics of single CD49b$^-$ and CD49b$^+$ cells

revealed higher division tendency of CD49b$^+$ cells by day 2 as compared to CD49b$^-$ cells (Paper I, Fig. 2E), overall suggesting that the CD49b$^+$ subset is more proliferative.

Since ultimately the functional behavior of HSCs can only be evaluated *in vivo*, we performed competitive transplantations, both at bulk and single cell level, using a *Gata-1* eGFP mouse model as a donor strain (Drissen et al., 2016) to allow for the detection of platelets and erythrocytes with eGFP in addition to leukocytes (B, T, NK, and myeloid cells) (Paper I, Fig. S2A-S2B). Five donor cells from CD49b$^-$, CD49b$^+$, CD150^{int} and CD150$^-$ subsets were transplanted and followed for up to 6 months. While all the subsets reconstituted all lineages stably during this period, they differed in their potential to give rise to specific lineages and phenotypic HSC output at the end point (Paper I, Fig. 2A-2C). Specifically, CD150$^-$ highly generated B and T cells and showed no phenotypic HSC repopulation, in agreement with the previous studies (Morita et al., 2010). On the other hand, CD49b$^-$ and CD49b$^+$ subsets had similarly high level of lineage repopulation and phenotypic HSC reconstitution compared to CD150^{int} and CD150$^-$ (Paper I, Fig. 2B-2C). Additionally, CD49b$^-$ and CD49b$^+$ HSCs could also give rise to all mature lineages and progenitors in the BM (Paper I, Fig. S2C-S2F), overall indicating that CD49b$^-$ and CD49b$^+$ cells are the most durable HSC subsets.

The caveat of using bulk cell transplantations is the averaged output from multiple cells transplanted, hence causing limitations to assess the lineage bias and behavioral differences. Therefore, we transplanted a total of 139 mice, each with a single CD49b$^-$ or CD49b$^+$ HSC and followed them up to 6 months. Both fractions gave rise to stable repopulation and all lineages in accordance with five-cell transplantations (Paper I, Fig. 3A-3B). Based on the lineage pattern categorization in the PB according to the level of myeloid and lymphoid lineages observed, five classifications were detected. The CD49b$^-$ subset was enriched for M>L and M-L whereas CD49b$^+$ subset had more of L>M and ST/transient output indicating the differences in lineage preferences of the subsets. Importantly, CD49b$^-$ fraction also had mice with a P-E only pattern indicating the presence of Pl-bi HSCs within this fraction (Paper I, Fig. S4A-S4B).

The calculation of lineage bias within donor derived leukocytes (M, B, T and NK cells) was performed as previously described (Müller-Sieburg et al., 2002) and this demonstrated that the CD49b$^-$ fraction is mainly My-bi (78%) while the CD49b$^+$ fraction is Ly-bi (46%) (Paper I, Fig. 4C-4E and S4C) and that CD49b$^-$ HSCs can generate higher frequency of myeloid cells in the BM (Paper I, Fig. S4D). The lymphoid bias is mainly associated with ST-repopulating ability and limited self-renewal potential (Dykstra et al., 2007; Morita et al., 2010), therefore we assessed the durability of single transplanted CD49b subsets. In line with the patterns observed earlier in the PB (Paper I, Fig. S4A-S4B), 39% of CD49b$^+$ cells showed ST-activity. Of the 61% LT-activity detected, 39% was Ly-bi suggesting the presence of LT- and Ly-bi HSC within CD49b$^+$ subset. The CD49b$^-$ subset had mainly LT-activity of which 82% was My-bi (Paper I, Fig. 5A-5B and 5F). To test if LT-activity is sustained we followed the mice for a longer term (up to 9 months) which demonstrated no decline in lineage potential, therefore suggesting a maintained high self-renewal ability in both subsets (Paper I, Fig. 5D-5E). In addition, both populations generated BM HSC and progenitors albeit less efficiently in CD49b$^+$

subset (Paper I, Fig. 5C and S4E-S4F). Collectively, these data indicates that CD49b^{-} is more durable than CD49b^{+} cells.

The ultimate assessment of stemness and self-renewal potential of the HSCs rely on the secondary transplantations. Therefore, single CD49b^{-} and CD49b^{+} cells reconstituted in primary transplanted mice were secondary transplanted both as unenriched BM ($10x10^{6}$) and limited number (30-100) of donor-derived phenotypic HSCs. While both subsets were multilineage reconstituted in the PB of secondary mice and sustained their lineage biases, the level and efficiency to engraft mature lineages and phenotypic HSCs were robust in the CD49b^{-} fraction and lower in the CD49b^{+} subset. The correlation of myeloid bias to high self-renewal potential and lymphoid biasness to reduced durability was sustained in our experiments albeit we also show here the rare presence of LT-HSCs with Ly-bi within the CD49b^{+} subset (Paper I, Fig. 5F-5G, S5A-S5E). The hierarchical relationship between the subsets demonstrated that whereas both subsets repopulated phenotypic HSC subsets in secondary transplanted mice, CD49b^{+} was less efficient to do so (Paper I, Fig. 5H). All in all, this suggests that both subsets are durable and maintain lineage bias, but they differ in self-renewal potential.

