Early fate decisions in hematopoietic stem and progenitor cells. Through the lens of genomic and functional assays.

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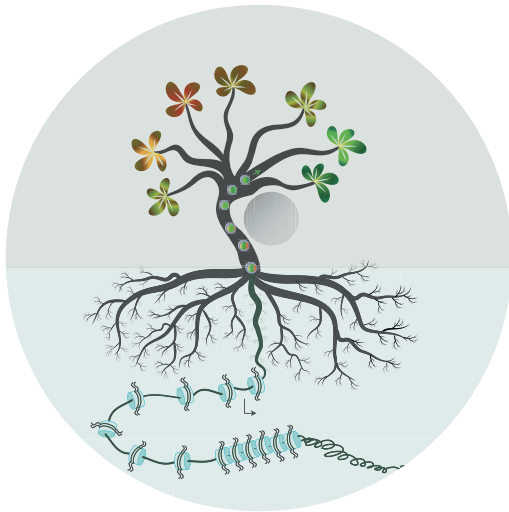
Through the lens of genomic and functional assays

FATEMEH SAFI
MOLECULAR HEMATOLOGY | FACULTY OF MEDICINE | LUND UNIVERSITY



The cover photo is influenced by the poetry of Rumi

"May be you are searching among the branches for what only appears in the roots"

This thesis is about hematopoiesis. The leafs on the branches represent different mature blood cells in our body which came from the very rare cells in the bone marrow called hematopoietic stem cells (HSCs, a colorful cell very close to the root of the tree).

Hematopoiesis is a dynamic non-linear ecosystem in which HSCs and HPCs that have chosen a fate can still 'change their mind' and differentiate into a different fate, this is called lineage priming. The production of different blood and immune cell types is undoubtedly influenced by intrinsic events that control fate options within HSCs/HPCs, such as the accessibility of genes and the binding of suitable transcription factors (represented in the root of the tree). Furthermore, extrinsic signals (represented here by a full moon & water around the root) may influence TFs and chromatin structure to regulate fate decision.

We will discuss this in the thesis
I hope you enjoy reading it
Mojgan Safi

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Abstract

Hematopoietic stem cells (HSCs) are rare cells on top of the differentiation hierarchy of hematopoiesis. HSCs are unique in their combined capacity to differentiate into all mature blood lineages and self-renew to maintain the HSC pool. Based on classical models of hematopoiesis in mouse, the self-renewal potential of HSCs is gradually and step-wise lost during the transition from long term (LT)-HSCs to multipotent progenitors (MPPs) accompanied by upregulated expression of the cell-surface marker FMS-like tyrosine kinase 3 (Flt3). The Flt3^{+} multipotent progenitors serve as developmental intermediates for hematopoietic lineage priming. Notably, the 25% highest Flt3^{+} cells, known as lymphoid-primed MPPs (LMPPs), have been defined as restricted, lymphoid primed cells with decreased megakaryocyte and erythroid (MegE) priming. However, recent single cell RNA sequencing (scRNA-seq) studies question the step-wise model of HSC differentiation and instead suggest a continuum model of the early hematopoietic hierarchy, where the first differentiation events occur in a low-primed cloud of HSPCs without sharply defined gene expression programs. In this model, no transition of different lineages from MPPs with intermediate gene expression occur, instead these progenitors are largely comprised of uni-lineage-primed cells. The overall aim of this thesis is to investigate how cellular-fate options emerge in the cloud of hematopoietic stem/progenitor cells (HSPCs), at what stage the multipotency gives way to lineage priming, and how this stage can be detected. For this aim, single-cell (sc) chromatin accessibility (ATAC-Seq), scRNA-Seq and sc-qPCR analysis were employed extensively to identify the transition of HSCs to lineage restricted multipotent progenitor cells and functionally validated using *in vivo* and *in vitro* assay.In paper I, scATAC-seq was used to map the accessibility of 571 TF-binding motifs as a measure of lineage priming along the Flt3 differentiation axis. The resulting data identified a transition point of highly lineage-primed cells within the continuum of HSPCs where self-renewal and multipotency was lost and lineage commitment initiated. This transition point is characterized by down-regulation of CD9 and up-regulation of Flt3 cell surface expression. Within the Flt3 intermediate population (Flt3^{int}), LSKFlt3^{int}CD9^{high} cells display co-incidental stem and multi-lineage primed chromatin states while the downstream LSKFlt3^{int}CD9^{low} contain an LMPP-like program. Also, this priming seems to initiate in the epigenome without being starkly reflected in the transcriptome. In order to validate the genomic data from the aforementioned analyses, we established *in vitro* culture systems to functionally examine the differentiation fates of cells at a clonal level (Paper II). The result confirms that LSKFlt3^{int}CD9^{high} cells generated more multilineage progeny compared to clones within the LSKFlt3^{int}CD9^{low} fraction. It has been shown extensive changes in heterogeneity of human hematopoietic cells with age. For example increase HSCs frequency and myeloid output while lymphoid output is decreased. However human immunophenotypic changes associated with aging have received little attention. To this end we in paper III examined CD9 cell surface expression in correlation with molecular programs and functional features of human HSPCs throughout life and in leukemia. Interestingly, only a small fraction of HSPCs expressed CD9 in neonatal hematopoiesis and in young adult bone marrow while CD9 expression substantially increased during situations of myeloid and megakaryocytic biased hematopoiesis, such as during ageing or in chronic myeloid leukemia (CML). Thus, CD9 represents an HSC marker for myeloid-biased hematopoiesis.

Key words: Hematopoiesis, HSCs, MPPs, singel cell, scRNA-seq, scATAC-seq, CD9, Aging

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"Maybe you are searching among the branches for what only appears in the roots"

**Rumi was a Persian poet, theologian, and a Sufi mystic (1207-1273)*

The leaves on the branches represent different mature blood cells in our body which came from the very rare cells in the bone marrow called hematopoietic stem cells (HSCs, a colorful cell close to the root of the tree). The production of different blood and immune cell types is undoubtedly influenced by intrinsic events that control fate options within HSCs/HPCs, such as the accessibility of genes and the binding of suitable transcription factors (represented in the root of the tree). Furthermore, extrinsic signals (represented here by a full moon & water around the root) may influence TFs and chromatin structure to regulate fate decision.

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MADE IN SWEDEN

To My Mother

& all the strong women and girls
who fight for their rights…
Woman, Life, Freedom

The one who falls and gets up is
so much stronger than he who never fell
Roy T. Bennett

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2022 may 10, volume 39, Issue 6, Cell Report.

Paper II

Fatemeh Safi, Parashar Dhapola, Eva Erlandsson, Linda Geironson Ulfsson, Ariana S. Calderón, Charlotta Böiers and Göran Karlsson. In vitro clonal multilineage differentiation of distinct hematopoietic progenitor populations.

2023 Jan 10, volume 4, Issue 1, STAR Protocols

Paper III

Fatemeh Safi, Parashar Dhapola, Mikael N.E. Sommarin, Göran Karlsson. CD9 marks myeloid/MegE -biased human hematopoiesis. *Manuscript*

Papers not included in this thesis

Mikael N.E. Sommarin*, Parashar Dhapola*, Fatemeh Safi, Rebecca Warfvinge, Linda Geironson Ulfsson, Eva Erlandsson, Anna Konturek-Ciesla, Ram Krishna Thakur, Charlotta Böiers, David Bryder, Göran Karlsson. *Single-Cell Multiomics Reveals Distinct Cell States at The Top of The Human Hematopoietic Hierarchy*

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Mikael N.E. Sommarin, Rebecca Warfvinge, Fatemeh Safi, Göran Karlsson Combinatorial Single-Cell Approach to Characterize the Molecular and Immunophenotypic Heterogeneity of Human Stem and Progenitor Populations. J.Vis.Exp: Jove .2018. (140)

List of Abbreviations

a-BM	aged bone marrow
ABC	atp-binding cassette
AGM	aorta-gonad-mesenephros
ALL	acute myeloid leukemia
AML	acute lymphoid leukemia
ATAC-seq	assay for transposase-accessible chromatin sequencing
BM	bone marrow
CB	cord blood
CD	cluster of differentiation
CFC	colony forming cell
CFU	colony forming units
CFU-E	colony forming units-erythroid
CFU-S	colony forming units-spleen
CITE-seq	cellular indexing transcriptomes & epitopes sequencing
CLOUD	continuum of low primed un differentiated
CLP	common Lymphoid Progenitors
CML	chronic myeloid leukemia
DC	dendritic cell
Er/E	erythroid
EMP	erythro-myeloid progenitors
EPO	erythropoietin
FL	fetal liver
Flt3	fms-related tyrosine kinase 3
Flt3L	fms-related tyrosine kinase 3 ligand
FACS	fluorescence-activated cell sorting
GFP	green fluorescent protein
GM-CSF	granolocyte macrophage colony –stimulating factor
GMP	granulocyte and macrophage progenitors
GP	granulocyte progenitors
HPC	hematopoietic progenitor cells
HSC	hematopoietic stem cells
HSPC	hematopoietic stem/progenitor cells
HVGs	highly variable genes
IL	interleukin

KO	knockout
Lin-	lineage negative
LMPP	lympho-myeloid progenitor populations
LSC	leukemic stem cell
LSK	lineage negative sca1posetive ckit positive
LT	long term
LT-HSC	long term hematopoietic stem cells
Ly	lymphoid
MEP	megakaryocyte and erythroid progenitors
MegE	megakaryocyte and erythroid
MK	megakaryocyte
MPP	multipotent progenitor populations
My	myeloid
NK	natural killer
NSG	nod/ltsz-scid/il2rg-/-
P	platelet
PB	peripheral blood
PMTs	photomultiplier tubes
Pol	polymerase/reverse transcriptase
RBC	red blood cell
RNA	ribonucleic acid
Sc	single-cell
sc-qPCR	single-cell quantitative real-time polymerase chain reaction
scRNA-seq	single-cell RNA sequencing
Sca1	stem cell antigen 1
SCF	stem cell factor
SLAM	signalling lymphocyte activating molecule
ST	short-term
ST-HSC	short term hematopoietic stem cells
TFR	treatment free remission
TF	transcription factor
TFBS	transcription factor binding sites
TKI	tyrosine kinase inhibitors
TPO	thrombopoietin
t-SNE	t-distributed stochastic neighbor embedding
TSS	transcription start sites
UMAP	uniform approximation and projection
UMIs	unique molecular identifiers
WT	wild type
y-BM	young bone marrow
YS	yolk sac

Nomenclature

Here a short note on certain nomenclature that used in this thesis.

Gene symbols in mice are italicized, with only the first letter in upper-case (e.g., *Gata1*).

Gene symbols in human are italicized, with all letter in upper-case (e.g., *GATA1*).

Protein symbols for both mice and human are not italicized, and all letters are in upper-case* (e.g., GATA1).

*Some cell surface protein in mice commonly presented as below which also used in this thesis.

Tyrosine-protein kinase or c-Kit receptor (c-Kit/K/CD117)

FMS-like tyrosine kinase 3 (Flt3/CD135)

Macrophage-1 antigen (Mac1/CD11b)

Stem cell antigen-1 (Sca-1; S)

CD is stand for cluster of differentiation

Popular summary

Over 2 million new bloods cells are produced every second in the human body by a process called hematopoiesis (Greek: to make blood). The blood cells that circulate in the vascular system (e.g erythrocytes, thrombocytes, lymphocytes, monocyte etc.) are usually the mature version of a progenitor that develops in the bone marrow from a small population of cells called hematopoietic stem cells (HSCs). HSCs are rare and quiescent cells that can both differentiate into fully mature blood cell as well as give rise to further HSCs through self-renewal, thereby maintaining the stem cell pool. This process, how mature functional cells came from their precursors in the bone marrow has been the focus of intensive research over the years.

The HSCs themselves are heterogeneous and are considered to have different capacity or "potency" for self-renewal and ability to give rise to different cells (called multipotent). They can vary from very stable long term hematopoietic stem cells (LTHSCs), that can give rise to any lineage of blood cell to more short term, and less potent cells such as short term hematopoietic cells (STHSCs) or multipotent progenitors (MPPs). After almost 70 years of ongoing research into the hematopoietic system, stem cells can now be isolated using fluorescence-activated cell-sorting (FACS) technology and functionally tested *in-vitro* and in-vivo. From this research, a hierarchical model of hematopoiesis is inferred, where differentiation of cells occurs in a stepwise process from multi-potent (multiple lineage output) to oligo-potent (lineage–restricted output) to uni-potent (single lineage output) progenitors finally leading to mature blood cells. Thus, the more cells mature the less capable they are of self-renewal and differentiation into different cells.

Each cell contains it´s genetic information encoded in DNA (deoxyribonucleic acid). When a gene needs to be read, it must go through a reading process (called; transcription). First, the region of DNA that needs to be read is made accessible by different factors (called; epigenetic factors). Then the accessible part of the DNA molecule is transcribed into an RNA molecule. The RNA molecule then serves as a recipe for the synthesis of a protein, which can alter the function of the cell. Recently, the view of the hierarchical model has been changed by reading information from individual RNA molecules from the hematopoietic stem cells pool, using single cell RNA sequencing technology. Based on these kind of observations in HSCs, there is no measurable transition of different lineages from

multipotent progenitors (MPPs) with the intermediate expression of genes. this suggested a continuum model for hematopoiesis in contrast to the stepwise model above.

In paper I, we ask how cellular-fate options emerge, at what stage the multipotency is lost and lineage priming is initiated, and how we can detect it. We used single-cell chromatin analysis (scATAC-Seq) to identify the accessible region of DNA. We successfully identified mixed-lineage states undetectable by scRNA-seq, and implying that lineage priming begins in the epigenome rather than the transcript level. Since, self-renewal potential is gradually lost with up regulation of FMS-like tyrosine kinase 3-(Flt3), the HSPCs are arranged along the Flt3 expression gradient. It contains a pool of cell population acts as a transitional bridge connecting multipotency and lineage restriction. $CD9^{high}$ cells in the population that express the Flt3, at the intermediate level, show massive changes in chromatin accessibility, co-incidental stem and multi-lineage primed chromatin state, and multi-potency in vivo and in vitro. We found the transition point in a cloud of HSPCs, in mice, where multipotency is rapidly lost as CD9 expression is down regulated and Flt3 expression is up regulated. Due to functionally validated single-cell genomics data (scATAC-seq and scRNA-seq), in paper II, an in-vitro co-culture system was developed that successfully assessed colonel differentiation of cells to the three major hematopoietic lineage trajectories (lymphoid (B cells), myeloid, and megakaryocyte/erythroid).

As we mention above, each individual HSC in the stem cell pool is heterogeneous and this heterogeneity increases during aging. While HSCs in young mice perform qualitatively similar, ageing is marked by an increased size of the HSC pool and substantial functional variation of individual HSCs. Moreover, aging leads to numerous adverse effects on hematopoiesis and immunity, as well as increased risk of certain hematopoietic diseases, like myeloid leukemia. Multiple sorting strategies have been developed to purify functionally different murine primitive HSCs based on cell surface markers heterogeneously expressed within the HSC population. In contrast to mouse, immunophenotypic changes associated with ageing effects are poorly explored in human. As a result, in Paper III, we examine how CD9 is expressed in humans and how cellular heterogeneity of CD9 expression changes during aging and in leukemia. We demonstrate that early in life, CD9 expression is infrequent, however, there is a significant increase in CD9 expression in HSPCs during situations of myelo-erythoid biased hematopoiesis, such as ageing or leukemia. Moreover, single-cell genomics data suggest that CD9 could mark myeloid/MegE biased hematopoiesis in human.

Populärvetenskaplig Sammanfattning

Över 2 miljoner nya blodkroppar bildas varje sekund i människokroppen genom en process som kallas hematopoes (grekisk översättning för att skapa blod). De blodceller som cirkulerar i kärlsystemet (t.ex. erytrocyter, trombocyter, lymfocyter, monocyter etc.) är vanligtvis den mogna versionen av en progenitorcell som utvecklas i benmärgen från en liten cellpopulation som kallas hematopoetiska stamceller (HSC). HSC är sällsynta och vilande celler som både kan differentiera sig till fullt mogna blodkroppar och ge upphov till ytterligare HSC genom självförnyelse, vilket gör att stamcellspoolen bibehålls. Denna process, där mogna funktionella celler kommer in i blodomloppet från sina föregångare i benmärgen, har varit föremål för intensiv forskning under årens lopp. Den vetenskapliga modellen för stegen i denna process uppdateras regelbundet genom nya framsteg och står i fokus för denna avhandling.

HSC är heterogena och anses ha olika kapacitet eller "potens" för självförnyelse och förmåga att ge upphov till olika celler (multipotenta). De kan variera från mycket stabila långvariga hematopoetiska stamceller (LTHSC) som kan ge upphov till alla blodcellslinjer till mer kortvariga och mindre potenta celler som kortvariga hematopoetiska celler (STHSC) eller multipotenta progenitorceller (MPP). Efter nästan 70 års forskning om det hematopoetiska systemet kan stamceller nu isoleras med hjälp av FACS-teknik (fluorescensaktiverad cellsortering) och funktionellt testas in-vitro och in-vivo. Från denna forskning har en hierarkisk modell för hematopoesi tagits fram, där differentiering av celler sker genom en stegvis process från multipotenta (med flera olika linjer) till oligopotenta (med begränsad linjeproduktion), till unipotenta (med en enda linjeproduktion) progenitorceller och slutligen mogna blodceller. Ju mer cellerna mognar, desto mindre är deras förmåga till självförnyelse och differentiering till olika celler.

Varje cell innehåller sin genetiska information kodad i DNA (deoxiribonukleinsyra). När en gen ska läsas måste den genomgå en läsprocess (transkription). Först görs delen av DNA som ska läsas tillgänglig av olika faktorer (så kallad epigenetik) och den tillgängliga delen av DNA-molekylen transkriberas till en RNA-molekyl. RNA-molekylen fungerar sedan som ett recept för syntesen av ett protein, vilket kan förändra cellens funktion. Synen på den hierarkiska modellen har nyligen ändrats efter att man har läst information från enskilda RNA-molekyler från den hematopoetiska stamcellsreserven med hjälp av RNA-sekvenseringsteknik för

enskilda celler. Denna typ av observationer av HSC tyder på att det finns ingen mätbar övergång av olika linjer från multipotenta progenitorceller (MPPs) med mellanliggande uttryck av gener, vilket tyder på en hematopoetiska hierarkimodell som bildar ett kontinuum i motsats till den stegvisa modellen ovan.

I artikel ett frågar vi oss hur valmöjligheterna för cellens öde uppstår och i vilket skede multipotentialen går förlorad och linjebestämning inleds, och hur vi kan upptäcka detta. Vi använde oss av kromatinanalys av en enda cell (scATAC-seq) för att identifiera den tillgängliga DNA-regionen. Vi har lyckats identifiera tillstånd med blandade linjer som inte kan upptäckas med scRNA-seq, och som innebär att linjebestämning börjar i epigenomet snarare än i transkriptet. Eftersom självförnyelsepotentialen gradvis går förlorad med uppreglering av FMS-liknande tyrosinkinas 3-(Flt3), arrangeras därför HSPC:er längs Flt3-uttrycksgradienten. Den innehåller en pool av cellpopulationer som fungerar som en övergångsbro som förbinder multipotens och linjebestämning. CD9-höga celler i den population som uttrycker Flt3 på mellannivå visar massiva förändringar i kromatin tillgänglighet, kromatin tillstånd som är förberett för stam- och flerlinjer och multipotens in vivo och in vitro-analyser. Vi fann att övergångspunkten i molnet av HSPCs hos möss är där multipotens snabbt förloras då CD9-uttrycket nedregleras och Flt3-uttrycket uppregleras. På grund av funktionellt validerade genomikdata från enstaka celler (scATAC-seq och scRNA-seq) utvecklades i artikel II ett in-vitro samodlingssystem som framgångsrikt utvärderade kolonidifferentiering av celler till de tre stora hematopoetiska linjebanorna (lymfoida (B-celler), myeloida och megakaryocyter/erytrocyter).

