From Department of Medicine, Huddinge - MedH
Karolinska Institutet, Stockholm, Sweden

HEMATOPOIETIC STEM AND PROGENITOR CELL FATE DECISIONS DURING FETAL DEVELOPMENT

Karin Belander Strålin

Karolinska Institutet

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Hematopoietic stem and progenitor cell fate decisions during fetal development

Thesis for Doctoral Degree (Ph.D.)

By

Karin Belander Strålin

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Principal Supervisor:
Professor Sten Eirik W. Jacobsen
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine (HERM)

Co-supervisors:
Assistant Professor Petter Woll
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine (HERM)

Professor Eva Hellström-Lindberg
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine (HERM)

Ph.D. Stefan Söderhäll
Karolinska Institutet
Department of Women's and Children's Health

Opponent:
Professor Katrin Ottersbach
The University of Edinburgh
Institute for Regeneration and Repair
Centre for Regenerative Medicine

Examination Board:
Associate Professor Mattias Carlsten
Karolinska Institutet
Department of Laboratory Medicine

Associate Professor Johan Flygare
Lund University
Department of Laboratory Medicine
Division of Molecular Medicine and Gene Therapy

Associate Professor Jenny Pyl Hansson
Lund University
Department of Experimental Medical Science
Proteomic Hematology

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science."

Albert Einstein

To my family

Popular science summary of the thesis

Since most mature blood cells are short-lived and critical for life, they must be continually produced to keep the blood system in balance. It is estimated that nearly 90% of the more than 100 billion cells replaced in the average adult human body each day, are blood cells. This life-long replenishment of blood cells is safe-guarded by hematopoietic stem cells (HSCs), which are rare cells capable of replenishment of all types of blood cells in the body. The process of blood production involves several stages whereby cells transition from stem cells to mature blood cells that circulate in the bloodstream. The intermediate stages between HSCs and mature blood cells are known as progenitor cells. As progenitor cells transition through these stages, they make stepwise decisions that determine which type of mature blood cell they eventually will become. Blood formation during fetal development primarily occurs in the liver and is generally less studied than adult blood formation, which takes place in the bone marrow. This thesis aims to further investigate fetal blood formation in mice and what distinguishes it from blood formation in the adult.

Previous studies have shown that adult mouse HSCs can be lineage-biased, meaning they have a preference for producing certain types of mature blood cells. For example, platelet-biased stem cells predominantly produce platelets.

In **Study I**, we investigated the presence of lineage-biased HSCs in the liver and bone marrow of mice at the time of birth. The HSCs at birth displayed a less frequent bias towards producing platelets and platelet-erythroid ("red blood cell")-myeloid (a type of "white blood cell") lineages compared to adult bone marrow HSCs. However, the development of platelet and platelet-erythroid-myeloid cell lineage bias had begun in a specific type of HSCs that expressed von Willebrand factor (a protein in the blood important for blood clotting). This study provides new insights into blood production from HSCs at birth.

Platelets are essential for preventing and stopping bleeding from injuries. Previous research has suggested but not identified an alternative, faster pathway for platelet replenishment from HSCs, bypassing the multiple steps of the ordinary pathway.

In **Study II**, we identified a distinct and faster pathway for platelet replenishment from HSCs in adult mouse bone marrow. This faster pathway is specialized for platelet production and originates from a specific type of platelet-restricted HSC

that expresses von Willebrand factor. After chemotherapy treatment, which lowers platelet counts in the blood, this pathway was utilized for a more rapid platelet production. If a similar alternative platelet pathway exists in human blood formation, it could potentially be used to rapidly stimulate platelet production in patients with low platelet counts.

The development of B cells, a type of "white blood cell", which plays a key role in the immune system, has been thoroughly investigated in the mouse. However, it remains unclear when and where the first fetal B cell progenitor cell, which can only develop into B cells, emerges.

In **Study III**, we investigated fetal B-cell development in the mouse, and discovered a progenitor cell in the fetal liver at day 12.5 of fetal development that could only develop into B cells and no other types of white blood cells. This fetal B cell progenitor was characterized by the expression of a specific gene called *Mb1*. We also found that both fetal and adult B cell progenitors can be divided based on *Mb1* expression, with the specific commitment to B cells having begun in the *Mb1*-expressing cells. These findings are of particular interest in research of the most common form of childhood cancer, B-cell precursor acute lymphoblastic leukemia, which is thought to originate in the earliest B-cell committed progenitor cells.

Populärvetenskaplig sammanfattning

Många mogna blodkroppar har en kort livslängd samtidigt som de är livsviktiga för olika funktioner i kroppen. Det gör att blodkropparna ständigt måste ersättas för att hålla blodets sammansättning i balans. Uppskattningar anger att nästan 90% av de mer än 100 miljarder celler som byts ut i kroppen varje dag är blodceller. Blodstamceller, även kallade hematopoetiska stamceller, är sällsynta celler som kan bilda alla olika typer av blodkroppar som finns i blodet. Dessa stamceller ansvarar för nytillverkningen av blodkroppar under hela livet. Under blodkroppsbildningen är det många steg på vägen från den ursprungliga stamcellen till en mogen blodkropp som cirkulerar i blodet. Blodcellens stadier mellan stamceller och mogna blodkroppar består av förstadier till blodkroppar som kallas för progenitorceller. Under övergången från ett cellstadium till ett annat fattar cellen stegvisa beslut som avgör vilken typ av mogen blodkropp som till slut kommer att bildas. Under fostertiden sker blodkroppsbildningen huvudsakligen i levern och är inte lika väl studerad som blodkroppsbildningen hos vuxna som sker i benmärgen. Studierna i denna avhandling har utförts i musmodell och undersöker blodbildning under fostertiden och nyföddhetsperioden och vad som skiljer den åt från vuxen blodbildning.

Tidigare studier har visat att vuxna blodstamceller kan vara så kallade ”lineage-biased” blodstamceller, vilket innebär att de i första hand bildar en viss typ av mogna blodkroppar, till exempel kommer ”platelet-biased” blodstamceller främst att bilda trombocyter (”blodplättar”).

I **Studie I** undersökte vi förekomsten av så kallade ”lineage-biased” blodstamceller i lever och benmärg vid födelsen. Blodstamcellerna vid födelsen visade mindre benägenhet att producera trombocyter, erytrocyter (”röda blodkroppar”) och myeloida blodkroppar (en sorts ”vita blodkroppar”) jämfört med tidigare undersökta vuxna blodstamceller. Vi såg också att benägenheten att bilda trombocyter, erytrocyter och myeloida blodkroppar hade börjat i en särskild typ av blodstamcell som utrycker von Willebrand-faktor (ett protein som finns i blodet). Denna studie gav nya insikter om blodstamcellernas förmåga att bilda blodkroppar under nyföddhetsperioden.

Trombocyter är nödvändiga för kroppens förmåga att förhindra och stoppa blödning. Tidigare forskning har föreslagit att det kan finnas en alternativ och snabbare väg för trombocyter att bildas från blodstamceller än den ordinarie vägen med många steg.

I **Studie II** visade vi att det finns en alternativ och snabbare väg för bildning av trombocyter från blodstamceller i vuxen benmärg. Denna snabbare väg är specialiserad på bildning av trombocyter och har sitt ursprung i en särskild typ av blodstamceller som utrycker von Willebrand-faktor. Efter behandling med cellgifter, som leder till lägre nivåer av trombocyter i blodet, kunde vi se att den alternativa snabbare vägen användes för att bilda trombocyter. I framtiden skulle denna kunskap kunna leda till utveckling av läkemedel som stimulerar bildning av trombocyter hos patienter med låga nivåer av trombocyter i blodet.

Uppkomsten av en av de så kallade ”vita blodkropparna”, B-cellerna, som är aktiva i immunsystemet, har varit föremål för mycket forskning i musmodeller. Trots detta saknas fortfarande kunskap om när och var det förstadium till B-cell som bara kan utvecklas till en B-cell (och inte till någon annan typ av blodkropp), först dyker upp under fostertiden.

I **Studie III** undersökte vi den tidiga utvecklingen av B-celler i musmodell, och fann i levern under dag 12.5 av fosterutvecklingen en progenitorcell som endast kunde utvecklas vidare till B-celler. Detta förstadium till B-cell kännetecknades av att cellen uttryckte en särskild gen, *Mb1*-genen. Vi såg också att förstadier till B-celler från lever under fostertiden och från vuxen benmärg kunde delas in baserat på om de uttryckte *Mb1*-genen eller inte. I de förstadier till B-celler som uttryckte *Mb1*-genen hade den specialiserade utvecklingen till B-celler påbörjats, vilket inte var fallet i de förstadier till B-celler som inte uttryckte *Mb1*-genen. Denna kunskap om förstadier till B-celler kan komma till nytta i forskning där man försöker hitta de celler som den vanligaste formen av barncancer, akut lymfatisk leukemi, uppstår i.

Abstract

Rare multipotent hematopoietic stem cells (HSCs) are responsible for the replenishment of blood cells throughout life. In the maturation process, from primitive HSCs to peripheral blood cells, the intermediate cells pass through multiple steps of increasingly lineage-restricted progenitors and cell fate decisions are made at each step. However, fetal hematopoiesis is more complex and less studied than adult hematopoiesis. The aim of this thesis was to further characterize and compare fetal, neonatal and adult mouse hematopoiesis including lineage-biased HSCs.

Single HSC transplantation studies have previously demonstrated that adult mouse HSCs are heterogeneous in their ability to replenish different blood cell lineages upon transplantation. It remains unclear to what degree the adult patterns of lineage-bias and restriction exist during fetal development.

In **Study I**, we investigated the potential lineage-bias of mouse HSCs from liver and bone marrow at the time of birth. Perinatal HSCs demonstrated less platelet (P)- and platelet-erythroid-myeloid (PEM)-bias/restriction and more consistent lymphoid reconstitution than adult HSCs. However, the development of P- and PEM-bias/restriction had already begun in perinatal HSCs in a distinct HSC subset expressing von Willebrand factor (*Vwf*-reporter^{+} HSCs). We compared the transcriptional landscapes of *Vwf*-reporter^{+} and *Vwf*-reporter^{-} liver perinatal HSCs through single-cell RNA sequencing and identified transcriptional lineage priming corresponding to their lineage bias upon transplantation. The study provides new insights into the blood reconstitution potential of HSCs at birth.

Platelets are critical for hemostasis and play a role in both the innate and adaptive immune systems. Previous studies have implied the presence of an alternative, faster differentiation pathway for platelet production, however it has not been convincingly shown that such an alternative pathway can originate from true HSCs.

In **Study II**, we demonstrated a non-hierarchical relationship between two different stem cell types in adult mouse bone marrow, *Vwf*-reporter^{-} multi-HSCs reconstituting all blood cell lineages and *Vwf*-reporter^{+} P-HSCs primarily reconstituting platelets. Single-cell RNA sequencing confirmed that platelet-restricted progenitors originating from *Vwf*-reporter^{+} P-HSCs are molecularly different from *Vwf*-reporter^{-} multi-HSCs. *Vwf*-reporter^{-} multi-HSCs use a slower

multipotent differentiation pathway, whereas *Vwf*-reporter^{+} P-HSCs use an alternative and faster platelet-restricted pathway. After hematopoietic challenges by chemotherapy, the faster platelet-restricted pathway was activated for platelet replenishment. These discoveries could be of importance for the development of new treatments aimed at stimulating platelet recovery in clinical settings.

B-cell development has been most extensively studied in mice, where the timing and anatomical location of the first embryonic hematopoietic cells with B-lymphocyte potential have been established. However, these cells represent multipotent stem or progenitor cells. In contrast, the timing, location, and properties of the first fetal B-cell restricted progenitors have yet to be determined.

In **Study III**, we demonstrated that expression of *Mb1* describes an earlier fetal stage of CD19^{-} B-cell restricted progenitors than previously reported. These progenitors emerge at embryonic day 12.5 in the fetal liver and are molecularly distinct from subsequent stages of fetal B progenitor cells. Mini-bulk RNA sequencing revealed that the previously defined fetal liver and adult bone marrow PreProB cells can be subdivided by *Mb1* expression. Progenitors expressing *Mb1* have activated a transcriptional program compatible with B-cell specification which is not initiated in PreProB cells lacking *Mb1* expression. These findings are relevant for understanding potential cellular targets and leukemia-initiating cells in the development of infant and childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL).

List of scientific papers

I. **Platelet and myeloid lineage biases of transplanted single perinatal mouse hematopoietic stem cells.**

Karin Belander Strålin*, Joana Carrelha*, Axel Winroth, Christoph Ziegenhain, Michael Hagemann-Jensen, Laura M. Kettyle, Amy Hillen, Kari Högstrand, Ellen Markljung, Francesca Grasso, Masafumi Seki, Stefania Mazzi, Yiran Meng, Bishan Wu, Edwin Chari, Madeleine Lehander, Rickard Sandberg, Petter S. Woll, Sten Eirik W. Jacobsen.

Cell Research 2023;33(11):883–886.

II. **Alternative platelet differentiation pathways initiated by nonhierarchically related hematopoietic stem cells.**

Joana Carrelha*, Stefania Mazzi*, Axel Winroth*, Michael Hagemann-Jensen, Christoph Ziegenhain, Kari Högstrand, Masafumi Seki, Margs S. Brennan, Madeleine Lehander, Bishan Wu, Yiran Meng, Ellen Markljung, Ruggiero Norfo, Hisashi Ishida, **Karin Belander Strålin**, Francesca Grasso, Christina Simoglou Karali, Affaf Aliouat, Amy Hillen, Edwin Chari, Kimberly Siletti, Supat Thongjuea, Adam J. Mead, Sten Linnarsson, Claus Nerlov, Rickard Sandberg, Tetsuichi Yoshizato, Petter S. Woll, Sten Eirik W. Jacobsen.

Nature Immunology 2024;25(6):1007-1019.

III. **Identification of a B-cell restricted progenitor emerging early in fetal development.**

Karin Belander Strålin*, Masafumi Seki*, Joanna C. A. Green, Stephen Loughran, Kari Högstrand, Ellen Markljung, Axel Winroth Amy Hillen, Edwin Chari, Charlotta Böiers, Emanuele Azzoni, Joana Carrelha, Tetsuichi Yoshizato, Petter S. Woll, Sten Eirik W. Jacobsen.

Submitted.

*Equal contribution

List of scientific papers not included in the thesis

I. **Efficacy and safety of azacitidine in pediatric patients with newly diagnosed advanced myelodysplastic syndromes before hematopoietic stem cell transplantation in the AZA-JMML-001 trial.**

Franco Locatelli, **Karin Belander Strålin**, Irene Schmid, Julián Sevilla, Owen P. Smith, Marry M. van den Heuvel-Eibrink, Marco Zecca, Christian M. Swaan, Alison Gaudy, Meera Patturajan, Jennifer Poon, Mathew Simcock, Charlotte M. Niemeyer.

Pediatric blood & cancer. 2024;71(5):e30931.

Contents

List of abbreviations

ABM	Adult bone marrow
AGM	Aorta-gonad-mesonephros
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
B	B cell
BAC	Bacterial artificial chromosome
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
BCR	B-cell receptor
BM	Bone marrow
cDNA	Complementary DNA
CFU	Colony-forming unit
CFU-E	Colony-forming unit-erythroid
CFU-S	Colony-forming unit-spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CP	Cyclophosphamide
Cre	Cre recombinase
CS	Carnegie stage
CSF1R	Colony stimulating factor 1 receptor
DC	Dendritic cell
DEG	Differentially expressed gene
DP	Double positive
E	Erythroid
ED	Embryonic day
EHT	Endothelial-to-hematopoietic transition
EMP	Erythro-myeloid progenitor
eMPP	Embryonic multipotent progenitor

EoBP	Eosinophil-basophil progenitor
EPCR	Endothelial protein C receptor
ESC	Embryonic stem cell
ETV6	ETS variant transcription factor 6
FACS	Fluorescence-activated cell sorting
FBM	Fetal bone marrow
FL	Fetal liver
FLT3	FMS-like tyrosine kinase 3
FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	Flourescence minus one
FSC	Forward light scatter
GFP	Green fluorescent protein
GM	Granulocyte-macrophage
GMP	Granulocyte-monocyte progenitor
HCT	Hematopoietic cell transplantation
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
HPC	Hematpoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
Ig	Immunoglobulin
IL	Interleukin
IL7RA	Interleukin-7 receptor alpha
ILC	Innate lymphoid cell
IT	Intermediate-term
IT-HSC	Intermediate term hematopoietic stem cell
KIT	Mast/stem cell growth factor receptor Kit
L	Lymphoid

L-bias	Lymphoid-bias
Lin	Lineage (collection of mature lineage markers)
LK	Lineage$^-$ SCA1$^-$ KIT$^+$ cells
LMPP	Lymphoid-primed multipotent progenitor
L-restricted	Lymphoid-restricted
LSK	Lineage$^-$ SCA1$^+$ KIT$^+$ cells
LT	Long-term
LT-HSC	Long-term repopulating hematopoietic stem cell
M	Myeloid
MEP	Megakaryocyte-erythroid progenitor
Mk	Megakaryocyte
MkP	Megakaryocyte progenitor
MPP	Multipotent progenitor
mRNA	Messenger RNA
MYB	Transcriptional activator Myb
NGS	Next-generation sequencing
NK	Natural killer cell
P	Platelet
PB	Peripheral blood
PCA	Principal component analysis
PDO	Postnatal day zero
PE	Platelet-erythroid
PEM	Platelet-erythroid-myeloid
PEMB	Platelet-erythroid-myeloid-B cell
pnHSC	Perinatal HSC
PreB	Pre B cell
Pre-BCR	Pre-B-cell receptor
PreGM	Pre-granulocyte-monocyte progenitor

Pre-HSC	Precursor hematopoietic stem cell
PreMegE	Pre-megakaryocyte-erythroid progenitor
PreProB	PrePro B cell
ProB	Pro B cell
PSC	Pluripotent stem cell
P-Sp	Para-aortic splanchnopleura
RAG	Recombination activating gene
RNA-Seq	RNA sequencing
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
scRNA-Seq	Single-cell RNA sequencing
SDF-1	Stromal cell-derived factor 1
SLAM	Signaling lymphocyte activation molecule
SP	Somite pairs
SSC	Side light scatter
ST	Short-term
ST-HSC	Short-term repopulating hematopoietic stem cell
T	T cell
TCR	T-cell receptor
TDT	Terminal deoxynucleotidyl transferase
TO	Thiazole orange
Tom/TOM	tdTomato
TSLP	Thymic stromal lymphopoietin
Vwf	von Willebrand factor
YFP	Yellow fluorescent protein
YS	Yolk sac
5-FU	5-fluorouracil

Introduction

Although hematopoiesis has been a field of intensive research for many decades, there are still numerous hematopoietic cell functions, properties and processes yet to be explored and characterized to achieve a better understanding of both normal and malignant hematopoiesis. In recent years, the development of new techniques has led to new discoveries of cell fates and cell functions, which has changed our view of hematopoiesis.

When conducting research in the field of hematopoiesis it is important to keep in mind the known functional differences between fetal and adult hematopoiesis, and recognize that there are still undiscovered differences. This knowledge is especially important for the understanding as well as development of treatment, and potentially prevention, of childhood leukemia.

The aim of this thesis is to further characterize and compare stem and progenitor cells in fetal, neonatal and adult hematopoiesis including lineage-biased HSCs.

1 Background

1.1 Hematopoiesis

Hematopoiesis is the hierarchically organized process of continuous blood cell formation in which all types of mature blood cells are produced, and the term hematopoiesis comes from the conjunction of the two Greek words haima (blood) and poiesis (to make). The functions of the blood and immune system are carried out by the different mature cell types that form the hematopoietic system, i.e. erythrocytes, platelets, innate immune cells (e.g. neutrophils and monocytes) and adaptive immune cells (lymphocytes, B cells, and T cells). Within the hematopoietic system, multiple mature cell lineages can be produced by rare multipotent hematopoietic stem cells (HSCs) with remarkable self-renewal capacity, responsible for the lifelong replenishment of all blood cell lineages.[1] Since most mature blood cells are short-lived, they must be continually produced to keep the hematopoietic system in balance. It is estimated that nearly 90% of the more than 100 billion cells replaced in the average adult human body each day, are blood cells.[2] From HSCs, intermediate progenitor cells are generated and have the capacity to, in multiple stages with increasing lineage-restriction, differentiate into a large number of mature blood cells with specialized functions.[1]

The majority of studies referenced in this literature review are conducted on mice, however most findings are similar between murine and human hematopoiesis.[3]

1.2 Hematopoiesis during fetal development

Understanding the differences between fetal and adult hematopoiesis requires attention to both the location and developmental timepoint for hematopoietic cell emergence. In contrast to adult hematopoiesis, which occurs in the bone marrow (BM), fetal hematopoiesis takes place in distinct anatomical locations and in temporally overlapping waves.[4,5] These anatomical niches includes both intra- and extra-embryonic locations, contributing to the complexity of fetal blood production. Furthermore, the different progenitor waves are controlled by distinct molecular mechanisms.[5] Importantly, it is only the intra-embryonic hematopoiesis which can sustain lifelong hematopoieis.[6–8] Figure 1 provides an overview of the emergence of hematopoietic stem and progenitor cells in the mouse embryo.