As the functional data showed differences in the lineage bias and durability of CD49b subsets, we investigated the transcriptomic and epigenetic landscape of the CD49b cells. Principal component analysis (PCA) of bulk and uniform manifold approximation and projection (UMAP) analysis of single RNA-seq data indicated no transcriptional differences among CD150^{+} fraction. The limited number of genes that were upregulated in CD49b^{-} are related to HSC quiescence such as *Dlk1* and *Gfi1* (Paper I, Fig. 6A-6C, S6A-S6D and S7A). Analysis of open chromatin regions using ATAC-seq revealed that although the CD49b^{-} and CD49b^{+} populations clustered closely, they formed distinct clusters indicating epigenetic differences between the subsets (Paper I, Fig. 6D). Important to note here is that chromatin accessibility of CD49b^{-} subset was different than other CD49b^{+}, CD150^{int} and CD150^{-} subsets and CD49b^{+} subset and downstream progenitors had similar profiles. This suggested a hierarchical order in which the CD49b^{-} population is in the upstream of the other subsets. (Paper I, Fig. 7A-7C). The genes that were found differentially expressed in RNA-seq data also showed up as more accessible regions in CD49b^{-} subset such as *Dlk1* and *Gfi1* (Paper I, Fig. 6B and S7B). In line with higher proliferation and cell cycle status of CD49b^{+} cells, gene ontology (GO) analysis of ATAC-seq demonstrated that processes involved in increased cell number, differentiation and activation were highly enriched in the CD49b^{+} subset, whereas the CD49b^{-} subset had genetic and maternal imprinting processes upregulated which are related to quiescence (Paper I, Fig. 7D). Lastly, foot printing analysis indicated that both subsets are mainly regulated by the same TFs (Paper I, Fig. 7F-7G).

3.1.2 Discussion and Future Directions

In this study we showed that further subfractionation of the CD150^{hi} subset with CD49b results in two functionally and molecularly distinct subsets. The CD49b^{-} subset demonstrating the highest durability and self-renewal ability and enriches for My-bi HSCs. On the other hand, CD49b^{+} subset is less durable, more proliferative and enriches for Ly-bi cells with a fraction containing LT Ly-bi HSCs. Although lymphoid dominance is associated with limited self-renewal activity as in the CD150^{-} fraction and in γ/δ cells (Dykstra et al., 2007; Morita et al., 2010), Ly-bi cell definition in those studies and our study differ in their longevity. The cells with lymphoid dominant pattern in our study are classified as ST/transient cells. Within CD49b^{+} subset, Ly-bi HSCs show LT-activity with high lymphoid but low myeloid output resulting in lymphoid bias. Although these cells are present, they are quite rare in YA BM. We also highlight that previously conflicted results in longevity of CD49b cells might partly be resolved by the addition of CD150 in the immunophenotypic definition (Benveniste et al., 2010; Wagers and Weissman, 2006; Zhao et al., 2019).

The addition of CD41 and CD229 combined with CD49b did not seem to further resolve the heterogeneity in cell cycle and cell proliferation analysis. As CD41 is suggested to mark My-bi HSCs (Gekas and Graf, 2013) and CD229 Ly-bi HSCs (Oguro et al., 2013), further investigations through in vivo experiments are needed.

It has been suggested that while both CD49b^{-} and CD49b^{+} subsets localize near the blood vessels (BVs) and Mk cells, CD49b^{-} preferentially localizes near the endosteal region of the BM (Zhao et al., 2019) and therefore are differentially protected from external effects such as chemotherapy or 5-FU treatment. In our experiments, we did not expose the subsets to such stress inducing treatments e.g., 5-FU and chemotherapy in their niche, rather both subsets were given the same opportunity to localize in the BM niche upon transplantation. However, since our study did not focus on the niche aspect of the cells investigated, we cannot exclude the fact that the cells are exposed to different external cues.

CD49b (Integrin α2 subunit, encoded by *Itga2*) is a member of integrin family and forms a heterodimer with β1 subunit (CD29) generating the α2β1 receptor (Bouvard et al., 2001). Even though both subunits can mediate ligand recognition, vWF factor type A domain is present only in the CD49b subunit, therefore ligand binding is mainly proceeded by CD49b. Integrin α2β1 is expressed on mast cells, neutrophils, platelets, NK cells and some T cells in addition to HSCs (Adorno-Cruz and Liu, 2019). Compete ablation of CD49b was shown to give viable and fertile mice with normal development but has problems with wound healing, platelet adherence and platelet aggregation (Chen et al., 2002; Kunicki et al., 1993; Zweers et al., 2007). Overall, so far there is no data in the literature showing the impact of the ablation of CD49b from the HSCs, therefore it would be of interest to block the CD49b cell surface marker and look at the functional differences of the CD49b^{-} and CD49b^{+} subsets.

