Human Bone Marrow Microenvironment in Health and Disease

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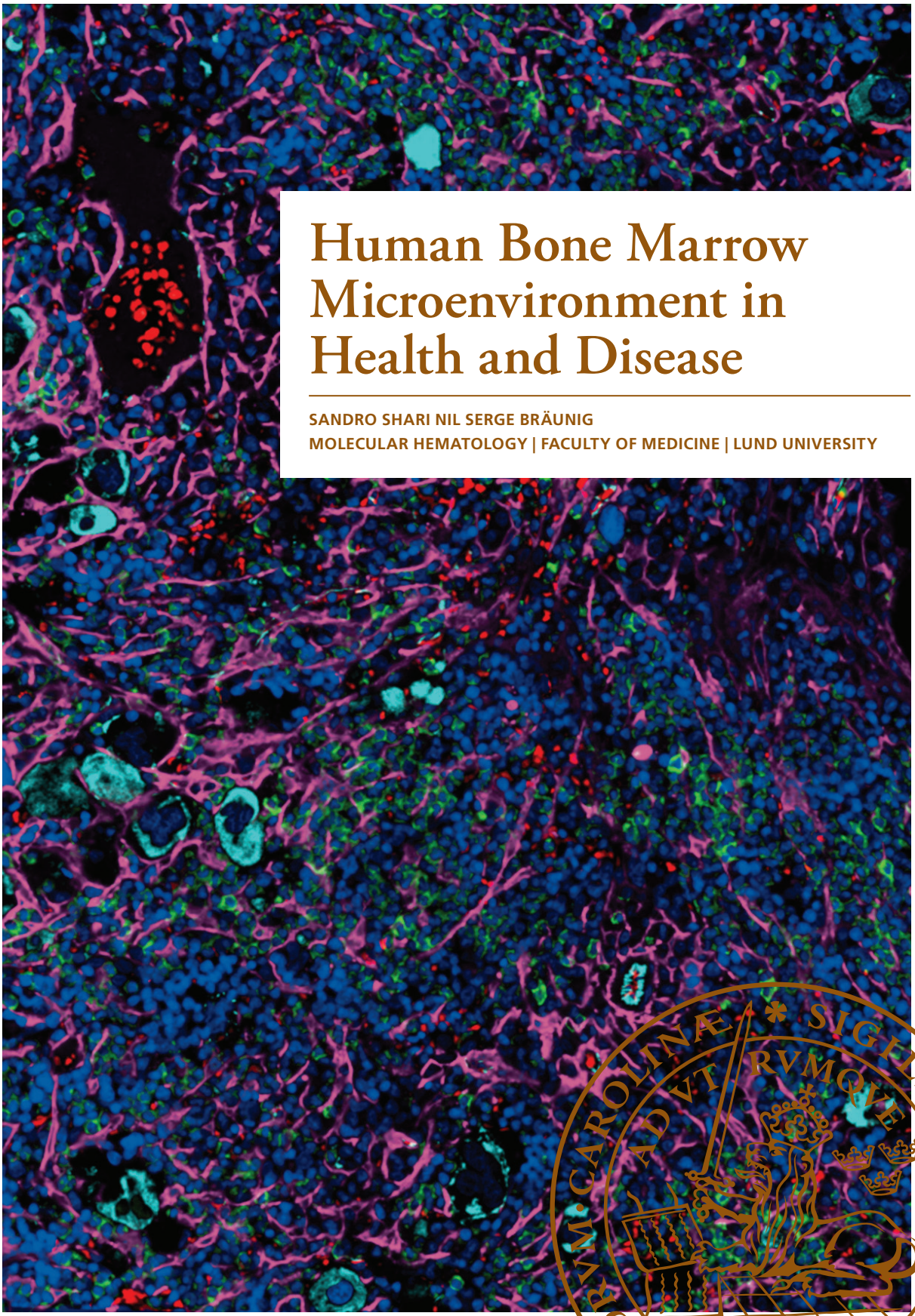
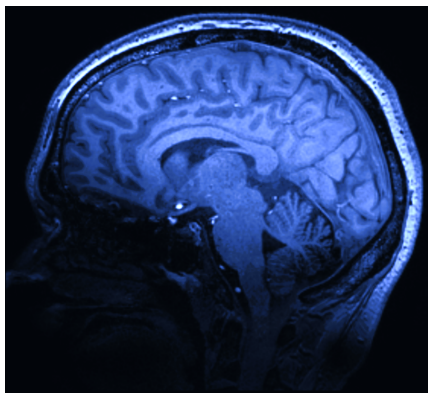


Human Bone Marrow Microenvironment in Health and Disease

SANDRO SHARI NIL SERGE BRÄUNIG
MOLECULAR HEMATOLOGY | FACULTY OF MEDICINE | LUND UNIVERSITY



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"Your own brain ought to have the decency to be on your side!"
— Terry Pratchett

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Sandro Shari Nil Serge Bräunig

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UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 24th November at 09.00 in Segerfalksal, BMC, Lund, Sweden

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Initially, we elucidated the role of the Early Growth Response 1 (*EGR1*) gene in bone marrow mesenchymal stem cells (MSCs). *EGR1* expression was found to be significantly elevated in specific BM MSC populations, particularly $lin^-CD45^-CD271^+$ $CD140a^-$ BM MSCs, exerting a pivotal role in hematopoietic stroma support. This support function was mediated through both cell-cell interactions and soluble factors, where *EGR1*-overexpressing BM MSCs exhibited enhanced secretion of chemokine ligand 28 and increased expression of vascular cell adhesion molecule 1, crucial for hematopoiesis support. *EGR1* also played a dual role in BM MSC proliferation regulation. Understanding these mechanisms can improve hematopoietic stem cell transplantation and regenerative medicine.

Further, employing single-cell RNA sequencing, we provided a comprehensive analysis of the cellular composition of human BM stroma, revealing diverse cell populations and stromal progenitors with varying differentiation capacities. We identified and characterized multipotent stromal stem cells (MSSCs), highly adipocytic gene-expressing progenitors (HAGEPs), pre-osteoblasts, and other stromal clusters, offering valuable insights into BM stromal heterogeneity and its structural organization. We employed *in silico* cluster interaction analysis and found that the different stromal populations are predicted to interact and support HSCs differentially in different niches.

Next, we introduced a meticulous methodology utilizing multicolor immunofluorescence staining and 3D analysis to investigate human BM architecture. We illustrated the potential of sequential staining, emphasizing specific structural changes associated with myeloproliferative neoplasms (MPNs) and their correlation with CD271 expression.

Finally, we investigated human BM architecture and cytokine expression patterns in patients with Acute Lymphoblastic Leukemia (ALL) and Primary Myelofib(PMF) with elaborate immunofluorescence and mRNA-based staining methods and compared them to healthy controls. We revealed significant alterations, including differential numbers of megakaryocytes, differences in cellularity, altered mesenchymal stem cell density, and distinct cytokine expressions offering critical insights into disease pathogenesis and progression.

Collectively, these findings illuminate various facets of the BM microenvironment, offering valuable insights into its critical role in hematopoiesis, stromal heterogeneity, and disease pathology, paving the way for potential therapeutic advancements in regenerative medicine and hematological disorders.

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„Don't let anybody tell you there is darkness.
Darkness is only the absence of light. “

Johannes Brecht Feat. Fesum - I Am Free,
Jan Oberlaender, Wildeburg, 2017

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Abstract

Hematopoietic stem cells (HSCs) are safeguarded from various threats such as stress, injury, or radiation within specialized microenvironments or niches within the bone marrow. In this thesis, we investigated various facets of the bone marrow (BM) microenvironment and its critical role in hematopoiesis and related disorders.

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Collectively, these findings illuminate various facets of the BM microenvironment, offering valuable insights into its critical role in hematopoiesis, stromal

heterogeneity, and disease pathology, paving the way for potential therapeutic advancements in regenerative medicine and hematological disorders.

Populärvetenskaplig sammanfattning

Hemligheterna i benmärgens mikromiljö

Benmärgen, en viktig aktör i vår kropps blodproduktionssystem, har länge fångat forskarnas nyfikenhet. I en rad viktiga studier har vi tagit oss djupt in i benmärgens mikromiljö för att avslöja dess gåtfulla värld och dolda komplexitet.

Del 1: Stromacellernas maestro

I den första delen av vår resa undersökte vi vilken roll en transkriptionsfaktor vid namn *EGR1* spelar i mesenkymal a benmärgsstromaceller (BMSC). Dessa celler är hematopoesens osjungna hjältar och orkestrerar ett finstämt stöd till blodstamcellerna. Det vi fann var intressant. Det visade sig att *EGR1* är maestron bakom BMSC-funktionerna. Den uttrycks i hög grad i dessa celler, men dess närvaro minskar under cellodling utanför benmärgen, och den är särskilt låg i specifika stromala celltyper. När vi inducerade överuttryck av *EGR1* i stromaceller blev dessa celler ett effektivt stöd för blodstamcellerna . Detta ledde till produktion av fler transplanterbara blodstamceller, ett avgörande genombrott i vår förståelse av benmärgens fysiologi. Vi upptäckte att *EGR1* är dirigenten i en genetisk symfoni som orkestrerar både proliferation och stödfunktioner för blodstamceller i BMSC . Detta avslöjande ger en djupare förståelse för hur BMSC fungerar i olika biologiska sammanhang.

Del 2: Dechiffrering av cellulär komplexitet

Vi fortsätter vår resa och ger oss in i den komplexa världen av cellulär mångfald i benmärgens mikromiljö. Vi kartlade noggrant de olika stromacellspopulationerna med hjälp av RNA-sekvensering i enskilda celler . Sex distinkta stromacellgrupper framträdde ur vår data, var och en med unika funktioner. Denna omfattande karaktärisering gjorde det möjligt för oss att avslöja differentieringshierarkin för dessa celler och identifiera nyckelfaktorer som dikterar deras övergångar. Dessutom upptäckte vi att dessa stromacelltyper lever i olika nischer i benmärgen, där var och en reglerar produktionen av blodceller genom specifika mekanismer. Denna insikt breddar vår förståelse för den cellulära komplexiteten i den mänskliga benmärgen och berikar vår kunskap om den intrikata kommunikationen mellan stroma och blodstamceller.

Del 3: En 3D-glimt in i benmärgen

Vårt nästa steg på denna resa innebar en banbrytande tredimensionell undersökning av benmärgens rumsliga organisation. Med hjälp av en innovativ antikroppsbaserad sekventiell fluorescensfärgningsteknik skapade vi detaljerade 3D-modeller av benmärgsarkitekturen och jämförde friska och sjukdomsdrabbade tillstånd, särskilt myeloproliferativa neoplasier (MPN). Denna metod avslöjade slående skillnader i BMSC-färgningens densitet och megakaryocyternas distribution i MPN-

benmärgen. Avgörande var att vi kunde definiera de rumsliga relationerna mellan olika celltyper, vilket kastar nytt ljus över cellinteraktioner i benmärgen. Denna innovativa metod har potential att fördjupa vår förståelse av benmärgspatologier och interaktioner.

Del 4: Att lösa fibrotiska mysterier vid hematologiska maligniteter

Vår slutdestination på denna resa ledde oss till att avslöja mysterierna med fibros i benmärgen, en faktor som förknippas med ogynnsam prognos vid hematologiska maligniteter . Med fokus på akut lymfatisk leukemi (ALL) och primär myelofibros (PMF) undersökte vi det spatiala uttrycket av de fibrotiska drivkrafterna *TGFB1* och *PDGFA1*. Intressant nog fann vi ett förhöjt uttryck av dessa i megakaryocyter inom ALL, vilket tyder på deras roll i initieringen och utvecklingen av denna sjukdom. Framför allt banar denna forskning väg för potentiella terapeutiska mål, vilket ger hopp om förbättrade behandlingsresultat.

Sammanfattningsvis har vår kollektiva resa genom dessa fyra artiklar avsevärt fördjupat vår förståelse av benmärgens mikromiljö, blodstamcellsinteraktioner och hematologiska sjukdomar. Dessa resultat utgör tillsammans ett betydande bidrag till området och erbjuder nya insikter och möjligheter till fortsatt forskning inom detta område.

Populärwissenschaftliche Zusammenfassung

Eine Reise zu den Geheimnissen des Knochenmarks

Das Knochenmark ist der wichtigste Akteur bei der Blutbildung und weckt schon seit langem die Neugier vieler Forscherinnen und Forscher. In einer Reihe von Studien haben wir uns tief mit dem Knochenmark und der dort befindlichen Zellen beschäftig, um die verborgenen Feinheiten der Interaktionen zwischen den verschiedenen Zellen des Knochenmarks aufzudecken. Von besonderem Interesse waren für uns die mesenchymalen Stomazellen, welche nicht zum blutbildenden System gehören, aber die Stammzellen des Knochenmarks durch verschiedenste Signale bei der Bildung von Blutzellen unterstützen.