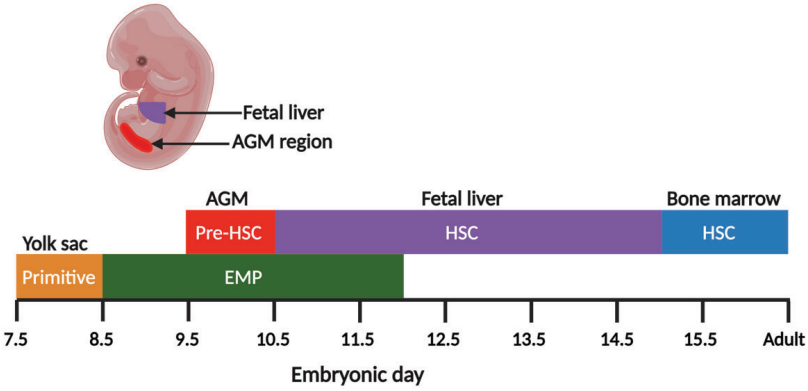

Figure 1. Overview of the emergence of hematopoietic stem and progenitor cells at different hematopoietic sites in the developing mouse embryo. AGM, aorta-gonad-mesonephros; EMP, erythro-myeloid progenitor; HSC, hematopoietic stem cell; Pre-HSC; precursor hematopoietic stem cell. Adapted from Elsaid *et al.*[9] Figure created with BioRender.com

1.2.1 Primitive hematopoiesis

The term primitive hematopoiesis has been inconsistently used in the literature.[10] In this text, the term refers to the development of mature blood cells from progenitors that emerge directly from the embryonic mesoderm without passing through an endothelial-to-hematopoietic transition (EHT).[5,10]

In mice, the first hematopoietic wave begins in the extra-embryonic yolk sac (YS) at embryonic day (ED) 7-7.5.[11-13] The first hematopoietic cells, detected in the formation of blood islands, are erythroid progenitors independent of the key transcription factors runt-related transcription factor 1 (RUNX1)[14] and transcriptional activator Myb (MYB).[15] Embryonic erythroblasts differ from adult erythroblasts in that they are large nucleated cells synthesizing embryonic globin. These erythroblasts temporarily expand in the YS until entering the circulation as cardiac contractions initiate at ED8.25.[16,17] In parallel with the erythroid progenitors, primitive megakaryocyte[18,19] and macrophage[12,20] progenitors also arise. The main functions of the first wave of primitive hematopoiesis are to provide the developing embryo with erythroid cells for oxygen transport, megakaryocytes for vascular integrity and macrophages for tissue remodeling.[5,10]

1.2.2 Transient hematopoiesis

The second wave of hematopoiesis starts in the YS around ED8.5.[5] It is transient and definitive, with the term definitive referring to progenitors emerging via EHT.[10]

This wave is characterized by the generation of erythro-myeloid progenitors (EMPs) through EHT, a process where individual hemogenic cells round up and detach from the endothelial layer.[21-24] These definitive EMPs are proposed to originate from a distinct hemogenic endothelium cell type that lacks the ability to give rise to HSCs.[25] The EHT process is RUNX1-dependent,[26] but in contrary to the emergence of definitive HSCs, not dependent on the transcription factor MYB when the EMPs in the YS differentiate into megakaryocytes and macrophages.[27,28] EMPs will hereafter relocate to the fetal liver (FL) and take part in the generation of erythrocytes, megakaryocytes, monocytes, granulocytes, macrophages, and mast cells until replacement of cells produced from HSCs.[28,29] Notably, this differentiation process is MYB-dependent.[30] Fate mapping models have shown that EMPs give rise to tissue-resident macrophages that last for life and are active contributors to the innate immune system, which also applies for their adult derived counterparts with specialized functions.[28,31-34]

Subsequent progenitors from the second wave of hematopoiesis emerge around ED9.5, possessing both lymphoid and myeloid potential prior to the emergence of definitive HSCs.[35,36] The development of cells with lymphoid potential is further described in section 1.6 (Fetal B lymphopoiesis).

Recent studies have also identified emerging multipotent progenitors (MPPs) in parallel with the emergence of HSCs. The earliest MPPs have been indicated at ED9-ED10 in the para-aortic-splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) region.[37] Similarly, a population of embryonic MPPs (eMPPs) have been identified in the YS and AGM region at ED10.5 with hematopoietic contribution into early adulthood.[7]

In mice, the change from primitive to definitive hematopoiesis is characterized by the change from embryonic to adult hemoglobin composition in red blood cells, which also enucleate in the FL before going into circulation.[38] Altogether, the two first waves of hematopoiesis emerging in the YS provide the embryo with important blood cells before the onset of HSC activity.[5,10]

1.2.3 Definitive hematopoiesis

A third and definitive wave of hematopoiesis is established within the mouse embryo in the AGM region.[5,6] In this wave, HSC activity, including self-renewal, multipotent differentiation and long-term (LT) repopulation capacity, is detected.

At this stage, blood circulation is established and the first definitive HSCs, derived from hemogenic endothelium in the ventral wall of the dorsal aorta,[21] emerge at ED10.5 in the mouse embryo and around 30 days post fertilization in humans.[39–41] Generation of HSCs by EHT has been visualized in live-imaging studies, showing how endothelial cells begin to expand into the arterial lumen before they detach into circulation.[22] HSC precursors (pre-HSCs), which will mature into HSCs, are also present.[42,43] Around the same developmental time point, at ED10.5-ED11, definitive HSCs are found in other intra-embryonic locations, such as the major arteries,[44] the head,[45] as well as the placenta.[46,47]

1.2.3.1 Fetal liver hematopoiesis

During fetal development, the FL is the major site for hematopoiesis until birth where HSCs expand and erythroid and lymphoid progenitors differentiate.[5] Seeding of the FL starts around ED9.5 with EMPs migrating through blood circulation.[28] HSCs generated in the AGM are detected in the FL from ED11, which is demonstrated by LT multilineage repopulation capacity.[39,40] However, HSCs with the ability to reconstitute myeloablated adult recipient mice are first detected in ED12.5 FL.[48] The HSCs will undergo maturation, differentiation and expansion in the FL between ED12-ED16,[48–51] after which the reconstitution capacity decreases as the HSCs start to migrate to the spleen and BM.[49,52,53] It is unknown why the FL is a temporary site for hematopoiesis, however the FL tissue does protect and support HSCs through several microenvironmental factors. Within the FL, the HSCs are situated adjacent to Nestin-positive perivascular cells, supporting HSC expansion in vivo.[54] FL progenitor cells have been reported to express stem cell factor (SCF) and sustain cultured HSCs in vitro.[55] In addition, FL bile acids provide protection to the expanding HSCs.[56]

1.2.3.2 Fetal bone marrow hematopoiesis

After maturation and expansion in the FL, HSCs re-enter the circulation and start to seed the spleen as well as the fetal BM at ED15.5.[52,53,57,58] HSC clonogenic

potential in fetal BM is reported at ED15.5, while the first transplantable HSCs are not detected until ED16.5 and remain very rare until the time of birth.[47,53,58,59] The understanding of the signals instructing or allowing HSCs to relocate from FL to fetal BM is still restricted. However, the chemokine stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 are important for attraction of HSCs to the BM.[52] This function clearly differs between FL and fetal BM, which is demonstrated by SDF-1 deficient embryos having normal numbers of FL HSCs and yet severely reduced colonization of the BM.[60] Upon reaching their final destination in the fetal BM, the HSCs are affected by environmental factors in the BM, the so-called HSC "niche". Both non-hematopoietic cells and hematopoietic cells contribute to the HSC niche, which impacts regulation of HSC activity and quiescence.[61,62] Fetal HSCs settle in the vascularized areas of the fetal BM and have been shown to require osteolineage cells to support their function.[53] HSC numbers continue to increase until 3-4 weeks post birth, the timepoint when the properties of fetal HSCs are considered to shift to quiescent adult HSC properties.[63] Adult HSCs mainly reside in a perivascular niche in the BM, where the niche provides important factors for maintenance of HSC quiescence.[61,62]

1.2.3.3 Fetal spleen hematopoiesis

The fetal spleen is less studied than other sites of fetal hematopoiesis. Seeding macrophage progenitors arrive in spleen at ED12[64,65], followed by reconstituting HSCs colonizing the spleen from ED15.5.[52,57] Spleen hematopoiesis can be sustained into adult life and can contribute to extramedullary hematopoiesis in situations of hematopoietic stress.[57,66] B cell progenitors have been detected in the spleen from ED13, though their origin has been uncertain.[65,67] One study demonstrated that fetal spleen stroma could promote B-cell differentiation, which indicated that already committed B cell progenitors from FL arrive in the spleen for further differentiation.[67]

1.3 Human fetal hematopoiesis

To investigate the human hematopoietic development and to make adequate comparisons, a staging system for the developmental age of an embryo is needed. Most published studies on early hematopoiesis and brain development use the established Carnegie stage (CS) classification system, in which the first 8

embryonic weeks are subdivided into 23 stages based on the size of the embryo and developmental description.[68]

Similar to the mouse embryo, hematopoiesis in the human embryo is first detected in the YS, where erythroid and myeloid progenitors emerge around CS7-CS8 (16-18.5 days after fertilization).[69,70] The first human HSCs, defined by their capacity of LT multilineage repopulation, are likewise first detected in the AGM at CS14 (comparable to ED10.5 in the mouse), and later in the YS and placenta around CS16.[3,41,71,72] Hematopoiesis relocates to the human FL, where low numbers of HSCs can be detected at CS17.[3,41,72,73] The human fetal BM is suggested to be colonized by myeloid progenitors at 8-10 weeks, and HSCs probably migrate to the human fetal BM around 12 weeks post-fertilization.[74] A recent study combining single-cell transcriptomics and HSC transplantations detected LT engraftable HSCs at 12 weeks post-fertilization.[75] For the rest of the human life, hematopoiesis occurs in the BM.[76]

1.4 Hematopoietic stem cells

In mice and humans, HSC transplantation assays have long been the gold standard for the functional definition of HSCs in vivo, where HSCs are commonly defined as cells with sustained self-renewal activity that possess multilineage reconstitution potential and can give rise to LT multilineage repopulation in myeloablated recipients also at the single cell level.[1,77,78] The self-renewal capability can be described as the HSCs ability to generate new HSCs by asymmetric or symmetric self-renewal division, while multipotency refers to the ability of HSCs to generate different mature blood cell lineages via an asymmetric or symmetric "commitment" HSC division and subsequent differentiation.[78,79] Studies have demonstrated that these functional properties are connected to different stem cell states: quiescent HSCs, which serve as a reservoir of HSCs, and active HSCs, which develop into progenitor cells.[78,79]

HSCs can be phenotypically defined by expression of a combination of immunophenotypic markers on their cell surface, however, several of these markers change during the different developmental stages of hematopoiesis. In recent years new methods, including lineage tracing of genetically modified mice and detection of molecular signatures, have opened up new ideas for the definition of functional HSCs.[78,79] For example, it has been suggested that a new

definition of HSCs could be cells capable of contributing to lifelong steady-state blood production.[51]

The fetal and the adult hematopoietic systems operate under very different conditions. While the fetal system is experiencing expansive changes and needs during a limited time span, the adult blood system mostly works under static or steady-states conditions. Building a blood system from the very beginning requires different processes in fetal hematopoiesis than in steady-state adult hematopoiesis. The faster passage through the blood system for fetal HSCs was convincingly shown with lineage tracing in a *Tie2* mouse model.[80] Just one week after birth, the progenitors originating from FL HSCs labeled at ED10.5 made a significant contribution to the peripheral blood (PB). This is in contrast to adult HSCs which took months to show considerable contribution.[80]

1.4.1 Models of the hematopoietic hierarchy

The prevailing view has been that HSC differentiation follows a linear hierarchy where LT-HSCs first evolve into ST-HSCs, which in turn form MPPs, followed by lineage commitment in which MPPs branch into oligo-potent and unilineage progenitors.

In general, the lineage potential of a cell is defined as the capacity to differentiate along a certain lineage, while lineage commitment is the process by which an HSC or multipotent progenitor cell starts to express the signature genes of one lineage and downregulates the expression of other lineage associated genes, and commits towards the generation of cells of a specific blood cell lineage. Lineage restriction is defined as the process by which a multipotent HSC or progenitor loses the ability to differentiate into one or more cell lineages, eventually becoming fully restricted towards one single blood lineage.[81]

Hematopoietic models have classically been depicted as a hierarchical tree of hematopoiesis, which subsequently has been modified based on new findings along with the introduction of other cell surface markers for definition of hematopoietic stem and progenitor populations.[78] In the initial version, the first division separated lymphoid potential from all other cell lineages (erythroid, myeloid and megakaryocytic lineage cells). The modifications have included identification of intermediate-term (IT) HSCs between the LT-HSCs and ST-HSCs,[82] myeloid fate decisions being moved to a later branchpoint,[83,84] earlier

megakaryocyte branching,[85,86] and the MPP compartment being fractionated into separate subpopulations.[85,86] Further complicating the described hierarchical tree is the functional and molecular heterogeneity seen among HSCs.[79] In recent years, the development of new techniques has further challenged the view of hematopoiesis as a clearly defined and stably organized structure. The most recent view can be described as a constellation of heterogenous hematopoietic populations, still in a hierarchical organization but with a continuum of differentiation instead of clear differentiation steps between the different progenitor populations.[78,79] Figure 2 shows two examples of hierarchical models of hematopoiesis.

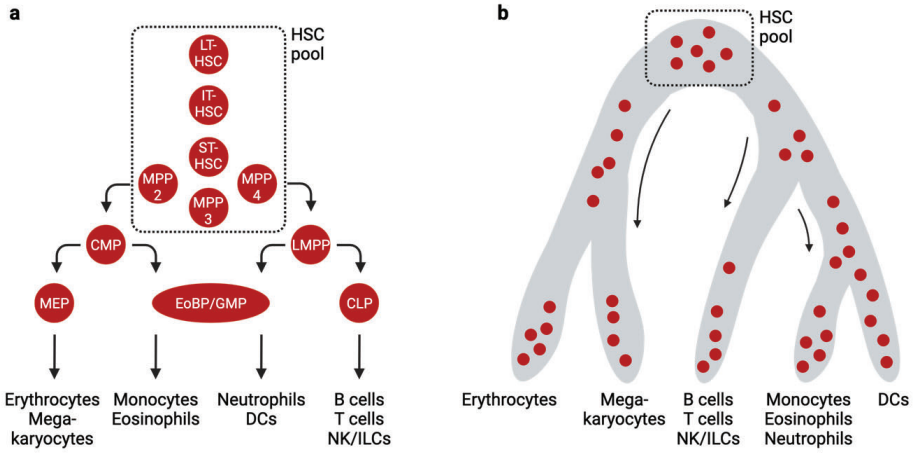

Figure 2. Models of the hematopoietic hierarchy. **a)** The well-known hematopoietic hierarchy model with hematopoiesis depicted as a branching tree with stepwise differentiation. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DCs, dendritic cells; EoBP, eosinophil–basophil progenitor; GMP, granulocyte–monocyte progenitor; HSC, hematopoietic stem cell; ILCs, innate lymphoid cells; IT-HSCs, intermediate-term HSCs; LMPP, lymphoid-primed multipotential progenitor; LT- HSC, long-term HSC; MEP, megakaryocyte–erythroid progenitors; NK, natural killer cells; ST-HSCs, short-term HSCs. **b)** Hematopoiesis depicted in a tree-like shape, but with a continuum of differentiation. The red dots represent single cells and their different differentiation trajectories. Adapted from Laurenti and Göttgens.[78] Permission obtained from the publisher. Figure created with BioRender.com

1.4.2 HSC transplantation assays functionally define HSCs

As previously mentioned, HSC transplantation assays have long been the gold standard for identification and functional characterization of HSCs.[1] By transferring a single donor cell together with support cells, into a recipient that lacks a functional hematopoietic system, the HSC-renewal and multilineage potential of the donor cell can be determined at the single cell level.[87]

When a functional HSC is transferred into a lethally irradiated (or immunodeficient) recipient mouse, the cell will engraft and present multipotent differentiation into mature blood cell lineages, and through their self-renewal ability also sustain LT hematopoiesis.[88] Serial transplantations can discriminate between HSCs with LT and short-term (ST) reconstitution by evaluating the self-renewal capacity.[88] In comparison, hematopoietic progenitor cells will upon transplantation only reconstitute the hematopoietic system to a limited degree and for a very brief period.[78] Different types of hematopoietic stem and progenitor cells (HSPCs) have been phenotypically and functionally defined based on their demonstrated reconstitution capacity and in vitro differentiation potentials.[78]

To monitor the ability of HSCs to replenish hematopoiesis, the transplantation assay is used in combination with the CD45-congenic system, which enables discrimination between white blood cells originating from the donor and the recipient. The method uses two different alleles of the cell surface protein CD45, CD45.1 and CD45.2, which can be separated by monoclonal antibodies. CD45 is expressed in B cell, T cell and neutrophil-monocyte lineages and can thereby distinguish these lineages.[77] With flow cytometric analysis, the progeny of single-cell transplanted HSCs can be traced, and this made it possible to demonstrate that single mouse BM HSCs (defined as $CD34^{-/lo}Lin^{-}SCA1^{+}KIT^{+}$) are multipotent and self-renewing.[87] Subsequently, the use of additional cell surface markers has enabled a higher purity when isolating functional HSCs in FL and adult BM, where most often the $LSKCD150^{+}CD48^{-}$ phenotype is used to purify HSCs,[89] as described in section 1.4.3. (Cell surface markers for identification of fetal and adult HSCs).

1.4.3 Cell surface markers for identification of HSCs

Advances in flow cytometry have made it possible to develop and refine the phenotypic identification of HSPCs based on their cell surface expression of immunophenotypic markers.[89]

Both adult BM and ED14.5 FL HSCs are found within the Lineage$^-$SCA1$^+$KIT$^+$(LSK) population.[90,91] Addition of CD48$^-$ and CD150$^+$ signaling lymphocytic activation molecule (SLAM) marker expression can be used for identification of FL as well as adult BM HSCs within the LSK compartment.[92,93] Whereas adult BM HSCs are negative for the myeloid lineage marker CD11b, a majority of FL HSCs are positive for CD11b.[91] The CD11b-positivity could be associated with a higher cycling activity, as shown after 5-fluorouracil (5-FU) treatment when HSCs upregulate their cell surface expression of CD11b and upon entry into active cell cycle.[94] For the adult BM HSC compartment a higher FMS-like tyrosine kinase 3 (FLT3) expression is linked to reduced self-renewal capacity and erythroid lineage potential.[83,95] This has also been demonstrated for most FL HSCs at ED14.5,[96] and both fetal and adult HSCs can be defined as FLT3-negative. Notably, lineage tracing has later suggested that there is a rare, but transient, FLT3-positive FL HSC population.[97] An important marker for identification of adult BM HSCs in the LSK population is CD34-negativity, in contrast to the CD34-positivity in FL HSCs which diminishes with developmental age.[98] Furthermore, the endothelial protein C receptor (EPCR/CD201) is expressed in both fetal and adult LT-HSCs and can be used to further select for HSCs in the LSKCD150$^+$CD48$^-$ cell population.[59,89] In addition, von Willebrand factor (*Vwf*) expression enriches for LT-HSCs.[99–101] *Vwf* is expressed in approximately 60% of adult BM LSKCD150$^+$CD48$^-$CD34$^-$ cells and almost all ED14.5 FL LSKCD150$^+$CD48$^-$ cells.[100]

The described alterations in cell surface marker expressions during development can reflect different functional properties together with distinct environmental factors between FL and adult BM. Of note, fetal BM hematopoiesis overall is less studied than FL and adult BM hematopoiesis. Importantly, the shifts in HSC cell surface markers during development complicates molecular comparisons between HSCs at different developmental timepoints.