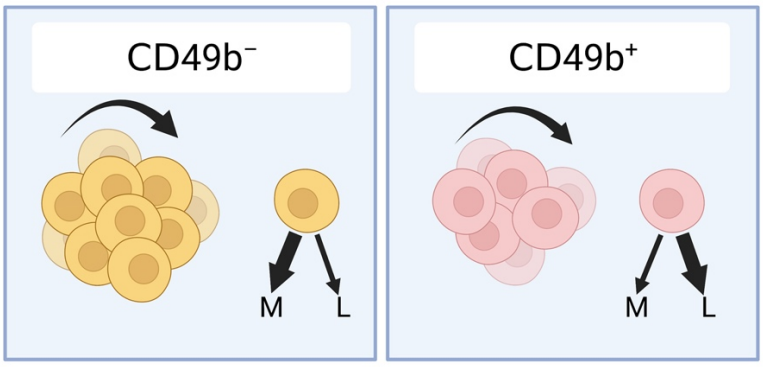

Figure 4. Graphical abstract of paper I. M: Myeloid, L: Lymphoid.

3.2 PAPER II

Hematopoietic aging has been associated with weakened and delayed innate and adaptive immune responses, anemia, and a higher risk of myeloproliferative disorders. The HSC population expands during aging, increasing the myeloid lineage output and reducing the cell polarity and self-renewal ability (Florian et al., 2012; Geiger et al., 2013; Rossi et al., 2008). The myeloid skewing throughout aging is proposed to be partly driven by My-bi HSCs (Cho et al., 2008) through epigenetic changes (Rossi et al., 2008; Sun et al., 2014).

In this paper, we explored the previously identified CD49b subsets in **paper I** and looked at the functional and epigenetic changes that occur during aging.

3.2.1 Results

In this study, we investigated the CD49b HSC subsets, characterized in young adult mice in **paper I,** during aging. To include the postnatal stage in our study, we included young (1 month old) mice in addition to young adult (YA, 2-4 months old) and old (1.5-2 years old) mice. Since the HSC pool size changes during aging, we assessed how this is reflected in $CD49b^-$ and $CD49b^+$ subsets in the three age groups. The total frequency and number of both subsets drastically increased in old mice as compared to young (Paper II, Fig. 1A-1C, S1A), while the significantly higher frequency of $CD49b^-$ remained the same throughout aging (Paper II, Fig. S1B). Evaluation of lineage differentiation potential *in vitro* at a single cell level for Mk, B and myeloid cells indicated that the lineage potential of the $CD49b^-$ and $CD49b^+$ subsets were preserved as all age groups in both subsets could efficiently generate Mk and myeloid cells while producing B cells at a lower level when compared to $CD150^-$ cells as expected (Paper II, Fig. 1D-1E, S1C-S1D).

Since we demonstrated in **paper I** that the $CD49b^-$ fraction was more quiescent and less proliferative compared to $CD49b^+$ cells in YA, here we investigated the cell cycle, cell division and proliferation kinetics in aging. Both subsets became increasingly more quiescent and less proliferative during aging. Whereas $CD49b^-$ maintained its significant quiescence as compared to $CD49b^+$ in old as in YA groups, both subsets were equally proliferative in young with a tendency to divide faster at day 2 (Paper II, Fig. 2A-2C, S1E-S1H). All in all, these results indicate that the $CD49b^-$ subset switch from an equally active with the $CD49b^+$ subset to a more quiescent state in the transitioning phase during young to YA and $CD49b^-$ is the most quiescent subset in aging.

Using the *Gata-1* eGFP mouse model as in **paper I** to permit the detection of platelets and erythrocytes in addition to leukocytes, we bulk transplanted the $CD49b^-$ and $CD49b^+$ cells from three age groups and followed their LT lineage output. To compensate for the reduced engraftment capacity of the old HSCs, 100 donor cells were transplanted from old mice against 5 cells in YA and young mice. Both subsets had stable engraftment in YA and old mice, with a significant and progressive increase in the $CD49b^-$ fraction compared to $CD49b^+$ in young in addition to being capable of multilineage repopulation during aging. Additionally, regardless

of the subsets, a higher B lineage output in young mice was shifted to a higher myeloid output in old mice, collectively indicating that age dependent changes occur in both subsets (Paper II, Fig. 3A-3B, S2A-S2C).

In **paper I**, we demonstrated that CD49b$^-$ is My-bi and CD49b$^+$ is mainly Ly-bi HSCs. Using the same lineage bias calculation (Müller-Sieburg et al., 2002) performed in YA (Paper II, Fig. S4A), here we evaluated the blood lineage distribution of CD49b$^-$ and CD49b$^+$ subsets in three age groups. In agreement with previous data in **paper I**, the most frequent Li-bi was My-bi in CD49b$^-$ and Ly-bi in CD49b$^+$ both in young and YA mice, whereas both subsets were highly My-bi in old which was also reflected in BM derived myeloid and lymphoid cells (Paper II, Fig. 3C-3E and S3A-S3B). We assessed the blood lineage distribution of CD49b$^-$ and CD49b$^+$ subsets calculated based on young and old unmanipulated mice. The blood profiles of the young CD49b$^-$ and young CD49b$^+$ transplanted mice were found to be categorized in a similar pattern as in YA group. On the other hand, while the blood profiles of the old CD49b$^-$ transplanted mice were mainly categorized in My-bi pattern, the old CD49b$^+$ transplanted mice were categorized in lineage balanced pattern (Paper II, Fig. 3D and S4A-S4D).