Som vi nämnde ovan är varje enskild HSC i stamcellsgruppen heterogen och denna heterogenitet ökar under åldrandet. Medan HSC i unga möss fungerar kvalitativt sett mer likartat, kännetecknas åldrandet av en ökad storlek på HSC-poolen och en betydande funktionell variation hos enskilda HSC. Stigande ålder leder även till ett flertal negativa effekter på hematopoesi och immunitet samt ökad risk för vissa hematopoetiska sjukdomar, som myeloisk leukemi. Flera sorteringsstrategier har utvecklats för att rena funktionellt olika primitiva HSC i musmodeller på basis av cellytmarkörer som uttrycks heterogent inom HSC-populationen. I motsats till möss är immunfenotypiska förändringar som är förknippade med åldrandet dåligt utforskade hos människan. Därför undersöker vi i Paper III hur CD9 uttrycks hos människan och hur den cellulära heterogeniteten i CD9-uttrycket förändras under åldrandet och vid leukemi. Vi visar att tidigt i livet är CD9-uttrycket sällsynt, men att det finns en betydande ökning av CD9-uttrycket i HSPCs under situationer med myelo-erytoid biaserad hematopoes, såsom åldrande eller leukemi. Dessutom tyder data från genomikdata för enskilda celler på att CD9 skulle kunna markera myeloisk/megakaryocyt biaserad hematopoes hos människor.

جمع بندی علمی

سلولهای بنیادی خون، سلولهای منحصر به فرد و کمیابی هستند که درون مغز استخوان قرار دارند. این سلولها تنها ۰/۱ درصد از سلولهای مغز استخوان را تشکیل میدهند. این سلولها توانایی تقسیم و بوجود آوردن یک سلول مثل خود را دارند، به این توانایی خود سازی گفته میشود. سلولهای بنیادی قابلیت تمایز دارند ومی توانند همه سلولهای خون را ، شامل گلبولهای سفید، گلبولهای قرمز و پلاکتها بوجود بیاورند. هر روز بالغ بر ۲ میلیون سلول خون در بدن انسان ساخته میشود که خود اهمیت این سلولهای بنیادی را بیش از پیش آشکار میکند.

بیش از ۷۰ سال تحقیق درزمینه جدا سازی سلولهای بنیادی به کمک تکنیک Flowcytometry (فلو سایتومتری)، و بررسی این سلولها *in vitro* (محیط آزمایشگاهی)، یا *in vivo* (در پروسه پیوند مغز استخوان موش) باعث طبقه بندی این سلولها به صورت پله ای شد. در راس این پلکان سلولهای بنیادی Multipotent (یاخته های بنیادی چند توان) که توانایی تبدیل شدن به هر سه گروه از سلولها خون را دارند/ قرار گرفته اند. در رده دوم سلولهای Oligopotent (یاخته های بنیادی دو توان) است که توانایی تبدیل به ۲ گروه از سلولها خون را دارند . رده سوم سلولهای Unipotent (یاخته های بنیادی تک توان) هستند که قابلیت تبدیل تنها به یک گروه از سلولها خون را دارند و در انتهای پلکان سلولهای تکامل یافته خون قرار دارند.

هرسلول دارای اطلاعات ژنتیکی به نام DNA است. DNA که به دور Histon (هیستون) پیچیده، در زمان رونویسی ژن باید قابل دسترس شود. فاکتورهای مختلفی از جمله Epigenetics (اپی ژنتیک) کمک میکنند تا از فشردگی DNA به دور هیستون کاسته و ژن را قابل دسترس کند تا رونویسی انجام گیرد. درنهایت مولکول RNA ساخته میشود که برای محتوای نهایی پروتیین مورد استفاده قرار میگیرد.

تعیین توالی ژنوم در درون یک سلول اخیراً بواسطه تکنیک Single cell RNA-(scRNA-seq) Sequensing انجام پذیر شده است. با این روش اطلاعات ژنتیکی از هزاران سلول گرفته میشود و سلولها بر اساس شباهت ملکولی طبقه بندی میشوند. این تحقیقات به تغییر ساختار پلکانی انجامید. در مدل جدید سلولهای بنیادی به صورت پیوسته از روی سرسره غلت میخورند وتغیرات بین سلو لی به صورت یکنواخت و تدریجی انجام می شود.این مدل به نام (پیوسته) continum شهرت دارد. متاسفانه در این مدل پیدا کردن سلولهای بنیادی چند توان از سلولهای بنیادی دو توان امکان پذیر نیست وبه همین دلیل ما این پرسش را مطرح کردیم که چرا این سلولها توسط تکنیک scRNA-seq تشخیص داده نمیشوند، در ادامه اینکه چطور سلولهای بنیادی چند توان به سلولهای بنیادی دو توان تبدیل میشوند و چگونه میتوان این سلولها را جدا کرد.

در مقاله اول با استفاده از تکنیک Single cell assay for transposase- accessible chromatin (sc-ATAC-seq)، توالی یابی کروماتین های در دسترس درتک سلول، یاخته های بنیادی دو توان شناسایی شدند. این تکنیک خیلی بهتراز scRNA-seq است، برای تشخیص سلولهای کمیاب و یا سلولهایی که ژن های انها به صورت قوی بیان نمی شوند است. استفاده از این تکنیک به همراهی sc-RNA-seq نشان داد که سلولهای بنیادی چند توان با بیان مارکر Flt3 و از دست دادن بیان مارکر 9CD به سلولهای بنیادی دو توان تبدیل می شوند. آخرین مرحله از سلولهای بنیادی چند توان که توانایی خود سازی ندارند ولی هنوز توانایی تبدیل به همه سلولها را دارند به واسطه این مارکرها $Lineag^{-}$ $Sca1^{+}ckit^{+}CD34^{+}Flt3^{int}CD9^{+}$ قابل شناسایی هستند. نتایج مولکولی بدست امده از این تحقیق با استفاده از تکنیکهای پیوند استخوان درموش و در کشت سلولی درآزمایشگاه تایید شد. ما در این مقاله نشان دادیم که هنوز مدل پلکانی درون ساختار پیوسته وجود دارد.

در مقاله دوم با راه اندازی ی کشت سلولی در شرایط آزمایشگاهی، تبدیل سلول بنیادی به همه سلولهای خون با دو روش متفاوت بررسی شد. در روش اول جدا سازی تک سلول بنیادی توسط دستگاه sorting Flowcytometry و نگه داری از آن در شرایط مناسب آزمایشگاهی انجام شد. در روش دوم سلولهای بنیادی به صورت گروهی جدا و با هر سلول با استفاده از تکنیک بار کدینگ نشان دارشد. در تکنیک دوم یک بار کد به DNA هر سلول اضافه می شود که هر سلول «دختر» که از این سلول ساخته میشود این بار کد را حمل میکنند. به این ترتیب میتوان تشخیص داد چه نوع سلول هایی از یک سلول اولیه بوجود آمده است.

در پروژه سوم به بررسی روند پیری در سلولهای بنیادی پرداختیم. در طول عمر عملکرد سلولهای بنیادی تغییر میکنند. با افزایش سن سلولهای بنیادی علی رغم اینکه از نظر تعداد زیاد می شوند ولی اغلب ناکارآمد هستند. سلولهای بنیادی مایولویئد با بالارفتن سن دربدن افزایش میابند همزمان ازتعداد سلولهای لینفوید کاسته میشود. در موش مارکرهایی پیداشده است که این سلولها را شناسایی می کنند. برای مثال مارکر CD86 می تواند سلولهای بنیادی لنفوسیت را شناسایی کند، که با طول عمراز بیان این مارکر در این سلولها کاسته میشود. متاسفانه تغییرات سلولهای بنیادی در انسان کمتر بررسی شده است.

در مقاله سوم ما نشان دادیم که بیان مارکر CD9 با افزایش سن بر خلاف سلولهای بنیادی جوان زیاد میشود. این روند افزایشی بیان مارکر CD9 درسرطان خون مثل لوسمی مزمن مایلوئیدی، Chronic (CML) myeloid Leukemia هم دیده شد. همه این نتایج نشان میدهد که مارکر CD9 می تواند برای جدا سازی سلولهای بنیادی مایولیئد با افزایش سن استفاده شود.

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
Marie Curie

Abstract

Hematopoietic stem cells (HSCs) are rare cells on top of the differentiation hierarchy of hematopoiesis. HSCs are unique in their combined capacity to differentiate into all mature blood lineages and self-renew to maintain the HSC pool. Based on classical models of hematopoiesis in mouse, the self-renewal potential of HSCs is gradually and step-wise lost during the transition from long term (LT)-HSCs to multipotent progenitors (MPPs) accompanied by upregulated expression of the cell-surface marker FMS-like tyrosine kinase 3 (Flt3). The $Flt3^{+}$ multipotent progenitors serve as developmental intermediates for hematopoietic lineage priming. Notably, the 25% highest $Flt3^{+}$ cells, known as lymphoid-primed MPPs (LMPPs), have been defined as restricted, lymphoid primed cells with decreased megakaryocyte and erythroid (MegE) priming. However, recent single cell RNA sequencing (scRNA-seq) studies question the step-wise model of HSC differentiation and instead suggest a continuum model of the early hematopoietic hierarchy, where the first differentiation events occur in a low-primed cloud of HSPCs without sharply defined gene expression programs. In this model, no transition of different lineages from MPPs with intermediate gene expression occur, instead these progenitors are largely comprised of uni-lineage-primed cells.

The overall aim of this thesis is to investigate how cellular-fate options emerge in the cloud of hematopoietic stem/progenitor cells (HSPCs), at what stage the multipotency gives way to lineage priming, and how this stage can be detected. For this aim, single-cell (sc) chromatin accessibility (ATAC-seq), scRNA-seq and sc-qPCR analysis were employed extensively to identify the transition of HSCs to lineage restricted multipotent progenitor cells and functionally validated using *in vivo* and *in vitro* assay.

In paper I, scATAC-seq was used to map the accessibility of 571 transcription factor (TF)-binding motifs as a measure of lineage priming along the Flt3 differentiation axis. The resulting data identified a transition point of highly lineage-primed cells within the continuum of HSPCs where self-renewal and multipotency was lost and lineage commitment initiated. This transition point is characterized by down-regulation of CD9 and up-regulation of Flt3 cell surface expression. Within the Flt3 intermediate population ($Flt3^{int}$), $LSKFlt3^{int}CD9^{high}$ cells display co-incidental stem and multi-lineage primed chromatin states while the downstream $LSKFlt3^{int}CD9^{low}$ contain an LMPP-like program. Also, this priming seems to initiate in the

epigenome without being starkly reflected in the transcriptome. In order to validate the genomic data from the aforementioned analysis, we established *in vitro* culture systems to functionally examine the differentiation fates of cells at a clonal level (Paper II). The result confirms that LSKFlt3^{int}CD9^{high} cells generated more multilineage progeny compared to clones within the LSKFlt3^{int}CD9^{low} fraction. It has been shown extensive changes in heterogeneity of human hematopoietic cells with age. For example increase HSCs frequency and myeloid output while lymphoid output is decreased. However human immunophenotypic changes associated with aging have received little attention. To this end we in paper III examined CD9 cell surface expression in correlation with molecular programs and functional features of human HSPCs throughout life and in leukemia. Interestingly, only a small fraction of HSPCs expressed CD9 in neonatal hematopoiesis and in young adult bone marrow while CD9 expression substantially increased during situations of myeloid and megakaryocytic biased hematopoiesis, such as during ageing or in chronic myeloid leukemia (CML). Thus, CD9 represents an HSC marker for myeloid-biased hematopoiesis.

Introduction

Brief on the stem cell theory

The invention of the microscope and Robert Hooke's discovery of cells in 1665 led to the beginning of cell research in the middle of the 17th century. However, it did not really take off until the 19th century,when Rudolph Virchow wrote "omnis cellula e cellula" (every cell from pre-existing cells), which introduced the concept of cell theory in 1858. Along with the work of Louis Pasteur and others, spontaneous generation of life was discarded and the search in to how complex life originates from a single cell was initiated (Mazzarello 1999). The term "stem cell" appears in the scientific literature as early as 1868 by Ernst Haeckel and later it was coined by Theodor Boveri and Valentin Haecker in 1892 to describe cells committed to giving rise to the germline. Artur Pappenheim later in 1905 created a diagram of stem cell fate (similar to what we see in textbooks today) that suggested the multipotent stem cell to be located in the very center of the figure, showing that one master cell, can give rise to other cells that can become master cells themselves and specialize further (Figure 1). We now know that his work on the blood stem cell theory, along with that of Alexander Maximow and Ernst Neumann, proposed the idea that all blood cells arise from a single ancestor cell (hematopoietic stem cells) (Ramalho-Santos and Willenbring 2007).

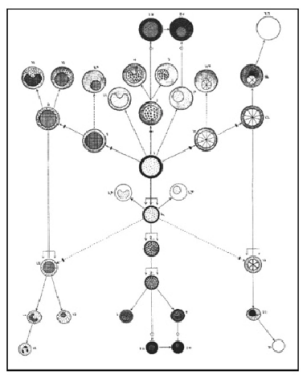

Figure 1- Artur Pappenheim's view of hematopoiesis from 1905, the central cell is thought to be the common progenitors of the entire blood network (Ramalho-Santos and Willenbring 2007).

Hematopoiesis

The process of blood cell generation through life is termed “Hematopoiesis” from Greek haima (αἷμα), meaning 'blood' and poises (ποιεῖν), meaning 'to make'. Hematopoiesis relies on hematopoietic stem cells (HSCs), a small number of multipotent and self-renewing cells that reside in the bone marrow (BM) and constantly supply mature blood cells by a complex multi-step differentiation process, during which intermediate progenitor cells are produced and have the capability to further differentiate to three main group of mature blood cells. Red blood cells (RBCs) or erythroid cells that collect oxygen from the lungs and transport to body’s tissue, thrombocyte or platelets which are essential for wound healing and blood clotting, and white blood cells (myeloid and lymphoid cells) contributing to immune system; that can be further divide to the innate and adaptive immune system. Cells involved in innate immune responses include granulocytes, monocyte/macrophages, dendritic (DC) and natural killer (NK) cells, which mediate a first/immediate line of defense against pathogens by phagocytosis and the induction of inflammatory responses. In contrast, adaptive immunity provides a slower and more specific response by destroying infected cells (T lymphocyte) or by producing antibodies against exogenous antigens (B- lymphocyte) (Dahlin et al. 2018). Remarkably over 2 million new bloods cells are produce every second in healthy human adults to maintain steady state level of peripheral circulation (Ogawa 1993). The extraordinary regenerative potential of HSCs gives them a critical role in the clinic to treat a wide range of hematopoietic disorders (Bryder, Rossi, and Weissman 2006; Copelan 2006).

Discovery of hematopoietic stem cells

There were many studies strengthening the concept of stem cells following the nuclear bombing of Hiroshima and Nagasaki in 1945, where observations of the survivors to initial explosion revealed that many individuals died later due to hematopoietic failure from exposure to gamma irradiation (Jacobson 1949). Later, it became clear that injection of cells from spleen or Bone Marrow (BM) of non-irradiated mice could rescue recipient mice from irradiation induced injuries. This observation suggested the presence of cells in these tissues that can reestablish the hematopoietic system following ablative injury (Jacobson 1949; Lorenz et al. 1951). E. Donnall Thomas performed the first successful bone marrow (BM) transplant between human identical twins in 1956. The leukemic patient accepted donated BM from his identical twin which created healthy blood cells in the patient (Thomas and Ferrebee 1962). He was later awarded the Nobel Prize for this discovery.

Originally, Till and McCulloch investigated the ability of generating progenitors from single cells in the early 1960s. They discovered that injected bone marrow

cells give rise to colonies in the spleen of irradiated mice. These colonies were named colony forming units in the spleen (CFU-S) (McCulloch and Till 2005). To investigate whether the spleen colonies originated from a single cell, Becker showed by introducing chromosomal marking by sublethal irradiation, that each colony had its own set of chromosal abnormality with the same karyotype change indicating that spleen colonies were indeed clonally derived (Becker, Mc, and Till 1963). However, it was later reported that CFU-S colonies are derived from hematopoietic progenitor cells (HPCs) and not HSCs, and could not maintain long-term hematopoiesis (Schofield 1978). Retroviral marking studies in 1980 proved the existence of common single hematopoietc cells of origin with multilineage capacity. Importantly, identical retroviral integration sites were detected among the different blood lineages in primary and secondary hosts. This was a strong indication of self-renewal capacity (Dick et al. 1985; Keller and Snodgrass 1990; Keller et al. 1985; Jordan and Lemischka 1990). Ultimately in 1996 isolation of single HSCs and succesful long-term reconstitution following transplantation into irradiated recipients was the ultimate proof for the self renewal and multilineage differentiation capacity of HSCs (Osawa et al. 1996).

Hematopoietic stem cell fate

Hematopoietic stem cells (HSCs) have been shown to interact with a wide range of cell types and extracellular components in their immediate vicinity.This differs in location in the body based on the developmental stage. The first definitive HSCs appear in both human and mouse in the aorto-gonad-mesonephros (AGM) region of the embryo, then migrate to the fetal liver (FL), where they proliferate and expand, before finally migrating to the bone marrow (BM) slightly after birth and enter a primarily quiescent state (Zhang et al. 2018). BM serves as the major niche for HSCs during the remaining life. Schofield was the first to introduce the term "HSC Niche" to describe the unique microenvironment in the BM that constantly evolves (Schofield 1978). The niche is a complex structure composed of the extracellular matrix and various populations of cells, including osteoblasts, sinusoids, endothelial cells, perivascular cells, mesenchymal stem cells, megakaryocytes, and macrophages, all of which secrete cytokines and growth factors to promote HSCs maintenance and self-renewal (Comazzetto, Shen, and Morrison 2021).

HSCs fate options include quiescence (G0, stay dormant), differentiation, apoptosis, proliferation, self-renewal, migration, and mobilization (Figure 2). Both intrinsic factors from within HSCs (Transcription factors and epigenetic) and extrinsic signals (molecular feedback) from surrounding niche cells combine to regulate these fate options. Self-renewing division in adult stem cells have two potential outcomes; asymmetric division resulting in the production of one daughter stem cell and one differentiated progenitor, while symmetric division results in the production of two

daughter stem cells. Alternatively, HSCs also can give rise to two progenitor cells (differentiation) where the self renewal potential is gradually lost (Morrison and Kimble 2006). Apoptosis acts as an effective quality control mechanism by removing dysfunctional and potentially harmful cells (e.g. prevention of malignant transformation) (Bergmann and Steller 2010). It has been shown that the balance between quiescence and proliferation is regulated by intrinsic and extrinsic signals to avoid excessive cell growth or exhaustion (Cho et al. 2019). Under homeostatic conditions, HSCs continuously move from the bone marrow niche towards the blood stream in a circadian manner. Also, circulating HSCs can migrate into the bone marrow after intravascular injection through a process called homing (Yuan et al. 2021).

The hematopoietic system has long served as a paradigm for stem cell systems in the body. This is mainly due to the capacity to prospectively isolate stem cells with developing of fluorescence–activated cell sorting (FACS) technologies coupled with single-cell *in vitro* and *in vivo* functional assays that effectively describe hematopoietic hierarchical relationships between HSCs and progenitor cells. This has also promoted the study of cell-intrinsic (e.g transcription factors, epigenetic) and cell-extrinsic (e.g cytokines and intracellular signaling pathway) regulators of cell fate in hematopoiesis.

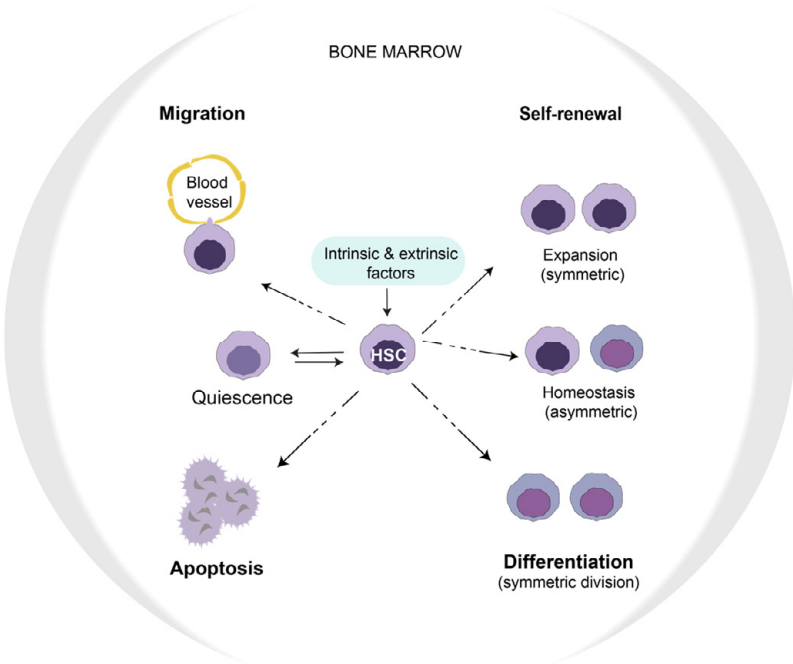

Figure 2. Hematopoietic stem cell fate options. HSCs can remain dormant or become active in response to intrinsic and extrinsic cues, allowing them to self-renew, migrate, apoptose or differentiate.