1.4.4 HSC properties change during development

Several studies have illustrated that functional characteristics of HSCs change during ontogeny and adulthood.[102,103] These characteristics, such as cycling activity and regenerative capacity, reflect different transcriptional and proteomic patterns during development.[104,105]

Linage tracing experiments show that the precursors of HSCs, termed pre-HSCs, labeled at ED9.5-ED10.5 are the major origin of adult BM HSCs[80,106,107] and further

generation of new HSCs after ED10.5 has not been demonstrated.[108,109] In the mouse hematopoietic system, a switch in HSC behavior has been described between 3 and 4 weeks of age. This change from a fetal to an adult HSC state includes a decrease in self-renewal, proliferation, and repopulation capacities.[63,102,110] At the same developmental time point, there is also a reported change from fetal to adult lymphopoiesis including a shift to interleukin-7 (IL7) dependence.[111–113]

Since there is no new generation of HSCs after the fetal period, the adult BM HSC population needs to balance its own population size through self-renewal.[108,109] This requires adjustment of differentiation in response to external signals with self-renewing divisions as well as cell death and transition of cells from the BM compartment. To enable HSC maintenance, a strict regulation of the numbers of HSCs is needed to safeguard the genome integrity during the whole life of an organism. Adult BM HSCs ensure their LT sustainment by staying in a quiescent state with low biosynthesis and few or no cell divisions.[78]

The reported alterations in transcriptional programs during development affect genes with crucial functions in fetal HSCs (that are down-regulated in adult HSCs), and genes with crucial functions in adult HSCs (that are down-regulated during fetal life), such as *Sox17*,[114,115] *Lin28b*,[116,117] *Hmga2*,[117] *Ezh2*,[118] *Pten*,[119] *Bmi1*,[120] and *Cebpa*.[121] Several of the reported changes occur during the same developmental period, between 3 and 4 weeks of age in the mouse.[102] Transcription factors highly expressed in fetal HSCs are often related to proliferation, in contrast to those with increased expression after the developmental switch, which are predominantly associated with preservation of HSC quiescence.[103]

1.4.5 Fetal HSCs show a high cycling activity

Some of the early studies comparing hematopoietic cells present in fetal and adult tissues examined colony-forming unit spleen (CFU-S), a multipotent transient amplifying cell. The fetal CFU-S showed high cycling activity, in contrast to the very low cycling activity in the unperturbed adult.[122] Later experiments with transplantation of CFU-S from mouse FL showed a similar result.[123]

Fetal HSCs display a high cycling activity consistent with fetal CFU-S,[91] while adult BM HSCs develop into a mostly quiescent population.[117,124] In the ED14.5 FL the estimated fraction of cycling HSCs is close to 100% compared to approximately

10% in the adult BM.[63] In accordance, FL HSCs demonstrate a high protein synthesis rate,[56] while adult BM HSCs have a very low protein synthesis rate.[125] At the same time, the FL proteome is less complex than in adult HSPCs.[105]

The cycling status of fetal mouse HSCs does not seem to be dependent on the location as the cycling status stays unaltered when the HSCs transition from the FL to the fetal BM. Instead, it has been established that the cycling activity changes 3 weeks after birth within a one week period.[63,110] The transcription factor C/EBPa is required for adult HSC quiescence, and a congruent change in the *Cebpa* gene expression pattern in HSCs is reported 2 to 4 weeks after birth.[121] The rate at which mouse HSCs expand post-transplantation also declines dramatically between 3 and 4 weeks of age.[110] In humans a possible correlation is a change in the rate of leukocyte telomere shortening is reported between one and 3 years after birth,[126,127] which can be considered as an indirect marker of HSC division history. Moreover, studies show that adult BM HSCs that have undergone fewer cell divisions in general have higher capacity for self-renewal and reconstitution after transplantation compared to HSCs with a history of less restricted proliferations.[124,128] Nevertheless, FL HSCs have both a higher self-renewal and repopulation capacity than adult HSCs and concurrently demonstrate active cycling.[129,130]

1.4.6 Fetal HSCs show a higher regenerative activity than adult HSCs

ED14.5 and ED18.5 fetal HSCs are distinct from adult BM HSCs in their capacity to repopulate transplanted irradiated recipients.[110] One hypothesis is that the higher regenerative activity seen in fetal HSCs depends partially on a higher in vivo self-renewal capacity of the FL HSCs, where FL HSCs show a higher regenerative activity independent of the numbers of initially transplanted HSCs.[131] The high regenerative activity declines in parallel with the decline in proliferative activity between 3 and 4 weeks of age.[110]

Previous studies have demonstrated a high expansion of HSCs in the FL,[49,52,63,91] however, the results from these studies have lately been questioned.[62,109] The studies demonstrating high expansion of HSCs were based on limiting dilution transplantation assays, which functionally assessed HSCs by their reconstitution potential.[49,52,63,91] When transplanting FL cells into an adult BM niche, which is different from a perinatal BM niche, this could affect the HSC reconstitution potential.[62] It was demonstrated that transplantation of AGM-derived cells into

neonatal mice resulted in higher numbers of the measured repopulating units than transplantation into adult recipients, suggesting that the developmental stage of the niche is significant for the experimental outcome.[132] In addition, this type of transplantation model will always measure the potential capacity of the cell instead of the cell function in an unperturbed setting. A study using Confetti allele-based in vivo lineage tracing indicated that HSCs only had a limited expansion at the FL stage, and that the investigated FL HSCs were designated to differentiate rather than to self-renew, in comparison to subsequent BM HSCs[51]. In line with these findings, a study from Rybtsov *et al.*[48] suggested that the increase of repopulating HSCs between ED11.5 AGM and ED12.5 FL is more likely an effect of maturation of immature HSCs than rapid expansion in the FL.

Nonetheless, other studies have indicated that the distinct regenerative capacity of fetal and adult HSCs could be controlled by a reduced expression level of *Lin28b*, which is a negative regulator of let-7-miRNA biogenesis and consequently the let-7-target Hmga2.[116,117] Interestingly, *Lin28b* is also reported to be involved in the developmental switch between fetal and adult B-lymphopoiesis.[113]

1.4.7 Concept of lineage-biased and lineage-restricted HSCs

1.4.7.1 Heterogeneity in HSC differentiation

Single-cell HSC transplantation experiments in combination with cellular barcoding experiments have demonstrated heterogeneity in HSC differentiation with a more myeloid-biased (neutrophil-monocyte) or lymphoid-biased (B cell and T cell) output, which resulted in the concept of lineage biased HSCs.[133-136]

Dykstra *et al.*[134] categorized HSCs into 4 subtypes (named α, β, γ and δ) based on their relative contributions to the total number of myeloid versus lymphoid PB cells found 4-6 months after single HSC transplantation. Through secondary transplantations they could establish that the different contribution patterns were preserved by daughter HSCs, which indicated that the distinct patterns are intrinsically maintained within the individual stem cell and its progeny.[134] Single cell transplantations, non-limiting transplantations and DNA barcode labeling[137-139] verified the HSC differentiation pattern described by Dykstra *et al.*,[134] but deviations from the original HSC pattern have also been reported in serial single HSC transplantations.[59] Notably, only if the transplanted single cell contributes to continued production of cells of the short-lived mature myeloid blood lineages,

does it fulfill the functional definition of an HSC with long-term self-renewal ability, which also can be sustained in secondary transplants.[133,134,140] Overall, these findings could correspond to an original epigenomic signature that replenishes similar lineage patterns from new generations of HSCs.[141]

1.4.7.2 HSC erythrocyte and platelet reconstitution give new insights into HSC dynamics

The development of transgenic donor mice with fluorescent labeled cells made it possible to also follow erythrocyte and platelet reconstitution in transplantation experiments,[100,101,140,142] which have contributed to new insights into HSC dynamics. Yamamoto *et al.*[140] identified a group of myeloid-restricted repopulating progenitor cells that only regenerate cells within the platelet, platelet-erythrocyte or platelet-erythrocyte-myeloid lineages. These repopulating progenitor cells reconstituted distinct parts of the hematopoietic system, but did not display reconstitution in serial transplantations.[77,101,140] Other studies established that myeloid restriction can also occur within functional HSCs.[101] Lineage-bias should be distinguished from lineage-restriction, where the myeloid-biased HSCs are multipotent HSCs that give a dominant myeloid output while myeloid restricted stem cells only produce myeloid progeny. Importantly, it is only the platelet lineage that always corresponds to HSC self-renewal capacity measured by serial transplantations.[101,140]

Sanjuan-Pla *et al.*[100] demonstrated that expression of *Vwf* identifies HSCs with higher platelet output. These *Vwf*-positive HSCs includes both α-HSCs (lymphoid deficient/myeloid biased HSCs) and β-HSCs (balanced HSCs) as well as platelet-biased and even platelet-restricted HSCs.[100,101] Moreover, *Vwf*-negative HSCs contains multipotent and lymphoid-biased but not platelet-biased or platelet-restricted HSCs.[100,101] In the study by Sanjuan-Pla *et al. Vwf*-positive HSCs gave rise to *Vwf*-negative HSCs and not vice versa,[100] which implicates a hierarchical relationship between the HSC subtypes and corresponds to the prior finding that α- and β-HSCs can generate γ/δ-HSCs but not the opposite.[134]

1.4.7.3 HSC lineage replenishment changes during development

In serial transplantation experiments the distinct differentiation patters originating from a single HSC are reported to be different when transplanting fetal or adult HSCs. Benz *et al.*[59] investigated the developing FL in mouse with a single HSC

transplantation approach and found that the α-HSCs (lymphoid deficient/myeloid biased HSCs) constituted less than 5% of all HSCs and less than 10% of the LT-HSCs. Interestingly, just before birth α-HSCs were much more prevalent in the initial HSC population in the fetal BM.[59] During adulthood and aging the composition of the HSC compartment continues to change and is reflected in the PB by a successive decrease in mature lymphoid cells.[59] In comparison, when transplanted into immunodeficient mice, human HSCs from aged people give rise to a higher proportion of myeloid relative to lymphoid blood cells than younger HSCs do.[143] The reason for change in lineage bias of fetal HSCs around birth is unknown, but a higher probability of survival of α-HSCs compared to β-HSCs when exposed to the BM niche has been discussed together with generation by transformation from other HSC subtypes.[103]

Moreover, the erythroid and platelet outputs from single fetal and neonatal HSCs have not been studied. However, one study investigated LT-reconstitution, including erythroid and platelets, after transplantation of low numbers of perinatal liver or BM HSPCs in comparison with adult BM adult, analyzing the function of the fetal BM niche.[58]

1.4.8 HSC contribution to native hematopoiesis

The improvement of cellular barcoding and lineage-tracing tools have enabled important observations of functional HSC activity and lineage output in an unperturbed (non-transplantation) setting.[80,107,144,145] These studies have suggested that BM HSCs give very limited contribution to steady-state hematopoiesis and indicated that phenotypic progenitor populations (especially MPPs) are the major contributors to blood formation in native hematopoiesis.[80,145] In addition, investigation of all five blood lineages within a molecular barcoding system revealed some HSCs to exclusively regenerate platelet precursors (megakaryocytes) in unperturbed hematopoiesis, with an estimated contribution of half of the megakaryocytes via a suggested "myeloid-bypass" pathway.[145] More recent studies investigating the native embryonic hematopoiesis have also implicated that hematopoietic progenitor cells can sustain embryonic hematopoiesis independently of HSCs.[7,8] Together these findings suggest that hematopoietic progenitor cells under native conditions can sustain steady-state hematopoiesis independently of HSCs. However, this has in part been questioned based on contrasting findings and due to technical limitations of the models

applied.[81,146,147] Figure 3 provides a schematic picture of a suggested time window of lineage contribution of hematopoietic progenitor cells (HPCs) and hematopoietic stem HSCs.[148]

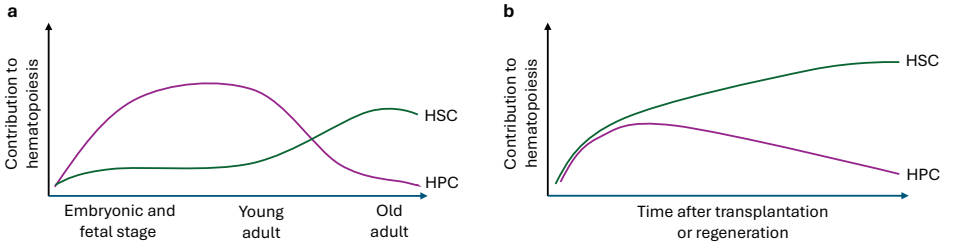

Figure 3. Lineage contribution of hematopoietic progenitor cells and hematopoietic stem cells. Schematic presentation of contribution to hematopoiesis (y-axis) from hematopoietic progenitor cells (HPCs, purple line) and hematopoietic stem cells (HSCs, green line) under **a)** native conditions, and **b)** hematopoietic stress conditions such as hematopoietic cell transplantation. X-axis represents different developmental ages. Adapted from Gao *et al.*[148] Permission obtained from the publisher.

1.5 Fetal B-cell development

The adult adaptive immune system consists of many different lymphocyte populations with distinct antigen receptor profiles, varying functions, life span and tissue location. Together they form an efficient system for immune protection and self-regulation. Some of the mature lymphocyte subsets are produced by BM HSCs throughout life, while other arise exclusively during a limited developmental period in fetal and early postnatal life and are thought to replenish by self-renewal.[149,150] The developmental changes in B-cell lymphopoiesis affect immune regeneration and may also be involved in vulnerability for leukemic development during early development.[151]

1.5.1 B-cell development is antigen dependent or antigen independent

B-cell development can be separated into two phases, an antigen independent and an antigen dependent phase. In the antigen independent phase, HSCs will differentiate through distinct progenitor stages into mature B cells expressing a functional B-cell receptor (BCR) with immunoglobulin (Ig) heavy chain (H) and light

chain (L).[152] During the antigen dependent phase, the BCR will bind to an antigen. Hereafter, the B-cell clone will in a T-cell independent or T-cell dependent manner expand in a germinal center in the spleen.[150] Affinity maturation and Ig class switching precede the clonal expansion with production of large amounts of antibody secreting plasma cells.[150]

1.5.2 Mature B cells and their developmental origin

Mature B cells are separated into three main populations, B-1 B cells, follicular (FO) B cells and marginal zone (MZ) B cells. Both FO B cells and MZ B cells are known as B-2 cells, whereas B-1 B cells are further subdivided into B-1a cells ($CD5^+$) and B-1b ($CD5^-$) cells based on their CD5 expression.[152,153] Notably, B cell subsets that arise during different stages of fetal development create distinct parts of the adult antibody defense. Innate-like lymphocyte subsets, for example $\gamma\delta$ T cells and B-1a cells, emerge during fetal development and have important functions in the interface of adaptive and innate immunity.[151,154] These cells often display self-reactive antigen receptors, have a tissue resident localization and respond rapidly to antigen. B-1a cells mainly reside in the pleural and peritoneal cavities in mice and act against phosphatidylcholine found in cellular membranes and bacteria cell walls.[151] Independent of T cells, B-1a cells secrete natural IgM antibodies with the function to clear microbes and cellular debris.[152] The B-1a cell pool decreases after the neonatal period and is mostly sustained via self-replenishment. In contrast, follicular B-2 B cells are continually sustained via BM progenitors.[146,147]

1.5.3 B-cell staging

During the differentiation process from multipotent HSCs and progenitors into a fully mature B lymphocyte, rearrangements of the Ig H and L chains are required to generate functional BCRs. In utero, B-cell development occurs in the FL before migration to the BM just before birth,[155] while in the adult the first steps of B-cell differentiation take place in the BM. The staging of B-cell development is based on the molecular rearrangement status of the immunoglobulin loci.[156]

Development of multicolor flow cytometry enabled identification and characterization of different B-cell progenitor stages based on cell surface markers. In the "Philadelphia" B-cell classification, Hardy *et al.* originally sub-divided B cell progenitors based on cell surface expression of B220, CD43, CD24, BP-1 and IgM.[157] In the subsequent "Basel" B-cell classification, Melchers and

colleagues included the mast/stem cell growth factor receptor Kit (KIT) and CD25 into their B-cell staging and subsequently used a different nomenclature.[158,159] With further development of flow cytometry, more markers have successively been included in the identification and staging of B cell progenitors.[156,160,161] B-cell staging in the mouse has primarily been established in adult BM, however, the Hardy staging has been suggested to also apply to fetal B progenitor cells but has only been investigated at late developmental stages in the FL.[162,163] In this thesis the Hardy nomenclature is used. An overview of Ig gene rearrangements and expression of cell surface markers at the different B-cell developmental stages is provided in Table 1.

Table 1. Immunoglobulin gene rearrangements and cell surface marker expression during B-cell developmental stages.

Cell stage		Status of Ig genes	Surface Ig	AA4.1	B220	CD43	CD24	KIT	IL7RA	CD19
PreProB	Fraction A	Germ line arrangement	None	+	+	+	+	+	+	–
Early ProB	Fraction B	D_HJ_H	None	+	+	+	++	++	++	+
Late ProB	Fraction C	Some $V_HD_HJ_H$	None	+	+	+	++	++	++	+
Large PreB	Fraction C'	$V_HD_HJ_H$	Pre-BCR	+	+	+	+++	++	++	+
Small PreB	Fraction D	$V_HD_HJ_H$ & V_LJ_L arrangement	Decreased Pre-BCR	+	+	–	+++	–	++	+
Immature B	Fraction E	$V_HD_HJ_H$ & V_LJ_L	IgM-BCR	+	+	–	+++	–	–	+
Mature B	Fraction F	$V_HD_HJ_H$ & V_LJ_L	IgM-BCR IgD-BCR	–	+	–	+	–	–	+

Ig, immunoglobulin. AA4.1, B220, CD43, CD24, KIT, IL7RA, and CD19 are commonly used cell surface markers in B cell staging. The plus signs denote the degree of detected cell surface expression, while the minus signs denote the absence of detected cell surface expression of the indicated markers. Adapted from Rolink and Melchers[164], Hardy and Hayakawa[165], and Hardy *et al.*[156]

1.5.4 B-cell progenitor differentiation

In brief, common lymphoid progenitors (CLPs)[166] (Figure 2) differentiate into the first $CD19^+$ B progenitor cells, called ProB cells, as they go through VDJ recombination.[157,161] The VDJ recombination starts with rearrangement of the diversity (D) and joining (J) regions of the IgH gene and proceeds with

rearrangement of the variable (V) region to the recombined DJ region. This recombination process is primarily controlled by the recombination activating gene enzymes RAG1 and RAG2 and has also been demonstrated during earlier stages of B-cell development.[167-169] In addition, the established Hardy staging includes the CD19$^-$ PreProB cell stage in the differentiation step between CLPs and ProB cells.[170] The PrePro B cells have a cell surface marker expression similar to CLPs and in addition express low levels of B220.[165] This CD19$^-$ PreProB cell population contains progenitors with additional lineage potentials.[156,161,170] However, expression of B-lineage specific genes including *Mb1* (*CD79a*) encoding the Ig-alpha signaling subunit of the B-cell antigen receptor suggests that they might also contain early B-cell restricted progenitor cells, although this has not been further investigated.[161]

ProB cells continue to differentiate into PreB cells, which are characterized by the loss of cell surface expression of CD43 and completion of VDJ rearrangement (Table 1).[156,164,165] The PreB cells express the Pre-BCR, which is comprised of the rearranged IgH, a surrogate L chain (encoded by the *lambda5* and *VpreB* genes), and the Ig-alpha *CD79a* (encoded by the *Mb1* gene) and Ig-beta *CD79b* (encoded by the *B29* gene) subunits.[157,171] Expression of the *Rag1* and *Rag2* genes is downregulated once the signaling through the Pre-BCR is initiated, thereby completing the recombination process and leading to proliferation of the cells.[172,173]