Long-term myeloid engraftment in PB reflects the self-renewal potential of HSCs. Assessment of the frequency of myeloid repopulated mice in primary transplantation at month 6 demonstrated higher frequency of CD49b$^-$ transplanted mice with myeloid engraftment compared to CD49b$^+$ cells in young. Whereas in old, both populations had equal frequency of myeloid reconstituted mice (Paper II, Fig. 4A). In agreement with this, the frequency of GMP and MkP engrafted mice was higher from CD49b$^-$ (both 12/13, 92%) than CD49b$^+$ (12/22, 54% and 10/22, 45% respectively) in young with no discernable differences in old populations as well as CLP or LMPP in general (Paper II, Fig. S4E-S4H). Additionally, the frequency of phenotypic HSC repopulated primary mice and reconstitution levels of HSC subsets in young and old groups was similar. Furthermore, the engraftment level of phenotypic HSCs in the CD49b$^-$ subset was significantly higher compared to CD49b$^+$ subset in old. This suggested that the CD49b$^-$ subset had higher durability (Paper II, Fig. 4B-4C, S4I-S4J). Assessment of myeloid repopulation in secondary transplantation indicated no differences between the subsets in both young and old groups but a drastic decrease was observed in the frequency of mice secondary engrafted with phenotypic HSCs in the CD49b$^+$ subset as compared to the CD49b$^-$ subset (Paper II, Fig. 4D-4E), strengthening the argument that CD49b$^-$ is the most durable subset in all age groups.

As functionally CD49b$^-$ and CD49b$^+$ subsets were functionally different *in vivo* during aging, we analyzed their chromatin landscape using Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) to understand the molecular basis behind it. Principal component analysis (PCA) showed three distinct clusters for LMPP, GMP and HSC as expected. In the HSC cluster, while cells were grouped in an age dependent manner, CD49b$^-$ and CD49b$^+$ subsets could also be distinguished (Paper II, Fig. 5A). Gene ontology (GO) analysis of PC1 and PC2 exhibited differences in biological processes that regulate immune responses between HSC and progenitor populations as anticipated (Paper II, Fig. 5B).

Evaluation of differentially accessible regions (DARs) between the subsets revealed 242 DARs in young (Paper II, Fig. 5C) and 854 DARs in old (Paper II, Fig. 5D). The limited number of DARs detected in young, suggested high similarity in their chromatin accessibility in accordance with the similar cell cycle and proliferation status of the subsets in young. In GO analysis of old however, CD49b$^-$ was enriched in biological processes related to negative regulation of cell cycle whereas CD49b$^+$ was associated with leukocyte activation and migration (Paper II, Fig. 5E). All in all, functional cell cycle and proliferation differences were also reflected in the open chromatin landscape highlighting their regulation at an epigenetic level.

Due to significant functional differences observed during aging between the subsets, we investigated the epigenetic changes in CD49b$^-$ and CD49b$^+$ cells by age. PCA clustering was indicative of age specific assembling (Paper II, Fig. 6A) therefore we explored DARs between young and old CD49b$^-$ and young and old CD49b$^+$ subsets. We found 2764 regions with decreased accessibility and 7361 regions with increased accessibility during aging (Paper II, Fig. 6B). Additionally, regions that were accessible in young lost their accessibility upon aging and regions that were more accessible in old were not accessible in young indicating the de novo open chromatin regions in old (Paper II, Fig. 6C). GO biological processes in old were associated with myeloid homeostasis, interferon-gamma regulation, response to reactive oxygen species and negative regulation of lymphocyte activation although at differing levels between the CD49b$^-$ and CD49b$^+$ subsets suggesting that subtle changes in the chromatin accessibility might be playing a major role in functional behavior (Paper II, Fig. 6D). Overall, these data suggests that massive chromatin accessibility changes occur during aging towards an increased accessibility of chromatin landscape in both CD49b$^-$ and CD49b$^+$ subsets.

3.2.2 Discussion and Future Directions

In this study we demonstrated that CD49b$^-$ and CD49b$^+$ HSCs are subjected to age dependent intrinsic changes impacting their functional, behavioral, and molecular patterns that is already initiated at the postnatal stage. The effect of murine hematopoietic aging is mostly studied by using young adult (YA, 2-4 months) and old (18-24 months) counterparts without considering the postnatal stage when HSCs acquire an adult phenotype (1 month). Here we took that into account and demonstrated that CD49b$^-$ and CD49b$^+$ subsets, switching from more active state in young, progressively acquire quiescence during aging. Additionally, we show that throughout aging CD49b$^-$ enriches for My-bi HSCs and becomes even more My-bi in old group, whereas CD49b$^+$ exhibits enrichment in Ly-bi HSCs in young and YA but Bala HSCs in old mice. Furthermore, increased open chromatin regions observed during aging in both subsets is in agreement with the literature (Itokawa et al., 2022) and the differences between the subsets are subtle. It is of interest to understand the lineage-bias shift that occurs in old CD49b$^+$ in more detail and this might be more exclusively concluded when the chromatin landscape of the donor-derived HSCs is analyzed from young and old. Lastly, as behavioral differences were assessed on a population level, it will be of importance to assess the subsets at the clonal level.