Teil 1: Der Maestro der Stromazellen

Im ersten Teil haben wir die Rolle des Transkriptionsfaktors namens *EGR1* in den mesenchymalen Stromazellen des Knochenmarks (BMSC) untersucht. Stromazellen unterstützen die empfindlichen Blutstammzellen und orchestrieren die Blutzellbildung. Es stellte sich heraus, dass *EGR1* der Dirigent hinter den Funktionen der BMSCs ist. Er wird in diesen Zellen in hohem Maße gebildet. Dieses hohe Niveau nimmt aber während der Kulturvierung im Labor ab, und wird in bestimmten Stromazelltypen nur gering produziert. Als wir eine Überproduktion von *EGR1* in BMSCs induzierten, konnten Sie im Labor für eine höhere Produktion an transplantierbaren Blutstammzellen sorgen als nur mit geringen Mengen an *EGR1*. Wir entdeckten wir, dass *EGR1* der Dirigent einer genetischen Symphonie ist, der sowohl die Proliferation als auch die Unterstützungsfunktionen für Blutstammzellen in BMSC orchestriert. Diese Entdeckung verhalf zu einem tieferen Verständnis dafür, wie BMSCs in verschiedenen biologischen Zusammenhängen funktionieren.

Teil 2: Entschlüsselung der zellulären Komplexität der Knochenmarksnische

Desweitern untersuchten wir die komplexe Welt Stromazellen ein, die die Stammzellen des Knochenmarks umgeben. Mit Hilfe der Einzelzell-RNA-Sequenzierung haben wir die verschiedenen Stromazellpopulationen genaustens bestimmen können. Unsere Daten ergaben sechs verschiedene Zellgruppen, mit jeweils einzigartigen Funktionen. Diese umfassende Charakterisierung ermöglichte es uns, die Differenzierungshierarchie dieser Zellen aufzudecken und die Schlüsselfaktoren zu identifizieren, die ihre Differenzierung bestimmen. Darüber hinaus entdeckten wir, dass verschiedene Stromazelltypen unterschiedliche Nischen im Knochenmark besiedeln und jeweils über spezifische Mechanismen die Produktion von Blutzellen regulieren. Diese Erkenntnisse erweitern unser Verständnis der zellulären Komplexität im menschlichen Knochenmark und bereichern unser Wissen über die komplexe Kommunikation zwischen den Stromazellen und den Stammzellen des Knochenmarks.

Teil 3: Ein dreidimensionaler Blick in das Knochenmark

Unser nächster Schritt war eine dreidimensionale Untersuchung der räumlichen Organisation des Knochenmarks. Mit Hilfe einer neuen, auf Antikörpern basierenden sequenziellen Fluoreszenzfärbetechnik erstellten wir detaillierte 3D-Modelle der Knochenmarkarchitektur und verglichen gesunde und erkrankte Zustände, insbesondere von Patienten mit Myeloproliferativer Neoplasie (MPN). Diese Methode zeigte auffällige Unterschiede in der Dichte der mesenchymalen Stromazellfärbung und der Verteilung der Megakaryozyten im Knochenmark von MPN-Patienten. Entscheidend ist, dass wir damit die räumlichen Beziehungen zwischen den verschiedenen Zelltypen definieren konnten, was ein neues Licht auf die zellulären Interaktionen im Knochenmark wirft. Diese innovative Methode verspricht, unser Verständnis der Pathologien und Interaktionen im Knochenmark zu revolutionieren.

Teil 4: Fibrotische Geheimnisse bei hämatologischen Malignomen

Unser letztes Ziel auf dieser Reise war es, die Geheimnisse der Fibrose im Knochenmark zu lüften, ein Faktor, der mit ungünstigen Krankheitsverläufen bei hämatologischen Krankheiten in Verbindung gebracht wird. Wir konzentrierten uns auf die akute lymphoblastische Leukämie (ALL) und die primäre Myelofibrose (PMF) und untersuchten die räumliche Expression der Fibrosetreiber *TGFB1* und *PDGFA1*. Interessanterweise fanden wir eine erhöhte Expression dieser Faktoren in Megakaryozyten bei ALL, was auf ihre Rolle bei der Entstehung und dem Fortschreiten dieser Krankheit hindeutet. Diese Forschungsergebnisse ebnen den Weg für mögliche therapeutische Ansätze und geben Hoffnung auf bessere Behandlungsergebnisse.

Zusammenfassend lässt sich sagen, dass unsere gemeinsame Reise durch diese vier Arbeiten unser Verständnis des gesunden und erkrankten Knochenmarks, seiner zellulären Zusammensetzung und der Interaktionen von Stromazellen mit Blutstammzellen erheblich vertieft hat. Insgesamt stellen diese Ergebnisse einen bedeutenden Beitrag zum Fachgebiet dar und bieten neue Einblicke und Möglichkeiten für weitere Untersuchungen in diesem wichtigen Forschungsbereich.

List of Papers

Paper I

Li H, Lim HC, Zacharaki D, Xian X, Kenswill KJG, Braunig S, Raaijmakers MHG, Woods NB, Hansson J, Scheding S. Early growth response 1 regulates hematopoietic support and proliferation in human primary bone marrow stromal cells. Haematologica (2020) May;105(5):1206-1215

Paper II

Li H, Bräunig S, Dhapolar P, Karlsson G, Lang S, Scheding S. Single-cell RNA sequencing identifies phenotypically, functionally, and anatomically distinct stromal niche populations in human bone marrow. eLife (2023). Mar 6;12:e81656. doi: 10.7554/eLife.81656

Paper III

Bräunig S, Karmhag I, Li H, Enoksson J, Hultquist A, Scheding S. Three-dimensional spatial mapping of the human hematopoietic microenvironment in healthy and diseased bone marrow. Cytometry A (2023), Jul 8. doi: 10.1002/cyto.a.24775.

Paper IV

Bräunig S, Dencker C, Hultquist A, Enoksson J, Li H, Scheding S. In situ spatial mRNA analysis shows differences in *TGFB1* and *PDGFA1* expression in fibrotic pediatric acute lymphoblastic leukemia and primary myelofibrosis bone marrow. (manuscript)

Abbreviations

3D	Three-dimensional
AML	Acute myeloid leukemia
B-ALL	B cell acute lymphoblastic leukemia
BM	Bone marrow
CCL28	Chemokine ligand 28
CML	Chronic myelogenous leukemia
CXCL12	Stromal-derived factor-1 alpha [SDF-1α]
CXCR4	C-X-C chemokine receptor type 4
DAPI	4′,6-diamidino-2-phenylindole
ECM	Extracellular matrix
ECs	Endothelial cells
EFS	event-free survival
EGR1	Early growth response 1
ET	Essential thrombocythemia
HME	Hematopoietic microenvironment
HSC	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
IF	Immunofluorescence
JAK2	Janus kinase 2
LEPR	Leptin receptor
LNGFR/CD271	Low-affinity Nerve Growth Factor Receptor
LSC	Leukemic stem cells
MDS	Myelodysplastic syndrome
MKs	Megakaryocytes
MPN	Myeloproliferative neoplasms
MSC	Mesenchymal stromal cells
MSSCs	Multipotent stromal stem cells
OBCs	Osteoblasts

OPN	Osteopontin
PBS	Phosphate-buffered saline
PDGFA1	Platelet-derived growth factor alpha
PMF	Primary myelofibrosis
PV	Polycythemia vera
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
SCF	Stem cell factor
scRNAseq	single-cell sequencing
T-ALL	T cell acute lymphoblastic leukemia
TGFβ	Transforming growth factor β (TGFB1)
TNF-α	Tumor necrosis factor-α
UMIs	Unique molecular identifiers
VCAM1	Vascular cell adhesion molecule 1

Introduction

Hematopoiesis

Hematopoiesis, derived from Greek, literally means "to make blood," representing the process of generating all blood cells throughout one's life. In humans, an astounding two million new blood cells are created per second, underscoring one of the highest rates of cellular turnover in the body (Ogawa 1993). This continuous production of mature blood cells relies on a small group of multipotent and self-renewing hematopoietic stem cells (HSCs) residing in the bone marrow (BM) of adult mammals. The remarkable regenerative capacity of HSCs is vividly demonstrated by their ability to reconstruct the entire blood and immune system following myeloablation, a milestone first achieved in mice in 1949 (Jacobson, Marks et al. 1949) and later in humans in 1957 (Thomas, Lochte et al. 1957). Since then, the transplantation of HSCs derived from BM has emerged as a life-saving procedure for treating various hematological and immunological disorders such as leukemia, anemia, and autoimmune conditions. HSC transplantation is the sole stem cell therapy routinely practiced in clinical settings (Marks, Witten et al. 2017).

In broad terms, the hematopoietic system encompasses cells contributing to innate and adaptive immunity (myeloid and lymphoid cells, respectively), oxygen transportation (erythroid cells), and wound healing (platelets). HSCs generate cells falling into the lymphoid (B, T, and natural killer cells), myeloid (granulocytes, monocytes/macrophages, and dendritic cells), and megakaryocyte-erythroid (megakaryocytes [MKs]/platelets, erythrocytes and MK/erythrocyte-progenitors) lineages through a sequential multi-step differentiation process (Velten, Haas et al. 2017). This process involves a progressive reduction in self-renewal capacity and includes intermediate populations of hematopoietic stem and progenitor cells (HSPCs). While the precise arrangement of the human hematopoietic hierarchy is a topic of discussion, recent discoveries propose a continuum of differentiation rather than distinct steps, emphasizing primed undifferentiated HSPCs with varying differentiation potential (Figure 1; Velten, Haas et al. 2017; Buenrostro, Corces et al. 2018).

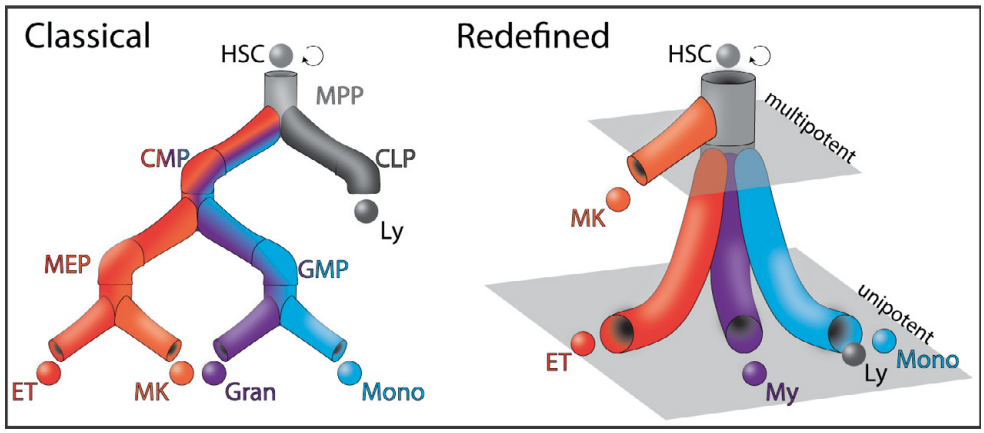

Figure 1: Human blood stem cell differentiation
The classical model postulates that oligopotent progenitors like CMP play a vital role as an intermediate stage, giving rise to My/ER/Mk differentiation. In contrast, the revised model suggests a continuum of differentiation rather than distinct steps, emphasizing primed undifferentiated HSPCs with varying differentiation potential. HSC: hematopoietic stem cell, MPP: multipotent progenitors, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, Ly: lymphoid cells MEP: megakaryocyte-erythroid progenitors, GMP: granulocyte/macrophage progenitor, ET: erythrocytes, MK: megakaryocytes, Gran: granulocytes, Mono: monocytes. The shaded planes symbolize hypothetical differentiation stages. Adapted from Notta, Zandi et al. (2016).

The stem cell niche

In 1978, Raymond Schofield introduced the "stem cell niche" concept, proposing it as a meticulously regulated spatial and functional entity that supplies all essential signals for stem cell survival and differentiation (Schofield 1978). His theory was built upon several fundamental principles:

- Stem cells occupy a fixed location within a particular tissue.
- This specific location enables stem cell self-renewal and maintenance.
- In the niche stem cells remain undifferentiated.
- The niche confines the number of resident stem cells to a finite limit.
- A slightly more differentiated cell can regain its stem cell characteristics within a niche.

Initially met with controversy among his contemporaries, Schofield's theory gained credibility as experimental evidence from other tissue niches keeping stem cells in an undifferentiated state emerged from studies in Drosophila gonads, C. elegans, and various mammalian tissues (Papayannopoulou and Scadden 2008). This concept has since laid the groundwork for an exciting field in scientific research.