PreB cells develop into immature B cells when the second Ig rearrangement is finalized and express a complete BCR of IgM isotype (Table 1).[157] Immature B cells will differentiate to mature B cells through a negative selection process in which cells with autoreactivity are deleted or go through editing of the BCR.[174] In the end, the mature B cells with IgM and IgD expression will move to the spleen and secondary lymphoid organs for their final maturation process and receive their role in the adaptive immune system.[150,175]

1.5.5 Differences between fetal and adult lymphopoiesis

It is well established that transplantations of unsorted FL cells replenish B-1a cells in irradiated recipients unlike adult BM cells.[176,177] Later independent studies with transplantation of adult hematopoietic stem and progenitor cells verified these findings and collectively showed that adult BM cells do not efficiently replenish B-1a cells in a transplantation setting.[163,178] It is hypothesized that fetal LT-HSCs slowly lose their capacity to give rise to B-1a cells in adult BM[179] and later studies have

suggested epigenetic modifications in the aging LT-HSCs.[116] Further studies using single cell transplantation assays showed that single adult BM LT-HSCs transplanted into lethally irradiated recipients fully reconstituted follicular B cells, marginal zone B cells, B-1b and PB B cells, but were not able to reconstitute tissue-resident B-1a cells.[180] Similarly, ED15 FL LT-HSCs specifically did not reconstitute B-1a cells but did regenerate all other mature blood lineages.[181] It is possible that the adult BM niche does not support B-1a development, but this is not considered likely since B-1a cells are generated in adult recipients after transplantation of unsorted FL cells.[177] To address this question, Ghosn *et al*.[181] transplanted sorted LT-HSCs into newborn recipients before the generation of host B-1a cells and co-transplanted sorted LT-HSCs together with unsorted FL cells.[181] In both conditions the LT-HSCs failed to reconstitute B-1a cells, which indicate that the ability of LT-HSCs to reconstitute may involve an intrinsic genetic program instead of an environmental response.[181] Other groups have investigated the origin of B-1a cells with other methods. By inducible genetic labeling of adult LT HSCs in a non-transplant setting, it has been demonstrated that LT-HSCs in unperturbed hematopoiesis fully reconstitute the hematopoietic system with exception for microglia and B-1a cells.[146,147] Another study used HSC-deficient transgenic embryos that could not develop LT-HSCs and showed that a minor part of B-1a cells can be regenerated without the presence of LT-HSCs.[182] Similarly, studies in embryonal tissues have established that B-1a progenitors can be detected before LT-HSCs emerge by demonstrating that ED9 mouse YS, P-Sp and AGM region cells preferably generate innate-like B-1a and MZ B cells and do not generate FO B cells.[183–185]

1.5.6 HSC independent B-cell lymphopoiesis

Heterogeneity in precursor origin is considered to be an important factor for developmental changes in lymphocyte production.[186] During the fetal period there are different waves of HSPCs that are active during overlapping windows of time and the process engages several sites of the hematopoietic system.[9,187] Several studies have verified that there are lymphoid cells of extra-embryonic and as well as intra-embryonic origin before the emergence of HSCs,[7,8,35,182,185] including yolk sac progenitors with lympho-myeloid restricted lineage potential,[35] and similarly, Yoshimoto *et al*.[185] demonstrated yolk sac progenitors with B-1a/MZ B-cell potential. It is still unclear if and in what way this heterogeneity in origin affects the properties of differentiated B cells.[9,36,187] In addition, recent lineage tracing studies

suggest that the contribution from HSC-independent MPPs to lymphoid lineages last into young adulthood and may be larger than previously assumed.[7,188]

1.5.7 HSC function during B lymphopoiesis

FL HSCs are the major origin of B cells after birth.[187] Around 3 weeks of age, the described switch of HSC properties takes place in mouse hematopoiesis.[63,110,117] These changes occur at the same time as there is a decrease in B-1 cell production in B lymphopoiesis,[112] which could indicate a link between loss of B-1 potential and changes in HSC properties. Kristiansen *et al.*[186] used cellular barcoding to track changes in the HSC plasticity and B-1a and B-2 cellular output over time. Single ED14.5 FL LSK-defined HSPCs were labeled with DNA barcodes that were inherited to all progeny and assessed for multilineage contribution in primary and secondary transplantations. In this clonal setting they could show functional FL HSCs that initially were able to produce both B-1a and B-2 cells but develop into B-2-restricted HSCs during development.[186] It should be noted that Kristiansen *et al.*[186] used different cell marker expressions for cell sorting at the first LSK labeling and the later transplantation experiments, which could affect the results and allows other interpretations of the findings.[187] Regardless, the findings from Kristiansen *et al.*[186] are in line with other clonal studies demonstrating that B-1a and B-2 cells have a common progenitor at the time when embryonic HSCs emerge.[107,188,189]

1.5.8 HSC heterogeneity and fetal B-cell output

A number of studies have investigated the differentiation of fetal HSCs with respect to fetal-like B-cell output.[97,181,186] Kristiansen *et al.*[186] demonstrated that only a minor part of the transplanted ED14.5 FL clones with serial reconstitution showed a detectable reconstitution of B-1a cells in the transplantation setting. Beaudin *et al.*[97] performed lineage tracing in a *Flt3*-expressing mouse model and identified a developmentally restricted FL HSC subtype labeled by cell surface expression of FLT3. This rare HSC population showed B-1a potential when transplanted but could not be traced after birth in a non-transplant setting.[97] Notably, the HSC subset identified by Beaudin *et al.*[97] did not fulfill the generally accepted definition of HSCs. Moreover, Ghosn *et al.*[181] used transplantation of ED15 FL cells and demonstrated that FL HSCs failed to reconstitute B-1a cells. Another study

reported that B-1a cells emerge from different fetal progenitors and indicated that B-1 and B-2 B cells have separate sensitivity to the transcription factor PU.1 depending on developmental origin.[190] This result strengthens the idea of fetal and adult B cells following different programs for their development. Together these studies suggest that fetal HSCs are heterogenous regarding their B-1a cell output and the long-standing question of HSC contribution to the long-lived B-1a cell pool still remains to be resolved.

1.5.9 Emergence of B-cell potential in the mouse embryo

To investigate where HSCs first emerge in the mouse embryo, lymphoid potential has been extensively studied using organ culture and stromal cell co-culture, as it was previously considered an indicator of HSC presence.[191,192]

An early study reported the emergence of multipotent hematopoietic cells with lymphoid cell potential in YS and P-Sp already at ED8.5.[193] The contribution of the YS to lymphoid development has for a long time been debated. At ED9.5, several studies have detected B-cell potential in YS.[35,185,194] The data from Yoshimoto *et al.*[185] did not support B-lineage restriction and a separate publication showed that the ED9.5 YS cells also possessed T-cell potential.[185,195] Moreover, other studies demonstrated that an early progenitor found at ED9.5 co-expressed myeloid and lymphoid lineage programs and gave rise to both myeloid and lymphoid lineages of the immune system.[35] In ED12.5 FL, a progenitor was previously identified possessing both B cell and macrophage potential, but no other myeloid potential.[196] This study did not investigate T cell potential, while in a follow-up study the identified progenitors could generate T cells which suggested multipotency.[197]

A main point of discussion has been whether the demonstrated lymphoid potential originates in the YS tissue or originates from the embryo proper given it is always detected after the start of circulation. Studies investigating embryos in a mouse model lacking circulation have detected lymphoid potential in the YS,[195] however these results do not agree with previous studies that indicate only the AGM and not the YS possesses B-, T-, or NK-lineage potential, also before the start of circulation.[6,198] Recently published fate-mapping studies indicate that the first progenitors with lymphoid potential arise in the AGM approximately one day before HSCs are detected.[7,8]

1.5.10 Emergence of B-cell restricted progenitors in the mouse embryo

Although B lymphopoiesis has been extensively studied over the years, the timing, location, and properties of the first fetal B-cell restricted progenitors remain unknown. The B-cell development process is regulated by transcription factors, which become activated in early B-cell ontogeny and continue to be activated during the life-span of the B cell.[199] In the FL and BM, HSPCs differentiate into B cells through stepwise restriction. When the progenitor becomes B-cell restricted, it has lost its ability to generate all other lineages and thus unipotent. B cell restriction is considered to be associated with cell surface expression of CD19, which is regulated by the transcription factor PAX5.[200-202] The first $CD19^+$ B progenitor cells are detected in the mouse FL at ED13.5 and increase in frequency thereafter,[203,204] while cell surface expression of IgM is detected at low levels at ED17.5.[205] One early study detected $CD19^+$ progenitor cells in AGM and FL at ED11.5,[206] but this has not been verified in other publications. Kawamoto *et al.*[207] used in vitro culture of FL cells and detected progenitors emerging between ED11 and ED12 that only produced B cells or T cells, but did not isolate any pure populations based on cell surface phenotype. To establish lineage restriction, the studied population must be prospectively purified and assessed in molecular and functional assays, including optimized conditions to reveal all possible lineage outputs.

Studies of adult BM have reported the potential existence of distinct B cell restricted progenitors prior to CD19 cell surface expression.[161,208,209] This includes the $CD19^-$ PreProB cell stage in the Hardy staging of B cell progenitors in adult BM.[156,161] The vast majority of the PreProB cells show DJ recombination at the IgH locus and transplantation experiments show predominant B cell repopulation.[161] Additional lineage potentials are however detected in the early $CD19^-$ PreProB cell progenitor cells, precluding the demonstration of a B-cell restricted progenitor. Moreover, expression of B-lineage specific genes suggests that they might also contain early B cell restricted progenitor cells.[156,161,162] These B-lineage specific genes include *Mb1*, which encodes the Ig-alpha signaling subunit of the BCR and shows high expression early in the B-cell development before the completion of VDJ rearrangement.[161,162]

1.5.11 Different properties of fetal and adult B cell progenitors

It is well known that myeloid programs are sustained longer in fetal than adult B cell progenitors. A subset of the first CD19^{+} ProB cells found in ED13.5 FL express colony stimulating factor 1 receptor (CSF1R) and was shown to retain some myeloid potential, while the corresponding population was absent in the adult BM ProB population.[210] The immunophenotypic ED14.5 fetal counterpart of the adult CLP population was also demonstrated to retain myeloid macrophage potential.[211] Moreover, fetal and adult B progenitor cells show different responses to cytokines. For example, adult BM cells require IL7 for their development while FL cells are not IL7-dependent.[212,213] Additionally, fetal but not adult ProB cells proliferate in response to thymic stromal lymphopoietin (TSLP).[214] These findings indicate that not only are the fetal and adult B progenitor cells different in their intrinsic properties, they also have different requirements and react differently to microenvironmental factors.

1.5.12 Fetal B cell progenitors and developmental susceptibility to childhood leukemia

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common childhood cancer.[215] It is hypothesized that fetal B cell progenitors are more susceptible to preleukemic or transforming translocations during a certain developmental period, and it has been suggested that BCP-ALL is initiated in a fetal restricted progenitor compartment.[215,216] An early fetal B-cell restricted progenitor has been hypothesized to be the cell-of-origin for childhood ALL,[217,218] but the exact identity of such a progenitor has yet to be established. Many studies have investigated ETS variant transcription factor 6 (ETV6)-RUNX1 positive BCP-ALL, since the ETV6-RUNX1 fusion is the most prevalent genetic alteration in children with BCP-ALL.[215] However, lack of polyclonal IgH DJ and/or T-cell receptor (TCR) rearrangements in ETV6-RUNX1 positive cells indicates that the preleukemic cells are not maintained by a RAG negative progenitor population of HSC type.[218,219]

Recently the first arising B cells were characterized in human embryos at 41 days post fertilization (CS17, corresponding to ED12.5-ED13 in mice), and a fetally restricted CD19^{-}IL7RA^{+} progenitor population which changes from myeloid to lymphoid lineage programs during development was identified.[220] Together these studies point to distinct developmental (fetal versus adult) differences in the lineage differentiation of B cell progenitor cells, which could be an explanation to

a developmental susceptibility to preleukemic genetic lesions such as ETV6-RUNX1 translocation. There are also other studies suggesting genetic lesions have different impacts on cell fates at different developmental stages.[221–224] Therefore, identification of the first fetal B-cell restricted progenitor would be of great importance for further understanding fetal B-cell development, for modeling childhood BCP-ALL and for an improved understanding of developmental susceptibility to childhood leukemia.

1.6 Modeling hematopoiesis

1.6.1 Modeling hematopoiesis in mouse models

Mouse models have been widely used in hematopoietic research as they provide genetically homogenous in vivo systems where experiments can be repeated in the same setting. The first knock-out mouse model was generated in 1989 by introducing distinct gene modifications into the mouse genome using embryonic stem cells (ESCs).[225] Since then, phenotypic characterization of cell populations in inbred mouse strains, along with investigation of gain or loss of function and other genetic alterations, have identified molecular and cellular mechanisms in a wide range of biological processes. This includes different medical conditions with inherited or acquired mutations in humans. Additionally, the introduction of inducible knock-out or knock-in models using the Cre/lox and FLP/FRT systems (both with origin in other organisms) has made it possible to turn off or induce the expression of a gene of interest at a chosen time point or within a specific cell or tissue.[226–230] Importantly, mouse models have been used to demonstrate the ability of a single HSC to regenerate the whole hematopoietic system in a transplantation setting.[87] The use of different isoforms of CD45, which is expressed on the surface of all nucleated hematopoietic cells, allows for the discrimination between donor and recipient cell origin. This type of model also enables investigation of gene function in a transplantation context.[87]

Generation of mouse models of different types of leukemia, including ALL and acute myeloid leukemia (AML), has been crucial for revealing mechanisms for disease onset and leukemia initiating populations.[231,232] However, modeling of childhood BCP-ALL has been challenging, perhaps reflecting the importance of targeting the relevant preleukemic or leukemic translocation to the relevant fetal progenitor cells. Similarly, the development of immunodeficient mice, such as NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ), has refined xeno-transplantation models for

studies of normal and malignant human hematopoiesis.[233] In parallel, humanized mouse models with expression of human genes have been constructed for modelling of the human hematopoiesis and immune system in vivo in the mouse.[234]

1.6.2 Modeling hematopoiesis in human cell systems

While many preclinical studies in hematopoietic research are primarily conducted on mice or other animal models, the results also need to be verified in human systems. When studying fetal hematopoiesis, the availability of human fetal hematopoietic tissue is limited, and the use of such tissues raises ethical concerns. Consequently, human cell models that replicate the properties of developmental hematopoiesis are of high interest.

Pluripotent stem cells (PSCs) are characterized by their ability to self-renew and differentiate into any cell type in the organism. The first human PSCs, the hESCs, were successfully derived in 1998 and have been used for developmental studies and different types of in vitro testing such as drug screening.[235] Hereafter, the discovery of reprogramming human somatic cells into human induced PSCs (hiPSCs) was made in 2007[236] and followed by further development of different models. The hiPSCs can capture the full genome of a patient and with their properties be a continuous source of cells for modeling development and various human diseases.[237,238]

Although hPSCs provide many opportunities, several limitations remain, including ethical considerations regarding long-term use of human-derived cells. When utilizing hPSCs to model early embryonic hematopoiesis, it remains unclear which hematopoietic hierarchy they mimic. hPSCs preferentially generate cells with expression of embryonic hemoglobin, and previous studies aimed at generating definitive HSCs with transplantable properties have shown conflicting results.[237] A recent publication demonstrated the generation of long-term engrafting multilineage cells differentiated from hiPSCs,[239] making hiPSCs potentially more attractive for future studies of fetal and adult human HSCs. Such studies could verify previous results from studies in other models and serve as a basis for primary studies of human HSCs. Additionally, the recent findings of long-term ex vivo expansion of mouse and human hematopoietic stem cells offer new options for studying HSPCs.[240,241]

2 Research aims

The overall aim of this thesis was to further characterize and compare fetal, neonatal and adult mouse hematopoiesis including lineage-biased HSCs.

This thesis investigated early fetal B lymphopoiesis and lineage biases in perinatal HSCs as well as explored the hematopoietic hierarchy and function of platelet-biased HSCs previously discovered in the adult mouse hematopoiesis.

The studies included in the doctoral project were performed with the specific aims to:

1. Characterize perinatal HSC lineage biases, by establishing the distribution and characteristics of lineage-biased perinatal HSCs in liver and bone marrow in the mouse at the time point of birth (Study I)

2. Investigate whether adult mouse HSCs with distinct lineage biases have hierachical or non-hierarchical relationships, and whether they utilize distinct platelet progenitor pathways downstream of HSCs (Study II)

3. Characterize fetal B-cell progenitor development by identification and characterization of the earliest B-cell restricted progenitor in the mouse embryo and through molecular profiling compare them to the corresponding progenitor stage in adult bone marrow (Study III)

3 Materials and methods

Detailed descriptions of materials and methods are provided in the three individual studies included in the thesis (Study I, Study II, and Study III). This chapter offers a general overview of the methods considered most important for these studies. The methods used for characterizing mouse hematopoietic stem and progenitor cells in this thesis are summarized in Table 2. Additionally, Table 3 provides a comprehensive overview of the transgenic mouse strains used in Studies I-III.

All tables in this chapter (Materials and methods) are presented at the end of the chapter.

3.1 Mouse models

In this thesis, different mouse models were used in all studies as relevant models for human hematopoiesis (Table 3). The decision to use mouse models was based on lack of availability of equally good or better methods to answer the research questions. There are no methods using human cell samples that allow for reliable in vivo studies with genetic labeling of fetal HSPCs at early timepoints and prospective long-term follow-up. Furthermore, no method yet exists to label and prospectively track lineage-biased HSCs in vivo in a human setting. Additional discussion regarding the use of mouse models is found in section 3.8 (Ethical considerations).

3.1.1 Cre-recombinase and Cre-reporter mouse strains

Cre-recombinase mouse strains were used in Study II and Study III in combination with fluorescent reporter strains. The *Flt3*-Cre strain[242] was used in Study II (in combination with other transgenic mouse strains) for donor cells in single HSC transplantations, as well as fate-mapping and in vivo treatment experiments (Table 3). The *Vav*-Cre strain[243] was also used in Study II for fate-mapping and in vivo treatment experiments. The *Mb1*-Cre strain[244] was used in Study III for fate-mapping of early fetal B cell progenitors.

Cre recombinase (Cre) is an enzyme consisting of four subunits that binds pairs of short specific DNA sequences called loxP sites. Upon binding it creates a DNA

loop and subsequent excision or inversion of the DNA loop based on the orientation of the loxP sites.[226,245] The recombinase and the original loxP sequence were first detected in the bacteriophage P1[245] and later used in the development of Cre mouse strains.[228,230] By applying cell-specific Cre drivers, the Cre/lox recombination can be used to create conditional knock-out or knock-in models. Insertion of lox sequences will allow for genes to be manipulated in several different ways. In the mouse, control of Cre expression by a cell-specific promoter enables the inactivation, activation or mutation of genes of interest. In addition, the Cre/lox system makes it possible to control the tissue and timing of gene expression.[228,230]

Fluorescent reporter strains are often combined with Cre-models generating so called Cre-reporter strains. These reporter strains have been designed to express a detectable marker, such as yellow fluorescent protein (YFP)[246] or tdTomato,[247] after the Cre-mediated removal of a loxP flanked stop sequence. When mice expressing Cre recombinase are bred to a fluorescent reporter strain, the double mutant offspring will express the detectable marker only in cells with Cre expression and in their offspring. These reporter strains are used for confirming Cre activity in expected tissues, and for excluding unwanted Cre activity in other tissues. In addition, the Cre-reporter strains can be utilized to create an origin of labeled cells to be used for fate-mapping in transplant experiments. Since the excision of the stop sequence is permanent, the reporter gene will also be expressed in all progenies of the cells where Cre was initially activated.

3.1.2 Transgenic fluorescent reporter mouse strains generated by BAC recombinant engineering

The *Vwf*-tdTomato,[101] *Gata1*-eGFP,[248] and *Vwf*-eGFP[100] mouse lines, generated through bacterial artificial chromosome (BAC) recombinant engineering, were used in Study I and Study II.

In the transgenic BAC mouse strains used in this thesis, transcription from the promoters of the *Vwf* and *Gata1* genes is reported by fluorescent proteins. These reporter stains were used primarily in single-cell transplantation experiments (Study I, Study II) and fate-mapping of HSPCs (Study II), and were used in combination with other mouse strains (Table 3).