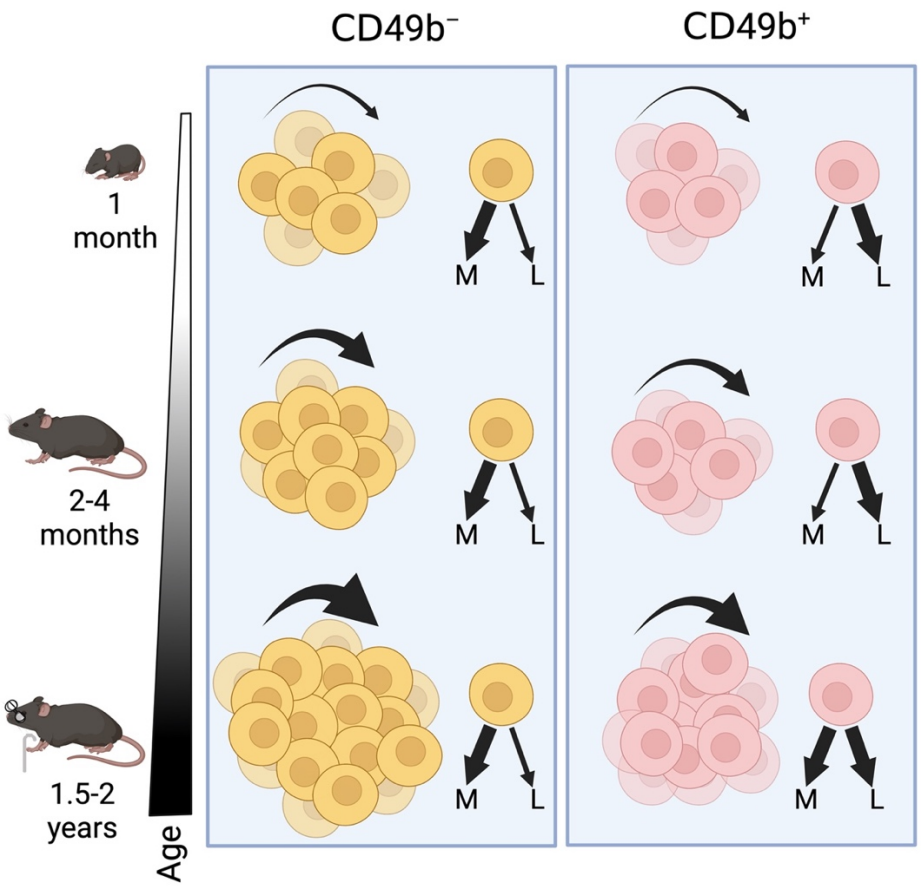

Figure 5. Graphical abstract of paper II. M: Myeloid, L: Lymphoid.

3.3 PAPER III

One aspect of resolving the heterogeneity within HSC compartment is to allow for the investigation of true LT-HSC behavior without the presence of progenitor and transient cells that might obscure the readout. There has been extensive research performed to identify BM progenitor subsets, yet within the immunophenotypically identified HSC compartment there are stills cells with ST activity. Therefore, in this paper we explored whether the addition of cell surface marker to the CD49b HSC subsets identified in **paper I** can purify LT-HSC by separating out those cells with ST activity.

3.3.1 Results

In **paper I**, we identified CD49b cell surface marker that can functionally subfractionate LSKCD34^{-}CD48^{-}CD150^{hi} (CD150^{hi}) cells into My-bi CD49b^{-} and Ly-bi CD49b^{+} subsets. As heterogeneity remained to some extent in both fractions with CD49b^{+} fraction also containing cells with ST/transient activity, we decided to test additional markers to further enrich the CD49b^{+} subsets. Since CD229 enriched for Ly-bi cells (Oguro et al., 2013), we analyzed the CD49b and CD229 expression. Within CD150^{hi} fraction i.e., CD49b^{-}CD229^{-}, CD49b^{-} CD229b^{+}, CD49b^{+}CD229^{-}, CD49b^{+}CD229^{+} (Paper III, Fig. 1A). Cell cycle and cell proliferation analysis demonstrated that while all subfractions were highly quiescent, CD49b^{+}CD229^{-} and CD49b^{+}CD229^{+} were significantly more in G1 phase and more proliferative regardless of CD229 expression in accordance with **paper I** (Paper III, Fig. 1B-1E).

As both CD49b and CD229 were suggested to enrich for Ly-bi fractions, we assessed their multilineage differentiation potential both *in vitro* and *in vivo*. Both subsets, evaluated at the clonal level, could give rise to B and myeloid cells *in vitro* (Paper III, Fig. 2A). As the lineage potential *in vitro* does not necessarily answer the lineage potential *in vivo*, we transplanted 5 cells from each fraction using *Gata-1* eGFP mouse model and analyzed platelets, erythrocyte, myeloid, B and T cells in the transplanted mice. Following the mice up to 6 months, all subsets demonstrated increasing donor leukocyte engraftment but had differing levels of contribution from each lineage (Paper III, Fig. 2B and S1A-S1C). Particularly, only CD49b^{+}CD229^{+} subset showed declining myeloid, platelet and erythrocyte engraftment with lowest efficiency to produce myeloid cells in PB. On the other hand, the CD49b^{-}CD229^{-}, CD49b^{-}CD229b^{+} and CD49b^{+}CD229^{-} subsets stably increased their individual lineage engraftment over time and had efficient myeloid engraftment. This suggested a limited self-renewal ability within only the CD49b^{+}CD229^{+} fraction (Paper III, Fig. 2C-2D and S2A-S2D). Indeed, when looking at myeloid and lymphoid (B, T and NK cells) in the PB at month 6, CD49b^{+}CD229^{+} cells demonstrated lymphoid lineage preference over myeloid which was also recapitulated in the BM. Interestingly, CD49b^{-}CD229^{+} subset also had stronger lymphoid preference in PB, but with a stable LT output instead (Paper III, Fig. 2E-2F). Moreover, the phenotypic HSC reconstitution level and frequency of mice that HSC reconstituted were the lowest in CD49b^{+}CD229^{+}. In further agreement, the frequency of CLP, LMPP, GMP and MkP