Hematopoietic stem cell niche

Definitive HSCs initially emerge in the Aorta-Gonad-Mesonephros region, then migrate sequentially to the yolk sac, placenta, fetal liver, spleen, and ultimately settle in the bone marrow, which serves as the central hub for adult hematopoiesis during homeostasis (Morrison and Scadden 2014). Hematopoiesis is a tightly regulated and vital process essential for sustaining life. HSCs are safeguarded from various threats such as stress, injury, or radiation within specialized microenvironments or niches within the BM (Mendelson and Frenette 2014). The BM as the postnatal residence for the HSC niche was driven by evolutionary pressures in terrestrial animals. This adaptation allowed HSCs to endure exposure to ultraviolet light while being shielded by the protective layer of cortical bone (Kapp, Perlin et al. 2018).

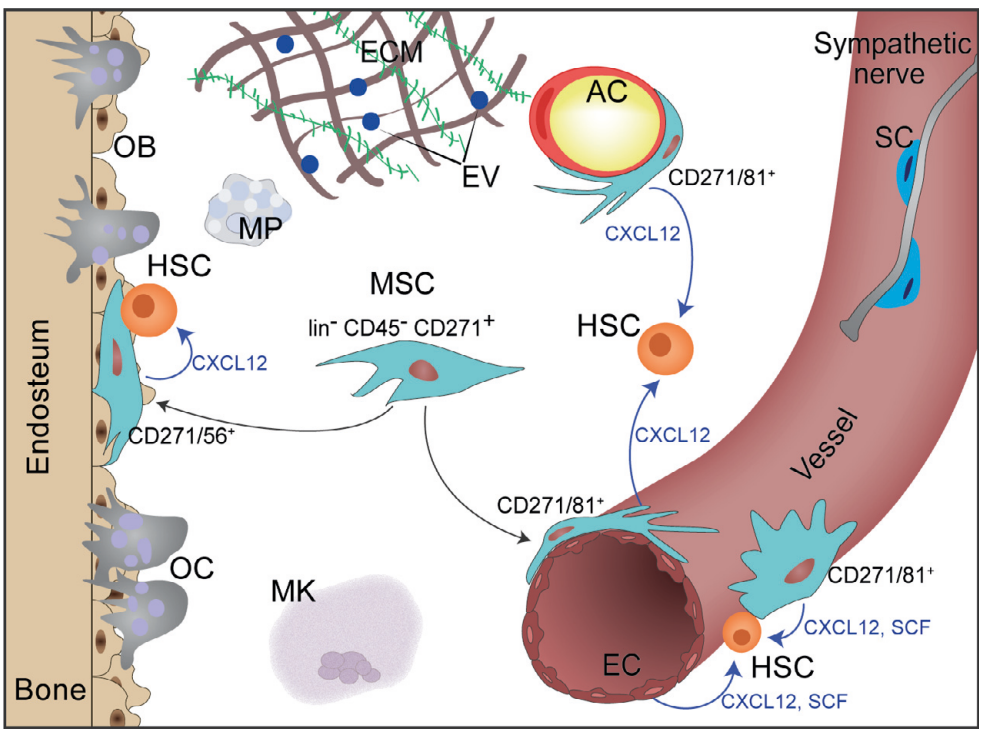

Figure 2: Healthy human bone marrow niche.
Depicting important components including MSC subsets (with CD marker expression) and their proposed cytokine interactions with HSCs. MSC: mesenchymal stromal cell, HSC: hematopoietic stem cell, OB: osteoblast, ECM: extracellular matrix, AC: adipocyte, EV; extracellular vesicles, MP: macrophage, SC: Schwann cell, OC: osteoclast MK: megakaryocyte

The HSC niche consists of both cellular and non-cellular elements (Figure 2). HSCs typically maintain a state of quiescence, a condition of metabolic dormancy in which they do not actively enter the cell cycle. This quiescent state is essential to prevent premature depletion of HSCs. However, HSCs have the remarkable ability to swiftly transition from quiescence to a proliferative state (Wilson, Laurenti et al. 2008; Pietras, Warr et al. 2011). Additionally, they can be mobilized from the BM to peripheral or extramedullary sites like the liver or spleen in response to signals from their surrounding microenvironment or systemic cues (Massberg, Schaerli et al. 2007).

The bone marrow microenvironment in health

Anatomical localization

The spatial arrangement of the HSC niche has gathered significant scientific interest because it involves the coordination of both anatomy and function at the tissue and organ level. The prevailing theory states that HSCs, which are mostly in a quiescent state, predominantly reside in endosteal niches in direct contact with bone-lining osteoblasts (OBCs). Simultaneously, most actively proliferating HSCs are situated in perivascular niches, contributing to the continuous production of blood progeny (Kiel, Yilmaz et al. 2005; Tormin, Li et al. 2011). This model categorizes the hematopoietic niche into distinct anatomical regions with specialized functions, providing a spatial perspective on HSC-niche interactions.

However, recent years have witnessed challenges to this theory due to advancements in imaging techniques and lineage tracing of native (non-transplanted) cells in mouse models. These innovations have enabled a more precise identification of the (sub)anatomical locations of HSCs and their adjacent niche cells (Mendelson and Frenette 2014). It's worth noting that the endosteal region is not clearly separated, but approximately 80% of HSCs are found within this region when the BM is divided into endosteal and central marrow cavity portions. Nevertheless, given the high degree of vascularization in the endosteal region, drawing strict boundaries between the endosteal and vascular niche becomes challenging (Hoggatt, Kfoury et al. 2016).

Endosteal niche

The endosteum is a delicate inner membrane that envelops both compact and trabecular bone tissue, encircling the medullary cavity. It is innervated by arterioles and small sinusoidal vessels and is a region of active bone growth and remodeling (Morrison and Scadden 2014). Early research indicated that cultures of cells from the osteolineage could support the growth of HSCs *in vitro* (Taichman and Emerson

1994). Subsequent *in vivo* studies revealed that the endosteal region houses a greater proportion of quiescent HSCs in comparison to other areas within the BM (Hoggatt, Kfoury et al. 2016). Additionally, research has demonstrated that the endosteal region serves as the preferred destination for HSC homing following transplantation in mice (Nilsson, Johnston et al. 2001). The cellular components of the endosteal compartment include OBCs, osteoclasts, endothelial cells (ECs), mesenchymal cells with the potential to become osteolineage cells, and macrophages. The region exhibits notably high concentrations of extracellular calcium ions, and variations in these levels are detected by the calcium receptor (CaR) in HSCs. Upon sensing these fluctuations, the CaR promptly signals for the retention of HSCs in the endosteal region of mice (Adams, Chabner et al. 2006). Additionally, numerous adhesion molecules, including integrins, laminins, osteopontin (OPN), stromal-derived factor-1 (CXCL12), angiopoietin receptor tyrosine kinase with immunoglobulin-like and EGF-like domains 2, and others produced by cells of the osteolineage, play a regulatory role in HSC anchorage within the niche (Hoggatt, Kfoury et al. 2016).

Vascular niche

The vascular niche is comprised of single-layered, thin-walled vessels forming arterioles and sinusoids, creating a dense network within the BM (Schepers, Campbell, and Passegue 2015). This network extends from the endosteum to the central canal and longitudinally spans from the proximal to the distal epiphysis in mice (Nombela-Arrieta, Pivarnik et al. 2013). As observed in murine hematopoiesis development, the formation of vasculature in the developing BM is vital for the migration of HSCs into the BM and the establishment of hematopoiesis (Coşkun, Chao et al. 2014). Moreover, the common origin of blood and vessels from mesodermal precursors in humans, as highlighted by the endothelial-to-hematopoietic transition (Choi, Vodyanik et al. 2011), underscores the critical role of the vascular niche in hematopoiesis. Within the perisinusoidal and periarterial vascular niches, cell types such as ECs, pericytes, mesenchymal stem cells, nerve fibers, and non-myelinating Schwann cells contribute to the microenvironment.

The discovery of transcortical vessels traversing the murine bone cortex (Grüneboom, Hawwari et al. 2019) challenges prior theories centered on a clear division between osteoblastic and vascular niches. Instead, it has given rise to a more nuanced concept known as endosteal-vascular niches in mice (Lassailly, Foster et al. 2013; Itkin, Gur-Cohen et al. 2016). In these niches, perivascular units likely unite contributions from various sources, including ECs, osteoprogenitor cells like mesenchymal stromal cells (MSCs), and perisinusoidal stromal cells, along with fully differentiated OBCs. This new understanding has refined the definition of HSC niches in the mouse, revealing a greater level of complexity (Itkin, Gur-Cohen et al. 2016; Ramasamy, Kusumbe et al. 2016; Asada, Takeishi et al. 2017; Zhang, Wu et al. 2021).

Niches of committed cells in the bone marrow

After examining the specialized niches for HSCs in the BM, potential niches for more differentiated cells where examined by the field. Ding et al. demonstrated that while HSCs are sustained by a perivascular niche consisting of Lepr-cre^{-} or Prx1-cre-expressing stromal cells and endothelium, early lymphoid precursors reside in the endosteal niche established by OBCs, supporting proliferation and differentiation of lymphoid precursors in mouse *in vitro* models. Furthermore, committed B-lineage progenitor cells were shown to be maintained by a distinct perivascular niche involving Prx1–cre-expressing stromal cells without ECs (Ding and Morrison 2013). The depletion of OBCs detrimentally impacted the lymphoid precursor population in mice (Visnjic, Kalajzic et al. 2004; Zhu, Garrett et al. 2007). Additionally, the absence of G protein alpha in OBCs was shown to reduce the B lymphoid precursor population (Wu, Purton et al. 2008).

Macrophage colony-stimulating factor-1 (CSF-1) is crucial for maintaining myeloid lineage cells. A study revealed that CSF-1 derived from BM ECs influenced the development of myeloid cells, particularly Ly6C^{-} monocytes, in both developmental and adult contexts, highlighting the significance of a distinct cellular niche for monocytes in the mouse BM (Emoto, Lu et al. 2022).

Erythroblastic islands, primarily found in the BM during regular erythropoiesis, expand in the fetal liver and adult spleen during stress erythropoiesis, as a response to inflammation and anemia (Mohandas and Prenant 1978; Paulson, Hariharan et al. 2020). These islands are composed of a central macrophage (termed erythroblastic island [EBI] macrophage) encircled by developing erythroblasts, acting as a supportive "nurse" cell. EBI macrophages aid erythropoiesis by fostering erythroblast differentiation through cell-cell interactions and secretion of supportive factors, facilitating erythroid cell maturation, enucleation, and engulfment of expelled nuclei (McGrath, Kingsley et al. 2008; Porcu, Manchinu et al. 2011; Lopez-Yrigoyen, Yang et al. 2019).

Cellular components of the bone marrow microenvironment

The types of cells present within the niche play a pivotal role in cell-cell contact-mediated responses and the production of vital signaling molecules like chemokines, cytokines, growth factors, and extracellular vesicles. These molecular components serve to regulate HSC functions and maintain overall homeostasis. While a significant amount of data elucidating niche functions has been acquired using transgenic mouse models, comparable studies in humans are still relatively scarce. Here, we introduce the primary cellular constituents of the human and mouse hematopoietic niche.

Mesenchymal stromal cells

MSCs are a rare non-hematopoietic cell population. These versatile skeletal stem cells possess the potential to differentiate into bone-lining OBCs, adipocytes, chondrocytes, fibroblasts, and stromal cells supporting hematopoiesis (Bianco, Cao et al. 2013). The identification of these distinctive cells dates to 1966 when Friedenstein and colleagues recognized large, spindle-shaped, fibroblast-like cells capable of forming osteogenic foci (Friedenstein, Piatetzky et al. 1966). Research at that time demonstrated the influential role of bone and stroma in directing the differentiation of HSCs (Owen 1978). Subsequent evidence from various research groups established that murine and human stromal cell preparations have the potential to give rise to osteogenic, adipogenic, and chondrogenic cells (Owen and Friedenstein 1988; Beresford, Bennett et al. 1992; Pittenger, Mackay et al. 1999).

The term "mesenchymal stem cell" was introduced by Caplan in 1991 and has since become prevalent in the scientific literature (Caplan 1991). However, the true "stemness" of MSCs was first convincingly demonstrated two decades ago through subcutaneous transplantation of human single-cell-derived colonies, resulting in bone formation in the murine system (Kuznetsov, Krebsbach et al. 1997). This pivotal discovery was followed by the groundbreaking work of Sacchetti and colleagues, providing critical insights into the *in vivo* self-renewal properties of MSCs (Sacchetti, Funari et al. 2007).