3.1.3 Limitations of mouse models

While studies in mouse models have been key to our improved understanding of human hematopoiesis, there are also limitations to modelling hematopoiesis in mice. Some limitations are general, while other limitations are specific to the type of model used, as exemplified below for Cre-recombinase models. Examples of general limitations include differences in cytokine requirements between mouse and human hematopoietic cells, as exemplified by FMS-like tyrosine kinase 3 ligand (FLT3L).[249] This, along with genetic differences[250] and variations in cell transformation ability between mouse and human, can lead to difficulties in interpreting experimental data from studies in the mouse. When using leukemic models, the mouse hematopoiesis is strongly biased toward the development of AML, which is different from human hematopoiesis. This difference in bias has long been an obstacle in efforts to model ALL development in mice.[231]

When performing experiments in a Cre mouse model, it is important to control for potential toxic effects of the Cre itself. Additionally, there can be inefficient and/or unspecific recombination depending on the loxP flanked locus.[251] When utilizing a Cre-reporter strain to detect gene mediated Cre-expression, inefficient recombination can result in an imbalance between reporter expression and actual gene expression in the tracked cell population.[251] Notably, the Cre recombinase gene should preferentially be carried by the male in the breeding pair to avoid potential germline expression of Cre recombinase.[252]

3.2 Flow cytometry and FACS

Flow cytometry, including fluorescence-activated cell sorting (FACS), is a technique used for analysis and isolation of viable cells based on expression of cell surface proteins.[253] This is done by staining the cells with fluorophore-conjugated monoclonal antibodies that bind to specific cell surface antigens before the cells are analyzed by passing them through the beams of different lasers, which excite the fluorophores bound to the cell surface.[253] The method can also detect fluorescent proteins expressed intercellularly, for example in genetically modified fluorescent reporter mice. Figure 4 shows an overview of a flow cytometry experiment.

Flow cytometry was a primary method in the three studies presented in this thesis. In these studies, flow cytometry was used for multiple purposes, such as

sorting of HSCs for single HSC transplantations, sorting HSPCs for RNA sequencing and other molecular or functional assays, analysis of PB and BM for fate-mapping, and analysis of early B progenitor populations in fetal hematopoietic tissues.

The development of flow cytometry methods has been crucial for the characterization of hematopoietic cells and the description of the hematopoietic hierarchy in both mice and humans.[254-256] Cell surface markers are generally well-defined for different hematopoietic populations, and flow cytometry detection of cell surface markers can be used to separate the different populations with high specificity.[253]

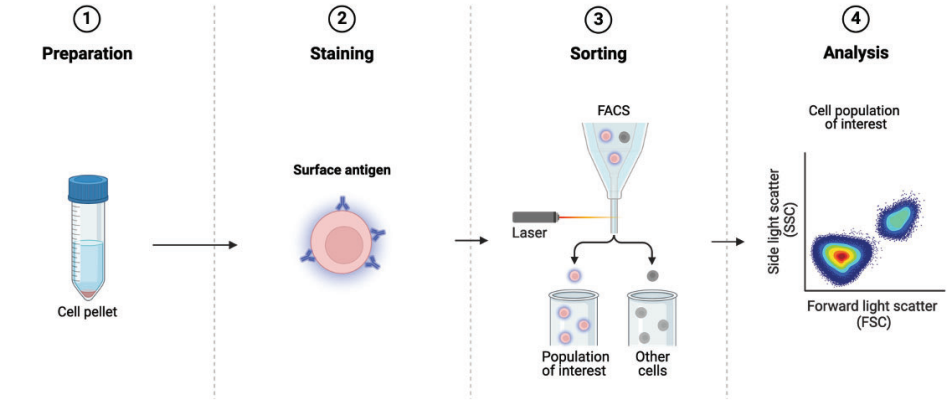

Figure 4. Overview of the different principal stages of a flow cytometry experiment including fluorescence-activated cell sorting. FACS, fluorescence-activated cell sorting; FSC, forward light scatter; SSC, side light scatter. Figure created with BioRender.com

Flow cytometry is a useful and efficient method, however it will only define the phenotype and not the function of the investigated cell. Therefore, cell populations defined by flow cytometry must also be assessed using methods that identify the functional properties of the cells. To ensure specificity in flow cytometry data analysis, it is critical to include experimental controls.[257] In the studies presented in this thesis, single-stained compensation controls and fluorescence minus one (FMO) controls[257] were always used, for some experiments isotype controls[257] were also included. Data analysis including the gating of cell populations was performed using FlowJo™ software. For the gating

procedure, a combination of FMOs, isotype controls, a known antigen-negative cell population, population-based gating and back-gating was used.[253,257] Phenotypic definitions of mouse HSPCs and mature PB lineages used in the thesis are specified in Table 4, Table 5, and Table 6.

3.3 Gene expression analysis with Real-Time Quantitative Reverse Transcription PCR

Gene expression analysis with Real-Time Quantitative RT-PCR (qRT-PCR) was used in Study III. This method assesses the patterns of gene expression at the transcriptional level in specified cells (or under specific conditions) by using an oligonucleotide probe tailored to hybridize within the target sequence.[258] The target specific product is detected by cleavage of the probe with Taq DNA polymerase during PCR and gives a reliable detection and quantification of messenger RNA (mRNA) present.[258] A limitation is that the method only detects the expression of the selected genes included in the assay, however it works well for verification of known genes. The method was used in Study III for confirmation of B-cell associated genes in early B cell progenitors.

3.4 RNA sequencing

RNA sequencing (RNA-Seq) was used in all three individual studies: single-cell RNA-Seq in Study I and Study II, and mini-bulk RNA-Seq in Study III. The RNA-Seq method uses high-throughput next-generation sequencing (NGS) to determine the presence and quantity of RNA molecules in a cell sample, which generates a dynamic picture of the gene expression in the sample.[259,260] The depicted gene expression pattern is known as the transcriptome. RNA-Seq has the advantage of detecting both known and novel transcripts in the same assay, and allows for the identification of transcript isoforms, gene fusions, and single nucleotide variants.[259,260] The method can provide resolution at the single cell level when performing single-cell RNA-Seq, which makes it possible to reveal the existence of rare cell types within a previously defined cell population.[260] Single-cell RNA-Seq was selected for Study I and Study II to examine RNA expression within specific subgroups of the HSC population. In contrast, mini-bulk RNA-Seq, which provides an average gene expression for a cell population, was chosen for Study III to investigate RNA expression profiles in B cell progenitor populations. A regular

RNA-Seq experiment involves RNA-isolation, conversion of RNA to complementary DNA (cDNA), preparation of the sequencing library, and sequencing on an NGS platform.[260] For reliable detection of transcripts in a sample, it is key to achieve adequate sequencing depth and coverage across the transcriptome. Notably, data generation artifacts (also called technical variance) can be introduced and must be controlled for during data analysis. In general, an RNA sequencing data analysis process includes quality control of raw data, read alignment and transcript assembly, expression quantification, and differential expression analysis.[259,260] It is important to note that RNA-Seq generates a large amount of data for each experiment, which can be challenging for both bioinformatic analysis and data storage.

3.5 Genetic lineage tracing

In lineage tracing, a heritable genetic marker is induced or targeted to specific cells of interest and will be passed on to all progenies of the initial cell.[261] Lineage tracing has been used in classical developmental biology and is a valuable tool in stem cell research. Genetic lineage tracing methods in hematopoietic research involve genetic labeling of HSPCs to assess the contribution of HSPCs to hematopoiesis.[88] Different types of genetic labeling are available, for example molecular barcoding and transgenic mouse models.[88,261]

In this thesis, genetic lineage tracing using transgenic mouse models was applied in the three studies. Study I and Study II utilized transgenic mouse models with fluorescent reporter expression to follow HSC progeny after single HSC transplantation. Study II also used genetic labeling with transgenic mouse lines for fate-mapping and hematopoietic stress experiments. In Study III, genetic labeling of *Mb1* gene expression was used to identify and fate-map early fetal B cell progenitors.

In molecular barcoding, either DNA barcodes introduced into HSCs or naturally occurring somatic mutations can be used to assess the HSCs' contribution to hematopoiesis.[261] This approach makes it possible to study the function of the labeled cell in steady state in situ, as opposed to its capacity in a transplantation setting or in vitro culture.[88] Another advantage of molecular barcoding is that it allows for studies of the entire organism. However, a limitation of molecular barcoding is that so many labeled HSCs contribute to hematopoiesis that

sensitivity can be below the detection level when assessing rare cell offspring.[88,261] Furthermore, the specificity of the method is lower than for single-cell transplantations as there is a risk that not all labeled cells are stem cells.[88] To address the research questions in this thesis, genetic lineage tracing via transgenic mouse models and in vivo transplantation assays were preferred to ensure single-cell resolution and high specificity in the identification of the labeled donor cell.

3.6 In vitro cell culture assays for evaluation of lineage potentials

To avoid relying solely on phenotypic definitions of HSPCs, it is necessary to combine them with an assay that evaluates the functional properties of the investigated cells. For this purpose, in this thesis, the assessment of the proliferation and differentiation ability of selected HSPCs was performed by investigating their capacity to produce cell colonies in vitro. In vitro evaluation of granulocyte/macrophage and megakaryocyte potential was used in Study II and in vitro evaluation of lymphoid potential was used in Study III, as described in the individual studies. Although these assays are based on cellular properties, they may not fully capture the functional capacity of the investigated cells, as they are conducted in vitro rather than within a complete tissue microenvironment. However, in vitro analysis is preferred for investigating progenitor cells, given their limited proliferative capacity, which makes them unsuitable for in vivo evaluation.

3.7 In vivo transplantation assays

Both single-cell HSC transplantations (Study I, Study II) and bulk BM transplantations (Study I) were used in the thesis. The principle behind these methods is to transplant HSCs into lethally irradiated or immunocompromised recipient mice to assess the LT repopulation capacity of the transplanted cells.[88] By using different isoforms of the cell surface marker CD45, the donor and recipient cell origin can be determined for white blood cells.[77] Additionally, the use of transgenic mouse models, such as the *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ mouse model,[88,101] allows for the labeling of platelets and erythrocytes, enabling the tracking of donor cell replenishment across all five blood lineages post-transplantation (Study I, Study II).[88] Notably, the single HSC transplantation setting allows a single HSC to demonstrate its LT repopulation capacity, while bulk BM

transplantation demonstrates the repopulation capacity of a group of transplanted cells. In single HSC transplantations, it is possible to backtrack the properties of each reconstituting individual HSC. However, in vivo transplantation assays will never be able to display the function of the transplanted cell in steady state.

A crucial part of the single HSC transplantation assay is the identification of HSCs before and after transplantation. Fetal and adult HSCs were phenotypically defined by flow cytometry (Table 4, Table 5), as described in the methods for Study I and Study II, and isolated by FACS for transplantation. HSC identity was functionally confirmed via the LT post-transplantation assessment of PB reconstitution and terminal analysis of BM and thymus reconstitution (Table 4, Table 5), in combination with secondary transplantations for evaluation of self-renewal capacity. Figure 5 demonstrates the principle for single HSC transplantations for identification of stem cell type in Study I.

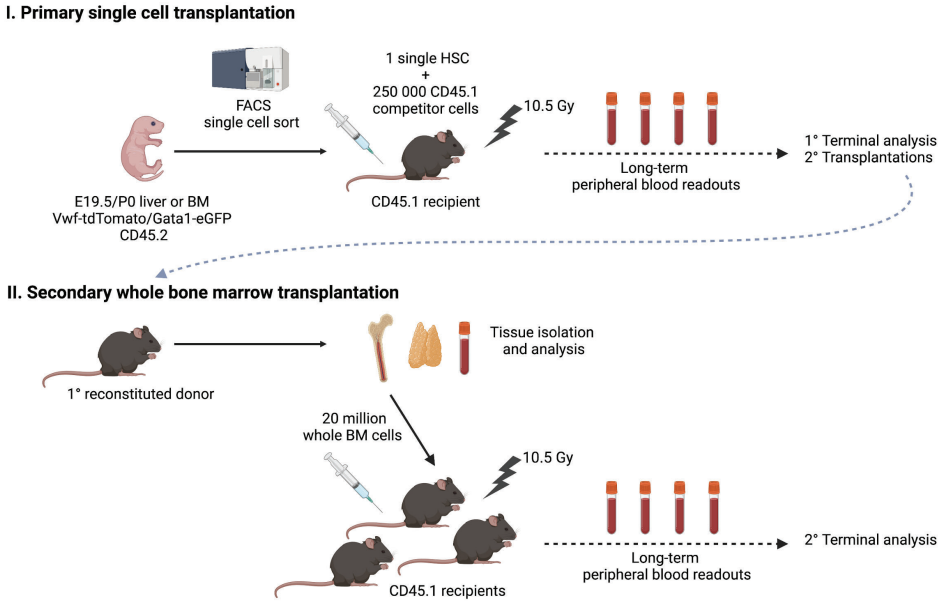

Figure 5. Overview of transplantation experiments with single hematopoietic stem cells from embryonic day 19.5/postnatal day 0 mouse liver or bone marrow in Study I. BM, bone marrow; FACS, fluorescence activated cell sorting; HSC, hematopoietic stem cell; E19.5/P0, embryonic day 19.5/postnatal day 0. Figure created with BioRender.com

The thresholds set for cell detection and reconstitution are important for interpreting the reconstitution analyses. Based on a previous single HSC transplantation study performed by the Jacobsen research group, which also used the *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ mouse model, detection level was set to 0.01%.[101] Recipients in Study I were considered to be reconstituted by potential LT-HSCs if the donor contribution to any PB lineage was ≥0.1% at ≥ 16-18 weeks post-transplantation. In Study II, recipients were considered to be reconstituted by HSCs if the donor contribution to platelets was ≥0.1% at ≥ 16-18 weeks after transplantation.

3.8 Ethical considerations

3.8.1 Ethical considerations on the use of animal models

Mouse experiments performed at Karolinska Institutet were carried out in accordance with regulations from the Swedish Board of Agriculture (Jordbruksverket), with approval of the Stockholm Ethical Committee (Stockholms djurförsöksetiska nämnd). Mouse experiments carried out at the University of Oxford were performed in accordance with regulations from UK Home Office, with the approval of the Ethical Review Committee of the Oxford University Medical Sciences Division.

Conducting scientific experiments involving laboratory animals always presents a moral dilemma: is it right or wrong to expose animals to potential harm in pursuit of research questions and findings that may contribute to the research field and improve human health? When using animal models, as well as when you are using human materials in your research, the benefits and the risks must be carefully considered and thoroughly evaluated. The research questions addressed in this doctoral thesis, could not have been answered through studies performed in a human setting or in cell lines in vitro. For this reason, in vivo mouse models were selected to enable the studies.

When performing scientific studies using animal models, the principles of the 3Rs (Replacement, Reduction and Refinement) must always be considered. These principles were first defined by Russel and Burch in 1959 in their publication "The Principles of Humane Experimental Technique" as a guideline for humane animal research. Since then, the principles have been further developed and remain valid

in modern research practices as a framework for conducting science of high quality.

Replacement strategies should be applied when possible, such as using in vitro systems instead of animal models. Nonetheless, in vivo evaluation is sometimes unavoidable to address a question. This is often the case for HSC studies, where the definition of an HSC requires proof of engraftment and long-term reconstitution, which can only be tested in vivo. Reduction of the overall number of animals used in research is an important goal that can be achieved through careful planning and experimental design to maximize data collection from the animals included in experiments. Refinement refers to minimizing pain and distress for the laboratory animals, including improving experimental procedures and providing good animal husbandry. This will contribute to a better animal welfare for the animals still used in research.

When conducting the studies within this thesis work, ethical considerations were continually taken into account. Each experimental protocol was thoroughly discussed within the research group to avoid unnecessary experiments or inaccurate experimental setups. In parallel, the experimental procedures including animal work were planned in close collaboration with the veterinarian and the trained staff at the animal facilities. In all experiments, the experimental mice were closely monitored for expected side effects as well as unexpected adverse effects.

3.8.2 Ethical considerations on the reproducibility of research data

Another ethical concern is the reproducibility of research data. It has become clear that many previously conducted scientific studies cannot be reproduced with the same results.[262] To ensure the quality of published research data, all experimental data (experimental protocols, raw data, and analysis of data) included in this thesis work has been systematically and independently reviewed and reanalyzed by other scientists in the research group before publication.

Table 2. Methods used in this thesis for the characterization of mouse hematopoietic stem and progenitor cells.

Assay	Description	Advantages	Limitations	Used in
Flow cytometry	Detects cell size, optical and fluorescent properties of the cells by passage of cells via different lasers. Uses fluorescent conjugated monoclonal antibodies binding to specific cell surface antigens or detects fluorescent reporter expression in genetically modified mice.	Fast method. Can be used for identification of cells for analysis and cell sorting for further assays.	Identifies the cell phenotype but not the functional or complete molecular properties of the cell.	Study I, Study II, and Study III
Gene expression analysis with Real-Time Quantitative Reverse Transcription PCR	Assessment of the patterns of gene expression at the transcriptional level in a specified cell (or under specific conditions). Uses an oligonucleotide probe tailored to hybridize within the target sequence. The target specific product is detected by cleavage of the probe with Taq DNA polymerase during PCR.	Reliable detection and quantification of generated mRNA.	Will only detect gene expression of the selected genes included in the assay.	Study III
RNA sequencing	Uses next-generation sequencing to determine the presence and amount of RNA molecules in a cell sample, generates a snapshot of gene expression in the sample (known as the transcriptome). Can be performed both as single cell RNA-Seq (used in Study I and Study II) and bulk RNA-Seq (used in Study III).	Detects both known and novel properties in the same assay. Resolution at single cell level when performing single-cell RNA-Seq.	Time dependent. A certain sequencing depth and coverage is required for reliable detection. Data generation artifacts (technical variance) can be introduced. Complex data management, large amount of data can cause storage issues.	Study I, Study II, and Study III

Genetic lineage tracing	Uses genetic labeling of HSPCs to assess the contribution of HSPCs to hematopoiesis. Different types of genetic labeling are available, for example molecular barcoding and transgenic mouse models.	Possible to study HSPC contribution in a non-transplantation setting.	Sensitivity and specificity can be a limiting factor when assessing rare cell offspring.	Study I, Study II, and Study III
In vitro cell culture assays for evaluation of lineage potentials	Assessment of the proliferation and differentiation ability of selected HSPCs by their capacity to produce cell colonies in vitro. For example, in vitro evaluation of granulocyte/ macrophage and megakaryocyte potential (used in Study II) and in vitro evaluation of lymphoid potential (used in Study III).	Time efficient. Based on cellular properties. Cell colonies can be collected for further analysis. Single-cell analysis of progenitors only possible in vitro due to limited proliferative ability.	Does not assess the significance of tissue microenvironment.	Study II and Study III
In vivo transplantation assays	Transplantation of HSPCs into lethally irradiated (or immunocompromised) recipient mice for assessment of the long-term repopulation capacity of the transplanted cells. Quantification of the capacity can be performed via competition with a CD45 syngeneic mouse.	Gold standard for functional identification and functional characterization of HSCs. Resolution at single cell level when performing single-cell transplantations. In vivo model with more applicable microenvironmental conditions. Self-renewal capacity can be verified by secondary transplantations.	Time consuming, experiments running over a long time (minimum 16 weeks). Assesses the capacity of the transplanted cell rather than the cell function in an unperturbed setting. High experimental cost. Primarily applicable to HSCs and not progenitors due to their limited proliferative potential.	Study I and Study II

HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; mRNA, messenger RNA; RNA-Seq, RNA sequencing.

Table 3. Transgenic mouse models used in this thesis for the characterization of hematopoietic stem and progenitor cells.