reconstituted mice in the CD49b^{+}CD229^{+} fraction was the lowest suggesting that the CD49b^{+}CD229^{+} population is rather enriching for highly proliferative MPP population with ST activity (Paper III, Fig. 2G-2L).

3.3.2 Discussion and Future Directions

In this paper, we show that subfractionation of CD150^{hi} cells with CD49b and CD229 allows for the detection of a MPP population within CD49b^{+}CD229^{+} cells with ST-activity. Using CD229, we show that LT-HSCs can be separated from MPPs which further resolves the heterogeneity within the CD49b^{+} fraction.

CD229 has been shown to mark Ly-bi HSCs. When CD229^{-} and CD229^{+} HSCs are co-transplanted, CD229^{-} was found to outcompete CD229^{+} subset suggesting that CD229^{-} HSC is LT-HSC whereas CD229^{+} HSC is IT-HSC (Oguro et al., 2013). As CD49b also marks Ly-bi HSCs one expectation would be to detect true Ly-bi HSCs within CD49b^{+}CD229^{+} fraction. Although we did not see that, the reason might be due to the averaged output of bulk cell transplantation. Therefore, it is of importance to perform single transplantations to detect the lineage bias at the clonal level. Secondly, even though it is already clear that the proportion of mice that have myeloid reconstitution in CD49b^{+}CD229^{+} subset is the lowest and has the ST-activity in line with Oguro *et al*., it will be important to see the self-renewal potential of the cells in secondary transplantations.

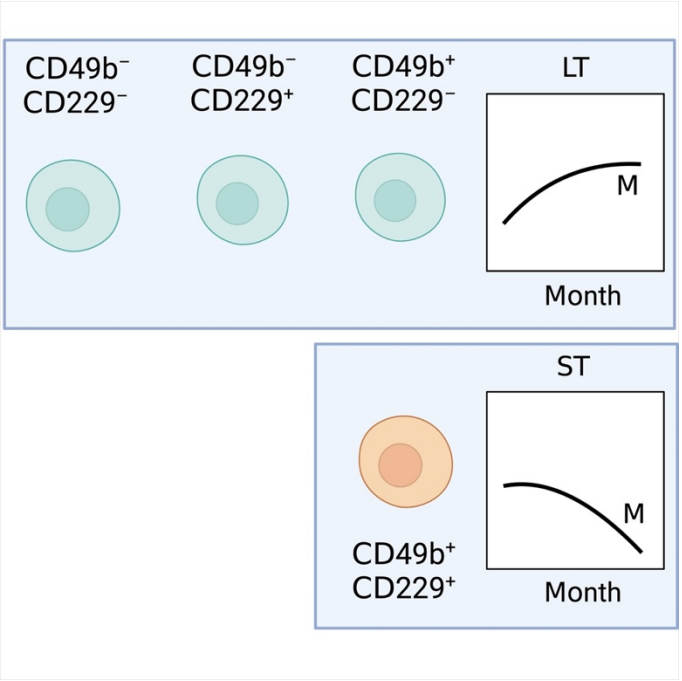

Figure 6. Graphical abstract of paper III. M: Myeloid, LT: Long-term, ST: Short-term.

3.4 PAPER IV

In this study, the impact of FOXO1 and/or FOXO3 TFs on B cell development was explored using the Vav-iCre mouse model by their conditional deletion (*Foxo1ko, Foxo3ko and Foxodko*) throughout the hematopoietic system.

3.4.1 Results

To understand the impact of FOXO1 and FOXO3 deletion, BM and spleen organs were analyzed. *Foxo3ko* had normal cellularity in both BM and spleen with reduction in the BM B cells. FOXO3 is identified to be crucial for B cell precursors yet is dispensable for the mature B cells as *Foxo3ko* mice exhibited reduction in BM Kit^- proB, preB and immB cells and spleen located trB cells while the mature B cell subsets were unaffected (Paper IV, Fig. 1B-1E). Previously unknown but essential role of FOXO3 is also uncovered in lymphoid branching of HSPCs as its deficiency resulted in diminished HSC, LMPP, ALP and BLP compartments (Paper IV, Fig. 2A-2C). Overall, the effect of FOXO3 in the hematopoietic hierarchy is larger than it was thought before.