Traditionally, defining MSCs has heavily relied on various *in vitro* cultured preparations of BM mononuclear cells. Due to the absence of specific markers for precise MSC identification and the increasing number of studies on MSCs, the International Society for Cellular Therapy addressed this by publishing a position paper in 2006. The paper outlined a set of minimum criteria for characterizing *in vitro* expanded human MSCs (Dominici, Le Blanc et al. 2006). These criteria encompass three main aspects:

1. Adherence to plastic surfaces.
2. Expression of specific cell surface markers (CD73, CD90, and CD105), while lacking hematopoietic markers (CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR).
3. Demonstrating differentiation potential into three lineages *in vitro*: adipogenesis, osteogenesis, and chondrogenesis.

While these criteria laid the groundwork for standardizing cell preparations, they have faced significant criticism. Many argue that meeting these criteria doesn't necessarily denote cells reflecting the native properties of BM-MSCs. Moreover, proving stemness necessitates more rigorous testing (Frenette, Pinho et al. 2013).

One of the most reliable markers for primary human MSC identification is the expression of LNGFR/CD271 (Low-affinity Nerve Growth Factor Receptor).

Various studies have explored the potential use of this marker alone or in conjunction with others to identify genuine human MSCs (Jones, Kinsey et al. 2002; Quirici, Soligo et al. 2002; Bühring, Battula et al. 2007). LNGFR/CD271 has been demonstrated to be expressed in all colony-forming progenitors in the human BM (Li, Ghazanfari et al. 2016). A summary of all established human MSC markers is given in Table 1.

Table 1. Positive and negative marker expression in human BM-MSCs

Symbol / Cluster of Differentiation	Description	Publication
Marker expression in primary human adult BM-MSCs		
Stro-1	Stro-1 Cell Surface Antigen	Simmons and Torok-Storb (1991)
MCAM / CD146	Melanoma Cell Adhesion Molecule	Sacchetti, Funari et al. (2007)
Thy-1 / CD90	Thy-1 Cell Surface Antigen	Campioni, Lanza et al. (2008)
VCAM1 / CD106	Vascular Cell Adhesion Molecule 1	Gronthos, Zannettino et al. (2003)
5NTD / CD73	Ecto-5'- Nucleotidase, SH3, SH4	Veyrat-Masson, Boiret-Dupré et al. (2007)
EGLN / CD105	Endoglin, SH2	Aslan, Zilberman et al. (2006)
MSCA1	Mesenchymal Stem Cell Antigen 1	Vogel, Grünebach et al. (2003)
FZD9 / CD349	frizzled-9	Bühring, Battula et al. (2007)
PDGFRB / CD140b	Platelet-Derived Growth Factor Receptor-Beta	
NCAM / CD56	Neural Cell Adhesion Molecule	Sivasubramaniyan, Lehnen et al. (2012)
LEPR / CD295	Leptin Receptor	Li, Ghazanfari et al. (2014)
ITGAV / CD51	Integrin Alpha 5	Pinho, Lacombe et al. (2013)
SUSD2	Sushi Domain containing 2	Sivasubramaniyan, Harichandan et al. (2013)
PRNP / CD230	Prion Protein	Mohanty, Cairney et al. (2012)
Low to not expressed in primary human adult BM-MSCs		
PECAM-1 / CD31	Platelet endothelial cell adhesion molecule	Liu, Sun et al. (2006)
CD34	transmembrane phosphoglycoprotein	
CSPG8 / CD44	Chondroitin Sulfate Proteoglycan	Qian, Le Blanc et al. (2012)
PTPRC / CD45	Protein tyrosine phosphatase, receptor type, C	Jones, English et al. (2006)

As previously mentioned, a vital function of MSCs is to actively support HSPCs by producing essential cytokines and growth factors that facilitate HSC functions and adapt responsively to their needs. Notably, stem cell factor (SCF), angiopoietin-1,

and thrombopoietin-1 play a crucial role in regulating HSC quiescence in the mouse (Qian, Buza-Vidas et al. 2007). Additionally, CXCL12 is pivotal for retaining HSCs within the BM, while vascular cell adhesion molecule 1 (VCAM1) facilitates cell adhesion. Extracellular matrix proteins like fibronectin, hyaluronic acid, and E-selectin play a key role in guiding HSC homing within the niche. Furthermore, various stromal-secreted factors, including interleukin-7 and notch ligands, are essential for HSC proliferation and differentiation (Schepers, Campbell et al. 2015).

Despite the crucial role that BM MSCs play in hematopoiesis and the advancements in primary BM MSC identification, there remains a significant gap in our understanding of how these vital niche cells' functions, such as proliferation, differentiation, and hematopoietic support, are precisely regulated. Studies have indicated that elevated levels of reactive oxygen species (ROS) can both promote and inhibit MSC activities. Increased ROS levels have been shown to stimulate cell proliferation, differentiation, and survival in MSCs, but excessively high levels can induce senescence and inhibit the osteoinductive effect of Wnt signaling (Kobayashi and Suda 2012; Atashi, Modarressi et al. 2015; Denu and Hematti 2016). Furthermore, research from our group has revealed that the early growth response 1 (EGR1) gene is a pivotal dual regulator of human BM MSCs. *EGR1* expression levels significantly influence hematopoietic stroma support, with high expression promoting hematopoiesis-supporting genes but suppressing BMSC proliferation. Conversely, the downregulation of *EGR1* enhances BMSC proliferation but compromises the hematopoietic support function (Li, Lim et al. 2020). In the realm of molecular regulators, the retinoic acid-inducible gene I (RIG-I) has emerged as a substantial player in regulating the stromal niche for hematopoietic reconstitution. Its expression is modulated by all-trans retinoic acid and inflammation stress, impacting the clonogenicity, the bone-forming ability of BM MSCs, and their supporting function in the stromal niche (Lou, Jiang et al. 2022). Additionally, antioxidant therapy has shown promise in protecting HSC function and improving hematopoietic stem cell transplantation (HSCT). It was revealed that antioxidant therapy can rescue the damaged BMSC niche, providing further benefit to HSCT (Hu, Cheng et al. 2014; Kong, Wang et al. 2019). Furthermore, studies have shed light on specific subpopulations of BM MSCs. A small subset of BM MSCs expressing N-Cadherin was found to regulate osteogenesis and support chemoresistant regenerative HSCs in mice (Zhao, Tao et al. 2019). These findings collectively emphasize the intricate and multifaceted regulatory mechanisms governing the behavior and functions of BM MSCs within the hematopoietic niche.

Several findings in mice indicate that MSCs show phenotypical and functional differences based on their spatial localization in the BM. Perivascular MSCs are positioned near arterioles and sinusoids, delivering crucial factors such as SCF and CXCL12, as well as extracellular matrix (ECM) proteins that significantly influence HSC function (Schepers, Campbell et al. 2015). Perivascular MSCs are often referred to as pericytes and are primarily identified by the expression of leptin

receptor (LEPR) in the sinusoids and Neural/glial antigen 2 (NG2) marker in arterioles lining the mouse bone (Ding, Saunders et al. 2012; Kunisaki, Bruns et al. 2013; Zhou, Yue et al. 2014) and expression of CD146 in humans (Tormin, Li et al. 2011). Remarkably, quiescent HSCs tend to coexist with peri-arteriolar NG2-expressing MSCs while proliferating HSCs are near perisinusoidal MSCs expressing LEPR in mice (Krause and Scadden 2015).

Megakaryocytes

Murine mature MKs are typically found close to HSCs and sinusoids, with their primary role being platelet production (Kokkaliaris, Kunz et al. 2020). Within the murine BM, they play a pivotal role in supporting HSCs. This support is essential for maintaining HSC quiescence through transforming growth factor β (TGFβ) signaling and activating HSCs in response to stress via fibroblast growth factor 1 (Zhao, Perry et al. 2014). HSC quiescence in the mouse is facilitated by megakaryocytic platelet factor 4 (Bruns, Lucas et al. 2014) and the membrane protein C-type lectin-like receptor-2 (Nakamura-Ishizu, Takubo et al. 2015). If MKs are depleted from the murine BM niche a notable surge in HSC numbers can be observed, further indicating that MKs are playing a role in regulating HSC quiescence (Pinho, Marchand et al. 2018).

Endothelial cells

ECs constitute a crucial component of the perivascular niches and play a vital role in maintaining and regenerating HSCs post-radiation in a mouse model (Chen, Liu et al. 2019). These cells line both arterioles and sinusoid vessels, forming distinct niches where dormant and active HSPCs co-localize (Medyouf 2017). BM ECs exhibit crucial growth factors necessary for HSC maintenance and regeneration, such as SCF, CXCL12, and Pleiotrophin. Conditional deletion of any of these factors from murine ECs results in a reduction of HSCs within the BM (Comazzetto, Shen et al. 2021). Of significant note, ECs are the exclusive producers of E-selectin, an adhesion molecule that actively promotes HSC proliferation (Winkler, Barbier et al. 2012). E-selectin is specifically expressed by ECs in regions where both healthy and leukemic HSPCs engraft within the mouse BM (Barbier, Erbani et al. 2020; Godavarthy, Kumar et al. 2020). In $CD105^{+}$ human ECs, interleukin-33 is recognized as a promoter of HSPCs, ECs, and osteogenesis (Kenswil, Jaramillo et al. 2018), while murine endothelial cell-derived angiopoietin-like 2 is important for the maintenance of HSC activities in the BM niche (Yu, Yang et al. 2022). Particularly, sinusoidal ECs play a critical role in supporting HSC self-renewal and expansion while preventing their exhaustion *in vivo* through notch signaling as shown in mouse models (Boulais and Frenette 2015; Xu, Gao et al. 2018).

Adipocytes

Adipocytes are abundant cells in the BM with increasing numbers in old individuals. In mice, they have been shown to originate from a subset of BM MSCs (Tikhonova, Dolgalev et al. 2019). Adipocytes have long been viewed as negative regulators of hematopoiesis due to their negative correlation with hematopoietic function, especially following radiation or chemotherapy in mice (Naveiras, Nardi et al. 2009). However, recent renewed interest in this cell type has uncovered a potential role in murine HSC and hematopoietic recovery post-radiation, particularly for BM adipocytes, which express high levels of SCF (Zhou, Yu et al. 2017). Moreover, subjecting purified HSPCs to BM adipose tissue conditioned media, comprising both soluble factors and factors derived from exosomes/microvesicles, resulted in the increased proliferation and an augmented potential for granulocyte-monocyte differentiation in a translationally relevant rhesus macaque model (Robino, Pamir et al. 2020). Further studies have shown that suppressing BM adipocytes during acute myeloid leukemia (AML) in mice can hinder erythro-myeloid maturation, a condition that can be ameliorated through the induction of adipogenesis (Boyd, Reid et al. 2017). A comprehensive understanding of the functions of BM adipocytes is likely to shed more light on the hematopoietic niche under conditions of homeostasis, aging, and disease.

Sympathetic nerves and non-myelinating Schwann cells

The perivascular niches exhibit rich innervation by sympathetic nerves, safeguarded by non-myelinating Schwann cells. The circadian rhythm in mice governs the release of adrenergic signals from these sympathetic nerves, orchestrating the movement of HSCs between the BM and the periphery (Méndez-Ferrer, Lucas et al. 2008). Moreover, a subset of non-myelinating Schwann cells, marked by the expression of glial fibrillary acidic protein, has been observed to activate latent TGFβ, playing a role in promoting HSC quiescence in mice (Yamazaki, Ema et al. 2011). Recent publications found an interface in mice involving cholinergic signaling and the skeletal nervous system stimulates bone formation and regulates MSC differentiation during postnatal growth and exercise (Hu, Lv et al. 2020; Gadomski, Fielding et al. 2022).

Macrophages

Macrophages represent a diverse group of fully differentiated cells present in various tissues, including the BM. They play vital roles in maintaining tissue equilibrium by engaging in functions such as tissue remodeling, clearing deceased cells, and generating angiogenic factors (Davies, Jenkins et al. 2013). They stabilize CD82 on murine long-term HSCs, a key factor in regulating HSC quiescence (Hur, Choi et al. 2016). Murin macrophages were shown to control the self-renewal and expansion of HSCs *ex vivo* (Luo, Shao et al. 2018).

Osteoblasts

OBCs form a cellular layer within the endosteum, coexisting with osteoclasts. This OBC population comprises both primitive osteoblastic progenitors and mature bone-forming cells. Murine genetic ablation studies have utilized different promoters (e.g., Sp7 or Runx2 for progenitors, and Collal for mature OBCs) to elucidate their distinct roles within the niche (Schepers, Campbell et al. 2015). OBCs play a crucial role in regulating HSC function, as evidenced by their numbers correlating with HSC numbers. This regulatory interaction is mediated through Notch signaling in mice (Calvi, Adams et al. 2003). Notably, depletion of OBCs in a mouse model resulted in a reduction of B lymphoid progenitors, suggesting their involvement in directing HSC differentiation (Wu, Purton et al. 2008).