Mouse model (common name)	**Characteristics of the model**	**Labeled cell populations and type of experiments**	**Used in**
***Flt3*-Cre**[242] Full name: B6(129X1)-Tg(Flt3-cre)#CCb/leg	Cre expression is under the control of the *Flt3* promoter. When mice expressing Cre are bred to mice carrying a loxP flanked fluorescent reporter gene, the fluorescent reporter is detected in the Cre-expressing cells as well as their offspring in the double mutant mice.	Labeling of HSPC populations expressing *Flt3* and their progeny. Used for BM donor cells for single HSC transplantations in combination with labeling of *Vwf* and *Gata1* expression. Used for fate-mapping, CP and 5-FU treatment experiments, anti CD42b treatment experiments, and analysis of reticulated platelets with TO.	Study II, primarily in combination with *R26*^{TOM}
***Mb1*-Cre**[244] Full name: B6.C(Cg)-*Cd79a*$^{tm1(cre)Reth}$/EhobJ	Cre expression is under the control of the *Mb1* promoter. When mice expressing Cre are bred to mice carrying a loxP flanked fluorescent reporter gene, the fluorescent reporter is detected in the Cre-expressing cells as well as their offspring in the double mutant mice.	Labeling of B cell progenitor populations expressing *Mb1* (*CD79a*). Used for labeling of early B progenitor cells, PreProB cells, ProB cells, and Early PreB cells.*	Study III, in combination with *R26*^{TOM} or *R26*^{YFP}
***Vav*-Cre**[243] Full name: B6.Cg-Tg(VAV1-cre)1Graf/MdfJ	Cre expression is under the control of the *Vav* promoter. When mice expressing Cre are bred to mice carrying a loxP flanked fluorescent reporter gene, the fluorescent reporter is detected in the Cre-expressing cells as well as their offspring in the double mutant mice.	Labeling of all hematopoietic cells in the BM and blood. Used for fate-mapping of platelets and CP treatment experiments.	Study II, in combination with *R26*^{TOM}
***R26*^{TOM}** (Ai9 or Ai14)[247] Full name: Ai9: B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J Ai14:B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J	Mice carrying the fluorescent reporter gene tdTomato. When bred to mice expressing Cre, the fluorescent reporter is expressed in the Cre-expressing cells as well as their offspring in the double mutant mice.	Depending on the Cre model.	Study II (Ai 9 or Ai14), in combination with *Flt3*-Cre or *Vav*-Cre Study III (Ai14), in combination with *Mb1*-Cre

***R26*^{YFP}**[246] Full name: B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J	Mice carrying the fluorescent reporter gene YFP. When bred to mice expressing Cre, the fluorescent reporter is expressed in the Cre-expressing cells as well as their offspring in the double mutant mice.	Depending on the Cre model.	Study III, in combination with *Mb1*-Cre
***Gata1*-eGFP**[248] Full name: B6.129P2-Tg(Gata1-EGFP)Cn	Transcription from the promoter of the *Gata1* gene is reported by the fluorescent eGFP protein.	Labeling of *Gata1* expressing cells including platelets and erythrocytes. Used for BM donor cells for single HSC transplantations in combination with labeling of *Vwf* (Study I, Study II) or both *Vwf* and *Flt3* (Study II) expression. Used for steady state fate-mapping and anti CD42b treatment experiments in combination with labeling of *Vwf* and *Flt3* expression (Study II).	Study I, in combination with *Vwf*-tdTomato Study II, primarily in combination with *Vwf*-tdTomato or *Vwf*-eGFP
***Vwf*-eGFP**[100] Full name: B6.Tg(VWF2-eGFP)#2	Transcription from the promoter of the *Vwf* gene is reported by the fluorescent eGFP protein.	Labeling of *Vwf* expressing cells including platelets. Used for BM donor cells for single HSC transplantations in combination with labeling of *Gata1* or both *Gata1* and *Flt3* expression. Used for steady state fate-mapping and anti CD42b treatment experiments in combination with labeling of *Gata1* and *Flt3* expression.	Study II, primarily in combination with *Gata1*-eGFP
***Vwf*-tdTomato**[101] Full name: Not registered	Transcription from the promoter of the *Vwf* gene is reported by the fluorescent tdTomato protein.	Labeling of *Vwf* expressing cells including platelets. Used for BM donor cells for single HSC transplantations in combination with labeling of *Gata1* (Study I, Study II) or both *Gata1* and *Flt3* (Study II) expression. Used for single-cell RNA-Seq of liver pnHSCs (Study I).	Study I, in combination with *Gata1*-eGFP Study II, in combination with *Gata1*-eGFP or *Gata1*-eGFP and *Flt3*-Cre

BM, bone marrow; CP, cyclophosphamide; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cells; pnHSCs, perinatal HSCs; RNA-Seq, RNA sequencing; TO, thiazole orange; 5-FU, 5-fluorouracil. *For phenotypic definition of B progenitor populations, see Table 6.

Table 4. Cell surface markers used to define mouse hematopoietic cell populations in Study I.

Population	Tissue	Cell surface markers
pnHSC*	Liver FBM	$Lin^-SCA1^+KIT^+FLT3^-CD150^+CD48^-$
LT-HSC	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^+CD48^-$
ST-HSC	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^-CD48^-$
MPP2	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^+CD48^+$
MPP3	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^-CD48^+$
MPP4	ABM	$Lin^-SCA1^+KIT^+FLT3^+$
MkP	ABM	$Lin^-SCA1^-KIT^+CD150^+CD41^+$
PreMegE	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^+CD105^-$
CFU-E	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^-CD105^+$
PreGM	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^-CD105^-$
GMP	ABM	$Lin^-SCA1^-KIT^+CD41^-CD150^-CD16/32^+$
ProB cells	ABM	$Lin^-B220^{low}CD19^+KIT^+IgM^-$
DP T cell prog	Thymus	$Lin^-CD4^+CD8a^+$
Platelets	PB	$TER119^-CD150^+CD41^+$
Erythrocytes	PB	$TER119^+CD150^-CD41^-$
Myeloid cells	PB	$NK1.1^-CD41^-CD4/CD8a^-CD19^-CD11b^+$
B lymphocytes	PB	$NK1.1^-CD41^-CD4/CD8a^-CD11b^-CD19^+$
T lymphocytes	PB	$NK1.1^-CD41^-CD11b^-CD19^-CD4/CD8a^+$

HSC, hematopoietic stem cell; pnHSC, perinatal HSC; LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; MkP, megakaryocyte progenitor; PreMegE, pre-megakaryocyte/erythroid progenitor; CFU-E, colony forming unit-erythroid; PreGM, pre-granulocyte/monocyte progenitor; GMP, granulocyte/monocyte progenitor; DP T cell prog, thymic double positive T cell progenitor; FBM, fetal bone marrow; ABM, adult bone marrow; PB, peripheral blood.

Lineage (Lin) exclusion markers for $Lin^-SCA1^+KIT^+$ (LSK) and $Lin^-SCA1^-KIT^+$(LK) panels: TER119, CD11b, Gr1, B220, CD4, CD8a, and CD5; for ProB cells: TER119, Gr1, F4/80, CD3e, NK1.1, and CD11c; for DP thymocytes: TER119, CD11b, Gr1, B220, NK1.1, CD11c, and CD19.
Cells within each population were considered donor-derived cells when $CD45.2^+CD45.1^-$ or when *Gata1*-$eGFP^+$ in the case of CFU-E cells due to their low CD45 expression. Platelets were considered donor-derived when *Vwf*-$tdTomato^+$*Gata1*-$eGFP^+$. Erythroid cells were considered donor-derived when *Vwf*-$tdTomato^-$ *Gata1*-$eGFP^+$.

*Donor pnHSCs were single $CD45.2^+Lineage^-$(TER119, Gr1, B220, CD8a, CD5)$LSKCD150^+CD48^-$ cells index-sorted from *Vwf*-$tdTomato^{tg/+}$ *Gata1*-$eGFP^{tg/+}$ CD45.2 mice.

Table 5. Cell surface markers used to define mouse hematopoietic cell populations in Study II.

Population	Tissue	Cell surface markers
Donor HSC*	ABM	$Lin^-SCA1^+KIT^+CD34^-CD150^+CD48^-$ or $Lin^-SCA1^+KIT^+CD34^-CD150^+CD48^-CD201^+$
LT-HSC	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^+CD48^-$
ST-HSC	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^-CD48^-$
MPP2	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^+CD48^+$
MPP3	ABM	$Lin^-SCA1^+KIT^+FLT3^-CD150^-CD48^+$
MPP4	ABM	$Lin^-SCA1^+KIT^+FLT3^+$
MkP	ABM	$Lin^-SCA1^-KIT^+CD150^+CD41^+$
PreMegE	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^+CD105^-$
CFU-E	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^-CD105^+$
PreGM	ABM	$Lin^-SCA1^-KIT^+CD41^-CD16/32^-CD150^-CD105^-$
GMP	ABM	$Lin^-SCA1^-KIT^+CD41^-$ $CD16/32^+CD150^-$
Platelets	PB	$TER119^-CD150^+CD41^+$
Erythrocytes	PB	$TER119^+CD150^-CD41^-$
Myeloid cells	PB	$CD11b^+NK1.1^-CD19^-CD4/CD8a^-$
B lymphocytes	PB	$CD19^+NK1.1^-CD4/CD8a^-CD11b^-$
T lymphocytes	PB	$CD4/CD8a^+NK1.1^-CD11b^-CD19^-$

HSC, hematopoietic stem cell; LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; MkP, megakaryocyte progenitor; PreMegE, pre-megakaryocyte/erythroid progenitor; CFU-E, colony forming unit-erythroid; PreGM, pre-granulocyte/monocyte progenitor; GMP, granulocyte/monocyte progenitor; ABM, adult bone marrow; PB, peripheral blood.

Lineage (Lin) exclusion markers for $Lin^-SCA1^+KIT^+$ (LSK) and $Lin^-SCA1^-KIT^+$ (LK) panels: TER119, CD11b, Gr1, B220, CD4, CD8a, and CD5.

*Donor HSCs were sorted as *Vwf*-tdTomato$^-$ and *Vwf*-tdTomato$^+$ LSK*Gata1*-eGFP$^-$$CD34^-CD150^+CD48^-$ or *Vwf*-tdTomato$^-$ and *Vwf*-tdTomato$^+$ LSK*Gata1*-eGFP$^-$$CD34^-CD150^+CD48^-CD201^+$
Cells within each population were considered donor-derived cells when $CD45.2^+CD45.1^-$ or when *Gata1*-eGFP$^+$ in the case of CFU-E cells due to their low CD45 expression. Platelets were considered donor-derived when *Vwf*-tdTomato$^+$*Gata1*-eGFP$^+$ for *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ donors and *Vwf*/*Gata1*-eGFP$^+$ for *Flt3*Cre$^{tg/+}$*R26*$^{TOM/+}$ *Vwf*-eGFP$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ donors. Erythroid cells were considered donor-derived when *Vwf*-tdTomato$^-$*Gata1*-eGFP$^+$ for *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/}$ donors, and *Vwf*/*Gata1*-eGFP$^+$ for *Flt3*Cre$^{tg/+}$ *R26*$^{TOM/+}$ *Vwf*-eGFP$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ donors.

Table 6. Cell surface markers used to define mouse hematopoietic B cell progenitor populations in Study III.

Population	Tissue	Cell surface markers
YFP$^+$ progenitor	ED11.5 – ED14.5 FL	CD45$^+$B220$^+$CD19$^-$IL7RA$^+$KIT$^+$FLT3$^+$YFP$^+$
TOM$^-$ PreProB	ED14.5 FL ABM	Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^-$
TOM$^+$ PreProB	ED14.5 FL ABM	Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^+$
FLT3$^+$ ProB	ED14.5 FL ABM	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^+$
FLT3$^-$ ProB	ED14.5 FL ABM	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^-$TOM$^+$
KIT$^-$ ProB	ABM	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^-$FLT3$^-$TOM$^+$
Early PreB	ABM	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{high}AA4.1$^+$IL7RA$^+$KIT$^-$FLT3$^-$TOM$^+$

ED, embryonic day; FL, fetal liver; ABM, adult bone marrow.

YFP or tdTomato (TOM) expression represent ongoing or past *Mb1* expression; *Mb1* expression drives expression of Cre recombinase which through recombination removes a loxP flanked stop cassette to allow expression of the fluorescent reporter.

Lineage (Lin) exclusion markers for B progenitor panels: CD3e, CD11c, F4/80, Gr1, NK1.1, Ter119. For sorting of ED14.5 FL YFP$^+$ progenitors for evaluation of lineage potentials Lin exclusion markers for B progenitor panels were used. For some experiments for detection of B cell emergence no lineage panel or a simplified lineage panel consisting of Gr1 and Ter119 was used.

4 Results and Discussion

4.1 Study I - Platelet and myeloid lineage biases of transplanted single perinatal mouse hematopoietic stem cells

HSCs, classically defined by their ability to self-renew and their potential to replenish all mature blood lineages throughout life, are molecularly and functionally heterogeneous.[79] In mammals, definitive LT-HSCs emerge during embryonic development and expand in the FL before seeding the BM prior to birth. These fetal HSCs are also the origin of HSCs in the adult BM.[109] By transplanting single HSCs isolated from adult BM, the Jacobsen research group previously uncovered a framework of ST and LT lineage-biased and lineage-restricted HSC fates, including frequent platelet-biased and rare platelet-restricted HSCs.[101] The lineage-biased fate of each HSC is sustained in secondary transplantations and consequently considered to be a mainly cell intrinsic property. However, it is still unknown to what degree the adult patterns of lineage-restriction, lineage-bias, or alternative distinct lineage-biases and restrictions, are established during fetal development.

Earlier single-cell transplantation studies of fetal HSCs have only investigated white blood cell lineages, and these studies demonstrated myeloid (M)-biased and lymphoid (L)-biased HSCs in ED14.5 and ED18.5 FL.[59] It remains unknown whether fetal and perinatal HSCs exhibit other lineage-biased and lineage-restricted HSC fates, such as platelet-biased and platelet-restricted LT-HSCs, which have been demonstrated in adult BM.[100,101,145] In this study we aimed to investigate perinatal HSC lineage-biases and restrictions, by establishing the distribution and characteristics of lineage-biased perinatal HSCs (pnHSCs) in the liver and BM of mice at the time of birth, including their contribution to the replenishment of platelet (P) and erythroid (E) lineages.

Using a *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ mouse model,[101] we sorted single embryonic day 19.5/postnatal day 0 (ED19.5/PD0) pnHSCs from liver and BM by flow cytometry. Notably, cells were not sorted based on the expression of von Willebrand factor (*Vwf*)-tdTomato. However, the cells were index-sorted and therefore could be retrospectively categorized based on their *Vwf*-tdTomato expression. The sorted single HSCs (and supporting BM cells) were transplanted into lethally irradiated recipient mice and the contribution to PB cell lineages were tracked over time. The goal of using single HSC transplantations is to achieve definitive clonal tracing of blood lineage contribution over time from a single HSC

with broad and LT self-renewal ability. Importantly, this model allowed us to follow the reconstitution of all five blood lineages in PB: P, E, M, B, and T cells. The sorted and investigated cell populations are defined in Table 4 in Chapter 3 (Materials and methods). For comparisons with adult mouse BM HSCs, we used published data from a single-cell transplantation study by the Jacobsen research group which utilized the same single-cell transplantation mouse model.[101]

In our current study, 22% of primary transplanted mice demonstrated ≥ 0.1% LT contribution to at least one blood cell lineage after 25–26 weeks. A significant majority (>90%) of the reconstituted single pnHSCs from liver or BM reconstituted all blood lineages, compared to less than 50% of single adult BM LT-HSCs.[101] Surprisingly, we found no pnHSCs with P- or PEM-restriction, whereas one third of the single adult BM LT-HSCs gave exclusively rise to PEM lineages.[101] Furthermore, while half of the adult LT-HSCs demonstrated P/PEM-bias,[101] only a single pnHSC from BM (and none from liver) exhibited P-bias, and a small fraction (<15%) exhibited PEM-bias. At 25-26 weeks post-transplantation, the only lineage restriction observed was PEMB-restriction from a small number of pnHSCs. In contrast to adult HSCs, where a majority of P- and PEM-restricted HSCs maintained their restriction in primary recipients,[101] most PEMB-restricted pnHSCs lost their restriction and achieved T cell reconstitution over time in our study. Figure 6 demonstrates the LT reconstitution patterns in PB upon primary transplantation of single pnHSCs.

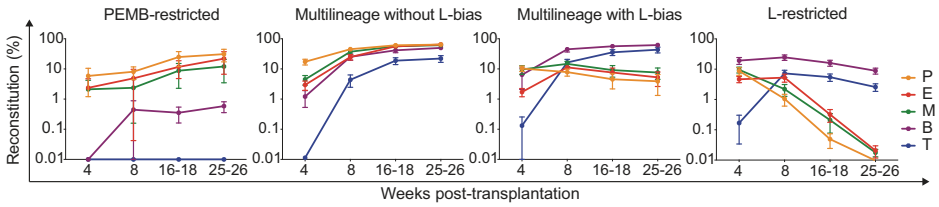

Figure 6. Long-term peripheral blood reconstitution patterns after primary transplantation of a single perinatal HSC sorted from mouse liver or bone marrow on ED19.5/PD0 (n = 252 recipients). Mean ± SEM for each lineage. PEMB-restricted, n = 4; multilineage without L-bias, n = 22; multilineage with L-bias, n = 8; L-restricted, n = 21. ED, embryonic day; PD, postnatal day; L, lymphoid; P, platelet; E, erythrocyte; M, myeloid; B, B lymphoid; T, T lymphoid. Figure reprinted from Belander Strålin *et al.*[263] (Study I) with permission from the publisher.

Further, we carried out secondary transplantations of unsorted bulk BM cells to verify the self-renewal of LT reconstituted pnHSCs and observe the secondary reconstitution patterns. At the same time point as the secondary transplantations, we performed phenotypic analysis by flow cytometry to characterize BM HSPCs replenished from the primary transplanted liver and BM pnHSCs. Figure 5 in Chapter 3 (Materials and methods) gives an overview of the performed transplantation experiments.

Most multilineage pnHSCs demonstrated no marked lineage bias, upheld LT replenishment of all PB lineages, and reconstituted HSPCs in both primary and secondary transplantations. A large portion of the pnHSCs demonstrated L-biased and L-restricted blood replenishment, in line with previously published data using ED18.5 HSCs.[59] The L-bias of multilineage pnHSCs only became evident weeks or months after the transplantation, with all pnHSCs that became L-restricted having shown PEM lineage reconstitution at earlier time points. Furthermore, L-biased and L-restricted HSCs displayed low and unpredictable reconstitution of HSPCs in primary recipients and no HSPC reconstitution in secondary recipients. Therefore, the L-biased or L-restricted blood reconstitution most likely reflects the long half-life of mature lymphocytes compared to the short half-life of myeloid lineage cells,[264] instead of lymphoid-biased LT-HSCs.

In connection with our findings regarding L-bias and L-restriction in pnHSCs, a recent study using clonal tracking of native hematopoiesis indicated that myeloid and lymphoid cells primarily originate from eMPPs and not from HSCs, particularly during the first months of life.[7] Furthermore, eMPPs persist as the main source of lymphoid cell production throughout life.[7] In our experiments, we demonstrated that many pnHSCs, which were found to be enriched in HSCs lacking expression of von Willebrand factor (*Vwf*-dTomato$^-$ HSCs), provided LT stable replenishment mainly of lymphocytes. Additional analysis of the BM in reconstituted recipients showed that the described L-biased/restricted cells were not actual LT-HSCs, but corresponded more to the properties of ST-HSCs or eMPPs.

Similar to adult BM HSCs, approximately half of the liver and BM pnHSCs expressed *Vwf*-dTomato (*Vwf*-dTomato$^+$ HSCs).[100,101] While P-and PEM-bias in pnHSCs were less frequent than in adult HSCs, these biases were only observed in *Vwf*-dTomato$^+$ pnHSCs. When comparing lineage-bias and restriction of *Vwf*-dTomato$^+$ and *Vwf*-dTomato$^-$ pnHSCs, no significant differences were detected between those originating from the liver and BM. This indicates that the

development of P-and PEM-bias/restriction has begun in *Vwf*-dTomato^{+} pnHSCs. Moreover, multilineage *Vwf*-dTomato^{-} pnHSCs did not stably reconstitute *Vwf*-dTomato^{+} BM LT-HSCs in primary transplanted recipients. The Jacobsen research group has previously shown that all ED14.5 FL HSCs express *Vwf*.[100] Together with our present data, this implies that the previously established hierarchical relation between *Vwf*-reporter^{+} and *Vwf*-reporter^{-} adult HSCs,[100,101] where *Vwf*-reporter^{+} HSCs can produce *Vwf*-reporter^{-} HSCs (but not the opposite), is present at the time of birth.