Classical model of mouse adult hematopoiesis

Only 0.01-0.005 % of all cells in BM are HSCs, and they were purified for the first time in 1988 by Weissman and colleagues using Florescence Activated Cell Sorting (FACS) and monoclonal antibodies (Spangrude, Heimfeld, and Weissman 1988). Using similar approaches, committed progenitor populations were isolated leading to the development of a hierarchical model of hematopoiesis. HSCs are rare quiescent cells at the top of the hematopoietic hierarchy. They are unique in that they possess extensive self-renewal capacity to maintain the HSC pool, as well as the potential to differentiate to all blood lineages at the bottom of hierarchy. This differentiation process include a range of increasingly lineage-restricted progenitor cells with progressively decreasing self-renewal capacity. In this section, the murine model will be discussed, while the models for human hematopoiesis is explained further down in the method section.

It has been previously shown that HSCs can be classified into two-populations: Long-term (LT) HSCs and Short-term (ST) HSCs (Ikuta and Weissman 1992; Ogawa 1993; Morrison, Shah, and Anderson 1997; Morrison, Uchida, and

Weissman 1995). LT-HSCs have a long-term reconstitution potential beyond four months and secondary transplantation (Christensen and Weissman 2001), while ST-HSCs have a short-term reconstitution capacity of 8 to 12 weeks with the potential to quickly restore the hematopoietic system post-transplantation (Yang et al. 2005; Morrison, Shah, and Anderson 1997). ST-HSCs give rise to multipotent progenitors (MPPs) with high cell-cycle progression frequency, more robust differentiation activity and limited self-renewal capacity compared to HSCs (Zhang et al. 2018). These three populations are found in the LSK compartment where cells don't express mature lineage markers (L) while being positive for stem cell antigen-1 (Sca-1; S) and the c-Kit receptor (K) (Okada et al. 1992). LT-HSCs and ST-HSCs have been isolated and characterized by the combined expression of CD34 (Osawa et al. 1996), FMS-like tyrosine kinase 3 (Flt3) (Adolfsson et al. 2001), CD105 (Endoglin) (Chen et al. 2002), CD201 (EPCR) (Balazs et al. 2006) or CD48, CD150 (SLAM markers, signaling lymphocyte activated molecule) (Kiel et al. 2005) and CD9 (Karlsson et al. 2013) (Table 2). The most quiescent LT-HSCs exist in the LSK $CD34^{-}$ $CD150^{+}$ $CD48^{-}$ $Flt3^{-}$ fraction (Wilson et al. 2008) with a frequency of 1 out of 3 cells (Kiel et al. 2005; Yilmaz, Kiel, and Morrison 2006). However, under activating conditions, they leave quiescence and enter the cell cycle (Schoedel et al. 2016). In the MPP population self-renewal capacity declines as Flt3 and CD34 are co-expressed (Adolfsson et al. 2001). Downstream of MPPs, the cells can be separated to the common myeloid progenitor (CMPs) (Akashi et al. 2000) and common lymphoid progenitor (CLP) (Kondo et al. 2003). More restricted myeloid progenitors subsequently undergo further branching into bipotent granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) (Nakorn, Miyamoto, and Weissman 2003; Tober et al. 2007). CLPs further form T, B, natural killer and dendritic cells, while GMPs differentiate into granulocytes/monocytes and MEPs generate megakaryocytes/ erythrocytes (Figure 3). According to this model, these populations form a tree-like and balanced hierarchy model, in which key transcription factors (TFs) and cytokines precisely instruct the stepwise differentiation of HSCs to mature blood cells (Cheng, Zheng, and Cheng 2020).

The balance in the hierarchical model has been shown to change in the leukemia, causing an accumulation of immature, poorly functioning hematopoietic cells known as leukemia blasts, which result in severely compromised blood and immune systems (Slany 2009). Therefore, understanding normal hematopoiesis is essential to comprehend leukemia.

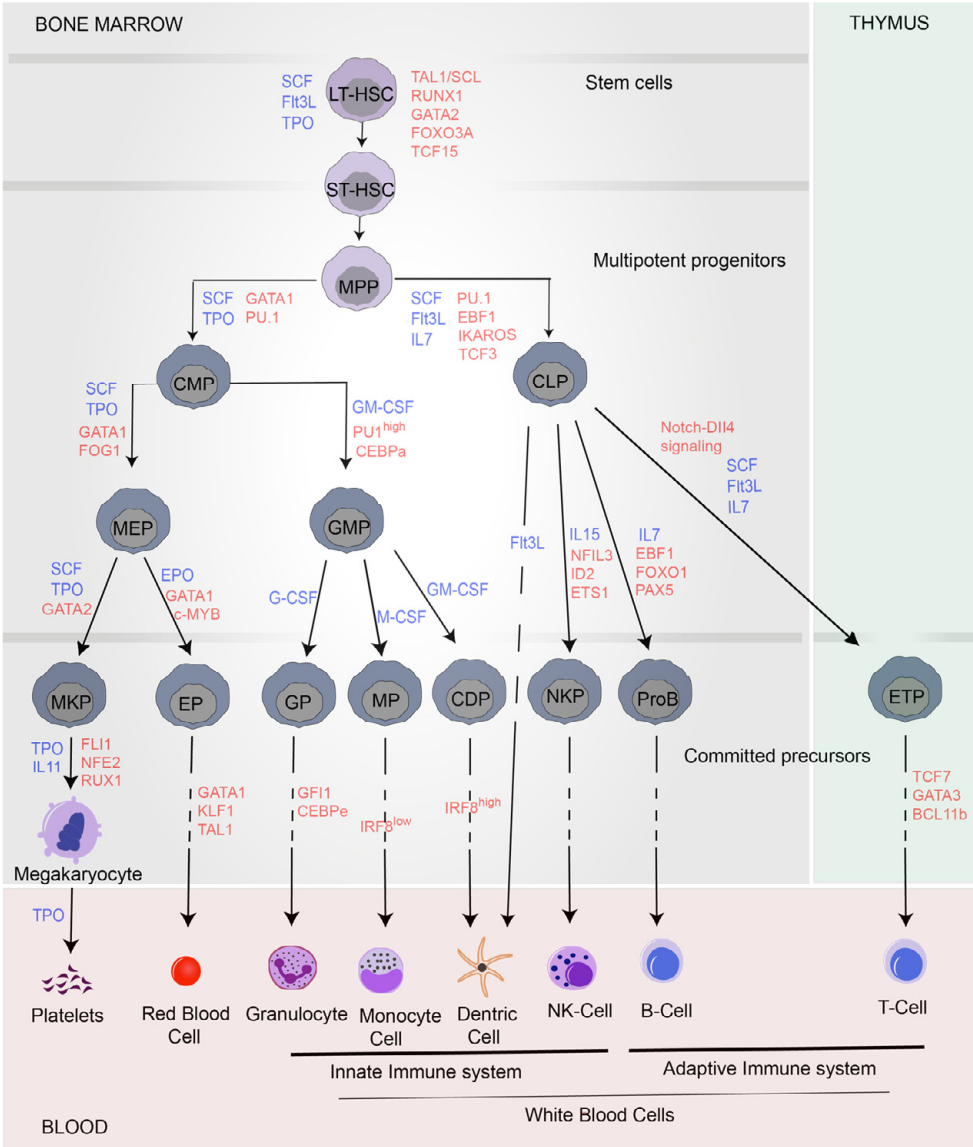

Figure 3. The classical model of adult hematopoiesis. In this model, LT-HSCs are at the top of the hierarchy and are differentiated into ST-HSCs, and subsequently to MPPs with reduced self-renewal capacity. The first step in lineage commitment occurs when MPP separates into CMPs and CLPs. CMPs further generate MEPs and GMPs. CLPs give rise to lymphocytes and dendritic cells. MEPs differentiate to platelets and erythrocytes. GMP produces granulocytes, macrophages, and dendritic cells. Hematopoietic differentiation is controlled by extrinsic cytokines (blue) and intrinsic transcription factors (red). Adapted from (Cheng, Zheng, and Cheng 2020; Comazzetto, Shen, and Morrison 2021).

Advances in the hematopoietic hierarchy model

The classical hierarchical model was derived based on experiments with cell populations that were initially thought to be homogeneous. However, studies of paired daughter cells, single cell transplantation, and more recent single cell 'omics' (*e.g.,* genomics, transcriptomics, epigenomics, and proteomics) have allowed for more detailed understanding of cellular heterogeneity (Psaila and Mead 2019). The heterogeneity of HSPCs is emphasized by the heterogeneity of cell-surface marker within progenitor populations. Combinations of these can be used to prospectively isolate cells that functionally vary in their capacity for self-renewal(Muller-Sieburg et al. 2004; Guenechea et al. 2001) and lineage differentiation output (Müller-Sieburg et al. 2002; Dykstra et al. 2007). Single cell genomics has provided unprecedented insight into regulatory heterogeneity in individual cells, revealing gene expression patterns that suggest a fate bias in HSCs (Martin-Rufino and Sankaran 2021). However, chromatin architecture at lineage-specific gene loci might process differently and exhibit lineage-specific priming, which likely determines their future fate (Yu et al. 2016). Therefore, based on different methodological insights, the current view of hematopoiesis includes extensive heterogeneity of lineage potential, bias and priming toward specific differentiation pathways in HSCs, which has slowly changed the understanding of the hierarchical model, as we will discuss here. The terminology used in the field of hematopoiesis has some overlap, as shown in Table 1. This is due to limitations of experimental systems, whether we are testing lineage bias, differentiation potential, and self-renewal capacity, or unperturbed hematopoiesis.

Table1: Definitions of commonly used stem cell terms, adapted from (Jacobsen and Nerlov, 2019).

Term	Definition	Assays
Lineage priming	It is referred to cells that express lineage-defining genes, but are still not committed to any particular population.	Single cell genomics
Lineage bias	Multi- or oligo-potent cell that specifically gives rise to a single lineage but retains potential for alternative differentiation	Single-cell transplantation or other clonal in vivo assay
Lineage potential Lineage commitment (restricted)	The capacity of a cell to differentiate into a particular lineage (or lineages). Oligo or uni-potent cells that have lost lineage potential and are restricted to a subset of its original potential	Clonal *in vitro* assay, colony forming ability, or *in vivo* transplantation
Lineage fate	The lineage that a stem cell or progenitor cell will give rise to	Lineage fate is unique to a specific condition and can only be measured under this condition (mostly *in vivo*). Lineage fate may change if the conditions are changed.

Heterogeneity of HSCs in lineage output

Using limiting dilution and single cell transplantation assays it was first discovered that lymphoid and myeloid lineage output could be heterogeneous between HSCs *in vivo*. These studies discovered that not all phenotypic HSCs equally contributed to the different blood lineages, however, the majority of HSC clones produced a higher proportion of myeloid (My biased) or lymphoid (Ly biased) cells than the average HSC population (Müller-Sieburg et al. 2002; Muller-Sieburg et al. 2004; Dykstra et al. 2007; Ema, Morita, and Suda 2014). However, mature erythroid (ER) and platelet (P) cells were not captured in these studies due their lack of CD45.1 or CD45.2 expression, which are routinely used in transplantation settings to discriminate between donor, host, and competing cells using the standard C57BL/6 mouse strain. This only allowed for the specific tracking of donor cells for myeloid (Granulocyte-Monocyte) or lymphoid (B and T) cells (Jacobsen and Nerlov, 2019). Later, the contribution of single HSCs to all five blood cell lineages was demonstrated *in vivo* by developing a transgenic mouse strain in which HSCs and their progeny expressed a fluorescent protein ubiquitously (Yamamoto et al. 2013; Carrelha et al. 2018). According to these observations, a high proportion of LT-HSCs (previously classified as "My biased") are platelet biased (Yamamoto et al. 2013; Sanjuan-Pla et al. 2013; Carrelha et al. 2018). Carrelha et al. identified conclusively that these LT-HSCs were fully and stably restricted to P, P-ER, P-ER-My, or P-ER-My-B fates (but not any other lineage combination) (Carrelha et al., 2018). MK-biased HSCs are also present in unaltered adult hematopoiesis (Rodriguez-Fraticelli et al. 2018).

In mouse different lineage biased HSCs can be prospectively isolated by multiple cell-surface markers (Yang and de Haan 2021; Konturek-Ciesla and Bryder 2022). For example, lymphoid-biased HSCs can be isolated based on CD86 expression (Shimazu et al. 2012), whereas, myeloid/platelet-biased HSCs are marked by CD150 (Beerman et al. 2010; Morita, Ema, and Nakauchi 2010), CD41(Gekas and Graf 2013), CD61 (Mann et al. 2018), c-Kit (Shin et al. 2014) and vWF (using reporter mice (Sanjuan-Plaet al. 2013). Furthermore, heterogeneity in self-renewal capacity has been demonstrated in HSCs as well (Ema et al. 2005). For example $CD150^{high}$ (Morita, Ema, and Nakauchi 2010) and vWF^{+} (Sanjuan-Pla et al. 2013; Grover et al. 2016) display high self-renewal capacity and can generate other lineage-biased HSCs. Placing these cells at the top of the hierarchy. Overall, individual HSCs are heterogeneous in terms of lineage output, engraftment, and self-renewal capacity.

Heterogeneity in progenitors

Based on the classical model of LT-HSCs differentiation to MPPs, self-renewal potential is gradually lost with up regulation of FMS-like tyrosine kinase 3 (Flt3/CD135, encoded by *Flt3* gene, also called *Flk2*) in the LSK compartment (Adolfsson et al. 2001). $Flt3^{+}$ multipotent progenitors serve as developmental intermediates for hematopoietic lineage priming (Boyer et al. 2011; Buza-Vidas et al. 2011; Beaudin, Boyer, and Forsberg 2014; Adolfsson et al. 2001; Buza-Vidas et al. 2009). Even *Flt3* expression within the $LSKCD48^{-}CD150^{+}$ HSCs is a step towards differentiation and loss of stem-like gene expression program (likely to represent contaminating progenitors cells) as well as self-renewal capacity (Mead et al. 2017; Mooney et al. 2017). The Jacobson group discovered defined the lymphoid-primed MPPs (LMPP) population within the 25% highest $Flt3^{+}$ cells which gives rise to granulocyte/macrophage and lymphoid lineages but not to megakaryocyte and erythroid (MK/ER) cells (Adolfsson et al. 2005). They proposed that MK and ER lineages may arise directly from Flt3- cells such as the LT-HSCs and ST-HSCs (Adolfsson et al., 2005). This model was supported by another report that suggested that HSC daughter cells could directly differentiate into MK/ER progenitors (Takano et al., 2004). Moreover *in vivo* molecular barcoding of LMPP cells observed a biased or restricted lineage fate to myeloid, dendritic and B cells (Naik et al. 2013). However, these views have been challenged by transplanting high numbers of LMPPs and lineage tracing, suggesting that LMPPs are capable of thrombopoiesis *in vivo* (Forsberg et al., 2006). However, these different outcomes may be attributed to the cell dose used in these experiments. Nevertheless, the acquisition of MK/ER potential in the LMPP population is a rare event, and as cells differentiate from LT-HSCs to LMPPs, MK/ER-priming is down regulated whereas lymphoid priming starts to increase (Mansson et al., 2007). It was proposed that the minimal MK/ER potential of LMPPs coincided with the transition of MPPs from $PU.1^{low}$ to $PU.1^{high}$ and from MPL^{high} to MPL^{-} states within the $Flt3^{high}$ LSK cells (Arinobu et al., 2007; Luc et al., 2008). PU.1 is a transcription factor known to be important for myeloid and lymphoid development (Scott et al. 1994), while thrombpoietin receptor (MPL) is known to be important for MK development (Alexander et Al., 1996).

Similarly, functional assays confirmed that there is heterogeneity among the MPP population as well. The Trumpp and Passegué groups further classified MPPs into, MPP1, MPP2, MPP3 and MPP4 base on Flt3, CD150, CD48 (Pietras et al. 2015) and CD34 expression (Table 2) (Wilson et al. 2008; Cabezas-Wallscheid et al. 2014; Woolthuis and Park 2016; Tsapogas et al. 2017). These MPPs have their own distinct immunophenotype, BM abundance, cell cycle state and differentiation (Cabezas-Wallscheid et al. 2014). Interestingly, MPP1 has multilineage reconstitution capacity for up to four months during the first transplant, similar to previously defined ST-HSCs. On the other hand, MPP2/3/4 only have short-term

myeloid reconstitution capacity of less than 1 month. More importantly, MPP2, MPP3, and MPP4 produce more megakaryocyte (MK bias), myeloid (My bias) and lymphoid (Ly bias) cells *in vivo*, respectively (Pietras et al. 2015; Cheng, Zheng, and Cheng 2020). Immunophenotypically, LMPPs share similar cell surface markers with MPP4 (Pietras et al. 2015). Recently, a comprehensive characterization of the complete LSK compartment determined MPP5 and MPP6 also based on CD34 expression in ST-HSCs/MPP1 ($Flt3^-$, $CD150^-$, $CD48^-$). MPP5 is similar to MPP1 with more myeloid production whereas MPP6 is a subset with higher multilineage potential. They are functionally located after HSCs and before MPP2-4 (Sommerkamp et al. 2021).

High heterogeneity has been reported in the classical CMPs population as well. Pronk et al. identified four newly defined subpopulations, including pre-MegE, pre-GMs, colony forming unit-erythroid (CFU-Es), and pre-CFU-E (Pronk et al. 2007). Pre-GMs lie developmentally upstream of the GMPs and have a similar lineage output to the GMPs while pre-MegE cells produce Mk, ER, and mixed Mk/ER. In contrast, pre-CFU-Es give rise to ER exclusively (Pronk et al. 2007). Consistent with these results, molecular barcoding of CMPs showed that the majority of CMPs assumed either an erythroid or myeloid fate *in vivo* (Perié et al. 2015). Taken together, this result indicates that there is functional heterogeneity within phenotypically defined progenitor populations. Therefore, the classical road map of hierarchy was gradually revised between 2005 to 2015 due to more accurate cell isolation procedures and precise functional clonal assays (Figure 4).

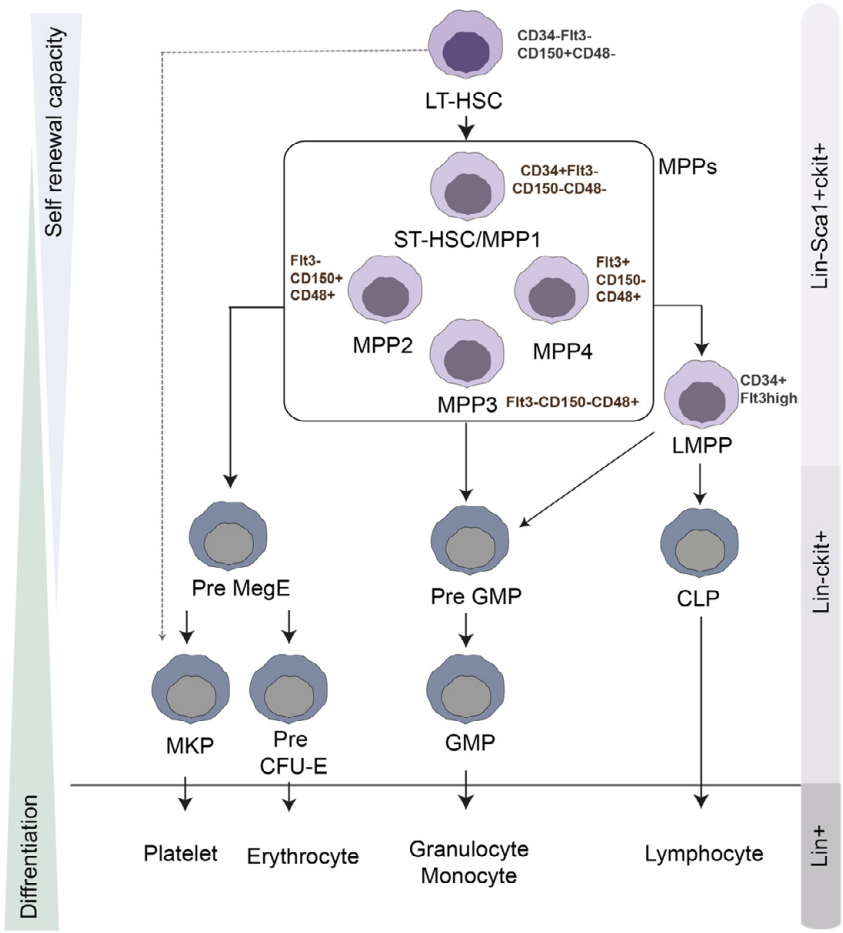

Figure 4. The revised model of hematopoiesis, In revised model of adult hematopoies, rare HSCs give rise to ST-HSC/MPP1, which subsequently produce MPP2, MPP3 and MPP4. MPP4 Immunophenotypically have shared similar surface marker with LMPP. MPP2 are MK/ER bias and can generate pre-MegE, which give rise to pre-CFU-E and MKP. MPP3 are My bias and can give rise to pre-GMP and GMP. MPP4/LMPP are Ly bias and can generate CLP. Adapted from (Cheng, Zheng, and Cheng 2020; Laurenti and Gottgens 2018).