Osteoclasts

Osteoclasts, originating from the monocyte/macrophage lineage, are endosteal cells. Like adipocytes, osteoclasts were historically believed to be non-essential for the functionality of the HSC niche (Mendelson and Frenette 2014). However, osteoclasts engaged in bone resorption within the endosteum are now recognized to likely support HSPCs (Miyamoto 2013). They play a role in promoting HSPC maintenance, proliferation, and their transition into circulation, especially during both homeostatic and physiological stress conditions (Kollet, Dar et al. 2007).

Other mature hematopoietic niche cells

Plasma cells (PCs) play a central role in humoral immunological memory, affording proactive antibody defense against pathogens (Khodadadi, Cheng et al. 2019; Lightman, Utley et al. 2019). They stem from terminally differentiated B cells, and their maturation, ranging from proliferative short-lived PCs ($CD138^+$ $B220^+$ or plasma-blasts) to enduring long-lived PCs (LLPCs), is a gradual process. These LLPCs, identified by their extended presence in the body and expression of a mature PC phenotype, reside predominantly in the BM, offering lasting humoral immunity as shown in mouse models (Manz, Thiel et al. 1997; Slifka, Antia et al. 1998). While the exact organization of the BM niche for PCs remains uncertain, recent mostly murine findings suggest that BM PCs demonstrate mobility and distinctive migratory patterns that are altered with age (Benet, Jing et al. 2021).

Regulatory T cells (Tregs) are a type of immune cell crucial for immune regulation. They are notably found in significant numbers near HSCs and HSPCs on the BM's endosteal surfaces in mice, a placement that appears essential for preventing autoimmunity and facilitating successful engraftment of allogeneic HSPCs without the risk of rejection (Fujisaki, Wu et al. 2011).

Neutrophils play a vital role in innate immunity despite their short lifespan. Originating in the BM, they quickly enter circulation and tissues before being cleared from the BM, liver, and spleen a few hours later. Histamine, a significant

component in the BM, primarily stems from neutrophils. Imaging analyses in mice revealed a close histamine-based association between neutrophils and myeloid-biased HSCs (Chen, Deng et al. 2017). Furthermore, neutrophils contribute to murine niche regeneration by aiding vessel and hematopoietic recovery through tumor necrosis factor-α (TNFα) secretion after myeloablation (Bowers, Slaughter et al. 2018).

Non-cellular components of the bone marrow microenvironment

Extracellular matrix proteins

In general, the ECM constitutes a three-dimensional (3D), intricate, and highly dynamic noncellular framework comprised of various components, including (1) proteoglycans; (2) fibrous proteins such as collagens, fibronectins, elastins, tenascins, vitronectin, and laminins; (3) glycosaminoglycans, like hyaluronic acid, chondroitin sulfate, heparan sulfate, keratan sulfate, dermatan sulfate, and heparin; and (4) matricellular proteins such as osteocalcin and periostin, which play a role in connecting other ECM proteins with cellular receptors. The predominant proteins found in the BM ECM encompass fibronectin, collagens I, II, III, IV, and X, laminin, tenascin, thrombospondin, and elastin. Additionally, glycoproteins from the sialomucin family, whether soluble or membrane-bound, such as platelet–selectin glycoprotein ligand (PSGL1/CD162) and intercellular adhesion molecule 1 (ICAM1; CD54), are closely linked with the ECM (Klamer and Voermans 2014). The ECM is instrumental in regulating the structural framework, notably influencing tissue stiffness, deformability, and overall tissue equilibrium. Furthermore, it acts as a significant reservoir for proteases and growth factors (Zanetti and Krause 2020). For instance, heparan sulfate, a proteoglycan, plays a role in retaining HSPCs in mouse BM (Coutu, Kokkaliaris et al. 2017). OPN, a vital constituent of the bone matrix, is primarily recognized as a protein secreted by osteolineage cells and MSCs. It plays a role in guiding the migration of HSCs towards the endosteum, aiding the adhesion of HSPCs through β1 integrins, and reducing their cycling rate, as shown in a mouse model (Nilsson, Johnston et al. 2005).

Lipids

While ECM components and signaling molecules like cytokines are well-known regulators of HSC behavior, lipids, non-protein metabolites, also play a role in HSC regulation in mice and humans (Hoggatt, Singh et al. 2009). In addition to OPN and CXCL12, OBCs produce prostaglandin E2 (PGE_2), a lipid from the eicosanoid family, known to stimulate bone resorption via activation of osteoclasts through the RANK-RANKL axis in mice (Inada, Matsumoto et al. 2006). HSCs express receptors for PGE_2 (EP1-4), and exogenous PGE_2 treatment has been shown to directly stimulate HSC expansion, enhance HSC engraftment in zebrafish and mice,

and accelerate hematopoietic recovery following irradiation (North, Goessling et al. 2007; Goessling, North et al. 2009; Porter, Georger et al. 2013). Another lipid affecting HSCs is sphingosine 1-phosphate (S1P), which acts via the S1P1 receptor. S1P was reviewed as a potent chemoattractant, influencing HSC trafficking, homing, and retention within the BM (Ratajczak, Kim et al. 2012). Interestingly, even at low levels in peripheral blood (PB), S1P creates a chemoattractive gradient from BM to PB, facilitating HSC mobilization in mice (Golan, Vagima et al. 2012). Studies in Sphk1$^{-/-}$ mice revealed that while S1P deficiency did not affect hematological homeostasis, it impaired the homing and engraftment of wild-type or C-X-C chemokine receptor type 4 (CXCR4)-deficient HSCs (Ratajczak, Lee et al. 2010). This underscores the role of S1P in HSC homing and mobilization but not in HSC maintenance. Overall, accumulating evidence emphasizes the importance of naturally occurring lipids as a vital family of niche molecules with diverse roles in HSC regulation.

Metal ions

Bone serves as a major reservoir for essential mineral ions such as calcium, magnesium, manganese, zinc, and copper (Saltman and Strause 1993). Osteoclast-mediated bone resorption releases calcium ions, creating concentration gradients from the endosteal surface to the central medullary cavity, as shown in a mouse model (Cao, Zhang et al. 2016). The abundance of extracellular calcium in the endosteal murine BM is believed to aid in HSC localization and engraftment, mediated through the CaR expressed by HSCs (Adams, Chabner et al. 2006). While calcium is a well-known metal ion in the BM niche, magnesium and manganese ions have also been detected at elevated levels in the endosteal region of mice (Cao, Zhang et al. 2016). These, along with calcium, are recognized modulators of integrin activity *in vitro* (Pepinsky, Mumford et al. 2002). Specifically, the higher calcium, magnesium, and manganese content in the murine endosteal region has been demonstrated to differentially activate α4β1 and α9β1 integrins expressed by endosteal HSCs (Cao, Zhang et al. 2016). Although the physiological role of this differential integrin activity on BM HSCs hasn't been fully determined, considering the crucial roles integrins play in HSC retention, migration, and homing (Sahin and Buitenhuis 2012), it's conceivable that the regulation of integrin function by divalent cations near bone may contribute to improved HSC functions.

The bone marrow microenvironment in disease

Disease initiation

There have been several investigations into how the dynamic interplay between stroma and HSPCs preserves the equilibrium within the BM. Persistent investigations revolve around alterations in these relationships within the BM in the context of diseases. It is of great interest how the dynamic control of the niche facilitates the proliferation of malignant cells at the cost of their healthy hematopoietic counterparts.

For a long time, it has been understood that certain diseases with clonal expansion, such as myeloproliferative neoplasms (MPN), harbor cytogenetic abnormalities that propel the expansion of malignant cells (Schepers, Campbell et al. 2015). These abnormalities induce the production of factors that modify the microenvironment to favor abnormal growth—a concept termed "bad seeds in bad soil" (Bousse-Kerdilès 2012). The disease-initiating stem cells, in leukemia and MPN termed leukemic stem cells (LSC), possess full stem cell capabilities. LSCs exploit signals from the vascular niche, such as CXCL12 or CD44, for homing and exhibit lower reliance on specific signals crucial for the survival and function of healthy HSCs (Korn and Mendez-Ferrer 2017).

However, numerous reports have contested this notion and the potential role of the niche in driving or facilitating malignancy was proposed more than twenty years ago (Dührsen and Hossfeld 1996). Recent evidence from mouse disease models has demonstrated that defects originating solely from the microenvironment can instigate a disease state in the BM. One of the early reports came from Walkley and colleagues, showcasing that defects in Retinoblastoma or Retinoic acid receptor y within the microenvironment could induce a myeloproliferative-like condition in mice (Walkley, Olsen et al. 2007; Walkley, Shea et al. 2007). Raaijmakers and colleagues reported that a deficiency of Dicerl, a miRNA processing enzyme, in early OBCs, could generate a myelodysplastic syndrome (MDS) and secondary leukemia in mice (Raaijmakers, Mukherjee et al. 2010). Furthermore, activating mutations in protein tyrosine phosphatase, non-receptor type 11 in mesenchymal stem/progenitor cells and osteoprogenitors could induce an exacerbated MPN in a murine model via macrophage inflammatory protein-1 alpha and a cascade of inflammatory signals in the BM (Dong, Yu et al. 2016). Likewise, Kode and colleagues demonstrated in a mouse model that mutations leading to activation of β-catenin in OBCs could give rise to AML by altering the differentiation of lymphoid and myeloid progenitors (Kode, Manavalan et al. 2014). The study also revealed that activated β-catenin and Notch signaling were common features in MDS and AML patients, suggesting that stroma-directed therapies could be effective in these patients. A rare type of donor-cell leukemia in transplanted

patients supports the notion that defects inherent to the niche may promote leukemogenesis (Wiseman 2011).

Moreover, there are reports highlighting the malignant cells' contribution to actively engaging or reprogramming the microenvironment to aid the tumorigenic process. In one study, it was demonstrated that MPN myeloid cells in an animal model "instructed" MSCs to produce OBCs with defective functions generating a “self-reinforcing niche" (Schepers, Pietras et al. 2013). In another model of MDS, MSCs were similarly primed by the malignant cells to preferentially support MDS expansion (Medyouf, Mossner et al. 2014).

However, niche environment or stem-cell-induced disease initiation doesn’t exclude the other, bringing research into niche-targeted therapies to the forefront of new therapeutic approaches.

Niche changes during disease initiation

The initial stem cell niche hypothesis proposed that the niche employs mechanisms to protect steady-state hematopoiesis, even against threats like mutant clones of HSCs (Hoggatt, Kfoury et al. 2016). However, a growing body of evidence indicates that mutant or malignant cells can manipulate the microenvironment's resources to support their own needs at the expense of healthy cells (Schepers, Pietras et al. 2013; Medyouf, Mossner et al. 2014). Altering the requirements for microenvironmental factors in malignant cells presents, therefore, a unique therapeutic opportunity. It is widely recognized that tumor cells interact with niche cells using various cell adhesion molecules, with specific interactions varying based on the disease context. For instance, the CXCR4/CXCL12 axis has been particularly implicated in leukemic progression, and inhibiting CXCR4 has shown beneficial effects in overcoming drug resistance in AML models (Sipkins, Wei et al. 2005; Zeng, Shi et al. 2009). However, in a chronic myelogenous leukemia (CML) mouse model, functional deficits in integrin B1 and CXCR4 by Philadelphia-chromosome positive stem cells are overcome by a higher dependence on the alternative pathway involving the hyaluronan receptor CD44 compared to healthy cells (Krause, Lazarides et al. 2006). This alternative pathway enhances the homing of CML stem cells by increasing their selectin binding activity. CD44 is essential for CML but not for B lymphoblastic leukemia development, and blocking CD44 could inhibit leukemic stem cell homing and engraftment in mice (Jin, Hope et al. 2006; Krause, Lazarides et al. 2006). Additionally, cultivating patient-derived MDS cells alongside BM MSCs *in vitro* leads to an excess production of specific cytokines. This excess facilitates the engraftment and proliferation of MDS in mice (Poon, Dighe et al. 2019).

As mentioned earlier, malignant cells may expand niche cells to enhance their survival; for example, the growth of osteolineage cells induced proinflammatory,

myelofibrotic conditions in the niche in an MPN mouse model (Schepers, Pietras et al. 2013). On the other hand, malignant or mutant cells may favor the inhibition of niche expansion, thereby impeding stromal support for healthy HSPCs and the niche's defense mechanisms against them. This is evident in the reduction of osteoblastic cells in human and murine MDS/AML, which correlated with an increased leukemia burden and a differentiation bias toward myelopoiesis (Zhou, Yue et al. 2014).