Next, we performed single-cell RNA sequencing[265] to compare the transcriptional profiles of *Vwf*-dTomato^{+} and *Vwf*-dTomato^{-} liver pnHSCs. Dimensionality reduction analysis did not show clear clustering related to *Vwf*-tdTomato expression. However, a small number of differentially expressed genes (DEGs; 32 DEGs) were identified in the comparison between *Vwf*-dTomato^{+} and *Vwf*-dTomato^{-} pnHSCs, and multiple genes correlated with the expression level of *Vwf*-dTomato. For this reason, we further compared *Vwf*-dTomato^{-} and *Vwf*-dTomato^{high} pnHSCs and found a higher number of DEGs (162 DEGs), with generally higher fold changes that better separated subclusters of pnHSCs.

DEGs more highly expressed by *Vwf*-tdTomato$^{+/high}$ than *Vwf*-dTomato^{-} pnHSCs contained the maternally imprinted long-noncoding RNAs *H19*, *Meg3*, and *Rian*. These are found in the *H19–Igf2* and *Dlk1–Dio3* loci shown to be highly expressed in LT-HSCs and crucial for support of embryonic and adult HSCs.[266,267] Additionally, DEGs contained genes associated with lineage-biased adult HSCs, which generated enrichment in *Vwf*-tdTomato$^{+/high}$ pnHSCs of previously reported molecular signatures for LT-HSCs,[268] serial engraftment,[269] and megakaryocyte (Mk) lineage bias.[269] Notably, *Flt3* was one of the genes with the largest increase in expression in *Vwf*-dTomato^{-} compared to *Vwf*-dTomato$^{+/high}$ pnHSCs. High *Flt3*-expression is characteristic for eMPPs,[7] and its high expression in our comparison supports the idea that L-biased *Vwf*-dTomato^{-} pnHSCs are enriched in HSPCs associated to eMPPs.

It is important to note that our kinetic fate-mapping was performed in a single-cell transplantation model with myeloablated recipients, therefore, reconstitution occurs under hematopoietic stress conditions. This model is more indicative of a clinical hematopoietic cell transplantation (HCT) setting rather than steady-state conditions. However, similar to counterpart studies of adult HSCs,[101] the lineage-bias patterns remained unchanged several months post-transplantation when

hematopoiesis had returned to a more steady-state like setting. The study gives new information on the blood reconstitution potentials of definitive HSCs at birth in comparison to adult HSCs. Different from other studies,[59] we did not observe an increasing M-bias in BM HSCs during late gestation since no significant differences in lineage patterns between liver and BM pnHSCs were demonstrated. Nevertheless, our single-cell transplantation data align with previously published molecular data[270] showing that pnHSCs from liver and BM possess similar transcription patterns and primarily cluster by developmental age.

Our study did not investigate when P-bias/restriction first arises, though the rarity of P- and PEM-bias/restriction found in pnHSCs suggests that these patterns mainly arise after birth. The functional and transcriptional data, obtained at single-cell resolution, demonstrated that the proportion of P-and PEM-bias/restriction is very low in pnHSCs in comparison to adult HSCs. These data are consistent with recently published data on Mk priming.[271] Furthermore, we showed that this activity had already started in pnHSCs in a pattern resembling adult HSCs. Notably, the lineage biases we identified (P-, PEM-, and PEMB-lineage bias) were the same as those previously found in adult HSCs, and the P-and PEM-biases were only observed in *Vwf*-dTomato$^+$ HSCs, which is also characteristic for the P- and PEM-restricted HSCs in adult BM.[101]

We have not yet examined to what extent the described lineage-biases of pnHSCs are intrinsic or extrinsic, which will require further investigation. Since adult[100,101,269] and perinatal LT-HSCs exhibit consistent lineage patterns after primary and secondary transplantations, it is likely that the observed lineage bias is at least in part an intrinsic HSC property. Additionally, their transcriptional lineage priming is consistent with their lineage bias after transplantation, further supporting a robust and intrinsic HSC property. However, the demonstrated epigenetic programming in the P-bias of HSCs[272] suggests that there could be an interaction between intrinsic and extrinsic properties.

In essence, Study I identified the extent to which adult patterns of lineage bias and restriction are established in HSCs already at the time of birth. Unlike adult BM HSCs, and consistent with previous reports on fetal HSCs, the blood replenished by single liver and BM pnHSCs was largely multilineage-balanced or L-biased. However, we demonstrated that this L-biased blood reconstitution reflects the activity of ST-rather than LT-HSCs. Most notably, P-biased output was very rare, and the LT P-restricted and PEM-restricted HSCs found in adult BM were absent

from the perinatal HSC pool. Nonetheless, the process of P- and PEM-lineage-biases had initiated in a subset of pnHSCs, and only in *Vwf*-tdTomato^{+} HSCs.

4.2 Study II – Alternative platelet differentiation pathways initiated by nonhierarchically related hematopoietic stem cells.

Platelets are critical for survival as they are essential for blood clotting and the prevention of bleeding. Decreased platelet counts pose a risk for spontaneous bleeding and complications with excessive bleeding after injury.[273] Additionally, platelets take part in both the innate and adaptive immune system.[274] Low platelet counts are often seen in connection with BM depletion caused by chemotherapy and HCT.[275] Traditional models of hematopoiesis describe a single pathway where multipotent HSCs differentiate stepwise into all types of blood cells through multiple progenitor stages.[78] However, in recent years, this view has been challenged with some studies suggesting an additional and faster differentiation pathway for platelet production.[142,276,277] Nevertheless, it has not yet been convincingly shown that a rapid platelet pathway can originate from a true HSC. This is also because HSCs, by their functional definition, can only be verified through LT hematopoietic reconstitution.[1]

The Jacobsen research group previously characterized in the adult BM a distinct HSC subset with expression of von Willebrand factor (*Vwf*-reporter^{+} HSCs).[101] When transplanted into recipient mice, this *Vwf*-reporter^{+} HSC subset demonstrated selective and stable platelet reconstitution. *Vwf*-reporter^{+} HSCs can reconstitute all blood lineages, but they display a P-restricted or P-biased reconstitution pattern. So far, only hierarchical relationships between HSCs with different lineage biases have been demonstrated,[101,134,278] suggesting common instead of separate pathways for blood lineage production. In this study we sought to investigate whether adult BM HSCs with P-bias or P-restriction have non-hierarchical relationships with multilineage HSCs, and whether they utilize distinct platelet progenitor pathways downstream of the HSCs for platelet production.

Multilineage HSCs, as well as P-biased and P-restricted HSCs, can only be identified by their unique functional characteristics, therefore to be able to track all lineages, we used the *Vwf*-tdTomato$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ mouse model.[101] *Vwf*-tdTomato^{+} HSCs from adult BM are enriched for P-biased and P-restricted HSCs (*Vwf*-tdTomato^{+} P-HSCs), and HSCs without *Vwf*-dTomato expression (*Vwf*-

tdTomato⁻ HSCs) are enriched for multilineage HSCs (*Vwf*-tdTomato⁻ multi-HSCs).[101] The single-cell sorted HSCs were transplanted into recipient mice and the reconstituted PB cell lineages were followed over time. Once the lineage pattern was determined, we characterized and sorted HSPCs reconstituted from *Vwf*-tdTomato⁺ P-HSCs or *Vwf*-tdTomato⁻ multi-HSCs. These sorted HSPCs were used in subsequent global RNA sequencing and secondary transplantation experiments. The sorted and investigated cell populations are defined in Table 5 in Chapter 3 (Materials and methods).

Our hypothesis was that if there are parallel differentiation pathways for platelet production downstream of HSCs, they would be used separately by *Vwf*-tdTomato⁺ P-HSCs and *Vwf*-tdTomato⁻ multi-HSCs reconstituting blood cell lineages in a balanced or L-biased pattern.

Reconstitution analysis with flow cytometry showed that single *Vwf*-tdTomato⁻ multi-HSCs consistently reconstituted all investigated phenotypic HSPC populations. On the contrary, single *Vwf*-tdTomato⁺ P-HSCs invariably replenished only LT-HSCs and megakaryocyte progenitors (MkPs), and more rarely also MPP2 and pre-megakaryocyte–erythroid progenitors (PreMegEs). Three specific subsets of LSK progenitors (MPP3, MPP4 and ST-HSCs) were always stably replenished by *Vwf*-tdTomato⁻ multi-HSCs, but not by *Vwf*-tdTomato⁺ P-HSCs. In addition, functional assessment using in vitro single-cell clonal analysis established that all MPP subsets reconstituted by *Vwf*-tdTomato⁻ multi-HSCs had megakaryocyte potential, indicating that *Vwf*-tdTomato⁻ multi-HSCs may use a separate pathway with additional progenitor stages for platelet reconstitution in comparison to *Vwf*-tdTomato⁺ P-HSCs.

Next, we investigated if there was a hierarchical relationship between *Vwf*-tdTomato⁻ multi-HSCs and *Vwf*-tdTomato⁺ P-HSCs. We observed that phenotypic HSCs in the BM of recipients from a single transplanted *Vwf*-tdTomato⁻ multi-HSC remained only or primarily *Vwf*-tdTomato⁻. Instead, when reconstituted by a single *Vwf*-tdTomato⁺ P-HSC, the generated HSCs remained primarily *Vwf*-tdTomato⁺, however they often included a minor part of *Vwf*-tdTomato⁻ cells. To functionally verify the HSC identities, we conducted secondary transplantation experiments, by transplanting *Vwf*-tdTomato⁻ and *Vwf*-tdTomato⁺ cells isolated from the BM of primary recipients reconstituted by a single *Vwf*-tdTomato⁻ multi-HSC or *Vwf*-tdTomato⁺ P-HSC. These experiments demonstrated that both single *Vwf*-tdTomato⁻ multi-HSCs and *Vwf*-tdTomato⁺ P-

HSCs possessed self-renewal capacity, but not the ability to give rise to the other. The results illustrated that *Vwf*-tdTomato⁻ multi-HSCs and *Vwf*-tdTomato⁺ P-HSCs have a non-hierarchical relationship. Flow cytometry analysis of HSPCs along with in vitro lineage potential experiments verified that *Vwf*-tdTomato⁻ multi-HSCs differentiated via multiple stages of multilineage progenitors before becoming platelet-restricted progenitors. In contrast, the *Vwf*-tdTomato⁺ P-HSCs were demonstrated to circumvent these stages. Figure 7 illustrates the HSPC hierarchies reconstituted by single transplanted *Vwf*-tdTomato⁻ multi-HSCs and *Vwf*-tdTomato⁺ P-HSCs.

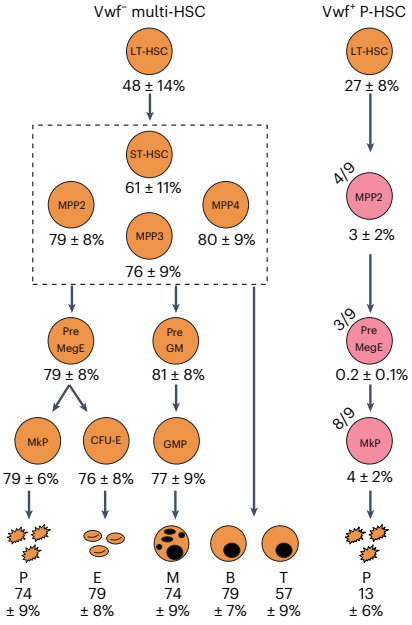

Figure 7. HSPC hierarchy reconstituted by a single transplanted *Vwf*-tdTomato⁻ multi-HSCs (left, n = 8) or *Vwf*-tdTomato⁺ P-HSCs (right, n = 9). The numbers shown are the mean ± s.e.m. percentage contributions to each detected population. Orange, reconstitution in all mice. Pink, reconstitution in some mice (the fraction of reconstituted mice is indicated in the upper left of each circle); mean of positive mice. LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; MkP, megakaryocyte progenitor; PreMegE, pre-megakaryocyte/erythroid progenitor; CFU-E, colony forming unit-erythroid; PreGM, pre-granulocyte/monocyte progenitor; GMP, granulocyte/monocyte progenitor; P, platelets; E, erythrocytes; M, myeloid cells (granulocytes and monocytes); B, B cells; T, T cells. Reprinted from Carrelha *et al.*[279] (Study II) with permission form the publisher.

MkPs represent the final progenitor stage before differentiation into mature megakaryocytes, which are responsible for platelet production. To investigate whether the MkP populations generated by the different HSC types are molecularly distinct, we carried out single-cell RNA sequencing.[280] This analysis showed that the MkPs reconstituted from *Vwf*-tdTomato^{+} P-HSCs and *Vwf*-tdTomato^{-} multi-HSCs were molecularly different from each other. Furthermore, these results suggested that *Vwf*-tdTomato^{+} P-HSCs and *Vwf*-tdTomato^{-} multi-HSCs utilize molecularly separated platelet progenitor pathways for platelet production.

A prominent difference identified by single-cell RNA sequencing was the lack of *Flt3* RNA expression at all HSPC differentiation stages in the progenitor pathway reconstituted by *Vwf*-tdTomato^{+} P-HSCs, compared to the high *Flt3* expression in the pathway initiated by *Vwf*-tdTomato^{-} multi-HSCs. Previous studies have suggested that all blood lineages pass through a primitive progenitor stage characterized by *Flt3* mRNA expression.[281] Using a *Flt3*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ mouse model,[242,247] with Cre expression under the control of a *Flt3* promoter to label all cells that have expressed *Flt3*, we verified that erythrocytes, granulocytes, B and T lymphocytes exhibited nearly 100% *Flt3*-Cre-tdTomato positivity in steady-state *Flt3*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ mice. However, a small part (10%) of the platelets were invariably *Flt3*-Cre-tdTomato negative, implying that steady state platelets may be partially replenished through a *Flt3*-negative pathway, potentially via *Vwf*-tdTomato^{+} P-HSCs.

To investigate if a part of the steady-state platelets could be produced via a *Flt3*-negative pathway, we used a *Flt3*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ *Vwf*-eGFP$^{tg/+}$ *Gata1*-eGFP$^{tg/+}$ mouse model [100,242,247,248] for fate-mapping of single-cell transplanted *Vwf*-GFP^{-} multi-HSCs and platelet-restricted *Vwf*-eGFP^{+} P-HSCs. These fate-mapping experiments verified that all blood lineages originating from *Vwf*-eGFP^{-} multi-HSCs pass through a progenitor stage with *Flt3* expression, which included the platelets reconstituted from *Vwf*-eGFP^{-} multi-HSCs. At the same time, we demonstrated that *Vwf*-eGFP^{+} P-HSCs only use an alternative progenitor pathway without *Flt3* expression for their platelet production. These results further established a non-hierarchical relationship between *Vwf*-eGFP^{-} multi-HSCs and *Vwf*-eGFP^{+} P-HSCs, and demonstrated a clear separation between *Vwf*-eGFP^{-} multi-HSC and *Vwf*-eGFP^{+} P-HSC platelet production. Figure 8 shows *Flt3*-Cre-tdTomato-labeling in platelets and other blood lineages.

Using the *Flt3*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ mouse model[242,247] we proceeded with lineage-tracing of platelet replenishment after chemotherapy treatment with cyclophosphamide and 5-FU, which causes a sharp reduction of platelet progenitors in the BM. The *Flt3*-Cre lineage-tracing after hematopoietic challenge showed as hypothesized that platelets were reconstituted faster through the alternative *Flt3*-negative progenitor pathway than through the *Flt3*-expressing progenitor pathway.

Our study was conducted in mouse models to enable the described single-cell transplantations and genetic lineage-tracing methods that are not feasible in the human setting. However, additional studies are needed to verify whether similar progenitor pathways exist in the human hematopoiesis. Recent reports on findings congruent with human P-HSCs reinforce the likelihood of alternative platelet progenitor pathways also in human hematopoiesis.[282,283]

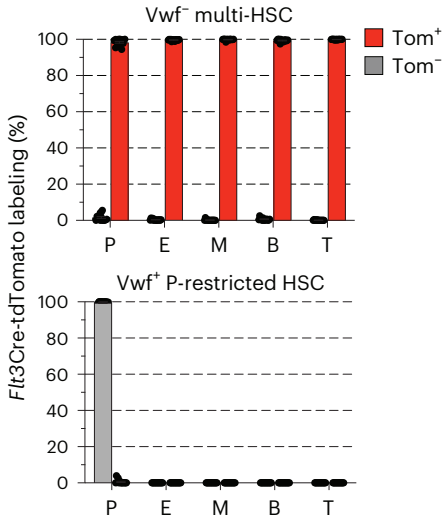

Figure 8. Fate-mapping of alternative platelet replenishment pathways. *Flt3*-Cre-tdTomato-labeling in platelets and other blood lineages replenished by a single *Vwf*-eGFP^{-} multi-HSC (top, n=14) and by a *Vwf*-eGFP^{+} platelet-restricted HSC (bottom, n=14), 18–21 weeks after transplantation, which demonstrates replenishment of platelets through a *Flt3*-positive and *Flt3*-negative pathway, respectively. LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; MkP, megakaryocyte progenitor; PreMegE, pre-megakaryocyte/erythroid progenitor; CFU-E, colony forming unit-erythroid; PreGM, pre-granulocyte/monocyte; GMP, granulocyte/monocyte progenitor; P, platelets; E, erythrocytes; M, myeloid cells (granulocytes and monocytes); B, B cells; T, T cells. Reprinted from Carrelha *et al.*[279] (Study II) with permission from the publisher.

If the faster alternative platelet progenitor pathway would be confirmed in humans, it could presumably be used to improve platelet recovery after cancer chemotherapy with drugs severely depleting platelet production. Moreover, it would be of importance to perform platelet pathway *Flt3*-Cre fate mapping in steady-state conditions, as well as after various types of hematopoietic challenges, to further validate the reliability of platelet progenitor fate-mapping in this model. Equally important would be to investigate if the platelets produced by the two distinct progenitor pathways differ in molecular and functional aspects. A potential functional difference could be dysregulated platelet function leading to a higher risk of thrombosis, as indicated in a recent study demonstrating an expanding alternative *Flt3*-negative platelet replenishment pathway in aging mice.[284] Furthermore, it has not yet been explored if other short-lived lineages could be produced through alternative and potentially faster progenitor pathways.

While this study demonstrated a non-hierarchical relationship between *Vwf*$^-$ multi-HSCs and *Vwf*$^+$ P-HSCs, we hypothesize that these HSC subsets share a common origin in an upstream HSC, which may be found in the Vwf$^+$ multi-HSC population.[100] Future studies are required to explore this hypothesis.

In summary, study II demonstrated at single-cell resolution the non-hierarchical relationship between two distinct stem cell types, *Vwf*-tdTomato$^-$ multi-HSCs, which reconstitute all blood cell lineages, and *Vwf*-tdTomato$^+$ P-HSCs, which primarily reconstitutes platelets. Platelet-restricted progenitors originating from *Vwf*-tdTomato$^+$ P-HSCs and *Vwf*-tdTomato$^-$ multi-HSCs are molecularly distinct, with *Vwf*-tdTomato$^+$ P-HSCs exclusively using an alternative *Flt3*-negative progenitor pathway for platelet replenishment. After hematopoietic challenges, such as chemotherapy, the faster *Flt3*-negative pathway is used for platelet production. The identification of the different progenitor pathways for platelet replenishment is significant for understanding and potentially finding treatments enhancing platelet production in patients with low platelet counts.

4.3 Study III – Identification of a B-cell restricted progenitor emerging early in fetal development

It is well known that BCP-ALL is the most common childhood cancer.[215] The initiating translocations causing childhood leukemia often occur before birth, during fetal hematopoiesis, and differ from mutations causing adult leukemias.[285]

Although ALL subtypes frequently correspond to distinct stages of normal B-cell development, the cell-of-origin remains unknown. It has been speculated that the first oncogenic event might occur at a different developmental cell stage than the one to which the overt leukemic cell most closely corresponds.[216] Acquisition of new characteristics in preleukemic cells or a differentiation arrest at a later cell stage could position the leukemic cells upstream or downstream of the actual cell-of-origin. Another possibility is that childhood leukemias originate in developmentally restricted progenitor cells that emerge during fetal hematopoiesis and wane during later stages of normal hematopoiesis.[215,216] The transcriptional programs controlling the developing hematopoiesis are likely involved in and affect the initiation of leukemia.[216] If we could identify the distinct fetal progenitors in B-cell development, we could potentially also identify cells vulnerable to leukemogenesis and subsequently find new targets for treating infant and childhood leukemia.

Despite the long-standing research efforts to find early fetal progenitors with B-cell potential in the mouse, the timepoint and hematopoietic tissue for emergence of the earliest fetal B-cell restricted progenitor remain unclear. In this study we aimed to investigate where and when in the mouse embryo the first fully B-cell restricted progenitor cells emerge, to characterize these progenitor cells both functionally and molecularly and to compare them with their adult counterparts.