On the other hand, *Foxo1ko* had diminished cellularity in both BM and spleen with reduced BM B cells and total lack of splenic B cells. FOXO1 is found to be critical for pro-B to preB transition as *Foxo1ko* mice demonstrated a complete block at the pro-B cell stage with severe depletion in the cell number in line with the BLP expansion (Paper IV, Fig. 1B-1E and 2C). Although phenotypic analysis of the *Foxo1ko* strain indicated the presence of residual $CD19^+$ splenic B cells (Paper IV, Fig. 1D), these cells were not a result of inefficient *Foxo1* deletion, and therefore were identified as B cell progenitors. This was because they clustered adjacent to pro-B cells in their transcriptional profiling in the principal component analysis (PCA), lacking expression of *Rag* genes therefore incapable of V(D)J rearrangement of pre-BCR receptor and phenotypic cell surface markers specific for B cell maturation (Paper IV, Fig. 1F-1G).

Combinatorial deletion of FOXO1 and FOXO3 exhibited normal cellularity in the BM and spleen accompanied with an absence of B cells, which was confirmed by transplantation. Interestingly, increase in the HSC numbers followed by reduction in LMPP and ALP with normal BLP output suggested a block at the BLP stage (Paper IV, Fig. 2-3, S3A-S3B and S3E-S3F). While *Foxodko* CLP, LMPP and HSC populations sustained their cellular identity by clustering together with their control counterparts, their transcriptional programming was found to be significantly different within progenitors when developmental order (PC1) was plotted against the genotype (PC2) (Paper IV, Fig. 4A-4B). A total of 319 differentially expressed genes (DEGs) were detected in *Foxodko* ALP and BLP as compared to WT (Paper IV, Fig. 4C). A significant number of DEGs upregulated in ALP to BLP transition was shared in *Foxodko* CLPs. Hierarchical clustering of DEGs showed that while *Foxo1ko* presents gene expression changes more drastic than *Foxo3ko*, loss of both genes has the most aggravated alterations (Paper IV, Fig. 4D and S4A). This indicates that FOXO1 and FOXO3 works

collaboratively in regulation of DEGs. Gene ontology analysis revealed that downregulated genes in *Foxo1dko* were associated with B cell activation, primary immunodeficiency, and response to oxidative stress (Paper IV, Fig. S4B). Specifically, downregulation occurred in such genes that are indispensable for B cell development such as BCR formation (*Cd79a, CD79b, Cd19, Vpreb, Igll1*) and TFs (*Ets1*, *Rag1*, *Ebf1* and *Pax5*) further confirmed the block at the BLP stage (Paper IV, Fig. 4D,4F and S4A). In line with the previously reported loss of B cells in *Foxo1ko* mice due to the abruption in EBF1-FOXO1 positive feedback loop (Mansson et al., 2012), almost complete absence of *Ebf1* gene expression in *Foxodko* mice (Paper IV, Fig. 4F) suggests that FOXO3 also plays a crucial role in that circuit. Overall, the FOXO1 and FOXO3 deficiency cause incomplete of B-cell commitment at the BLP stage.

To decipher whether the epigenetic changes are the result of transcriptional alterations in *Foxodko* CLPs, chromatin landscape was investigated with ATAC-sequencing. *Foxodko* HSC, LMPP and CLP fractions also sustained their cell identity epigenetically similar to transcriptome landscape as *Foxo1dko* populations clustered together with WT counterparts but exhibited differences (PC2) when developmentally ordered (PC1) (Paper IV, Fig. 5A). Assessing the changes in chromatin accessibility of *Foxodko* ALP and BLP against WT pinpointed 297 and 1068 differentially accessible regions (DARs) with most of them being distal to promoter regions (Paper IV, Fig. 5B-5C). Additionally, FOXO1 and FOXO3 were found to be critical for the regulation of pro-B transition from CLPs as the more accessible DARs in *Foxodko* CLPs had reduced accessibility in WT pro-B cells and *vice versa*. The DARs with decreased accessibility in *Foxodko* CLPs but more accessible in WT pro-B cells were localized near the genes specific for B-cell commitment such as *Cd19*, *CD79a*, *Pou2af1*, *Rag2* and *Ebf1* (Paper IV, Fig. 5D). Assessment of TFs controlling DARs with decreased accessibility in *Foxodko* BLPs by motif enrichment analysis exhibited TF binding sites of EBF, FOXO, ETS, RUNX and E-box proteins. EBF1, FOXO cut-profiles were either diminished or absent further supporting the lack of their binding to DARs with decreased accessibility (Paper IV, Fig. 5H). DARs with increased accessibility (IRF, RUNX, ETS and CEBP) did not show any transcriptional upregulation of these genes (Paper IV, Fig. 5I) indicating the presence of altered binding of TFs in *Foxodko* CLPs.

When looking at whether the loss of FOXO1 and EBF1 in *Foxodko* BLPs is the cause for the inability to form accessible chromatin regions near *Foxo1* and *Ebf1* genes, it was found that these areas remained open in *Foxodko* as compared to WT suggesting that FOXO1 and EBF1 do not play a role in establishing the accessibility of the chromatin region. Rather, they bind to pre-determined open chromatin to initiate positive feedforward loop. These open chromatin regions were already established at the HSPC level independent of FOXO1, FOXO3 and EBF1 (Paper IV, Fig. 6).

Lastly, FOXO1 and FOXO3 deficiency does not limit myeloid potential in BLPs since the assessment of differentiation potential of *Foxodko* ALPs and BLPs *in vitro* demonstrated that *Foxodko* CLPs generate low proliferating clones with increased myeloid related transcripts

in ALP, but no upregulation of myeloid TFs such as *Pu.1* and *Cebp*-family or myeloid growth receptors (Paper IV, Fig. 7).