Hematopoiesis under physiological condition

Despite the fact that the suggested hematopoietic hierarchy models have been useful guides for genetic and pharmacological perturbation investigations, they have also highlighted fundamental technical limits within the field's methodology (Jacobsen and Nerlov 2019; Zhang et al. 2018). For example, the whole idea that LT-HSCs sit on top of hierarchy and reconstitute or maintains the entire hematopoiesis comes from evidence *in vitro* colony assay and *in vivo* transplantation studies. In both techniques,

HSCs maintenance and differentiation occur under non-physiological (non-steady state) conditions. For example, in transplantation assays, donor HSCs are forced to proliferate under stress to rebuild the entire hematopoietic system after myeloablation, which may additionally have alter the niche (Matteini, Mulaw, and Florian 2021). On the other hand, in *ex vivo* assays, the cells are isolated and maintained in culture being exposed to high concentrations of cytokines and non-hypoxic environment, etc. (Mantel et al. 2015).

Various lineages–tracing approaches have been performed to assay lineage contribution of HSPCs in unperturbed hematopoiesis. Lineage tracing can be performed at the clonal level (such as by delivering complex barcode libraries), or alternatively, by documenting the collective fate of a population of cells (such as by delivering a common label to the cell population)(Pucella, Upadhaya, and Reizis 2020). It has been shown by the Camargo group, using a doxycycline-induced Sleeping Beauty transposon tagging approach, that MPPs, rather than HSCs, are the main drivers of steady–state hematopoiesis (Sun et al. 2014). A follow-up study by the same group showed that LT-HSCs can directly give rise to platelets, bypassing several other multipotent progenitors (Rodriguez-Fraticelli et al 2018). While these findings have a huge impact on the field of hematopoiesis we should consider that such labeling require cell division since HSC clones will rarely amplify during unperturbed hematopoiesis, thus making barcode detection less reliable. Another study using a fluorescent reporter system driven by the Tie2 locus (expressed in hematopoietic stem cells) for labeling hematopoietic lineages, confirmed a major replicative role of MPPs while also demonstrating a consistent contribution of HSCs (Busch et al. 2015). However, Sawai et al. recognized LT-HSCs as a major source of the multiple blood cells at steady state using fluorescent reporter system driven by the Pdzk1ip1 locus (which labels more purified LT-HSCs (Sawai et al. 2016)). This result, together with similar studies using direct florescent labeling of mouse HSCs under steady state condition, support the evidence that HSC contribute regularly and consistently to hematopoiesis (Sawen et al. 2018; Chapple et al. 2018; Pucella, Upadhaya, and Reizis 2020). Together these paradoxical observations might be due to the fact that different tracing methods and labeling efficiency of HSCs are used. Therefore further investigation and development of new approaches will be necessary to reconcile this controvercies.

Kinetics of lineage differentiation

Lineage tracing has allowed for an unbiased examination of HSC differentiation kinetics, at both population (Chapple et al. 2018; Sawen et al. 2018; Upadhaya et al. 2018) and clonal (Rodriguez-Fraticelli et al. 2018) levels. Studies revealed that MkPs and/or megakaryocytes or platelets were produced well before other lineages. MkPs emerged from labeled HSCs within one week and 2–3 cell divisions. This

was followed by erythroid and myeloid progenitors in two weeks, according to proliferation tracing, functional analysis, and scRNA-seq (Upadhaya et al. 2018). Lymphoid differentiation of HSCs, on the other hand, began long after myeloid differentiation (Busch et al. 2015, Chapple et al. 2018, Sawai et al. 2016, Säwen et al. 2016) and occurred at 3–6 weeks after HSC labeling (Pucella, Upadhaya, and Reizis 2020; Upadhaya et al. 2018).

The evolutionary origin, life span, vital necessity, and likelihood of depletion are key parameters that effect the econstitution of each lineage. Platelets, according to these criteria, are ancient, have a short lifespan (∼5 days), and are required for survival on the scale of minutes. They are also easily lost during massive coagulation during injury (Pucella, Upadhaya, and Reizis 2020).

It has long appreciated that HSCs and megakaryocytes share several features, (Huang and Cantor 2009), such as the expression of CD150 (Pronk et al. 2007; Kiel et al. 2005), CXCR4 (Wang, Liu, and Groopman 1998; Sugiyama et al. 2006), CD9 (Karlsson et al. 2013; Clay et al. 2001) and thrombopoietin receptor (MPL) (Yoshihara et al. 2007; Kimura et al. 1998). Megakaryocytes also act as a niche compartment for HSCs and tightly regulate their maintenance (Bruns et al. 2014; Zhao et al. 2014). T and B lymphocytes, on the other hand, are evolutionarily recent, extremely long lived, and required for long-term survival. They are difficult to lose due to their residence in lymphoid organs and tissues. Myeloid cells and erythrocytes differ in some of these parameters (for example, a life span of 1–2 days versus 120 days, respectively), but in terms of risk of a sudden loss, they appear to be intermediate to platelets and lymphocytes (Pucella, Upadhaya, and Reizis 2020).

Continuum model

It has long been believed that HSC differentiation undergoes several discrete intermediate steps to produce mature blood cells (Laurenti and Gottgens 2018). Early observations using single cell quantitative real time polymerase chain reaction (sc-qPCR) documented heterogeneity in immunophenotypically-defined populations (Teles, Enver, and Pina 2014; Guo et al. 2013; Månsson et al. 2007; Pronk et al. 2007). As an example, LMPPs were found to co-express myeloid and lymphoid genes, whereas in Flt3-negative MPPs the programs of megakaryocyte/erythroid and myeloid observed similar to HSC (Månsson et al. 2007). Even though, sc-qPCR is a highly sensitive approach, it is limited in detecting small number of cells and a limited number of predefined genes per cell. A breakthrough in single cell technology in 2016 made it possible to investigate the entire heterogeneity of HSPCs and changed the perspective of hematopoietic hierarchy both in mice and human (Velten et al. 2017). Applying the transcriptome-wide RNA sequencing in single-cells (scRNA-seq) technique on thousands of

progenitors, single HSPCs were ordered hierarchical pseudo-time based on similarity of transcriptional profiling among cells. This revealed the intrinsic differentiation process in hemtopoiesis in a whole new dimension (Figure 5A) (Paul et al. 2015; Olsson et al. 2016; Nestorowa et al. 2016; Velten et al. 2017). It was suggested that HSCs and their progeny, like MPP and LMPP, exist in a differentiation continuum of low-primed undifferentiated (CLOUD)-hematopoietic stem/progenitor cells (HSPCs) which give rise to unilineage-primed cells along the axes of differentiation without discrete multi-lineage single cell states (Figure 5B) (Giladi et al. 2018). This is partially in line with the Waddington model who suggests that stem cells can unidirectionally develop in to a mature differentiated stage and rolls down from the top the hill in a continuum model (Figure 5C) (Waddington 2014).

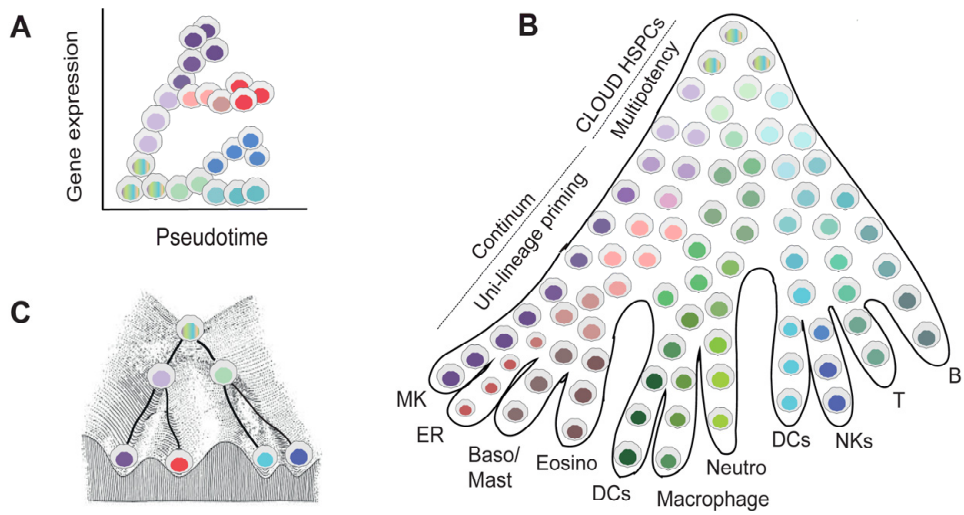

Figure 5. The continuum model of hematopoiesis, A) The computational methods of RNA sequencing orders thousands of single cells in the pseudotime trajectory, revealing the intrinsic differentiation process. Adapted from (Zhang et al. 2022). B) The coloration in the continuum model indicates that cells are variably lineage biased and emerge through a continuous process of differentiation with no punctuated transitions. Adapted from (Liggett and Sankaran 2020). C) Waddington's landscape (Waddington, 2014) illustrates the four different cell fates and two intermediate cell stages indicated by colured circles, which are highlighted by lines connecting differentiation and paths of all cell stages.

In this model, transcriptional lineage priming has become a standard measurement fot lineage potential. However, the transitional states are usually not detected since scRNA-seq has high sample noise, which likely skews the distribution of lowly expressed genes such as Transcription Factor (TF). It may also preclude the identification of rare transcripts that define cell identity and demarcate cell fate biases. Moreover, RNA levels is inherently noisy due to stochastic transcriptional processes and some intermediate cell states are exceedingly rare (Chen, Ning, and

Shi 2019; Jacobsen and Nerlov 2019). For example, a computational model of human scRNA-seq data suggest that all myeloid lineages derive from single GMPs, consistent with the continuum model (Velten et al. 2017). However, a combination of scRNA-seq and scqRT–PCR in mice revealed GMP heterogeneity based on *Gata1* expression. It was proposed that *Gata1*$^{+}$ progenitors with eosinophil, basophil/mast cell and megakaryocyte/erythroid potential co-segregate from *Gata1*$^{-}$ monocyte/macrophage, neutrophil and lymphoid potential very early during lineage determination (Drissen et al. 2016; Jacobsen and Nerlov 2019). In line with this finding using scRNA-seq in combination with FACS cell sorting and functional assays showed that basophils are produced in mice and humans from the erythroid-megakaryocyte branch (MEPs) (Tusi et al. 2018; Pellin et al. 2019). Therefore, cells of intermediate-stages could be detected by scRNA-seq in combination with FACS enrichment protocols, advanced computational methods, and functional assays (Jacobsen and Nerlov 2019).

Punctuated transitions continuum model

It is important to remember that scRNA-seq is an analytical method that only produces static snapshots of the transcriptional landscape and, as such, cannot provide conclusive information on the dynamics occurring during cell state transitions. In 2020, Weinreb et al. created a tool (LARRY) that used labeled cell clones with an scRNA-seq compatible barcode. By barcoding cells, letting them divide, and then sampling them immediately or after differentiation, it was now possible to link the initial states of cells with their differentiation outcomes and produce a map of cell fate bias on a continuous transcriptional landscape (Weinreb et al. 2020). The authors demonstrate that fate decisions cannot be reliably revealed using only the scRNA-seq method. They also proposed that cellular fate selection happens earlier than what current algorithms detect and that additional regulatory circuits (i.e. epigenetic state) most likely play a role in HSC fate determination (Weinreb et al. 2020). It has been shown that assessment of chromatin states at single-cell level can reveal destiny determinants that aren't visible at the transcriptome level and can reveal the regulatory networks underpinning cell type specification (Corces et al. 2016; Yu et al. 2017). Recently, single cell assay of transposase-accessible chromatin by sequencing (sc-ATAC seq) analysis have revealed different cell states among HSPCs through variations at gene regulatory level consistent with lineage biased toward different hematopoietic branches (Buenrostro et al. 2018; Granja et al. 2019). In the future, it will be crucial to consider the scRNA-seq limitations and combine this assay with functional perturbations and other multi-omic measurements like sc-ATAC-seq to thoroughly analyze the process of hematopoiesis. As a result, punctuated transitions across this

continuous gene expression landscape may be identified (Figure 6), representing functionally distinct groups of cells (Liggett and Sankaran 2020).

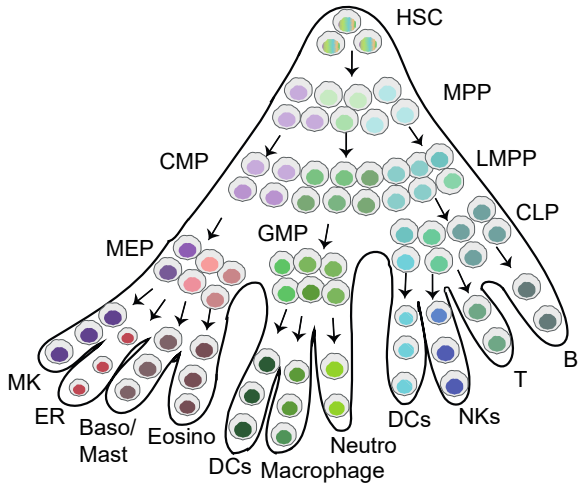

Figure 6. Punctuated continuum model, color variation in this model indicates that cells in each state are differently primed to give rise to different hematopoietic lineages. As cells progress through the differentiation hierarchy, the magnitude of this lineage commitment increases with color intensity, adapted from (Liggett and Sankaran 2020).

Lineage commitment and lineage priming

Lineage commitment is the point where cells become restricted irreversibly to a particular fate under steady state and looses the potential to differentiate into other cell types (Nimmo, May, and Enver 2015). Lineage commitment is governed both by intrinsic and extrinsic cues such as transcription factors and cytokines respectively as mentioned above. However, single-cell transcription experiments have shown that lineage-associated genes that become increasingly expressed during differentiation are expressed at low levels already in HSCs (Månsson et al. 2007; Hu et al. 1997). This is called lineage priming referring to that these cells express lineage-defining genes, but are still not committed to any particular population (Nimmo, May, and Enver 2015). Besides, single cell population studies (single cell transplantation and barcoding) has revealed lineage biases in multipotent cells (Rodriguez-Fraticelli et al. 2018; Sanjuan-Plaet al. 2013; Carrelha et al. 2018; Yamamoto et al. 2013), which are linked with differential lineage priming. These results suggest that lineage priming determines the cell's repertoire of possible fates. The dynamics of chromatin reorganization is thought to be responsible for lineage priming by recruiting the transcription network of the cells, while transcription programs associated with an alternative fate are prevented. This serves as a

platform for lineage commitment decision (Rodrigues, Shvedunova, and Akhtar 2020). Furthermore, environmental signals may influence TFs and chromatin structure to regulate fate decision (Brand and Morrissey 2020).

What governs the fate decision of HSPCs?

The process of hematopoiesis and differentiation is tightly controlled by both intrinsic (cytokines) and extrinsic signals (transcription factors and epigenetics). A large number of known regulators were discovered through traditional reverse genetic knockout studies, in which knocking out a gene caused decreased HSC activity (positive regulator) or increased HSC activity (negative regulator). However, many of the underlying regulatory mechanisms that govern fate decision are unknown. Recent advances in genomics have provided a new insight into the mechanisms underlying key hematopoietic fate decisions. Here, we give a brief overview of important cytokines, transcription factors and epigenetic regulators.

Cytokines

Cytokines are small proteins that are important extrinsic signals for cell communication. They are able to regulate hematopoiesis development and are synthesized systemically or locally within the bone marrow (Dinarello 2007). Stem Cell Factor (SCF) and Thrombopoietin (TPO) are important cytokines for maintainance and self-renewal of HSCs. They interact with the tyrosine kinase c-Kit receptor (CD117) and c-MPL receptor respectively (Ikuta and Weissman 1992; Qian et al. 2007; Thorén et al. 2008). TPO is mainly expressed by hepatocytes in liver and deleting TPO from hepatocytes depletes HSCs in the BM (Decker et al. 2018). Similarly, deletion of SCF or the gene encoding the C-Kit receptor, results in a loss of HSC self-renewal capacity (Waskow et al. 2002; Waskow and Rodewald 2002; Edling and Hallberg 2007). The ligand for Fms-like tyrosine kinase 3 (Flt3L), which binds to Flt3 (CD135), is an important growth factor for the development of hematopoietic stem cells and progenitors (HSPCs) (Lyman et al. 1993; Kikushige et al. 2008). The combination of SCF with TPO (Wilkinson et al. 2019; Sitnicka et al. 1996; Ramsfjell et al. 1996) and Flt3L (Oubari et al. 2015; Jacobsen et al. 1995; Hudak et al. 1995) or interleukin-11 (IL-11) (Oedekoven et al. 2021; Miller and Eaves 1997) is frequently used as soluble supplements in HSPCs expansions (Wilkinson, Igarashi, and Nakauchi 2020).

Important cytokines for myeloid development are colony-stimulating factors (CSFs), macrophage CSF (M-CSF or CSF1) and granulocyte macrophage CSF (G-CSF or CSF3). Studies using mice deficient for these cytokines show that in the steady state, M-CSF is required for the maintenance of the macrophage lineage,

whereas GM-SCF is important for the maturation of macrophages and G-SCF is required for neutrophil formation (Hamilton and Achuthan 2013).

Flt3L and interleukin 7 (IL7) are necessary cytokines for the survival and differentiation of lymphoid cells (Tsapogas et al. 2011; Miller et al. 2002; Kikuchi et al. 2005; Sitnicka et al. 2002). Mice deficient for the IL7 receptor (IL-7R/ CD127) or its ligand IL-7 show a block in T- and B-lymphocyte development (Peschon et al. 1994). Additionally, a constitutive loss of Flt3 or Flt3L induces the reduction of B and T cells (Mackarehtschian et al. 1995; McKenna et al. 2000; Sitnicka et al. 2003). Flt3L and IL7 have synergistic interaction for B cell commitment and differentiation (Veiby, Lyman, and Jacobsen 1996) and double deficient mice for Flt3L and IL7R almost completely lack mature B cells and B progenitors (Sitnicka et al. 2007).

The major extrinsic regulator for the erythroid lineage is the clinically relevant erythropoietin (EPO), which is produced by the kidney in an adult in response to hypoxia and interaction with its receptor, Epo.R (Koury and Bondurant 1990; Wu et al. 1995). TPO and its ligand c-MPL also play an important role in megakaryocyte progenitor differentiation (Kaushansky et al. 1995; Kaushansky et al. 1994; Vainchenker et al. 1998). TPO or c-MPL deletion reduces megakaryocyte numbers in the BM, as well as circulating platelets by 85 percent in mice (Alexander et al. 1996; Gurney et al. 1994; Murone, Carpenter, and de Sauvage 1998)

Transcription factors

Transcription factors (TFs) are a class of proteins that can bind to specific DNA sequences, usually in enhancer or promoter regions, to either positively (as an activator) or negatively (as a repressor) regulate the expression of target genes (Figure 6) (Sun, Wang, and Sun 2017; Lee and Young 2000; Lambert et al. 2018). The important TFs for HSCs emergence and regulation are RUNX1 (also known as AML1, CBFA2, PEBP2aB) (Chen et al. 2009; Imperato et al. 2015), TAL1 (SCL/TAL1, T-cell acute leukemia protein 1)(Vagapova et al. 2018), GATA2 (Strouboulis and Crispino 2020; Vicente et al. 2012), TCF15 (Rodriguez-Fraticelli et al. 2020) and the home-box (HOX) family of TFs. Uncommitted hematopoietic cells express HOXB3, HOXB4, and HOXA9 at high levels, whereas myeloid committed cells express HOXB8 and HOXA10 (Alharbi et al. 2013).