To explore where and when the first B-cell restricted progenitors emerge, we initially used flow cytometry to investigate when cell surface expression of the B-cell restricted marker CD19 appears in FL, which is the main hematopoietic site in the fetus after definitive HSCs have emerged at ED10.5.[5,40] At ED13.5 we identified a small population of cells co-expressing B220 and CD19, as previously described.[203,204] Next we explored additional sites of *de novo* hematopoiesis in the early embryo prior to ED13.5 in FL. To this end, we analyzed the YS and AGM, for possible detection of $CD19^+$ B cell progenitors, however we did not discover any $CD19^+$ cells in these sites or in the FL at ED12. Together these experiments showed that $CD19^+$ cells first emerge in the FL at ED13.5. These findings also reinforce that the previously reported B-cell potential in ED8.5 YS and AGM[193] represents multipotent stem or progenitor cells.[192]

The PreProB cell progenitor compartment in adult BM, as defined by the established Hardy staging,[156] includes B cell progenitors as well as progenitors with other remaining lineage potentials such as T-cell and NK-cell potential.[156,161] To

identify committed B cell progenitors prior to CD19 expression, we used a fate mapping approach to subdivide the PreProB cell compartment in the FL. The *Mb1* (*Cd79a*) gene encodes the Ig-alpha receptor, which is involved in signaling from the BCR and is expressed at high levels early in B-cell development, before the completion of productive VDJ arrangement.[162] Previous studies have shown that *Mb1* is expressed before CD19 cell surface expression in B cell progenitors.[161] Moreover, in pediatric BM the MB-1 protein is expressed before CD19 in progenitors co-expressing the DNA polymerase terminal deoxynucleotidyl transferase (TDT).[286] However, it is not known whether *Mb1*-expressing $CD19^-$ progenitor cells constitute B-cell restricted progenitors. To fate-map *Mb1*-expressing progenitor cells, we used a *Mb1*-$Cre^{tg/+}$ *R26*$^{YFP/+}$ mouse model,[244,246] in which cells expressing *Mb1,* as well as progeny derived from *Mb1*-expressing cells, are labeled with the fluorescent YFP reporter. In ED12.5 FL, prior to CD19 cell surface expression, we detected a small $B220^+CD19^-YFP^+$ progenitor population. This early progenitor population exhibited high expression of FLT3, KIT, and interleukin-7 receptor alpha (IL7RA), all of which are cytokine receptors essential for early lymphoid and B-cell development.[287-292]

To further examine the identity of the $B220^+CD19^-YFP^+$ progenitor cells, we isolated $B220^+CD19^-YFP^+$ progenitor cells with expression of FLT3, KIT and IL7RA by flow cytometry to perform B-lineage gene expression analysis by qPCR in ED12.5 and ED13.5 FL cells from *Mb1*-$Cre^{tg/+}$ *R26*$^{YFP/+}$ embryos. In ED13.5 FL cells, after the onset of CD19 expression, we compared the $B220^+CD19^-FLT3^+KIT^+IL7RA^+YFP^+$ progenitor cells to $B220^+CD19^+FLT3^+KIT^+IL7RA^+YFP^+$ progenitor cells. While the $B220^+CD19^-FLT3^+KIT^+IL7RA^+YFP^-$ progenitors showed almost no expression of *Mb1* or the other examined early B-cell commitment genes (*Cd79b* and *Pax5*), the *Mb1*, *CD79b* and *Pax5* genes demonstrated a clear upregulation in $B220^+CD19^-FLT3^+KIT^+IL7RA^+YFP^+$ progenitor cells in ED12.5 FL. As expected, in ED13.5 FL, all investigated B-lineage genes were expressed and a majority showed higher expression in the CD19-expressing $B220^+CD19^+FLT3^+KIT^+IL7RA^+YFP^+$ progenitor cells. Importantly, the transcription factor encoding gene *Pax5* did not demonstrate higher expression in $B220^+CD19^+YFP^+$ Pro B progenitors compared to $B220^+CD19^-YFP^+$ PreProB progenitor cells. Since the transcription factor PAX5 is known to be crucial for B-lineage restriction,[201] this finding supports that fetal $B220^+CD19^-YFP^+$ progenitor cells are B-lineage restricted.

Following this, in vitro lineage potential experiments were performed for functional assessment of B- and T-cell potential in ED14.5 FL $B220^+CD19^-KIT^+IL7RA^+FLT3^+YFP^+$

progenitor cells. While ED14.5 FL B220$^-$CD19$^-$FLT3$^+$KIT$^+$IL7RA$^+$YFP$^-$ control cells gave rise to both B and T cells, the B220$^+$CD19$^-$KIT$^+$IL7RA$^+$FLT3$^+$YFP$^+$ progenitor cells demonstrated robust B-cell potential but no detectable T-cell potential. This result, along with the onset of early B-cell commitment gene expression, including *Pax5* confirms that *Mb1*-expressing B220$^+$CD19$^-$YFP$^+$ progenitor cells represent the earliest B-cell restricted progenitors identified during fetal development.

To further characterize B-cell differentiation based on expression of *Mb1* and other key markers, we performed global RNA sequencing to compare the transcriptional profiles of corresponding B-cell differentiation stages in ED14.5 FL and adult BM from *Mb1*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ mice. In this mouse model, cells expressing *Mb1* are labeled with the fluorescent reporter tdTomato (TOM).[247] B220$^+$CD19$^-$FLT3$^+$KIT$^+$IL7RA$^+$TOM$^+$ *Mb1*-expressing PreProB cell progenitors were compared to B220$^+$CD19$^-$FLT3$^+$KIT$^+$IL7RA$^+$TOM$^-$ PreProB cells and later B-cell progenitor stages. The CD19-expressing ProB cells were further divided into a FLT3$^+$ and FLT3$^-$ fraction, based on the knowledge that PAX5 represses *Flt3* expression in the process of B-lineage commitment.[202] The investigated progenitor populations are defined in Table 6 in Chapter 3 (Materials and methods).

We observed that the relative frequencies of TOM$^-$ PreProB, TOM$^+$ PreProB, FLT3$^+$ ProB and FLT3$^-$ ProB cell populations (Table 6) were similar in ED14.5 FL and adult BM. RNA sequencing analysis verified that these progenitor populations constitute molecularly different B-cell differentiation stages in FL and equivalent stages in adult BM, which was established by the fact that biological replicates from each progenitor population and developmental stage (fetal and adult) clustered separately (Figure 9). In addition, principal component analysis (PCA) demonstrated that the corresponding fetal and adult B cell progenitor populations are transcriptionally close.

We then conducted a more detailed transcriptional analysis in which the transcriptional signature of TOM$^+$ B-cell restricted PreProB cells was correlated to those of the earlier TOM$^-$ PreProB cells and the first CD19$^+$FLT3$^+$ ProB cells in ED14.5 FL. Unsupervised hierarchical clustering discovered five gene clusters (C1-C5) with separate expression patterns at the different B-cell developmental stages in FL. The C4 cluster contained critical B-cell commitment genes (such as *Ebf1*, *Foxo1, Cd79a*, *Cd79b*, and *Pax5*), which all showed a clear increase in expression from TOM$^-$ to TOM$^+$ PreProB cells. In comparison, the C1 cluster included genes

important for mature B-cell differentiation and activation (such as *Cd19*, *Lef1*, *Tcf3*, *Vpreb1* and *Igll1*), with most genes showing distinct upregulation first at the FLT3$^+$ ProB stage and little or no increase from TOM$^-$ to TOM$^+$ PreProB cells. The C2 cluster comprised myeloid (*Mpo*, *Cd33*, *Cd34*, and *Csf1r*) and T-cell related (such as *Cd28* and *Il4ra*) genes that were downregulated from TOM$^-$ to TOM$^+$ PreProB cells.

Investigation of DEGs revealed that 6 out of 15 upregulated DEGs in fetal TOM$^+$ PreProB cells, compared to TOM$^-$ PreProB cells, were also upregulated in the adult corresponding population and enriched for B-cell commitment genes (*Foxo1*, *Cd79a*, *Cd79b*, and *Pax5*). Likewise, when comparing DEGs in FLT3$^+$ ProB cells to those in TOM$^+$ PreProB cells, many overlapping DEGs were found between fetal and adult progenitors (15/20 upregulated DEGs and 23/42 downregulated DEGs).

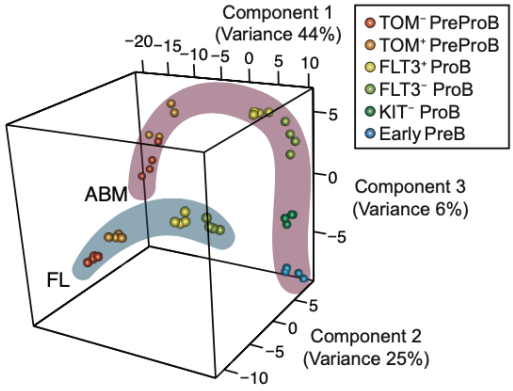

Figure 9. Three-dimensional principal component analysis of global RNA sequencing data from fetal (blue shading) and adult (pink shading) B cell progenitor populations (n = 4/cell population), from *Mb1*-Cre$^{tg/+}$ *R26*$^{TOM/+}$ ED14.5 FL and 8 weeks adult bone marrow. Note that KIT$^-$ ProB and Early PreB progenitors have yet to emerge in ED14.5 FL. ABM, adult bone marrow; ED, embryonic day; FL, fetal liver.
TOM$^-$ PreProB: Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^-$;
TOM$^+$ PreProB: Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^+$;
FLT3$^+$ ProB: Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^+$TOM$^+$;
FLT3$^-$ ProB: Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^+$FLT3$^-$TOM$^+$.
KIT$^-$ ProB: Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL7RA$^+$KIT$^-$FLT3$^-$TOM$^+$;
Early PreB: Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{high}AA4.1$^+$IL7RA$^+$KIT$^-$FLT3$^-$TOM$^+$.
Figure from submitted manuscript Belander Strålin *et al.* (Study III).

Another key finding was that the expression of relevant genes in fetal and adult B progenitor populations resulted in higher signature scores when compared to several published HSC transcriptional signatures[268,269,293,294] in the TOM$^+$ PreProB cell population compared to FLT3$^+$ ProB cells. This suggests that TOM$^+$ PreProB cells still possess self-renewal potential and could potentially play a role in propagation of preleukemic or leukemic cell clones. In contrast, there were no significant differences in scores in cell cycle status transcriptional signatures[293] in the TOM$^+$ PreProB cells compared to FLT3$^+$ ProB cells. Moreover, the expression of B-cell signature genes[295] accelerated from TOM$^+$ PreProB cells to FLT3$^+$ ProB cells. Conversely, myeloid cell[295] and early T-cell[296] gene transcriptional signature scores were reduced from TOM$^+$ PreProB cells to FLT3$^+$ ProB cells. Altogether, these observations demonstrate that TOM$^+$ PreProB cells have a distinct transcriptional pattern and represent the first fetal progenitor cells secured in a B-cell restricted state.

Additionally, when comparing fetal and adult TOM$^+$ PreProB cells, a high number of DEGs were identified. These involved reported fetal specific genes such as *Lin28b*, *H19*, *Igf2bp1* and *Igf2bp3*. In this group, *H19* is specifically intriguing as it was included in cluster C4 alongside genes important for B-cell commitment and has been suggested to have a putative role in ALL development.[297]

The discovery of an early fetal CD19$^-$ *Mb1*-expressing B-cell restricted progenitor is of significance for an improved understanding of the identity and characteristics of fetal B progenitor cells, which are thought to be potential targets of in utero translocations that create preleukemic clones and may give rise to infant and childhood BCP-ALL. Our findings may be used for developing more adequate mouse models for modelling BCP-ALL, for example a model in which the translocations of interest can be conditionally targeted to the CD19$^-$ *Mb1*-expressing B-cell restricted progenitor cells via mice expressing a *Mb1*-Cre allele.

To summarize, study III demonstrated that expression of *Mb1* identifies an earlier fetal stage of CD19$^-$ B-cell restricted progenitors than previously reported in the literature. Fetal *Mb1*-expressing progenitors are first detected in the ED12.5 FL and display a distinct molecular pattern compared to later stages of fetal B progenitor cells. The previously defined fetal and adult PreProB cells can be further sub-fractionated by *Mb1* expression. Notably, progenitors expressing *Mb1* have started a transcriptional program consistent with B-cell specification, which has not yet been initiated in PreProB cells lacking *Mb1*-expression and possessing additional

lineage potentials. The finding of an early fetal *Mb1*-expressing CD19^{-} B-cell restricted progenitor is another step towards an improved understanding of fetal B-lymphopoiesis.

5 Conclusions

The aim of this thesis was to further characterize and compare fetal, neonatal and adult mouse hematopoiesis including lineage-biased HSCs.

In Study I we explored potential lineage-bias of mouse HSCs at single-cell resolution in liver and BM at birth, and investigated to what extent the adult patterns of lineage restrictions and biases are already established. We concluded the following:

- pnHSCs display less P- and PEM-bias/restriction and more consistent lymphoid reconstitution than adult HSCs
- P- and PEM-bias/restriction has initiated in *Vwf*-reporter$^+$ HSCs at the time of birth
- Transcriptional lineage priming in pnHSCs corresponds to their lineage bias upon transplantation

In Study II, we investigated lineage-biased mouse HSCs at single-cell resolution in adult BM, with focus on *Vwf*-tdTomato$^-$ multi-HSCs and *Vwf*-tdTomato$^+$ P-HSCs. We concluded the following:

- There is a non-hierarchical relationship between *Vwf*-tdTomato$^-$ multi-HSCs, reconstituting all blood cell lineages, and *Vwf*-tdTomato$^+$ P-HSCs mainly reconstituting platelets
- Platelet-restricted progenitors originating from *Vwf*-tdTomato$^+$ P-HSCs are molecularly distinct from corresponding progenitors from *Vwf*-tdTomato$^-$ multi-HSCs
- *Vwf*-tdTomato$^-$ multi-HSCs use a multilineage *Flt3*-expressing pathway, while *Vwf*-tdTomato$^+$ P-HSCs use a faster *Flt3*-negative progenitor pathway for platelet replenishment

In Study III, we explored early fetal B-cell progenitor development to identify and characterize the earliest B-cell restricted progenitor. We concluded the following:

- *Mb1* expression defines an earlier fetal stage of CD19$^-$ B-cell restricted progenitors than previously reported

- *Mb1*-expressing CD19^{-} fetal B cell progenitors emerge in FL at ED12.5
- Progenitors expressing *Mb1* have started a transcriptional program consistent with B-cell specification, which is absent in PreProB cells lacking *Mb1* expression

6 Points of perspective

The studies performed in this thesis have contributed to new knowledge on cell fate decisions in different parts of hematopoietic development.

Study I, which focused on exploring potential lineage-biased HSCs at the time of birth, provided new information on the blood reconstitution potential of transplanted definitive HSCs at this developmental stage. We demonstrated that the adult patterns of lineage-bias and lineage-restriction had all been initiated, though they were not fully developed. Compared to the adult, no new types of lineage biases were identified at birth. The study did not investigate when P-bias/restriction first arises, but the rarity of P- and PEM-bias/restriction seen in pnHSCs implies that these characteristics arise mainly after birth. Interestingly, there was in our study no significant difference in the bias/restriction pattern between HSCs from FL and BM, which could have been expected from the different niches the HSCs reside in. However, the lineage priming of the perinatal HSCs seems to be mainly an intrinsic factor, supported by our results from secondary transplantations and scRNA-Seq. Our study is the first single HSC transplantation study conducted at this developmental time point investigating all five main blood linages. It would be of interest to further explore the lineage biased HSCs at birth to determine if the lineage priming and lineage-bias/restriction patterns could play a role in the developmental susceptibility to preleukemic development. A recent study investigated clonal contribution from ED14.5 FL MPPs and HSCs, indicating that different multipotent subsets may contribute to LT hematopoieis.[298] This would be valuable to explore in both FL and BM at the time point of birth to gain a better understanding of hematopoiesis at this rarely studied developmental timepoint. In relation to leukemia susceptibility, it would also be of great interest to study lineage bias in human perinatal HSCs, which could be done in cord blood samples and potentially combined with investigation of early human B-cell restricted progenitors at this developmental stage.

Study II described the non-hierarchical relationship between *Vwf*-tdTomato$^-$ multi-HSCs and *Vwf*-tdTomato$^+$ P-HSCs, and demonstrated an alternative *Flt3*-negative platelet differentiation pathway. The study also showed that the alternative platelet pathway was activated under hematopoietic stress conditions induced by chemotherapy treatment. To further evaluate the described alternative *Flt3*-positive and *Flt3*-negative platelet progenitor pathways, it would

be valuable to carry out platelet pathway *Flt3*-Cre fate-mapping in steady-state conditions. Furthermore, this model could be used to assess these pathways after additional types of hematopoietic challenges in the absence of transplantation. Another key question to address is whether platelets produced by these alternative pathways could differ in molecular and functional aspects. A recent study indicated that platelets produced via an alternative and faster *Flt3*-negative platelet progenitor pathway, that was expanded during aging, might be more prone to cause thrombosis.[284] Moreover, it has yet to be investigated if other short-lived blood cells might also be generated by additional and potentially faster progenitor pathways.

The discovery of an alternative progenitor pathway for platelet production is relevant for improving our understanding of platelet replenishment and could potentially lead to ways to stimulate platelet production in patients with low platelet counts, for example after cancer chemotherapy. Complementary studies are needed to confirm corresponding progenitor pathways in human hematopoiesis. Recent findings of long-term ex vivo expansion of human hematopoietic stem cells[239] may provide new options for studying alternative HSPC pathways in humans, and potentially for expanding P-HSCs. By employing ex vivo expansion of HSCs, it would be possible to examine HSCs from different medical conditions as well as different developmental timepoints. For example, from newborns using cord blood samples, and from young and old adults via bone marrow samples. In human hematopoiesis, when investigating different platelet pathways, the functional and molecular aspects of platelets potentially produced via alternative pathways could also be investigated under different medical conditions, such as thrombocytopenia after chemotherapy treatment and thrombocytopenia associated with immunological disease.

While we established a non-hierarchical relationship between *Vwf*-reporter$^-$ multi-HSCs and *Vwf*-reporter$^+$ P-HSCs in this study, we presume that these HSC subsets have a shared origin in an upstream HSC, and that this HSC ancestor may reside in the *Vwf*-reporter$^+$ multi-HSC population.[100] Additional studies are required to explore this question, which could preferably include investigations of HSCs from earlier developmental stages, such as the pnHSCs examined in Study I. The identification of new subtypes of hematopoietic stem and progenitor cells in adult BM, including platelet-biased and platelet-restricted HSCs, may also have implications for tracking the cell-of-origin for different types of leukemias, but as noted remains to be investigated in fetal hematopoiesis.

Study III identified an early functionally and molecularly distinct fetal CD19$^-$ *Mb1*-expressing B-cell restricted progenitor. This finding is significant for enhancing our understanding of differences between fetal and adult hematopoiesis. It is also relevant for understanding the identity and characteristics of fetal B cell progenitors hypothesized to be targeted by translocations that create preleukemic clones and potentially initiate infant and childhood BCP-ALL. However, the *Mb1*-expressing PreProB cell may be a leukemia-initiating cell, while the target cell-of-origin could reside higher up in the hematopoietic hierarchy. This could likely be tested in mouse models of BCP-ALL in which the leukemia-initiating translocations of interest could be conditionally targeted to the discovered early fetal B-cell restricted progenitor cells via a *Mb1*-Cre allele.

To prospectively identify and characterize fetal specific hematopoietic cells in human embryos is both ethically and technically demanding, thus studies of developmental hematopoiesis in mice remain of great importance to the field. Further functional studies to identify the cell-of-origin for BCP-ALL and other childhood leukemias are required and need to be parallelled by the development of relevant models for studying the initiation processes of these diseases. With advances in ex vivo expansion of both mouse and human HSCs, new opportunities for ex vivo studies of HSCs and progenitor cells including leukemia models are likely to be developed as a complement to in vivo studies.

With improved understanding of fetal and adult hematopoiesis alongside the development of molecular precision medicine, new diagnostic and therapeutic strategies for hematological diseases can be developed.

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