3.4.2 Discussion and Future Directions

In this study, we show that FOXO1 and FOXO3 are essential for B cell development and the absence of these two transcription factors blocks the B cell development at BLP stage. Previous studies suggested that the absence of FOXO1 cause arrest at pre-B cell stage (Dengler et al., 2008) and later suggested to be at the BLP stage although there were remaining undefined B cell progenitor population (Mansson et al., 2012). In this study, we shed a light on those remaining FOXO1ko B cells and identify them as pro-B cells with no proper function due to the loss of *Rag* gene expression. Additionally, here we demonstrate that FOXO3 initiates the B cell lineage programming earlier than thought already at the LMPP stage therefore has a broader impact on B cell development. Adding onto the previous findings showing the essential role of FOXO3 during B cell commitment at the pre-B stage (Hinman et al., 2009), in this paper we exhibit that this starts even earlier at the pro-B cell stage and includes even transitional and marginal zone B cells. Lastly, the combinatorial effect of FOXO1 and FOXO3 then blocks the B cell commitment at the BLP stage. Since both FOXO1 and FOXO3 are known to be tumor suppressors (Karube et al., 2011; Yadav et al., 2018) one important aspect to further pursue would be to investigate the potential factors that take advantage of the failed B cell restriction and might cause aberrant cell growth of other types than B cells.

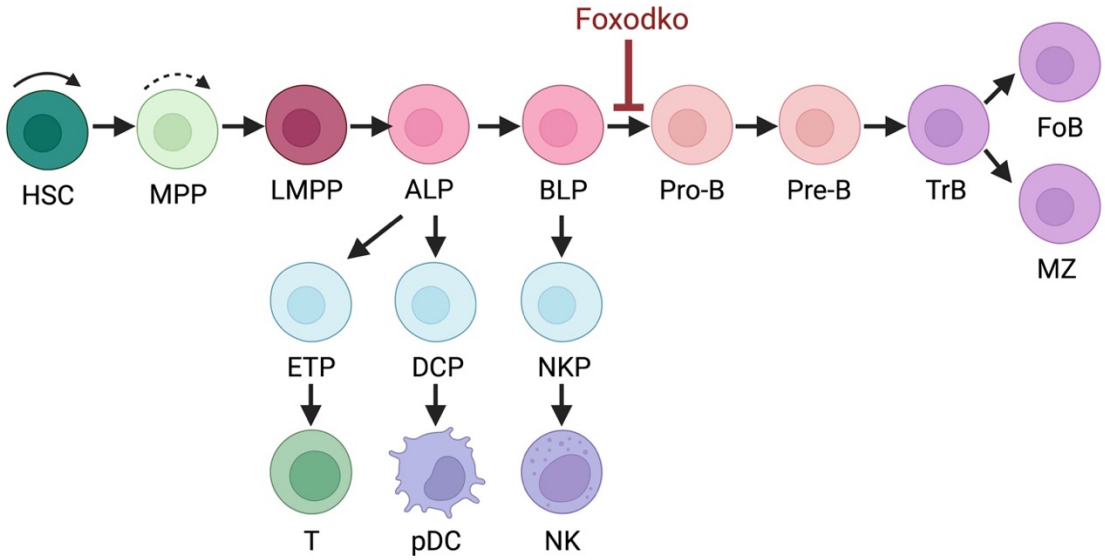

Figure 7. Graphical abstract of paper IV.

HSC: Hematopoietic stem cell, MPP: Multipotent progenitor cell, LMPP: Lymphoid-primed multipotent progenitor, ALP: All lymphocyte progenitor, BLP: B cell biased lymphocyte progenitor, TrB: Transitional B cell, FoB: Follicular B cell, MZ: Marginal zone B cell, ETP: Early thymic progenitor, DCP: Dendritic cell progenitor, NKP: Natural killer cell progenitor, NK: Natural killer cell, pDC: plasmacytoid dendritic cell, T: T cell, Foxodko: Foxo1 and Foxo3 knockout.

4 CONCLUDING REMARKS

The findings in this thesis shed a light on the importance of continuing efforts to identify cell surface markers to purify lineage-biased HSC subsets and investigating their molecular and epigenetic regulation during aging. Furthermore, we also reveal the impact of B cell specific transcription factors in during B cell development. We show that usage of an additional immunophenotypic marker CD49b in **paper I** identifies two HSC subsets as $CD49b^{-}$ and $CD49b^{+}$ with myeloid bias and lymphoid bias, respectively and that their functional behavior is regulated by epigenetic changes. In addition, in **paper II** we depict that throughout aging, both $CD49b^{-}$ and $CD49b^{+}$ HSCs gain aging related epigenetic signature and while $CD49b^{-}$ sustains its myeloid lineage-bias, $CD49b^{+}$ shifts towards lineage-balanced output. In **paper III** we depict that usage of CD229 as a phenotypic marker in addition to CD49b even further dissects the heterogeneity and identifies an MPP population within the $CD49b^{+}CD229^{+}$ fraction. Lastly, in **paper IV** we demonstrate that absence of FOXO1 and FOXO3 blocks the B-cell development at the BLP stage.

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