Niche cells possess the ability to influence tumor cell migration, promote metastasis to other sites, inhibit or evade apoptosis, and mediate signaling that induces drug resistance (Papayannopoulou and Scadden 2008). Various mechanisms contribute to these effects, either individually or in combination. Some of these mechanisms are potential targets for therapeutic interventions, with certain ones already being utilized in clinical settings. Notably, Notch signaling plays a crucial role in regulating the balance between HSC self-renewal and differentiation within the niche. Activation of Notch by osteolineage cells has been demonstrated to directly impact HSC numbers and promote diseases resembling MPN and leukemogenesis in mouse models (Kim, Koo et al. 2008; Kode, Manavalan et al. 2014). Another significant mechanism associated with self-renewal in steady-state hematopoiesis in mice and notch signaling is B-catenin signaling. This pathway is crucial not only for HSC self-renewal but also for the self-renewal and leukemic potential of CML granulocyte-macrophage progenitors (Jamieson, Ailles et al. 2004). Changes to the BM niche can potentially also support the onset of disease. For instance, restructuring of HSC niches in the BM due to aging can encourage the proliferation of myeloid cells as a first step for the initiation of myeloid malignancies (Ho, Del Toro et al. 2019).

Moreover, interactions between tumor cells and the niche can result in increased resistance to therapeutic agents. Various modes of chemoresistance have been identified. Tumor cells utilize hematopoietic microenvironment (HME) cues to evade immune surveillance, establish protective "niches", or secrete factors that enhance cell proliferation while inhibiting apoptosis (Krause and Scadden 2015). Cancer-associated fibroblasts (CAFs) have been identified as promoters of chemoresistance. In the context of solid tumors, their presence is associated with an unfavorable prognosis (Kieffer, Hocine et al. 2020). This phenomenon extends to B cell acute lymphoblastic leukemia (B-ALL), where CAFs originate from MSCs (Burt, Dey et al. 2019). Quiescence emerges as an alternate mechanism for maintaining the leukemic stem cell niche, providing both protection from chemotherapeutic insults and supporting long-term self-renewal (O'Reilly, Zeinabad et al. 2021).

Vascular remodeling is a prevalent characteristic of myeloid malignancies, indicating disease progression and a poor prognosis (Medyouf 2017). Malignant cells secrete proangiogenic factors such as VEGF, HGF, bFGF, and TNFa. Elevated endothelial cell (EC) activity may destabilize vessel integrity, and nitric oxide has

been implicated in this process. Recent findings in an AML model demonstrated that inhibiting nitric oxide normalizes vessel architecture and enhances drug responses *in vivo* (Passaro, Di Tullio et al. 2017). Tumor cells may exploit cell-to-cell communication pathways with niche cells, such as using exosomes. Exosomes, small extracellular vesicles of endosomal origin, are shed from the membrane of various cells in the HME, including tumor cells, MSCs, ECs, immune cells, macrophages, and mast cells (Sun, Gu et al. 2022). These exosomes contain coding and non-coding RNA, and their release is one of the most recently recognized modes of cellular communication. It is believed that exosomal information partly mediates the malignant niche transformation and disease progression (Prieto, Sotelo et al. 2017; Kumar, Garcia et al. 2018).

The bone marrow microenvironment in myeloproliferative neoplasms

Based on existing knowledge about the hematological niche and its involvement in hematological diseases, it is hypothesized that distinct diseases are associated with specific aberrations in the hematopoietic niche and its components (Méndez-Ferrer, Bonnet et al. 2020). This thesis particularly focuses on MPNs and ALL.

MPNs encompass a heterogeneous group of clonally expanded myeloid diseases. The subgroup of Philadelphia-negative MPNs or classic MPNs (CML is a Philadelphia-positive MPN) includes polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), and prePMF. Clinically, they manifest as an overproduction of cells contributing to the myeloid lineage following a mutational event in HSPCs. Individuals with MPNs face an increased risk of thrombotic and bleeding complications, extramedullary hematopoiesis (e.g., spleen, liver), and progression to acute myeloid leukemia (Mead and Mullally 2017). In 2005, a critical discovery was made—a single amino acid substitution of valine to phenylalanine resulting from a point mutation in the Janus kinase 2 (JAK2) autoinhibitory pseudokinase domain. This mutation led to the constitutive activation of JAK2 and was identified in about 95% of PV patients and approximately 60% of MF patients. It was found to be responsible for many constitutional symptoms of MPNs (Baxter, Scott et al. 2005; James, Ugo et al. 2005; Kralovics, Passamonti et al. 2005; Levine, Wadleigh et al. 2005). This groundbreaking discovery spurred numerous preclinical and clinical studies, culminating in the approval of Ruxolitinib by the FDA in 2011. Initially indicated for the treatment of intermediate and high-risk myelofibrosis, Ruxolitinib exhibited exceptional results in alleviating symptoms, notably in reducing spleen volume (Comfort I and II studies), and consistently improved survival (Verstovsek, Mesa et al. 2015). Since then, the investigation of the JAK2V617F mutation and other frequently mutated genes in MPDs, such as MPL, JAK exon 12, and calreticulin, which are mutually exclusive but all result in overly activated STAT signaling, has significantly enhanced our comprehension of these diseases concerning prognosis.

Advancements in understanding the hematopoietic niche under normal physiological conditions have underscored the indispensability of niche cells for the maintenance of HSCs. This progress has inevitably led to hypotheses concerning niche disruption in myeloid malignancies, including MPNs. Indeed, as discussed earlier in the "Disease Initiating Theories" section, various reports have indicated that niche defects alone can induce MPN phenotypes in mouse models (Walkley, Olsen et al. 2007; Walkley, Shea et al. 2007; Kim, Koo et al. 2008). However, niche disruption is likely a secondary event following a somatic or, less commonly, a germline mutation in long-term HSCs.

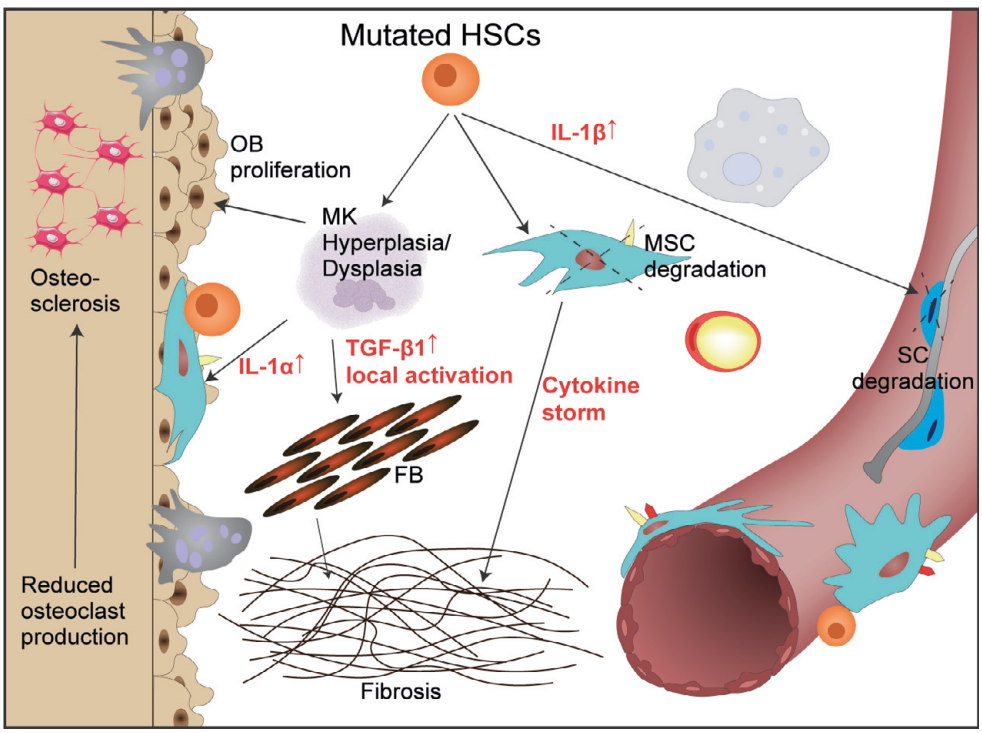

Figure 3: Hematopoietic microenvironment in primary myelofibrosis
In PMF, mutated HSC and their progenies induce HME changes. Fibrosis is viewed as a multifactorial process, involving aberrant cytokine production and different cell types. However, the exact mechanisms causing fibrosis in MPN have not yet been identified in detail.
HSC: hematopoietic stem cell, MSC: mesenchymal stromal cell, OB: osteoblast, MK: megakaryocyte, SC: Schwann cell, FB: fibroblast.

BM fibrosis, a prominent feature observed in PMF, certain MPNs, and occasionally in MDS and ALL, is a consequence of extensive remodeling of the mesenchymal niche. Fibrosis is closely linked to unfavorable disease prognosis (Medyouf 2017,

Figure 3). Recent studies have shed light on specific cell populations within the BM that play critical roles in fibrotic and angiogenetic processes following acute or chronic injury. For instance, a $Gli1^+$ MSC population expressing α-smooth muscle actin, located in both endosteal and perivascular niches of mice, was found to contribute to fibrosis (Kramann, Schneider et al. 2015; Schneider, Mullally et al. 2017). Additionally, investigations in thrombopoietin-overexpressing mice identified LEPR MSCs expressing *PDGFRA* and *PDGFRB* as the major source of myofibroblasts responsible for fibrosis in PMF (Decker, Martinez-Morentin et al. 2017). Fibrosis is closely connected to inflammation in the niche, Interleukin-1β (IL-1β), a key inflammation regulator, is implicated in MPNs, and its elevated levels and expression on hematopoietic cells correlate with the JAK2V617F mutant allele fraction in MPN patients; targeting IL-1β, either through hematopoietic cell knockout or anti-IL-1β antibody treatment in mice, reduces inflammation, preserves the niche, decreases myelofibrosis and osteosclerosis, presenting a potential therapeutic approach for MPNs, particularly when combined with ruxolitinib (Rai, Grockowiak et al. 2022).

Angiogenesis within the niche undergoes a surge in MPN patients, resulting in perturbed vessel architecture (Lundberg, Lerner et al. 2000). Intriguingly, the MPN-driving mutation JAK2V617F has been detected in ECs from a subset of MPN patients (Teofili, Martini et al. 2011) highlighting a potential role of ECs in MPN progression through the increased expression of P-selectin (Guy, Gourdou-Latyszenok et al. 2019). The tetraspanin CD9 has also been identified with high expression in $CD34^+$ cells and MKs of PMF patients, linking it to dysmegakaryopoiesis via AKT/MAPK signaling and abnormal stromal interactions *in vitro* (Desterke, Martinaud et al. 2015). Additionally, an *in vitro* investigation of MPN-derived MSCs showed a reduction in CD271 expression, altered localization of MSCs away from endosteal and vascular niches, interaction with dysplastic MKs, matrix remodeling marked by fibronectin upregulation, and impaired hematopoietic support (Schneider, Ziegler et al. 2014). Notably, MSCs from MPN patients have been found to confer chemoprotection to MPN cells against JAK2 inhibitor atiprimod primarily through paracrine production of soluble factors (Manshouri, Estrov et al. 2011). Sympathetic nerve fiber-regulated $nestin^+$ MSCs were shown to be integral for maintaining normal HSC function and disruption of this regulation is central to the pathogenesis of MPNs (Arranz, Sánchez-Aguilera et al. 2014). Furthermore, the MPN niche displays secretion of various chemotactic, pro-inflammatory, and fibrosis-associated cytokines from MSCs (Goulard, Dosquet et al. 2018).

In the context of MPNs, there is a growing interest in understanding how the niche contributes to the initiation and progression of the disease. A recent study in humans and mice revealed distinct BM niches for HSCs in different MPN subtypes, showing that JAK-STAT signaling plays a role in regulating niche interaction and mutant cell expansion (Grockowiak, Korn et al. 2023). The MPN subtype affected BM

niche remodeling, with potential acceleration in a prematurely aged BM microenvironment, emphasizing the influence of specialized niches on mutant cell expansion and clinical responses to JAK inhibitors. Insights into the role of the stromal components in MPNs may unveil novel therapeutic approaches or enhance existing ones, for instance, combining JAK inhibition with neuroprotection and/or neutralization of stromal cytokines.