The myeloid program is governed mainly by the TFs PU.1 (Zhang et al., 1999, Zhang et al., 2000), C/EBPα and ε (CCAAT/enhancer binding protein) (Rosenbauer and Tenen, 2007), IRF8 (Murakami et al., 2021) and GFI1(Rosenbauer and Tenen 2007). High expression of PU.1 commits cells to the myeloid lineage and intermediate expression of the TF PU.1 drives cells towards B lymphopoiesis (DeKoter and Singh, 2000). C/EBPα further differentiate the myeloid lineage to

GMPs. Mature myeloid generation is driven by GFI1, C/EBPε (Rosenbauer and Tenen 2007) and IRF8, which is highly important for regulation choice between neutrophils, monocytes and DCs. For instance, lack of IRF8 stimulates neutrophils while low and high level of IRF8 drive differentiation to monocytes and DCs respectively (Murakami et al. 2021).

B-cell development is governed by TFs; IKZF1(IKAROS), GFI1, TCF3 (E2A), FOXO1, EBF1 and PAX5, that act in synergy to promote the generation of B cells while simultaneously suppressing the myeloid program (Somasundaram et al. 2015). T cell generation happens primarily in the thymus where Notch1 signaling suppress B cell expression, while driving T cell differentiation. Subsequently, mature T cells are governed by GATA3 and TCF1 (Zhu and Emerson 2002; Yui and Rothenberg 2014).

The main transcription factors that defines the MEPs (erythroid and megakaryocyte progenitors) are GATA1 and FOG1 (Doré and Crispino 2011). The downsteam erythroid differentiation is governed by GATA1 (Stachura, Chou, and Weiss 2006), MYB (c-Myb) (Lu et al. 2008) and KLF (Bouilloux et al. 2008; Doré and Crispino 2011) while megakaryocyte maturation is dependent on GATA2, RUNX1, NFE2 and FLI1 (Doré and Crispino 2011; Noetzli, French, and Machlus 2019).

Epigenetics

Epigenetics (the root Epi means above (above genetics)) is the study of molecular mechanisms that cause heritable changes in gene expression without involving DNA sequence changes; this also called “epigenetic memory”. In 1957, Waddington proposed the concept of epigenetics to represent the process of cellular decision-making during development. The cell (represented by a ball) can take specific permitted trajectory, leading to different outcomes of cell fate (Figure 5C) (Waddington 2014). Chromatin or epigenetic factors can alter cell transcription output by turning on or turning off gene transcription without changing the underlying genetic sequence. Some of the processes for chromatin regulation involve chemical modifications to DNA and associated histone proteins like methylation, as well as changes in DNA accessibility, chromatin conformation and nucleosome remodeling. The chromatin environment that is created by some of these modifications and factors constitutes ‘’epigenetic landscape’’ (Rodrigues, Shvedunova, and Akhtar 2020). Lineage biases are thought to be established during hematopoiesis through priming of the epigenetic landscape (Rodrigues, Shvedunova, and Akhtar 2020; Yu et al. 2016). For example, Yu et al. demonstrated clearly that epigenetic characteristics such as DNA methylation and chromatin accessibility are strongly linked to the characetristics of individual clones and their

subsequent function (Yu et al. 2016). Among epigenetic mechanisms, in this thesis we discuss chromatin accessibility.

Chromatin accessibility

Transcription (or "gene expression") is the process of converting genetic information inscribed in DNA into RNAs and proteins. DNA is organized with the help of histones into compact particles called nucleosomes (around 147bp DNA encircled by an octamer of histone protein), which are inaccessible to many transcription factors. In the transcription of DNA to RNA, the chromatin must be accessible and TFs, chromatin re-modeling histones and chromatin-modifying enzymes control this accessibility. The landscape of chromatin accessibility across the genome is highly dynamic and reflects the network of permissible physical interactions through which enhancers, promoters, TFs and chromatin-binding factors co-operatively regulate gene expression (Figure 7) (Klemm, Shipony, and Greenleaf 2019).

Some transcription factors are described as pioneer factors because they can bind to the DNA sequence and open inaccessible chromatin, they recruit chromatin-remodeling factors to reposition nucleosomes and make the transcription start site (TSS) and gene body accessible, allowing other TFs to occupy nucleosomes later in development (Spitz and Furlong 2012; Lambert et al. 2018). The dynamic patterns of gene expression during hematopoietic differentiation are regulated through the TFs and integrated action of many cis-regulatory elements (non-gene coding area) including; promoters (proximal elements) located near gene transcription start sites (TSSs), as well as enhancers, silencers and insulators that are localized at greater distances from TSSs. Enhancers are major gene-regulatory elements found in the genome. These regulate cell-type-specific gene transcription programs, most commonly by looping over long distances to come into physical contact with the promoters of their target genes. Enhancers are short, specific DNA sequences that serve as operational platforms to recruit specific TFs through motifs and to drive expression in a given cell type (Spitz and Furlong 2012; Klemm, Shipony, and Greenleaf 2019). Therefore, the enhancer landscape (accessibility of enhancer is cell specific) reflects cell identity better than mRNA levels (Figure 7B)(Corces et al. 2016; Theilgaard-Mönch et al. 2022; Yu et al. 2017). For example, C/EBPα promotes lineage-specification by launching an enhancer-primed granulocytic differentiation program by direct activation of C/EBPε expression, which regulates cell cycle exit at the promoter level during *in vivo* granulocytic differentiation (Theilgaard-Mönch et al. 2022).

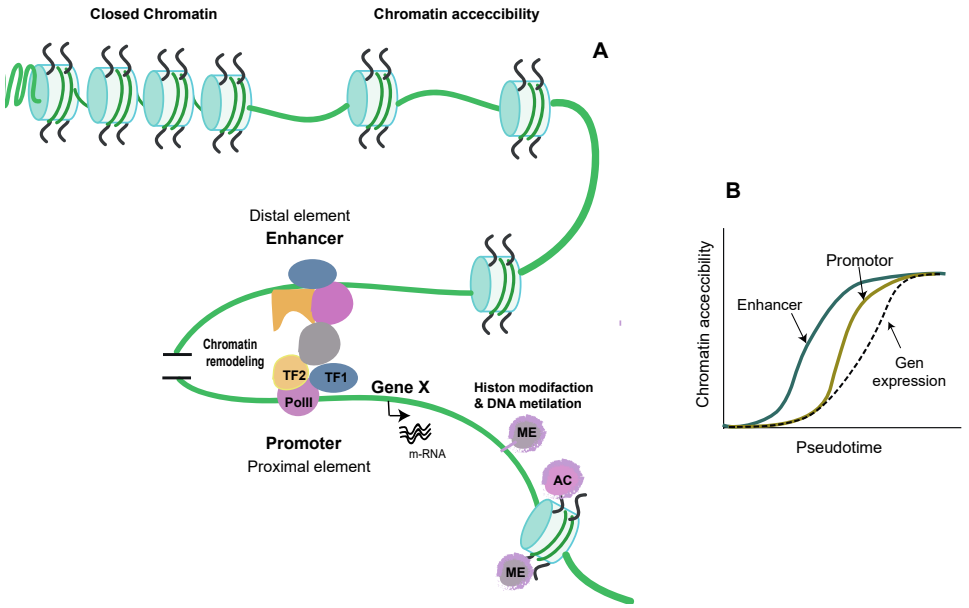

Figure 7. Chromatin accessibility, A) chromatin must be accessible, only then the enhancer could interact with promoter region of it's target gene by the formation of loop. This can initiate synthesis of messenger RNA by binding RNA polymerase to promoter at the transcription-binding site. Adapted from (Kim, Hemberg, and Gray 2015). B) Single cells can be ordered by their developmental progression through single-cell, genome-wide technologies. Adapted from (Shema, Bernstein, and Buenrostro 2019).

Chromatin accessibility is assessed by the vulnerability of its constituent DNA to enzymatic digestion or cleavage. Hewish and colleagues conducted the first evaluation of chromatin accessibility in 1973, revealing regular nucleosome phasing using DNA endonucleases (Rodrigues, Shvedunova, and Akhtar 2020; Wu et al. 1979). With advances in sequencing technology, genome-wide mapping of chromatin accessible regions has become more sensitive and a powerful tool to understanding the cell fate decisions along continuous trajectory. ATAC sequencing on HSCs and progenitors revealed that cis-regulatory elements are specifically active at different stages of differentiation, implying that cell type could be identified based on chromatin accessibility profiles rather than transcriptome (Buenrostro et al. 2018; Corces et al. 2016; Ludwig et al. 2019; Rodrigues, Shvedunova, and Akhtar 2020). Altogether, single-cell chromatin accessibility opens new opportunities for studying the molecular details that govern cell fate decisions (Shema, Bernstein, and Buenrostro 2019). Furthermore, it has been demonstrated that external signals can influence cell fate decisions via TFs and chromatin structure. For example, short-term exposure to an immunological challenge, such as bacteria, can cause C/EBPβ dependent alterations in chromatin accessibility, which prime HSCs to migrate to the myeloid compartment after secondary infections (de Laval et al. 2020). Changes in the niche compartment have

also been demonstrated to have a long-term impact on HSC chromatin accessibility, even after transplantation (Derecka et al. 2020). These findings imply that epigenetic memory plays a role in influencing HSC destiny decisions.

The impact of aging on hematopoiesis

Aging is a time-dependent, natural process that is associated with a number of changes in the hematopoietic system, which can have a number of negative effects on hematopoiesis and immunity. One of the key age-related changes to hematopoiesis is a decrease in the production of erythrocytes and lymphoid cells (B- and T-cells), contributing to age-associated anemia (Pang and Schrier 2012), and progressive decline in adaptive immune responses (Boraschi et al. 2013; Bron et al. 2015). Moreover, aging increases the risk of myeloid diseases (Lichtman and Rowe 2004) and cancer (Rossi, Jamieson, and Weissman 2008).

In mice, the number of immunophenotypic HSCs increase (Sudo et al. 2000; Rossi et al. 2005) while their capacity for regeneration declines with age (Sudo et al. 2000). Besides, the overall HSC differentiation during aging decreases. Importantly, the capacity of aged HSCs to produce lymphocytes is much lower than their capacity to produce myeloid cells, so the relative abundance of myeloid-to-lymphoid cells is higher, resulting in myeloid-biased hemtopoiesis with age (Kim, Moon, and Spangrude 2003; Rossi et al. 2005). The gradual functional decline in aged HSCs is referred to as stem cell exhaustion (de Haan and Lazare 2018). Interestingly, HSC function is not improved when transplanting aged HSCs into young recipients (Kuribayashi et al. 2021; Sudo et al. 2000; Morrison et al. 1996), indicating that changes in cell- intrinsic factors like TFs and epigenetics are crucial in HSC aging (de Haan and Lazare 2018; Saul and Kosinsky 2021; Chambers et al. 2007). Cell extrinsic alternations such as changes in BM niche also have an impact on age-related phenotypes (Kusumbe et al. 2016). A recent comprehensive single cell transcriptomics analysis confirms more heterogeneity in the aged HSCs pool compared to the young, and reveals the robust HSC transcriptomic aging signature (Flohr Svendsen et al. 2021). It has been shown that some markers used to purify HSCs are either up regulated or down regulated during aging in correlation with functional activity. For example, the up regulation of CD150 and down regulation of CD86 is consistent with the myeloid–biased phenotype of HSCs (Yang and de Haan 2021). CD86 has been shown to mark lymphoid-biased HSCs with high self-renewal capacity (Shimazu et al. 2012), this marker is down regulated with aging (Esplin et al. 2011). In contrast, myeloid biased HSCs ($CD150^{+}$) (Morita, Ema, and Nakauchi 2010) and platelet biased (vWF) (Sanjuan-Plaet al. 2013) HSCs with high self-renewal capacity are up regulated with aging (Esplin et al. 2011; Grover et al. 2016).

Similar to mice, aging increases the quantity of immunophenotypic human HSCs, which are less quiescent in comparison to young HSCs and have a higher cell division rate (Pang et al. 2011). This suggests that in order to maintain normal hematopoiesis in healthy elderly people; a larger population of HSCs may be required to adjust for loss of cellular potential (Pang, Schrier, and Weissman 2017). Furthermore, as we age, we develop more oxidative DNA damage, which provide a logical rationale for developing age-related disease (Yahata et al. 2011; Schumacher et al. 2021). Both of these factors may limit aged HSCs' ability to self-renew and rebuild the hematopoietic system on their own. Indeed, when aged human HSCs are xenotransplanted into immunodeficient mice, they have a lower per-cell engraftment rate than young HSCs (Kuranda et al. 2011; Pang et al. 2011). Moreover, aging skews the capacity of human HSCs to differentiate toward a myeloid bias. Gene expression profiling of human HSCs has shown that myeloid (Pang et al. 2011) and megakaryocytic/erythroid genes are mostly up regulated (Rundberg Nilsson et al. 2016) while lymphoid genes are mostly down regulated with age (Pang et al. 2011). Whether the increase in myeloid bias in human HSCs with age is due to a gradual overall functional change in HSC differentiation potential or to changes in HSC clonal composition, has yet to be directly demonstrated. ScRNA-seq and single HSC transplantation experiments in mice have demonstrated that as HSCs age, they become more biased toward producing platelets. These experiments discovered that when the HSC platelet programming was reduced, lymphoid output increased (via loss of the FOG-1 TF). As a result, increased platelet bias may play a role in the age-related decline in lymphopoiesis (Grover et al. 2016).

Furthermore, human HSC myeloid bias may have an impact on myeloid leukemia, which becomes more prevalent as people age. For example, transformation of hematopoietic stem cells by the BCR-ABL fusion gene, which is caused by a t9: 22, translocation, is the most frequent abnormality in chronic myelogenous leukemia (CML) (Klco et al. 2008). The BCR-ABL fusion, can transform young mouse HSCs into mixed myeloid and lymphoid leukemia, but it can only transform old mouse HSCs into myeloid leukemia (Signer et al. 2007). In addition, BCR-ABL mutation associated with CML is known to bias early HSC differentiation towards myeloid and megakaryocytic lineage (Hussein et al. 2017). This data suggests that myeloid bias is an important step to myeloid leukemia in the elderly. The BCR-ABL1 fusion protein is expressing a constitutively active tyrosine kinase, which is responsible for myelo-proliferative bias. CML was also one of the first cancers to be revolutionized by targeted therapy with Tyrosine kinase inhibitors (TKI) like imatinib (Longo 2017). Some patients respond well to the treatment and can achieve treatment free remission (TFR). However, the majority of patients relapse if tretament is withdrawn even if this was performed when the patients have undetectable BCR-ABL1 levels (Saussele et al. 2018). It is believed that leukemic stem cells (LSCs)

exist in CML and that a small percentage of leukemia initiating cells are TKI resistant, resulting in patient relapse (Corbin et al. 2011; Hamilton et al. 2012). LSCs are found in the phenotypic HSC compartment; and it has been shown that CML-LSCs are mostly transcriptionally distinct from normal HSCs (Giustacchini et al. 2017; Warfvinge et al. 2017), allowing for the identification of putative therapeutic targets that may have a differential impact on LSCs.

Researchers have now confirmed that the gradual accumulation of mutations and the development of clonal hematopoiesis with age significantly increases the risk of developing hematopoietic malignancies (Jaiswal and Ebert 2019; Genovese et al. 2014; Jaiswal et al. 2014; Pang, Schrier, and Weissman 2017). Clonal hematopoiesis is characterized by the over-representation of blood cells derived from a single clone. It has been demonstrated that the number of clones responsible for hematopoiesis decreases with age (Jaiswal and Ebert 2019; Holstege et al. 2014). Moreover, chronic inflammation increases with age, which may drive the selection of HSCs clones and the expansion of specific HSPC subsets (Hormaechea-Agulla et al. 2021). All together, this indicates that aging alters the clonal composition of the HSCs pool and increases the heterogeneity (de Haan and Lazare 2018). Thus arises the question of whether the HSC subpopulation, with heterogeneous function during aging, can be isolated prospectively in humans similar to mice. The immunophenotypic changes associated with aging-effects in humans have received little attention. As a result, further dissecting the human HSC population has the potential to improve our understanding of HSC function and heterogeneity during aging (paper III).

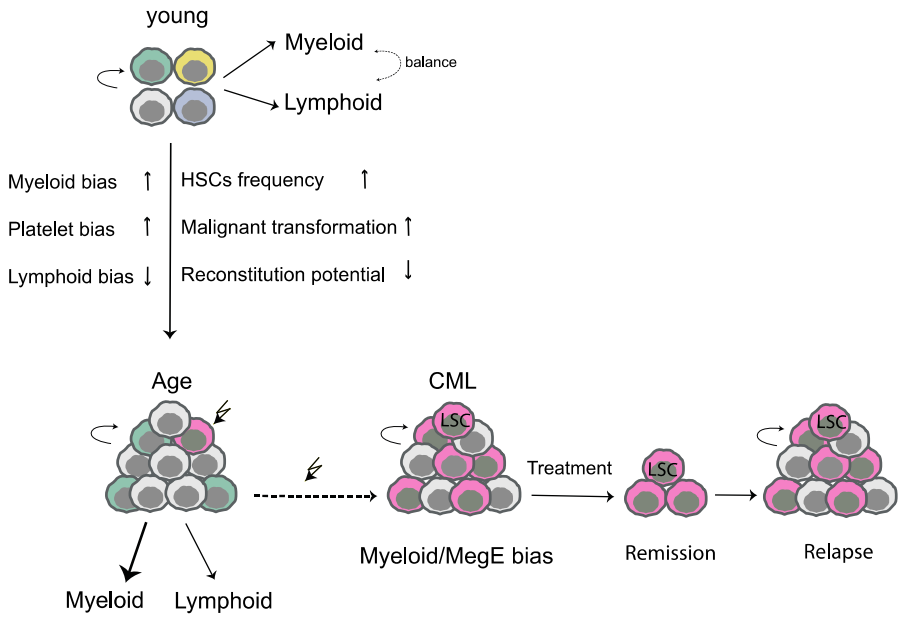

Figure 6. Age associated changes in HSCs, each cell (green, gray, blue, and yellow colors) represents a clone from a pool of young HSCs with balanced lymphoid- and myeloid differentiation potentials. Aging is associated with increased HSC frequency, heterogeneity and myeloid bias, but lower reconstitution capacity and lymphoid bias. More over, the number of clones that make the HSC pool decreases during aging (gray and green colors). Furthermore, HSCs acquire sporadic mutations as they age, and the mutant cells may clonally expand (pink color/ LSCs), which increases the risk of leukemic transformation (such as CML). CML is characterized by aberrant myeloid and megakaryocyte differentiation. The altered program results in differentiation blockage and the accumulation of blast cells (non-differentiated cells/ haven not been shown here). Some LSCs resistant to TKI treatment persist resulting in relapse.

Methods to study hematopoiesis

Flow cytometry

Hematopoietic stem cells can be identified and isolated by labeling cell surface antigens with monoclonal antibodies, conjugated with different fluorophores. Labeled cells are arranged into a liquid stream as single cells and pass the laser light of different wavelengths to detect the fluorophore molecules bound to cell surface antibodies. Detectors record the emitted light, and the data is gathered from thousands of cells and processed by a computer to identify specific marker proteins on the cell surface identifying each cell type. The most common flow cytometers can measure up to 20 different parameters (e.g. FSC, SSC, and 18 fluorescent detectors). One of the challenges of multi-parameter flow cytometry is compensation (erasing spectral overlap) between fluorophores (McKinnon 2018).

This issue is addressed in the new type of flow cytometry by replacing spectral analyzer with traditional Photomultiplier tubes (PMTs) (Sony, 2017). Another specific type of flow cytometry is called sorting, which separates cells by creating drops using an oscillating stream of sample liquid at a high frequency. The drops are then charged positively or negatively and directed to a specific collection vessel based on their charge as they pass through metal deflection plates. Tubes, slides, or a 96 well plate can be used as collection vessels (McKinnon 2018).

Over the last few decades, many hematopoietic cell surface markers have been discovered for separation of cells in stem cell pools using flow cytometry. In general, HSPCs do not express several lineage markers that are found on more differentiated hematopoietic cells. Therefore, the standard way to identify murine HSPCs has been to stain the BM with Mac1, Gr1, B220, CD3, and Ter119 (abbreviation L) (Okada et al. 1992). As we mention before, in mice, stem cells exist in a heterogeneous population called the LSK population, and can be further subdivided into subpopulations ranging from long-term HSCs (LT-HSC) to various MPPs using various markers such as CD34, and Flt3, as well as CD150, and CD48 according to Table 2 (Adolfsson *et al*., 2001; Christensen and Weissman, 2001; Kiel *et al.*, 2005; Osawa *et al.*, 1996). Novel HSC markers are being identified all the time, such as CD9 (Karlsson et al. 2013), CD105 (Endoglin) (Chen et al. 2002), CD201 (EPCR) (Balazs et al. 2006). It has also been demonstrated that CD9 can capture all engrafting LT-HSCs, even if they may not follow the classical immunophenotypical definition of LT-HSCs, such as $CD150^-$ or $CD34^+$ cells (Karlsson *et al.*, 2013).