The bone marrow microenvironment in ALL

ALL encompasses a diverse group of malignant blood disorders originating from either B-cell (B-ALL) or T-cell (T-ALL) progenitors. The majority of ALL cases (around 80%) fall under the B-ALL category. While B-ALL can manifest at any age, it predominantly affects children, emerging as the most common malignancy in this age group, peaking around 2-5 years old. In children, the incidence stands at 3-4 cases per 100,000 each year. Conversely, in adults, the annual frequency is lower, approximately 1 case per 100,000. Over the past two decades, there has been a remarkable improvement in the outcomes for pediatric B-ALL patients, with survival rates exceeding 80% at the 5-year mark. However, the prognosis for adult B-ALL patients is notably more grim (Pui, Mullighan et al. 2012; Parikh and Litzow 2014). This discrepancy is partially attributed to the presence of the BCR-ABL1 fusion protein in 25-30% of adult B-ALL patients, which is associated with a worse prognosis (Mullighan 2012). In contrast, only 2-10% of pediatric B-ALL patients exhibit this protein. Moreover, less than half of BCR-ABL1 negative adult patients maintain their remission at the 5-year mark (Parikh and Litzow 2014).

T-ALL constitutes approximately 10-15% of pediatric and 25% of adult cases of ALL (Pui, Mullighan et al. 2012). With the advancement of intensified multi-agent chemotherapy protocols, the 5-year event-free survival (EFS) for children diagnosed with T-ALL ranges from 70% to 75%. In contrast, adults under 60 years of age experience an EFS of 30-40%, while those over 60 years old have a significantly lower EFS of only 10% (Pui and Evans 2006). Unfortunately, the prognosis remains grim for T-ALL patients who exhibit primary chemoresistance or experience relapsed leukemia (Pui, Mullighan et al. 2012).

The development of chemoresistance and relapse in acute leukemias is attributed to a distinct population of LSCs, which are relatively rare and share critical characteristics with normal HSCs (Fregona, Bayet et al. 2021). Notably, LSCs tend to remain in a dormant state, evading the effects of chemotherapeutic drugs that primarily target actively dividing cells. Furthermore, LSCs possess the ability to self-renew and can also differentiate into progeny with limited self-renewal potential (Fragoso and Barata 2014; Pollyea, Gutman et al. 2014). Similar to healthy HSCs, LSCs rely, at least partially, on signals originating from the BM microenvironment (Dührsen and Hossfeld 1996). However, LSCs possess the ability to outcompete HSCs, effectively taking control of the BM

microenvironmental niches (Colmone, Amorim et al. 2008). Essentially, leukemic cells disrupt the normal BM niches, transforming them into what can be termed "leukemic" niches. This transformation is facilitated by leukemic cells releasing exosomes or microvesicles, inducing alterations in the BM microenvironment to support the persistence of leukemic cells (Huan, Hornick et al. 2013). These leukemic niches within the BM act as protective "sanctuaries," enabling LSCs to evade chemotherapy-induced cell death, develop drug resistance, and even acquire metastatic capabilities (Tabe and Konopleva 2014).

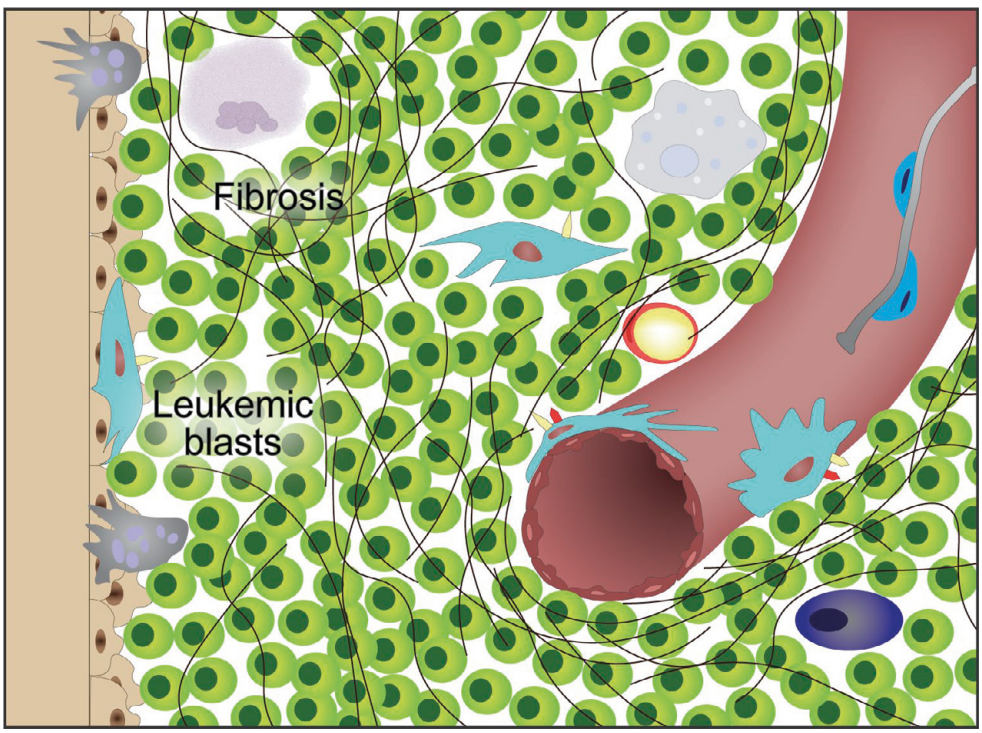

Figure 4: Hematopoietic microenvironment in fibrotic pediatric acute lymphoblastic leukemia
In fibrotic pediatric ALL the normal HME structure is disturbed by excess leukemic blasts and fibrosis. The mechanisms causing fibrosis in pediatric ALL are not known so far.

The detrimental effects of leukemia-induced bone complications are gaining recognition, affecting not only a patient's quality of life but also playing a significant role in the progression of the disease (Rajakumar and Danska 2020). In a recent research investigation, it was observed that both OBCs and osteoclasts experienced a notable reduction in BM trephine samples from children diagnosed with B-ALL (Nguyen, Melville et al. 2015). Notably, an *in vitro* study demonstrated that B-ALL

possesses the capacity to directly hinder osteogenesis. This was shown by the inhibitory effect of B-ALL on the osteogenic differentiation of MSCs (Yang, Xu et al. 2015). Finally, the creation of an immunocompetent syngeneic mouse model for BCR-ABL1$^+$ B-ALL, mirroring bone loss observed in patients, offers a valuable tool for in-depth exploration of the B-ALL microenvironment. Throughout the progression of leukemia in this model, a decline in the osteoblastic population and a reduction in serum osteocalcin levels were observed (Cheung, Tickner et al. 2018).

Conversely, the osteogenic niche also supports LSCs, OPN exhibits an ability to engage with B-ALL cells by interacting with very late antigen-4 (VLA-4), facilitating their binding and anchoring within the endosteal niche. Consequently, this interaction prompts an increase in both B-ALL and osteoblast-derived OPN, strengthening adhesion to the endosteal region and fostering a state of dormancy among leukemic cells (Boyerinas, Zafrir et al. 2013).

The initial homing of B-ALL cells to the central niche primarily occurs through interactions between B-ALL cells expressing CXCR4 and MSCs expressing CXCL12, underscoring the pivotal role of the CXCR4/CXCL12 pathway in leukemogenesis. B-ALL cells can displace HSPCs from the niche, disrupting normal hematopoiesis by reducing niche-derived CXCL12 (van den Berk, van der Veer et al. 2014; Balandrán, Purizaca et al. 2017; Ma, Witkowski et al. 2020) levels while elevating granulocyte colony-stimulating factor expression in MSCs (van den Berk, van der Veer et al. 2014; Balandrán, Purizaca et al. 2017). The significance of CXCR4/CXCL12 in B-ALL development is further demonstrated by findings linking higher phosphorylated CXCR4 expression in the BM with inferior treatment response and reduced overall survival in adults with B-ALL (Konoplev, Jorgensen et al. 2011).

Another notable aspect of the malignant niche is its disrupted inflammatory environment. In clinical settings, patients with B-ALL demonstrate heightened levels of pro-inflammatory cytokines in serum and plasma, such as TNF-α, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10, interleukin-12, interferon-γ, and monocyte chemoattractant protein-1 (CCL2) (de Vasconcellos, Laranjeira et al. 2011; Pérez-Figueroa, Sánchez-Cuaxospa et al. 2016). This inflammatory state is further confirmed at the cellular level, with upregulation of pro-inflammatory cytokines observed in both B-ALL cells (Vilchis-Ordoñez, Contreras-Quiroz et al. 2015) and B-ALL-associated MSCs (de Vasconcellos, Laranjeira et al. 2011; Balandrán, Purizaca et al. 2017). Specifically, MSCs derived from the BM of children with B-ALL exhibit elevated IL-1α, IL-6, interleukin-12p70, and TNF-α levels (Balandrán, Purizaca et al. 2017). Additionally, co-culture of patient-derived B-ALL cells and BM MSCs induces upregulation of IL-8 and CCL2 in MSCs. These cytokines play a crucial role in maintaining an inflammatory microenvironment that promotes leukemogenesis and sustains B-ALL malignancy within the central BM niche (de Vasconcellos, Laranjeira et al. 2011; Pérez-Figueroa, Sánchez-Cuaxospa et al. 2016).

Usually, long exposure to an inflammatory state leads to fibrosis in the affected tissue (Wynn 2007). BM fibrosis stands out as a severe form of HME remodeling, marked by an excessive accumulation of reticulin fibers (Figure 4). BM fibrosis has been linked to an unfavorable outcome in ALL, with high reticulin fiber density at diagnosis being associated with elevated levels of therapy-surviving leukemia cells, known as minimal residual disease, in B-ALL (Norén-Nyström, Roos et al. 2008). While not all studies have confirmed the negative impact of fibrosis on ALL prognosis (Bharos, Jong et al. 2010), it is widely acknowledged that BM fibrosis is a significant and prevalent feature, observed in approximately 80% of B-cell ALL cases (Wallis and Reid 1989). Recent data also indicates that fibrosis in ALL is linked to a poor response to CAR-T cell treatments in children (Anil, Lytle et al. 2022).

Historically, many therapies for leukemia focused on targeting intrinsic molecular attributes of leukemic cells at the molecular level, such as cell cycle regulatory proteins, constitutively activated tyrosine kinase or cytokine receptor signaling, and genetic alterations that drive malignancy. However, in recent years, growing evidence strongly supports the idea of therapeutically targeting interactions between leukemic cells and BM niche cells within the HME to enhance conventional chemotherapy (Kuek, Hughes et al. 2021). Various agents that target leukemic cell engraftment and chemoresistance, like CXCR4/CXCL12 inhibitors, Wnt signaling inhibitors, Notch inhibitors, as well as VLA-4 and E-selectin antagonists, have shown promising therapeutic efficacy in preclinical studies and/or clinical trials (Kuek, Hughes et al. 2021). Nevertheless, there is a notable absence of newly FDA-approved HME-targeted therapeutic agents or ones currently undergoing clinical trials for the treatment of ALL. Hence, it is critical to devise and test therapeutics targeting the HME of ALL to enhance the clinical outcome for patients, especially those with poor prognoses.

Aims of the thesis

The overarching theme of my graduate studies was to elucidate changes in the HME and the stromal compartment between the healthy and diseased state. Our group approached this on different levels: molecular, transcriptional, and *in situ* structural. The specific aims represented in our studies were:

- Investigation of the role of the immediate early response transcription factor *EGR1* in regulating the functions of BMSC, with a focus on its impact on stromal cell proliferation and hematopoietic support functions, and to understand how *EGR1* controls the genetic program of BMSC in various biological contexts (Paper I).
- Comprehensively characterize the different non-hematopoietic BM stromal cell populations in humans using single-cell RNA sequencing and investigate the regulatory principles governing these cells' differentiation and interactions with hematopoietic cells to contribute to a better understanding of the human hematopoietic niche (Paper II).
- Investigation of the spatial organization of the human BM HME, with a focus on comparing normal and MPN BM samples with our novel 3D immunofluorescence (IF) model to analyze changes in cellular architecture and to generate comprehensive 3D models of endosteal and perivascular BM niches (Paper III).
- Understand the mechanisms underlying BM fibrosis in ALL by comparing the cellular composition and the spatial mRNA expression of transforming growth factor beta (*TGFB1*) and Platelet-derived growth factor alpha (*PDGFA1*) in ALL BMs compared to PMF (Paper IV).

Description of Methods

Human tissue sampling

BM aspirates from the iliac crest bone were collected in the hematology clinic, at the University Hospital in Lund. Cord blood was collected from consenting donors at the hospital's maternity wards in Lund, Helsingborg, and Malmö, Sweden. Fetal long bones (median age: 18 gestational weeks, range: 15–20 gestational weeks) from elective abortions were obtained with informed consent at the Erasmus Medical Center, the Netherlands. BM biopsies from the iliac crest bone were obtained from the Division of Pathology (Department of Clinical Sciences, Lund). Sampling and sample preparation were approved by the Institutional Review Committee in Lund (Regionala Etikprövningsnämnden I Lund, approval no. 2014/776 and 2018/86). All procedures and handling of human samples were performed in accordance with our approved ethical permits and the Helsinki Declaration of 1975, as revised in 2008.