Like mice models, the first step in defining human HSCs is isolating BM cells that display lineage specific markers (abrevation LIN-). HSPCs are mostly found within the $CD34^+$ fraction, presenting around 1% of all mononuclear cells (MNCs) (Civin et al. 1984). However, this is a very heterogeneous population and can be further enriched using CD90 (Thy1)(Baum et al. 1992). Moreover, it has been shown that CD45RA and CD38 proteins are expressed more on progenitors within the $CD34^+$ population according to Table 2 (Bhatia, Bonnet, et al. 1997; Bhatia, Wang, et al. 1997; Lansdorp, Sutherland, and Eaves 1990). Integrin a6 (CD49f) can help further purify the HSC populations (Notta et al. 2016). Thereby, using a $Lin\text{-}CD34^+CD38^-CD90^+CD45RA^-CD49^+$ cell surface marker phenotype, provide enrichment of long term repopulating cells with a frequency of 1 out of 10 cells (Notta et al. 2011). In contrast, loss of CD49f expression identifies transiently engrafting MPPs (Notta et al. 2011). Interestingly, HSCs have also been reported to exist within the CD34 negative population (Bhatia et al. 1998). The $Lin^-CD34^-CD38^-CD93^{high}$ population not only possesses HSCs activity but also has been reported to be more quiescent than the putative $CD34^+$ HSCs. Additionally; $Lin^-CD34^-CD38^-CD93^{high}$ cells have the potential to regenerate $CD34^+$ HSCs in transplantation experiments (Anjos-Afonso et al. 2013). Recently, it was demonstrated that endomucin (EMCN) could

distinguish HSCs from multipotent progenitors much better than CD34 (Reckzeh et al. 2018).

The majority of adult HSCs are dormant and express a high proportion of ATP-binding cassette (ABC) transporters that can function as efflux pumps. Therefore, dye efflux properties such as rhodamine-123 (bertoncello *et al*., 1985) and hoechst-33342 (Goodell *et al.*, 1996; wolf *et al*., 1993) can be used as alternative cell surface markers to identify HSCs. These dyes will be retained in non-quiescent and cycling cells while the quiescent cells would pump them out. Committed progenitor and differentiated cells could be further isolated in mice and humans according to Table 2.

Table 2. Cell surface markers that are commonly used in this thesis to isolate HSC and progenitor cells in adult mice or humans, even though there are more markers are known to enrich various populations. This table includes data from (Pronk et al. 2007; Karlsson et al. 2013; Pietras et al. 2015; Adolfsson et al. 2005; Cheng, Zheng, and Cheng 2020; Doulatov et al. 2012). Lineage negative represent (L) in mice and (LIN-) for humans, while (LSK) represent Lineage$^-$ Sca1$^+$Ckit$^+$.

Primitive HSPC	**Mice**	**Human**
LT-HSC	LSKCD34$^-$Flt3$^-$CD9$^+$ or LSKCD150$^+$CD48$^-$Flt3$^-$	LIN$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$CD49$^+$
ST-HSCs/MPPS	LSKCD34$^+$Flt3$^-$ CD9$^+$	LIN$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$
MPP1	LSKCD150$^-$CD48$^-$Flt3$^-$	-
MPP2	LSKCD150$^+$CD48$^+$Flt3$^-$	-
MPP3	LSKCD150$^-$CD48$^+$Flt3$^-$	
MPP4	LSKCD150$^-$CD48$^+$Flt3$^+$	-
LMPP	LSKCD34$^+$Flt3^{high}	LIN$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$
Committed progenitors	**Mice**	**Human**
MEP	-	LIN-CD34$^+$CD38$^+$CD45RA$^+$CD135$^-$ CD10$^-$
MKP	LS$^-$K CD41$^+$CD150$^+$	-
pGM	LS$^-$K CD41^{low}CD150$^+$CD16/32$^{low/-}$ CD105$^-$	-
GMP	LS$^-$K CD41^{low}CD150-CD16/32$^+$	LIN$^-$ CD34$^+$CD38$^+$CD45RA$^+$CD135$^+$CD10$^-$
CLP	LS^{low}K^{low}Flt3^{high}IL7Ra$^+$	LIN$^-$CD34$^+$CD38$^+$CD45RA$^+$CD10$^+$
Differentiated cells	**Mice**	**Human**
T cells	CD3$^+$(CD4$^+$/ CD8$^+$)	CD3$^+$(CD4$^+$/ CD8$^+$)
B cells	B220$^+$ CD19$^+$	CD19$^+$
Myeloid	Mac1$^+$Gr1$^+$	CD15$^+$CD33$^+$
Erythroid	Ter119$^+$	CD235a$^+$

In vivo transplantation

While cell surface markers play an important role in identifying HSCs, the gold standard method to determine their stemness and function is through competitive and serial transplantation *in vivo* (Harrison et al. 1993; Jacobsen and Nerlov 2019). This method assesses HSC function by their capacity to reconstitute in an irradiated recipient and give rise to all blood lineages (lymphoid and myeloid). Transplantation in mice can be performed in an allogeneic or syngeneic way in which murine cells are injected into a recipient mouse or xenogeneic in which human cells are injected into an immunodeficient recipient mouse, respectively (Doulatov et al. 2012). The recipient mouse is subjected to lethal or sub-lethal radiation to eliminate the bone marrow prior to receiving cells of interest by intravenous injection. Competitive transplantation with a predetermined number of donor cells provides radioprotection, survival, and can be used to qualitatively evaluate the HSCs with high competitive repopulating capacity (Harrison 1980). Engraftment and long-term reconstitution of donor chimerism within the hematopoietic system of the recipient can be measured by flow cytometry at least 16 weeks after transplantation. The self-renewal capacity of HSCs can be evaluated through serial transplantation of bone marrow cells from the primary recipient into secondary recipients and monitored by HSCs chimerism analysis (Hétu-Arbour, Bouali, and Heinonen 2021).

In order to separate donor cells from host cells, two-mouse strains are routinely used that express different isoform of pan-hematopoietic cell surface marker CD45 (CD45.1 and CD45.2, respectively). These strains have also shown not to provoke an immune reaction after syngeneic or congenic bone marrow transplantation. Even though only leukocytes express CD45 antigen, mature erythrocyte and platelets replenishment could not be tracked in these models (Müller-Sieburg et al. 2002; Dykstra et al. 2007). Therefore, a transgenic florescent reporter mouse model was developed to track and label all lineage cells (lymphoid, myeloid, erythrocyte and platelets) by fluorescent protein detection in the recipient (Sanjuan-Pla et al. 2013; Yamamoto et al. 2013).

Currently, the most common mouse strain for xenogeneic transplantation of human cells is an improvement of non-obese diabetic mice crossed with immunodeficient mice (NOD/SCID) that carry a deletion of IL2rg gen (NSG model) (Ito et al. 2002; Shultz et al. 2005). This strain has complete loss of immune B, T and natural killer (NK) cells and also prevents leukemia initiation, thus they can support a robust long-term human engraftment (Ito et al. 2002; Shultz et al. 2005). Even though, humanized animal models have been extensively used to study HSCs function, it has a number of limitations, such as lineage skewing with lymphoid engraftment (lymphoid biased), lacking evaluating erythroid and platelet output or low permissiveness for modeling hematological malignancies (Doulatov et al. 2012). In

order to improve the lineage bias, several mouse models with transgenic human cytokines (*e.g* non cross-reactive cytokine such as IL3, GM-CSF, SCF) have been developed (Willinger et al. 2011). Despite all the progress that has been made in the study of human HSCs, it is important to be aware of differences between mouse and human physiology such age, size and genetic background that may causes artifacts in xenograft models.

In vitro cell culture

Even though, *in vivo* assays are necessary to assess self-renewal of HSCs, due to limited proliferative potential and distinct transient kinetics in lineage replenishment, evaluating lineage potential and fate *in vivo* for progenitors is challenging. Thus, several *in vitro* assays have been developed that allow ex-*vivo* interrogation of HSPCs self-renewal or differentiation. Above all, *in vitro* assays have financial and ethical advantages compared to *in vivo* assays. They eliminate the need for animal experimentations in line with 3R principles (explain farther in Ethics). Advanced *in vitro* cell culture systems can be utilized to mimic human or murine niche self-renewal or differentiation.

HSPC differentiation potential to myeloid (granulocyte/myeloid), erythroid or megakaryocyte can be studied by providing nutritional growth media and exogenous growth factor/cytokine to short term liquid or semi-solid cultures (Coulombel 2004). Colony forming efficiency assay (CFC assay) performed in growth media such as methylcellulose, which limits motility/migration of cells to investigate HSPCs colony forming capacity at the per-cell basis. However, most of the methylcellulose-based CFC cultures do not support lymphopoiesis (Purton and Scadden 2007). Microscopic analysis reveals progenitor cell proliferation and differentiation into burst-/colony-forming units erythroid (BFU-E, CFU-E) and colony-forming unit granylocyte/macrophage (CFU-GM) colonies. The size and type of cellular colonies can indicate the primitiveness of the progenitor cells; typically, the most primitive progenitors give rise to the largest and heterogeneous colonies. Cells that generate colonies are able to proliferate and differentiate in suspension cultures as well *(i.e*. without semi-solid support), in the presence of the same cytokines. However, identification of their progeny will require further advanced analysis by flow cytometry, which can be more time consuming and cumbersome.

To investigate lymphoid potential, more complex *in vitro* co-culture system with stromal cells can be used. To study immune B cells, theOP9 cell line, that is derived from the mouse BM of stroma cells, has been developed in 1994 (Nakano, Kodama, and Honjo 1994), and to study immune T cells, OP9 cells with ectopic expression of the notch delta like 1 (op9-DL1) was generated in early 2000's (Schmitt and

Zuniga-Pflucker 2002). This *in vitro* system allows discrimination of bio-potent lympho- myeloid and uni-potent lymphoid or myeloid restricted HSPS population. However; they might strongly promote myelopoiesis even in progenitors with lymphoid potential *in vivo* (Richie Ehrlich, Serwold, and Weissman 2011). As a result, these assays should be used to measure HSPCs differentiation rather than the steady-state function. In addition, the full lineage potential from individual progenitors may not be implemented under given conditions to demonstrate all the fate options. Thus, a combination of different single cell methods is required to obtain a comprehensive understanding of the potential of HSPCs *in vitro* (Jacobsen and Nerlov 2019).

Single cell transcriptomics

The traditional bulk transcriptomic approaches like polymerase chain reaction (PCR) or RNA-Sequencing provide an average state of gene expression across a large population. Single cell analysis approaches can be used effectively to study cellular heterogeneity, differentiation trajectory, and cell clonal relationships that are lost in bulk methods (Jacobsen and Nerlov 2019; Zhang et al. 2022). Single cell reverse transcription quantitative PCR (RT-sc-qPCR) was one of the techniques used to quantitatively study the heterogeneity of the hematopoietic systems. The commercial microfluidic platforms rely on small fluidic channels and reaction chamber to perform qPCR reaction, in which a maximum of 96 (cells) and 96 (primers) chips are used (Teles, Enver, and Pina 2014). Despite experimental analysis of limited number of cells and genes in this assay, the use of gene specific primers makes it highly sensitive method that even low expressing genes such as transcription factors or fusion genes in leukemia can be detected (Warfvinge et al. 2017).

To address the limitation of sc-qPCR, researchers have developed single cell RNA sequencing technique (scRNA-Seq), a high throughput method that un-biasedly detects all transcriptomes within each cell. However, due to nature of unbiased detection in this method, it is not considered as a sensitive assay and has dropouts, i.e., not detecting low-expressed lineage-specific transcription factors (TFs) likely precluding identification of early transitional states (Hicks et al. 2018; van Dijk et al. 2018). Similar to sc-qPCR, most scRNA-seq methods follow a general workflow in which RNA is reverse transcribed into cDNA. In contrast to sc-qPCR, which uses gene-specific primers to identify transcription, in scRNA-seq, universal primers are used to amplify all reverse transcribed RNAs. This makes it possible to impartially amplify every transcript. cDNAs are then broken down into smaller pieces and given sequencing adapters so that Next Generation Sequencing (NGS) can read the sequences. Moreover, before sequencing, the transcripts from distinct cells are additionally marked with cell-specific barcodes. Following sequencing, the

transcripts of each individual cell are separated based on the barcodes that are attached, allowing the transcriptomic profile of each cell to be examined. The method used to isolate individual cells and introduce cell barcodes has an impact on any single-cell library preparation protocol. Due to this, the different scRNA-seq methods are typically divided into three groups based on how cells are isolated: plate-based, droplet-based, and through combinatorial indexing. Among different plate-based scRNA-seq protocols that have been developed (Picelli et al. 2013; Jaitin et al. 2014; Hashimshony et al. 2012), the SMART-seq2 method has emerged as the most popular, owing to its high sensitivity (Ding et al. 2020) and ability to read full-length transcripts, allowing for isoform detection (Picelli et al. 2013; Ding et al. 2020). Index sorting is an advantage of plate-based techniques since it allows the integration of transcriptomic data and FACS index information at later stages of analysis (Schulte et al. 2015). However, the number of cells that can be captured using plate-based methods is limited. Therefore, combinatorial-indexing approaches such as Sci-RNA-seq (Cao et al. 2017) and SPLiT-seq (split-pool ligation-based transcriptome sequencing) (Rosenberg et al. 2018) were developed to increase plate-based cell throughput, which is more cost effective. Both methods use combinatonial indexing technique where RNAs are marked with barcodes that indicate their cellular origin (Hong et al. 2020).

The droplet-based techniques such as Drop-Seq (Macosko et al. 2015), in-Drops (Klein et al. 2015) and 10x (10x Genomics Chromium) (Zheng et al., 2017) were created to obtain a cost-efficient, robust and practical system to generate cDNA libraries for thousands of cells in a single run. To generate droplets, all methods use similar designs where on-bead primers with barcodes are used to differentiate individual cells in addition to a unique molecular identifier (UMI) for bias correction. Nanoliter droplets; contain cell lysis buffer, cDNA, amplification reagents and enzymes, along with oligo barcodes, to tag cDNA from the cells. The cDNAs with cell barcodes that have been synthesized are pooled by breaking the droplets and amplified to create a sequencing library (Zhang et al. 2019). Also, a molecular transcript-specific tag, UMI, secures the read's integrity by identifying PCR duplicates (Islam et al. 2014). Droplet-base techniques decrease the risk of collecting several cells packed into tiny emulsion droplets with the same barcode, which can no longer be distinguished. Afterwards the single cell suspension is loaded onto microfluidic chip with low concentrations, which means that most of the emulsion droplets remain empty, and the reagents are used very inefficiently. This issue is compensated by loading low concentrations of the single cell suspension onto the microfluidic chip, which leads to a high proportion of empty emulsion droplets and inefficient reagent usage. A recently developed ultra high throughput technique called "scifi-RNA-seq" (for single-cell combinatorial fluidic indexing), marks the RNA of numerous cells with unique barcodes before the cells are loaded on a microfluidic chip and their RNA is prepared for single-cell

sequencing. As a result, many cells can be added to the emulsion droplets simultaneously while still allowing for the analysis of single cells. Scifi-RNA-seq increased the output of individual cells 15 times more than popular 10x Genomics system (Datlinger et al. 2021).

Single cell epigenomics

Among multiple epigenomic features, such as histone modification, DNA methylation and chromosome organization, we focused on chromatin accessibility here. Chromatin accessibility profiles are able to identify cell types much more precisely than transcriptomes (Rodrigues Shvedunova and Akhtar, 2020). So far, many methods for investigating chromatin accessibility have been developed, including DNase I hypersensitive sites sequencing (DNAase-seq) (Boyle et al. 2008) and the Assay for Transposase-Accessible Chromatin Sequencing (ATACseq) (Buenrostro et al. 2015). Here we explain single cell ATACseq, that is a remarkable and commercially available method based on droplet (Lareau et al. 2019; Satpathy et al. 2019) or plate-based sequencing (Cusanovich et al. 2015). In droplet-based method, after isolation of cell nuclei in bulk, Tn5 transposase is used to fragment the DNA and intergraded the sequencing adaptors. Microfluidics can be used to separate single transposed nuclei and perform ATAC-seq reactions individually using gel beads in emulsions (GEM), each of which contains a unique 10x genomic barcode. Following the creation of GEM, the gel bead is broken and oligonucleotides for linear amplification are released. After amplification, GEM is broken down and barcoded DNA is pooled for generation of sequencing libraries to then visualize chromatin accessibility of the single cells (scATAC-seq). This allows inspection of chromatin dynamics during cell fate determination which is then suitable to study transcription foot printing (Lareau et al. 2019; Satpathy et al. 2019; Rodrigues, Shvedunova, and Akhtar 2020).

Analysis of single cell data

Multitudes of various single-cell analysis tools (over 1000 tools) have been developed to analyze scRNA-seq and scATAC-seq data (Zappia and Theis 2021). Therefore, the most important steps for analyzing such data will be briefed here. The raw output data from Illumina's sequencing is in BCL format, which is easily converted to FASTQ format by Cell Ranger mkfastq. The primary phase of single cell transcriptomic data analysis is to provide the two dimensions of the matrix; the cells and genomics features (including transcripts, accessible regions of the genome, surface proteins) via four major steps. Primarily, raw data should be mapped to the reference genome, grouped by genes, and given their original cellular barcode using tools like CellRanger-count or the STARsolo in scRNA-seq (Slovin et al. 2021).

CellRanger-ATAC pipeline that used BWA-mem (Li and Durbin 2010) or bowtie-2 (Langmead and Salzberg 2012) tools are used to align scATAC-seq read fragments to the reference genome. Further, the data is applied to peak calling using MACS2 algorithm (Zhang et al. 2008) or genomic bin assignment using SnapATAC (Fang et al. 2021).

Then, UMI correction is performed to rectify errors in sequencing data and eliminate duplicate reads. Thereafter, barcode de-multiplexing is performed to aggregate the UMI for individual cells. Then, the quality control (QC) of cells is performed wherein cutoff value of UMIs per cell is determined to distinguish cell barcodes from background barcodes. These steps result in a matrix wherein each row is a cell and each column is a feature (gene for scRNA-seq and peak for scATAC-seq), the values in the matrix are UMIs associated with the feature in the given cell.

Further, the single-cell downstream analysis toolkits can be used to analyze the data, for example, Seurat (R) (Butler et al. 2018), Scanpy (Python) (Wolf, Angerer, and Theis 2018), and Monocle (R) (Cao et al. 2019) for scRNA-seq data and SnapATAC (Fang et al. 2021) or ArchR (Granja et al. 2021) for scATAC-seq analysis. Upon loading the cell-feature matrix into these methods, the first step is to perform cell filtering. Cells can be filtered out, for example, based on high percentage of mitochondrial reads (Ilicic et al. 2016) or cells with low number of UMIs per barcode (i.e., coverage depth of a cell); also using stress and apoptosis marker genes, stressed and apoptotic cells can be removed (O'Flanagan et al. 2019). The next step is to perform data normalization might to reduce the impact of uneven signals captured from each cell. Normalization techniques such as count per million (CPM) in scRNA-seq or TF-IDF (term-frequency inverse-document-frequency) in scATAC-seq (Cusanovich et al. 2018) are two most commonly applied techniques. For RNA-seq UMIs, the data is also often transformed into log-scale and genes are scaled using z-transform (standard scaling).

Next, the normalized data is used to perform feature selection with twin objective of speeding up the processing of the data and filtering ‘uniformative’ genes (for example housekeeping genes and those that are ubiquitously expressed in all the cells). For scRNA-seq data, highly variable genes (HVG) are selected after correcting for mean-variance trend (Slovin et al. 2021). For scATAC-seq data, feature selection is performed by selecting the peaks that have highest normalized signal following TF-IDF normalization (Stuart et al. 2021).

The next step is to perform dimensionality reduction to decrease the redundancy of features and noise in the data (Moon et al. 2019). The PCA (Principal Component Analysis) is the most widely used linear dimensionality reduction algorithm used to transform scRNA-seq data into a new coordinate system (Jolliffe and Cadima 2016; Pearson 2010). Principal Components are orthogonal axes that capture the maximum variance in the data. For scATAC-seq, method like cisTopic (Bravo

González-Blas et al. 2019) reduces the dimensions of feature matrix by selecting the top topics based on the topic-cell distribution produced by latent Dirichlet allocation (LDA) (Blei, Ng, and Jordan 2003). LDA and its improved version LSI (latent semantic indexing) enhance cluster separation over PCA and identify open chromatin regions that are enriched in cellular populations. After running either PCA or LDA/LSI, the top 'n' dimensions are chosen for the next step of the analysis. The number of dimensions to be used can be determined based on the variance captured by each dimension.

Next, the dimension reduced data from both scRNA-Seq and scATAC-seq is used to generate a graph-representation of the data. In this graph representation of the data, each node of the graph represents a cell and each edge (connection between nodes) determines similarity between the cells. In this graph representation, instead of having number of cells (n) and feature (p) matrix, we obtain (n) number of cells and (k) number of nearest neighbors (KNN) for each sample matrix. Once we obtain *a* neighborhood graph*,* downstream data analysis can be performed including visualization the data, clustering, annotation, lineage trajectory, differential gene expression, etc.

To visualize the graph representation of the data, non-linear dimension reduction methods like t-Distributed Stochastic Neighbor Embedding (t-SNE), (Maaten and Hinton 2008) and the Uniform Manifold Approximation and Projection (UMAP) (McInnes et al. 2018) are used. UMAP preserve both global and local structure of the data (graph) while t-SNE only preserve the local data structure, therefore UMAP better captures data heterogeneity and at the same time clearly shows the cluster relationships in two/three dimensional space (Becht et al. 2018).

Unsupervised clustering is applied to define cell state that explains the cellular heterogeneity within the population, using some approaches like graph clustering in Seurat (Satija et al. 2015). In order to improve the accuracy of clustering and marker gene recovery, the technical artifacts can be removed such as cell cycle effects, mitochondrial contamination or batch effect (Slovin et al. 2021; Butler et al. 2018). Annotation of cell cluster is an important step to identify the group of cells and link them to their biological identity and states. Annotation can be performed manually based on existing knowledge about cell types and using the marker genes identified for each cell cluster. Machine learning based classifiers like Random Forest cab be trained on one dataset and then applied to other dataset to achieve automated annotation. Specialized single-cell methoids like scID (Boufea, Seth, and Batada 2020) or a mapping based approach, like Nabo (Dhapola et al. 2020), can be used to transfer labels from a reference dataset.

Biological mechanisms are a highly dynamic processes in which the cells can transit between several transcriptional states through cell autonomous factors and/or other external factors like influence of the cellular local environment within the niche. To

model cellular transition between states when a continuum biological model applies, trajectory inference methods like Slingshot (Street et al. 2018) have been developed. Such methods order cells along trajectories starting from a predefined point. There might be several trajectories within a dataset, for example, stem cells dividing into different fates. According to each cell's distance from the predefined starting point (root state), it is ascribed a "pseudotime'' value, which models the distance/time since the root state. The final result can be visualized on UMAP, based on their assigned pseudotime values. Cells are then given sizes and colors, and their trajectories are depicted as spline curves over the UMAP. This would quantify the relative activity or progression of the underlying biological process and reveal cell differentiation path, fate changes and lineage relationship (Zhang et al. 2022).

In scATAC-seq, following the peak calling step, peak annotation is performed which tags each peak based on its genomic context. For example, if a peak is close to or within a gene body or promoter then it can be called a gene-proximal element otherwise it would be a distal element (Baek and Lee 2020). Distal elements themselves can be further annotated, for example, to be regulatory distal elements if they are collocated with known enhancers. Following peak annotation, the downstream analysis can be carried out for all the peaks or only a subset of data based on annotation type. Our own results indicate that using distal peaks only in the analysis can lead to improved cell type identification (Safi et al Cell Reports, 2022). Further, the transcription factor binding motifs (TFBS) can be identified within the peaks. Motif search algorithms like FIMO can be used to find the TFBS using consensus binding sequence of transcription factors as available in JASPAR database (Castro-Mondragon et al. 2022). By summarizing the TFBS found across all the peaks, the cell-peak matrix can be collapsed into a cell-TFBS matrix, where each value represents the estimated global activity of a transcription factor in a given cell.

The peak set was divided in proximal or distal set base on peak's genomic location. Moreover, in scATAC-seq could reach short inaccessible chromatin that flanked by 2 peaks. This might detect the TF binding site called TF foot printing. The sequence of this inaccessible region can be linked to the sequence similarity to defined TF binding site (Vierstra and Stamatoyannopoulos 2016).

Ethical considerations

Certain ethical matters must be considered when working with animals and human samples. Animal experimens are a debated topic in society, with suggestions of abolishing these entirely or at least to reduce them to a minimum. However, animal models are required in some fields of medical research, including HSC research, as transplantation is the only way to prove HSC function. However, it is critical to

incorporate the 3R principles into the daily work with annimals; reduce, replace, and refine (Díaz et al. 2020). It is important to reduce the number of mice as much as possible in the experiment while also ensuring that animals are well-treated and suffer as little as possible to reduce stress and discomfort. When it comes to replacing animal experiment, alternative methods, such as *in vitro* assays, hold great promises in studying HSC lineage fate and expansion. Transplantation will most likely be required to functionally test HSCs for clinical applications in the near future. When it comes to human samples, donors must be well informed regarding the purpose of using their sample in medical research. Furthermore, all data generated from human samples must be anonymized and stored in a secure data storage facility to protect patients' identities and information privacy.

Aim of thesis:

The overall aim of this thesis was to investigate how cellular-fate options emerge in the cloud of hematopoietic stem/progenitor cells (HSPCs), at what stage the multipotency gives way to lineage priming, and how this stage can be detected. In this aim, single-cell (sc) chromatin accessibility (ATAC-seq), scRNA-Seq and sc-qPCR analysis were employed extensively to identify the transition of HSCs to multipotent progenitor cells. In order to validate the genomic data from the aforementioned analyses, we established *in vitro* culture systems to functionally examine the differentiation fates of single cells at a clonal level. We found that the transition point in the cloud of adult mouse HSPCs lies in lineage priming initiating with down-regulation of CD9 and up-regulation of Flt3 expression. Therefore, in paper III, we examined CD9 expression and its heterogeneity in human hematopoiesis as well as its changes during aging and in leukemia.

Aim1 - To understand how lineage committment emerge and to study the initiation of lineage priming in HSCs (Paper I-II).

Aim 2 - To characterize the molecular program underlining lineage specification and loss of multipotency in murine HSPCs (Paper I).

Aim 3 - To develop a new methods to allow functional assays of clonal multilineage differentiation (Paper II).

Aim 4 - To assess CD9 expression in HSPCs as a measure of heterogeneity during human aging (Paper III).

Paper I: Concurrent stem- and lineage-affiliated chromatin programs precede hematopoietic lineage restriction

Introduction

Hematopoietic stem cells (HSCs) are rare quiescent cells with long-term self-renewal capacity that divide asymmetrically and differentiate into all blood lineages (orkin and zon 2008). Based on classical models, the self-renewal potential is gradually lost during the transition from LT-HSCs to MPPs as FMS-like tyrosine kinase 3 (Flt3) is upregulated. The $Flt3^+$ multipotent progenitors serve as developmental intermediates for hematopoietic lineage priming (Boyer et al. 2011; Buza-Vidas et al. 2011; Akashi et al. 2000; Beaudin, Boyer, and Forsberg 2014; Kondo, Weissman, and Akashi 1997). In fact, it has been shown that the expression of *Flt3* mRNA in LT-HSCs ($LSKCD48^-150^+$) correlates with the loss of HSC-associated gene expression profile and the initiation of transition to MPPs (Mead et al. 2017; Mooney et al. 2017). MPPs can be divided into 4 sub-populations. The ST-HSCs (MPP1), MegE-biased (MPP2), and myeloid-biased (MPP3) populations are enriched within the $Flt3^-$ fraction, and lymphoid-biased MPP4 progenitors are included within the $Flt3^+$ pool (Woolthuis and Park 2016; Tsapogas et al. 2017). Notably, the 25% highest $Flt3^+$ cells, known as LMPPs, have been defined as restricted lymphoid primed with down regulation of MegE priming (Adolfsoon et al.2005, Mansson et al. 2007). This has brought about the idea that the progenitor with an intermediate level of Flt3 expression may bridge multipotency with lineage commitment. In contrast to this, recent single cell RNA sequencing (scRNA-seq) supports a continuum model of hematopoietic hierarchy with a low-primed cloud of HSPCs without sharply defined gene expression programs (Zhang et al. 2018; Giladi et al. 2018). Based on the observations in the field at the time we began paper I in 2016, there was no transition of different lineages from multipotent progenitors (MPPs) with intermediate gene expression; instead, these progenitors were a group of uni-lineage-primed cells.

In this study we aimed to investigate how cellular fate options occur, at which stem-like stage lineage priming is initiated, and whether this stage can be captured using a combination of single cell chromatin accessibility, RNA expression and cell surface markers.

Summary

Cellular fate options through the lens of single cell genomics

It has been shown that the cell potency aligns more closely with the epigenome rather than transcriptome (Paul et al. 2015; Yu et al. 2017) and fate decisions cannot be simply revealed by scRNA-seq (Weinreb et al. 2020). Therefore, we first performed single-cell assays for transposase-accessible chromatin sequencing (scATAC-seq) on the spectrum of Flt3 expression to identify the earliest stage of hematopoietic lineage priming. Our data suggests that the accessibility of the chromatin landscape is associated with continuous rather than stepwise differentiation. And the chromatin profiles of LSKFlt3^{int} fraction represent a heterogeneous population containing cells from multipotent-progenitors to LMPP-like cells. Next, we investigated potential markers to isolate the cells with the most primitive chromatin landscape within the LSKFlt3^{int} population. It has been shown previously that tetraspanin CD9 is a cell surface marker that marks HSCs and megakaryocytes in mice (Karlsson et al. 2013; Nakorn, Miyamoto, and Weissman 2003). Therefore, the scATAC-seq was performed on LSKFlt3^{int} CD9^{high} and CD9^{low} cells. Interestingly, the LSKFlt3^{int}CD9^{high} cells represented a continuum of cells with the boundary of chromatin accessibility signature between ST-HSCs and LSKFlt3^{int} cells, while the LSKFlt3^{int}CD9^{low} cells were located along with the LMPPs. To understand, transition stage from multipotency to lineage restriction and how it is reflected at chromatin level, cells were analyzed for transitions in motif accessibility by combining changes across the compendium of 571 TFBS profiles within the distal open chromatin regions, along a lineage differentiation trajectory according to pseudotime. LSKFlt3^{int}CD9^{high} showed unique accessibility of stem-like and lineage specific TF motifs within single cells (e.g FOXO, HOX-family and SPI1, ETV6, RUNX-family, and MECOM respectively) indicating a state of global lineage priming within LSKFlt3^{int}CD9^{high} cells. It was demonstrated that SPI1 motif accessibility became relatively higher than GATA1, only after the LSKFlt3^{int}CD9^{high} cells in the trajectory, indicating a likely loss of multipotency downstream of LSKFlt3^{int}CD9^{high}, consistent with loss of MegE potency in LSKFlt3intCD9low and LMPP. Altogether, these findings indicated massive changes in TF motif accessibility taking place during the transition from stem cells to lineage-specific progenitors in the LSKFlt3^{int}CD9^{high} cells. Importantly, individual cells at this transitional state simultaneously display a chromatin accessibility profile similar to HSCs, as well as gain of lineage-affiliated molecular programs associated with lympho-myeloid and MegE differentiation trajectories.

In line with this finding, scRNA-seq and multiplexed qPCR signature of the LSKFlt3^{int}CD9^{high} cells showed an enrichment for genes linked to stem cells, B and T lymphocytes, as well as MegE lineages while CD9^{low} cells gene expression profile aligned with LMPPs and pre-GMPs, corroborating the findings from scATAC-seq.

Finally, the single cell ATAC-seq and scRNA-seq datasets were integrated to assess the relationship between enhancer accessibility and the target gene expression. The integration showed that lineage-specific program of $CD9^{low}$ cells is likely seeded within primitive $CD9^{high}$ cells at the level of enhancer chromatin accessibility.

Cellular fate options through the lens of functional assay

To functionally validate the data from the sc-genomics experiments, $LSKFlt3^{int}CD9^{high}$ or $LSKFlt3^{int}CD9^{low}$ cells were isolated to perform competitive transplantation and several *in vitro* differentiation assays. Transplantation assays confirmed that $CD9^{high}$ cells were multipotent progenitors with short-term myeloid and long-term lymphoid engraftment potential but lacked self-renewal capacity. This data was in line with the observation from index sorting data in sc-qPCR, showing that most of the $LSKFlt3^{int}CD9^{high}$ are $CD150^{-}$, suggesting that they are MPPs. To evaluate the clonal capacity of $LSKFlt3^{int}CD9^{high}$ cells *in vitro*, a protocol was designed that supported the differentiation of all lineages (My, Ly and ER). This protocol, known as switch culture, allows all differentiation fates to be examined at a clonal level, as explained in detail in paper II. In general, the combination of switch culture and several other methods (like hierarchical experiment terasaki MK and ER, CFU, CFU-MK) determined the full capacity of the different cell fractions (Figure 7).

The results from all the *in vitro* experiments supported the idea that $LSKFlt3^{int}$ $CD9^{high}$ cells can generate multiple lineages including megakaryocytes and primitive erythroid (BFU-e) progenitor cells compared to the $CD9^{low}$ cells. Overall, functional data also supported the identification of a multilineage hematopoietic transitional state within the $LSKFlt3^{int}CD9^{high}$ cells. This multilineage capacity is rapidly lost as CD9 expression is down regulated (e.g. in $Flt3^{int}CD9^{low}$), Flt3 expression is up regulated (for example in $Flt3^{high}$ LMPP) and parallel chromatin changes occur consonant with increasing accessibility of lineage-associated TF motifs (Figure 7).

In conclusion, distinct chromatin landscapes separate hematopoietic stem and progenitor populations. $LSKFlt3^{int}CD9^{high}$ cells display concurrent stem- and multi-lineage chromatin status and act as a bridge between multipotency and lineage restriction. Accordingly, self-renewal capacity declines with the gain of the Flt3 immunophenotype, while multipotency is only lost with the loss of the CD9 immunophenotype.

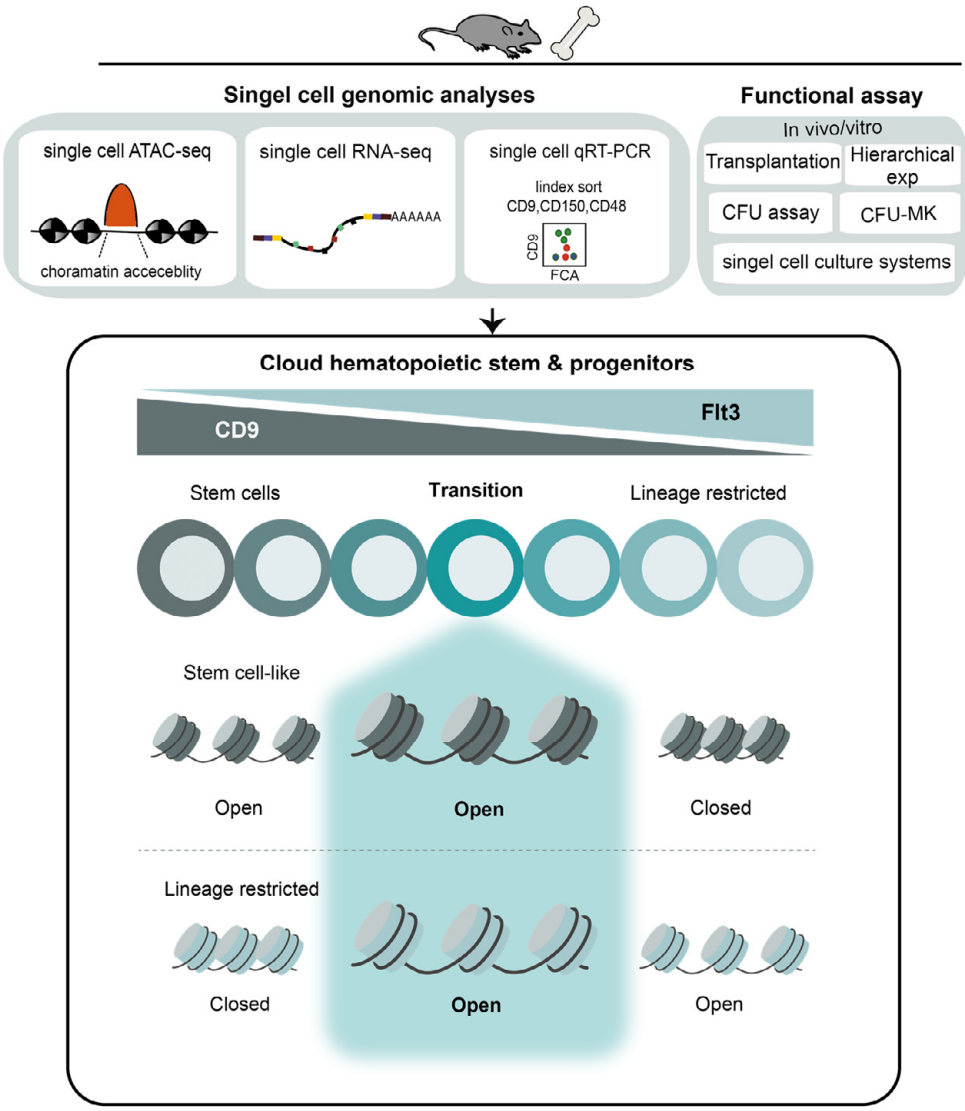

Figure 7. A schematic model of transition point in cloud of HSPCs in adult hematopoietic stem cell where multipotency is rapidly lost as CD9 expression is down regulated and Flt3 expression is up regulated. Adopted from (Safi et al. 2022).

Paper II: In-vitro clonal multilineage differentiation of distinct Murine hematopoietic progenitor populations

Introduction

One of the most important ways to evaluate lineage potential is at the single cell level, using a system that supports all potential cell fates (lymphoid, myeloid, megakaryocyte, and erythroid) at the clonal level. To study the lineage potential and fate of progenitors *in vivo* is challenging, due to limited proliferative potential and distinct transient kinetics in lineage replenishment of progenitor cells. Moreover, megakaryocytic and erythroid output of a candidate sub-population cannot be assessed in-vivo. Thus, *in-vitro* colony-forming assays and other clonal assays like (OP9/ OP9D co-culture system, teresaki assay, CFU assay) remain the preferred tool (Figure 8A), however, evaluating all lineage potential outcomes may be challenging. As full lineage potential from individual progenitors may not be implemented in certain conditions to demonstrate all the fate options, a combination of different single cell methods is required to obtain a comprehensive understanding of the potential of HSPCs *in vitro* (Jacobsen and Nerlov 2019).

The goal of this study was to develop an *in vitro* culture system that could be used to differentiate hematopoietic populations to all major hematopoietic lineages (lymphoid (B cells), myeloid, and megakaryocyte/erythroid), at the clonal level in order to validate single-cell genomics data.

Summary

Here, we designed both a sensitive single-cell switch-culture system and a less time-consuming alternative barcoding protocol that is more suitable for larger cell numbers.

Switch Culture method in Connection to paper I

In paper I, single-cell switch-culture protocols were developed to assess the combined myeloid, B, and erythroid potential of single LT-HSCs, LSKFlt3^{int}CD9^{high} cells, LSKFlt3^{int}CD9^{low} cells, and LMPPs. The switch-culture protocol is a sensitive co-culture system. However, due to many sensitive and laborious steps, working with more than 96 cells at a time is challenging. As a result, the quantity of sorted cells is a limitation of the switch-culture protocol. To perform the switch-culture, single murine cells were sorted into a 96-well plate containing the OP9-monolayer supplemented with complete OptiMEM and cytokines (50 ng/ml SCF and 50 ng/ml

Flt3L). The Op9 co-culture system was developed to study B cells (Nakano, Kodama, and Honjo 1994). After 4 days of co-culture, cells were divided and transferred to either confluent OP9 monolayer wells supplemented with the B cell differentiation condition or to SFEM medium with 1% Pen-Strep, 30% FBS supplemented with the erythroid differentiation condition, according to the (Figure 8B). Finally, following 21 days of culture, the clones were analyzed by flow cytometry for markers of erythroid ($CD45^{-}F4\text{-}80^{-}Mac1^{-}Gr1^{-}TER119^{+}$), B ($CD45^{+}Mac1^{-}CD19^{+}$), or myeloid cells ($CD45^{+}Mac1^{+}Gr1^{+}$ or $CD45^{+}Mac1^{+}F\text{-}480^{+}$). The clones were required to have >50-gated events of surface markers within the scatter profile and morphologically confirmed by May-Grunwald/Giemsa-staining to be counted as positive.

The results indicated that LT-HSCs could differentiate into all lineages (myeloid (My), macrophage (MΦ), lymphoid (B cells), and erythroid (ER)) using our *in vitro* co-culture system. In contrast, the LMPP population only produced B and myeloid cells. Surprisingly, $LSKFlt3^{int}CD9^{high}$ single cells generated more multilineage progeny compared to the clone with $LSKFlt3^{int}CD9^{low}$. Together, this data provided functional support for the identification of an $LSKFlt3^{low}CD9^{high}$ multilineage-primed progenitor as the transition stage between stem and lineage-committed cells.

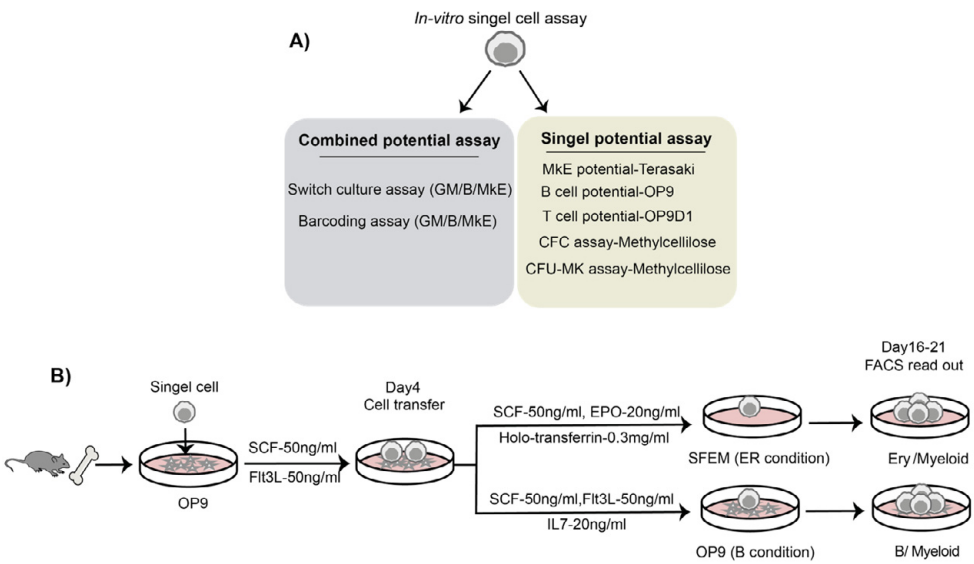

Figure 8. *In vitro* methods that used to functionally validated the genomic data. A) Assays that used to functionally study the single cell potential *in vitro* through a single potential assay (right panel) or a combined potential assay (left panel). B) Methodology and FACS read out for the switch culture protocol. Adopted from (Safi et al. 2023).

Alternative 2/barcoding protocol

Cellular barcoding is emerging as a powerful tool for addressing individual cell fates on a large scale by tagging individual cells of interest with unique heritable identifiers or barcodes (Naik, Schumacher, and Perié 2014). Here we improved the switch-culture system and added barcoding to enable higher throughput and bulk culture. The technology for this barcoding protocol was adapted from (Kristiansen, Doyle, and Yuan 2017) which was used to track the multilineage differentiation of LSK cells *in vitro* (Figure 9). This protocol enables the differentiation and growth of all lineages without a switch in culture conditions. This protocol allows for analysis of higher cell numbers however, it can introduce technical variations that may complicate analysis of purified long-term hematopoietic stem cells (LT-HSCs). This protocol consists of three major steps: First, bulk cells from LSK (Lin^-, $Sca1^+$, C-kit^+) and LMPP (LSK, $CD34^+$, $Flt3^+$) populations were sorted into 48-well plate containing the OP9-layer supplemented with complete OptiMEM and cytokines according to (Figure 9). Next, the cells were then transduced by adding optimized amount of lenti-virus (barcode labeling) to each well. After 12 hours, the cells were washed to remove any remaining virus and kept in culture with complete OptiMEM and cytokine according to (Figure 9). The transduction efficiency should be between 15-30% after 72 hours (Kristiansen, Doyle, and Yuan 2017), to reduce the risk of multiple barcodes integrating into a single cell as it affects the quantification of analysis, since they would counted as multiple progenitors with the same fate, instead of one cell labeled with two different barcodes (Naik, Schumacher, and Perié 2014).

In the second step, the cell-specific barcodes were recovered. Since the kinetics of differentiation is unique to each progenitor and can be influenced by virus transduction, we opted to sort GFP^+ cell from the lineage output (megakaryocyte, erythroid, myeloid (granulocyte and monocyte/macrophage) and lymphoid) from LSK cells at day 16 and day 21 and LMPP at day 8 and day 16, to ensure the discovery of clonal output based on distinct cell differentiation kinetics. After extracting the genomic DNA, liberally preparation and sequencing step was performed for each sample in two different technical replicates in order to avoid false positive overlap readout (Kristiansen, Doyle, and Yuan 2017).

In the third step, a list of reliable barcodes was generated for each sorted sample and combined with barcode read frequency to reflect the abundance of each clone within the population. To achieve this, a Python package called Bartid was designed. First, barcodes were automatically extracted by introducing the primer and sequence to the software. Next, the software detects and summarizes the barcode. The frequency barcode is available after the barcode has been corrected for sequence errors. Low-frequency barcodes (those below 20%) were eliminated. Then the barcodes were

compared between the samples, and all the combinations of the samples and the numbers of overlapping barcodes were visualized.

The results indicated that LSK cells can differentiate into all lineages (myeloid (My), macrophage (MΦ), B, megakaryocyte (MK) and erythroid (ER)) using our *in vitro* co-culture system after 21-days. In contrast, the LMPP population only produces B and myeloid cells.

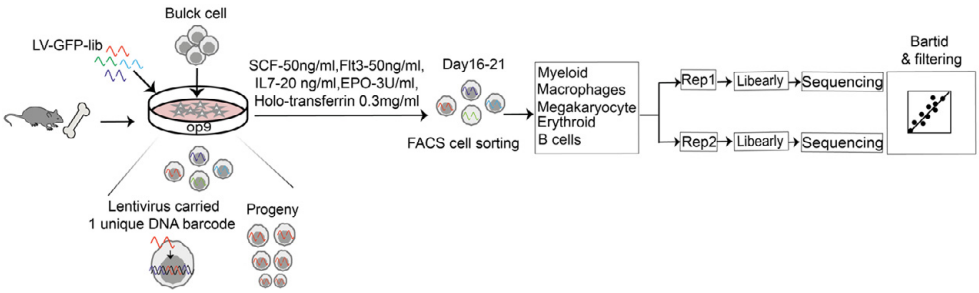

Figure 9. Summary of the barcoding protocol's experimental workflow, adopted from (Safi et al. 2023).

Paper III: CD9 marks myeloid/MegE-biased human hematopoiesis

Introduction

All hematopoietic stem cells have the ability to self-renew and differentiate to all blood cell lineages. (Osawa et al. 1996; Morrison et al. 1997; Pietras et al. 2015; Yamamoto et al. 2013). Individual HSCs in the stem cell pool, on the other hand, are functionally heterogeneous, and this increases with age. While HSCs in young mice perform qualitatively more similar, ageing is marked by an increased size of the HSC pool and decreased engraftment capacity (Dykstra et al. 2011; Sudo et al. 2000; Beerman et al. 2010; Liang, Van Zant, and Szilvassy 2005) as well as myeloid/platelet biased lineage differentiation (Dykstra et al. 2011; Sudo et al. 2000; Grover et al. 2016). Additionally, aging increases the risk of developing certain hematopoietic diseases like myeloid leukemia (Lichtman and Rowe 2004). To investigate the functional heterogeneity changes associated with age, several sorting strategies based on cell surface markers have been developed to purify functionally distinct murine primitive HSCs (Yang and de Haan 2021). In contrast to mice, human immunophenotypic changes associated with aging and its effects have received little attention.

The tetraspanin CD9 was identified as a marker for all murine HSCs (Karlsson et al. 2013) as well as for multipotency during the early stages of hematopoietic lineage commitment (Safi et al. 2022). Therefor, in this manuscript, we investigated the potential of CD9 as a human HSCs marker, as well as changes in its heterogeneity with aging.

Summary

CD9 expression was investigated in the $Lin^-CD34^+CD38^-$ HSPCs population from neonatal umbilical cord blood (CB) as well as in young Bone Marrow (yBM) and aged Bone Marrow (aBM) by flow cytometer. The results indicated that only a small proportion of CB and yBM HSPCs expressed CD9 as previously shown (Liu et al. 2021) however, the proportion of $CD9^+$ HSPCs significantly increased with age. Moreover, $CD9^+$ cells were primarily detected in multipotent progenitors (MPPs), and multi-lymphoid progenitors (MLPs/ LMPPs) and with low expression in the HSC population in CB and yBM. There was a shift in CD9 expression on HSPCs during aging, in which $CD9^+$ LMPPs were lost in conjunction with a relative increase in the MPP population, containing a larger fraction of $CD9^+$ cells.

Next, single-cell CITE-seq data generated in-house from $Lin^-CD34^+CD38^-$ of CB, yBM, and aBM (Sommarin et al. 2021) was used to directly compare CD9 expression across age groups and correlate CD9 expression with changes in heterogeneity. Data from all age groups were merged and analyzed. Our data indicated the enrichment of molecular HSCs and myeloid/MegE-biased clusters with age while the lympho-myeloid clusters decreased. These findings in the most primitive HSPCs are consistent with previous research on the effects of aging on hematopoiesis, which ranges from lympho-biased at birth to myeloid and MegE-biased in the elderly (Pang et al. 2011; Rundberg Nilsson et al. 2016; Kuranda et al. 2011). In order to study the heterogeneity, CITEseq enables direct integration of single-cell gene expression analysis and high-throughput immunophenotypic profiling. Visualizing the CD9 cell surface expression (CD9-ADT) in our data reveled that CD9 is mainly expressed between the HSCs and myeloid/MegE-biased clusters, in line with pervious finding that CD9 is up regulated during differentiation of MegE progenitors (Clay et al. 2001).

Furthermore, the number of $CD9^+$ and $CD9^-$ cells in each cluster was compared together during aging. The results of CITEseq analysis together with the data from sc-qPCR suggested that CD9 marks myeloid/MegE biased hematopoiesis. This is in agreement with the observed reduction of $CD9^-$ HSCs, and expansion of $CD9^+$ HSCs during myeloid-biased hematopoiesis associated with aging and myeloid leukemia. However functional assays, such as transplantation and CFU assays, revealed no significant differences between $CD9^+$ and $CD9^-$ cells when it comes to primitive

HSPC function, suggesting that CD9 expression is rather an indicator of molecular priming towards certain lineages. It has been demonstrated that the CML-associated BCR-ABL mutation biases early HSC differentiation towards myeloid and megakaryocytic lineages (Hussein et al. 2017). Therefore, CML is a good model to measure CD9 expression in HSCs with known myeloid/MegE bias. Indeed, CD9 was highly expressed in all CML patients as measured by flow cytometry analysis of HSCs, and on average 2.2 folds more CML HSPCs expressed CD9 compared to age-matched healthy controls ($p<0.01$). The data suggested that CD9 could be a human HSC marker candidate during phases of myeloid biased hematopoiesis (Figure 10).

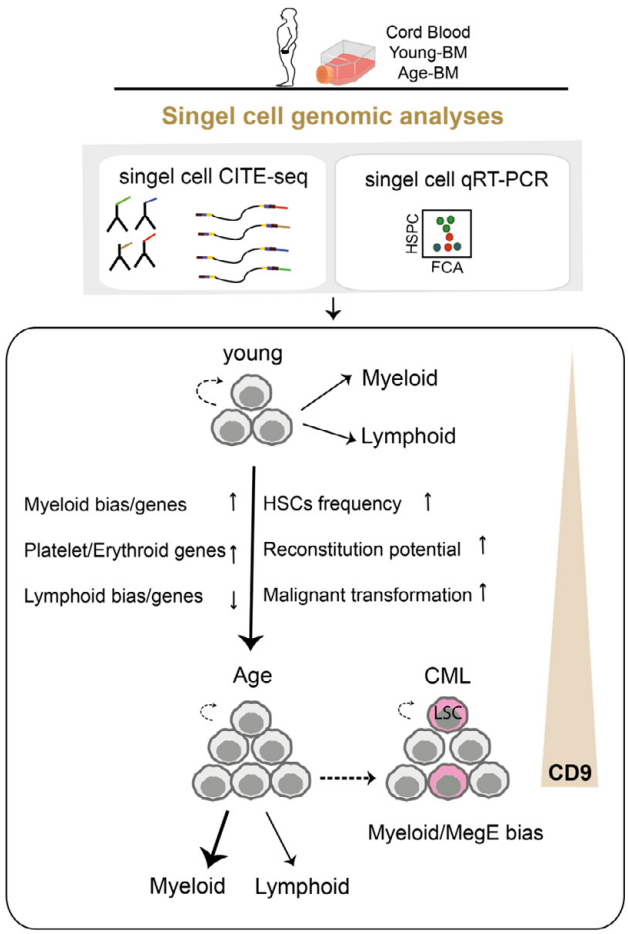

Figure 10. Summary of events that occur during aging in HSCs from mouse and human studies; here, we show that CD9 heterogeneity changes in humans as they age. While CD9 is expressed infrequently early in life, it increases during aging or CML, as our single genomic data suggests CD9 represents an HSC marker for myeloid-biased hematopoiesis.

General discussion and future plan:

In paper (I) we show that HSPCs arranged along the Flt3 expression gradient contain a pool of cells (LSKFlt3^{int}CD9^{high}) that acts as a transitional bridge connecting multipotency and lineage restriction. This is in contrast to the notion from recent scRNA-seq studies (Giladi et al. 2018) where transitional multipotent progenitors (MPPs) with simultaneous expression of genes of different lineages are not seen in adult hematopoiesis. There might be two reasons why the transitional states are usually not detected: first, scRNA-seq is inherently limited in precluding identification of early transitional states due to difficulty to detect low-expressed lineage-specific transcription factors (TFs), and second, such intermediary cells are relatively rare. Here we try to solve both problems by using scATAC-seq to map the accessibility of 571 TF-binding motifs as a measure of lineage priming bypassing the issue of dropouts, and combined it with capturing cells by Flt3 and CD9 surface markers that efficiently enriched the transitional cells.

Our results show that LSKFlt3^{int}CD9^{high} cells display a co-incidental stem and multi-lineage primed chromatin state, and represent a tipping point in the HSPC continuum where after different lineages segregate. Our findings address a critical but debated area in stem cell biology by demonstrating that single-cell chromatin analysis successfully identifies mixed-lineage states undetectable by scRNA-seq, and implies that lineage-priming initiates in the epigenome without being starkly reflected in the transcriptome. The results assume significance in view of recent reports that suggest that fate decisions cannot be reliably revealed by scRNA-seq alone (Weinreb et al. 2020), and the epigenome more closely aligns with cell potency rather than the coding transcriptome (Paul et al. 2015; Yu et al. 2017). The transition from HSC to specific lineages is multifaceted. Therefore, this data could be combined with single-cell multi-omics approaches in the future to extract several epigenetic features, such as DNA methylation and chromatin accessibility, to uncover the full extent of molecular changes (Argelaguet et al. 2019). Furthermore, to compare the transitional cell states and different underlying chromatin features across human and murine hematopoiesis, one could compare this work with Buenrostro et al work (Buenrostro et al. 2018). The data integrative analyses of scATAC-seq and scRNA-seq also identified putative enhancer-target gene pairs for LSKFlt3^{int}CD9^{high} and LSKFlt3^{int}CD9^{low} cells, and candidate regulators of stemness and lymphoid fate within these sub-populations. For example the transcription factor HOXB*5* and EGR3 were accessible in LSKFlt3^{int}CD9^{high} cells and LSKFlt3^{int}CD9^{low}, respectively. The *Hoxb5* gene has been previously demonstrated to be a key regulator of stemness (Chen et al. 2016), and is up regulated in CD9^{high} relative to the CD9^{low} cells. It is likely to contribute to the primitiveness of CD9^{high} cells. In comparison, the *Egr3* gene, previously shown to be involved in the development of T and B cells (Li et al. 2012), is up regulated in CD9^{low} vs. CD9^{high}

cells. Some novel TFs were discovered in integrative analyses, but studies on them are beyond the scope of this work as exploration of the many novel TF candidates is a major attempt including functional screening, perturbation experiments and ambitious functional validations. Still, such follow-up experiments could lead to important strategies for modulating hematopoietic lineage commitment, and potential insights into for example impaired differentiation in malignant disease.

Moreover, In paper (II) we designed an *in vitro* co-culture system that allows for examination of all differentiation fates at the clonal level, a method ideal for validation of single-cell genomics data, This protocol assesses differentiation to the three major hematopoiesis lineage trajectories (lymphoid (B cells), myeloid, and megakaryocyte/erythroid (MegE)). To further improve the utility of this protocol, it would be useful to evaluate potential outputs. For example for T cell differentiation by co-culture on OP9-DL1 could potentially be implemented in further optimization protocols (Schmitt and Zúñiga-Pflücker 2002). For example, to obtain both B and T cells at the same time, the previously suggested switch co-culture system from OP9 to OP9-DL1 could be used (Boiers et al. 2013). Furthermore, it would be intriguing to use this clonal culture system in conjunction with the LARRY system (Weinreb et al. 2020) to directly link the entire transcriptome description of cells to their future fate in order to generate a map of cell bias on a continuum transcriptional landscape.

It has been demonstrated that the hematopoietic system changes with age. Previous data from our lab indicate that hematopoietic heterogeneity in humans is characterized not only by increased HSC frequency, but also by a significant decrease in MPPs, an increase in MegE, and an increase in myeloid output (Sommarin et al. 2021; Scala and Aiuti 2019). However, immunophenotypic changes associated with ageing-effects are poorly explored in human. Therefore in paper (III) we investigated the correlation between changes in heterogeneity with the expression of the CD9 marker in human hematopoiesis. Single-cell genomic data suggested that CD9 could mark HSPCS during myeloid/MegE biased hematopoiesis, however transplantation assays and CFU assays revealed no significant differences between $CD9^{+}$ and $CD9^{-}$ HSPC function. To answer if CD9 expression marks priming of HSPCs to myeloid or MegE cells, for example during aging, it would be interesting to apply ATAC-seq analysis and motif identification in enhancer region, similar to what was done in paper I, and based on the idea that epigenetic priming establishes lineage biases in hematopoiesis (Rodrigues, Shvedunova, and Akhtar 2020; Yu et al. 2016). Since the enhancer landscape reflects cell identity more accurately than mRNA levels (Corces et al. 2016), ATAC-seq could be a sensitive method to prove if CD9 cells are primed for myeloid/MegE differentiation. Furthermore, the potential priming of CD9 could be functionally adressed in future studies by establishing the *in vitro* culture system from paper II in humans, and assess clonal lineage output of lymphoid (B cells), myeloid, megakaryocytes and erythroid cells. An alternative method for directly measuring

clonal lineage contribution is to use cell barcoding to trace cell fate and understand the extent of lineage commitment. Although the lenti-viral system Larry could be used in a human setting, it requires ex-vivo cell manipulation, precluding use in the native environment (Weinreb et al. 2020). The scATAC-seq itself provides an elegant solution by utilizing reads mapped to the mitochondrial genome to clonally track the lineages while simultaneously reading out the chromatin features within the same cell (Ludwig et al. 2019). Novel technology developing continues which led to new understanding from hematopoiesis system. More over development of myeloid leukemia have been shown to be closely correlated with age (Lichtman and Rowe 2004). Acute Myeloid Leukemia (AML) is a heterogeneous aggressive hematologic malignancy were one third of newly diagnosed patient have the Flt3 mutation (Kennedy and Smith 2020). It has been reported that CD9 is expressed in 40% of human AML samples (Touzet et al. 2019). Therefore study the heterogeneity of CD9 or Flt3 marker in AML could possibly improve treatment for leukemia or more efficient transplantation.

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because each has been sent as a guide from beyond''

-Rumi

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یک برگ از این دفتر تاریخ، سفید است این فرصت تاریخ، به پرواز پرنده است
اینجا من وتو، یک تن و یک فکر و صداییم
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(رها) محمد

'' what's the bravest thing you have ever said?'' asked the boy.

''Help,''said the horse.

'asking for help is not giving up,' said the horse, It is refusing to give up.

The Boy,the Mole,the Fox and the Horse

Charlie Mackesy

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