Flow cytometry and fluorescence-activated cell sorting (FACS)

In both Study I and Study II, lineage-depleted BM-MNCs underwent blocking in a buffer of DPBS (wo Ca^{2+} and Mg^{2+}), with 3.3 mg/ml of human normal immunoglobulin (Gammanorm, Octapharm, Stockholm, Sweden) and 1% FBS (Invitrogen). Subsequently, these cells were labeled with monoclonal antibodies, and sorting gates were established based on fluorescence-minus-one (FMO) controls. The actual cell sorting was conducted using either a FACS Aria II or Aria III instrument from BD Bioscience (Erembodegem, Belgium).

To ensure the exclusion of non-viable cells, 7-Amino-actinomycin (7-AAD, Sigma) staining was employed, and doublets were excluded by gating based on forward scatter height (FSC-H) versus forward scatter width (FSC-W) and side scatter height (SSC-H) versus side scatter width (SSC-W). The sorted cells were directly collected into phosphate-buffered saline (PBS) containing 0.04% BSA for subsequent sequencing analysis.

For the sorting of BM-MNCs intended for CFU-F or stromal culture, 4′,6-diamidino-2-phenylindole (DAPI) was used to exclude dead cells. These cells were then directly sorted into stromal cell culture medium, specifically StemMACS MSC Expansion Media for human cells (Miltenyi Biotec, Bergisch Gladbach, Germany).

Colony-forming unit - fibroblast (CFU-F)

The colony-forming unit–fibroblast assay was used in studies I and II to assess the clonogenic potential of MSCs. FACS-sorted cells were cultured at low plating densities depending on expected clonality Colonies (≥ 40 cells) were counted after 14 days of culture with medium change at day 7. Staining was done with 1% Crystal Violet (Sigma). Generally, assays were set up in duplicates or triplicates. For single-cell CFU-F assays, cells were sorted into 96-well plates, cultured in stromal cell culture medium, and colonies were counted after 3 weeks.

In vitro differentiation assays

Apart from employing surface marker analysis, the primary and dependable method to claim MSC status involves confirming their multipotent capabilities. MSCs exhibit the capacity to differentiate into adipocytes, OBCs, myocytes, and chondrocytes both *in vitro* and *in vivo* (da Silva Meirelles, Caplan et al. 2008). In papers I & II cultured MSCs were *in vitro* differentiated into adipogenic, osteoblastic, and chondrogenic lineages. For adipogenic differentiation cells were cultured for 14 days in AdipoDiff medium (Miltenyi) and cells were stained with Oil Red O. For osteogenic differentiation, cells were cultured in osteogenesis induction medium (standard medium plus L-ascorbicacid-2-phosphate, dexamethasone, and β-glycerophosphate) for 21 days and calcium depositions were detected by Alizarin Red staining. For chondrogenic differentiation, cells were pelleted and cultured for 28 days in chondrogenesis induction medium (DMEM-high glucose supplemented with dexamethasone, sodium pyruvate, L-proline, ascorbic acid, ITS + culture supplements, and TGF-β3). After the differentiation pellets were fixed in PFA and frozen in OCT. 5µm thick cuts were stained with Alcian blue with the nuclear counterstain Fast Red, or with an IF antibody against aggrecan and DAPI as a nuclear counterstain.

While various cell populations obtained from BM and other tissues in both humans and other species have been observed to transform into mesenchymal cell types during *in vitro* studies, it's crucial to note that there is evidence indicating that cells with a mature phenotype in their natural environment can regress to a more primitive state when cultured (Grogan, Mainil et al. 2005). Additionally, these cells can also

develop into different cell types when grown *in vitro* (Tallheden, Dennis et al. 2003), (Barbero, Ploegert et al. 2003). As a result, the definition of MSCs based on their operational characteristics following *in vitro* cultivation, such as their capacity for self-renewal and differentiation into mesodermal cell lineages, may potentially be influenced by experimental conditions rather than inherent characteristics.

Single-cell sequencing and analysis

In paper II we employed single-cell sequencing (scRNAseq) to elucidate heterogeneities in the BM MSCs. Therefore, we performed scRNAseq on FACS-sorted BM-MNCs. The analyzed populations included $CD45^{low/-}$ $CD235a^{-}$ cells from four donors, as well as $CD45^{low/-}$ $CD235a^{-}$ $CD271^{+}$ cells from another five donors. BM-MNC cells were individually encapsulated into emulsion droplets using the Chromium Controller from 10 X Genomics. ScRNAseq libraries were prepared using the Chromium Single Cell Gene Expression 3' v3 Reagent Kit following the manufacturer's guidelines. Individual libraries were then diluted to 1.5 nM and combined for sequencing. The pooled libraries were sequenced on a NovaSeq 6000 (Illumina) to achieve a sequencing depth of 50,000 reads per cell.

The scRNAseq data from both the $CD45^{low/-}$ $CD235a^{-}$ and $CD45^{low/-}$ $CD235a^{-}$ $CD271^{+}$ populations underwent several processing steps before being combined for subsequent analysis. Sequencing data were first demultiplexed and unique molecular identifiers (UMIs) were collapsed using the CellRanger toolkit (version 3.1, 10 X Genomics). To identify low-quality cells, standard quality control metrics were applied, including cell filtration, downsampling, gene filtration, and the exclusion of reads associated with mitochondria and ribosomal sequences. The reads were aligned to the human GRCh38 genome, paired with the gencode.v31 gene-level data, using the kallisto/bustools package (version 0.46.0 and 0.39.3, respectively). Count metrics generated by kallisto/bustools were then subjected to analysis using Python's scanpy/velocyto. Cells with high levels of mitochondrial and ribosomal reads were excluded from the dataset, as were cells with fewer than 1000 detected UMIs (representing diploid cells). Expression levels were normalized using the scanpy.pp.downsample_counts function to achieve a uniform count of 1000 UMIs per cell. Genes that were not expressed in at least 10 cells were also removed from the dataset before further analysis. To reduce dimensionality, we utilized gene expression data from a subset of the 3000 most variable genes. Subsequently, we generated a UMAP (Uniform Manifold Approximation and Projection) plot with Scanpy version 1.6.0, employing the default settings and considering the top 50 principal components.

We performed graph-based clustering on the PCA-reduced data, employing the Louvain method as implemented in Scanpy. For cell type annotation, we relied on

published marker gene expressions. For each cluster, we conducted differential expression analysis to identify genes with a significant fold change greater than 2 when compared to other clusters.

We analyzed the dynamics of BM stromal cells using the scVelo package (version 0.2.2) within Scanpy 1.6.0. To infer cellular trajectories using RNA velocity, we employed dynamical modeling, which extends the original RNA velocity method to characterize multiple transcriptional states. We computed latent time analysis, pseudotime analysis, and velocity confidence with default parameters. The extrapolated states were projected onto the UMAP embedding generated during the initial analysis.

ScRNAseq is a powerful method to investigate complex cellular organs. Given the fact that BM aspirates are easily processed into an MNC single-cell suspension, they are excellent samples to investigate by scRNAseq. As good as scRNAseq is to distinguish differences between cell populations it is omitting the spatial aspect and can just, like in our paper, hint towards possible cellular interactions.

Sequential staining

In papers III and IV we utilized sequential staining with subsequent bleaching steps to generate highly multiplexed IF images of human BM slides. 5 µm thick BM slides were cut from pathology-stored formaldehyde fixed paraffin-embedded (FFPE) BM biopsies. The sections were initially heated to 65°C for 45 seconds to enhance adhesion. Deparaffinization was carried out using neo-clear (Merck, Germany), followed by rehydration through a series of decreasing ethanol concentrations and distilled water. Antigen retrieval was performed at 98°C for 30 minutes using a target retrieval solution (Dako, Denmark). Antigen blocking was carried out by incubating the sections in a buffer containing 10% goat serum. In the first staining round, the specimens were stained with primary bound CD34-APC antibody at room temperature for one hour in the dark, followed by counterstaining with DAPI. The slides were scanned for the first time using an Olympus VS120 fluorescence scanning microscope. After this initial scan, the mounted coverslip was floated off in PBS to allow for subsequent staining. To prepare for the next staining, the slides were bleached in a solution containing 3% hydrogen peroxide and 20% sodium percarbonate. The sections were subsequently stained with primary bound CD31-AF647 antibody at room temperature for one hour in the dark. After a second round of scanning and bleaching the sections were incubated with primary CD271 and CD45 antibodies overnight at 4°C. Secondary antibody staining was performed with goat anti-mouse IgG-AF647 and goat anti-rabbit IgG-AF488 at room temperature for one hour in the dark. Finally, the slides were scanned once more, resulting in a total of three scans during the entire process. The sequential scans of each slide were

aligned and combined based on the localization of nuclei using Arivis Vision 4D software (version 3.5.1, Arivis AG, Rostock, Germany). For each sample, a total of 12 slides were stacked by employing volume fusion with landmark registration. Additionally, the z-position was adjusted, resulting in the creation of a 50 μm z-stack.

Sequential staining offers the possibility of multiplexing IF staining beyond the availability of different host animal-raised primary antibodies and spectral-resolved fluorophores. Using primary bound antibodies offers the possibility of reusing established antibodies from FACS experiments and with bleaching highly multiplexed staining beyond 5 markers can be archived.

Three-dimensional recapitulation of bone marrow niches

In paper III we extracted meaningful information from densely packed 50 μm z-stacks based on IF intensity, by creating 3D surface representations for specific cellular and extracellular niche structures. These surface representations were generated using pipelines within the Analysis Panel of Arivis Vision 4D. Pipelines are sequences of operations designed to process images, enhancing them and creating objects known as segments. These segments can undergo further processing, sorting, and filtering. In our analysis, we employed the Blob Finder segmenter, which identifies structures based on their approximate diameter and a probability threshold and can automatically separate objects that are in contact by adjusting the split sensitivity. The values for diameter, probability threshold, and split sensitivity were fine-tuned through visual assessment of the segmented objects to align them with the expected morphology of the cells of interest in the fluorescence images. The segmented objects were subsequently refined using the Segment Feature Filter, which considered channel intensities, volume, and sphericity. This ensured that the resulting objects accurately represented the visual definition of the cell types under scrutiny. To identify HSPCs, we began by segmenting $CD34^+$, $CD45^+$, and DAPI objects, saving them as objects. In the next step, we analyzed the compartmentalization of these objects, flagging $DAPI^+$ objects that overlapped with both CD45 and CD34 objects as potential HSPCs. From these, we selected objects representing small, round cells with clear surface expression of CD34/45, defining them as HSPCs. MKs were segmented as $CD31^+$ spherical objects with a minimum diameter of 20 μm. The resulting objects were filtered based on low CD34 intensity and a volume of more than1500 μm^3. MSCs were segmented as $CD271^+$ objects with a diameter of 4–6 μm. The resulting objects were filtered based on low intensity in CD45/31/34 channels. Blood vessels were segmented as objects using the intensity threshold segmenter, considering big structures and relying on CD34 expression. This choice was made because ECs lining the vessels are double-positive for CD31 and CD34, with CD34 providing

more accurate segmentation. The resulting objects were further filtered for a volume of greater than 900 μm^3. Trabecular bone structures were manually delineated layer by layer. Ultimately, all these objects were saved as 3D objects (iso-surfaces) and labeled according to their identity as cellular or niche structures.

Next, we analyzed the spatial distribution of cells in the generated 3D structures. The iso-surfaces representing vessels, trabecular bone, MKs, and HSPCs were exported as mesh objects (.obj) from Arivis Vision 4D. These mesh objects were then imported into Blender (version 3.0.1, Netherlands) for further analysis. It was observed that the exported mesh objects had a higher vertex density in areas with more complex topologies compared to mostly planar regions. To address this, a "remesh" modifier was applied in Blender. This modifier normalized the mesh by adjusting the distance between vertices to 0.5 μm, ensuring an even representation. To analyze the spatial distribution, one percent of the vertices were randomly selected from both the trabecular bone and vessel objects. Lines were drawn from these selected points to the nearest surface point on the mesh objects representing MKs or HSPCs. This was achieved using the "shrinkwrap" modifier in Blender. The shortest distance from either the vessel or bone to the MKs and HSPCs, respectively, was calculated for all MK and HSPC objects within the 3D model of the region. The calculated distance data were then exported as a .txt file. This processing was automated utilizing a Python script in Blender.

Through our method, we were able to create the first comprehensive 3D IF reconstruction of the human BM. Previously, similarly intricate BM representations had only been achieved for murine femoral bones (Gomariz, Helbling et al. 2018). Consequently, our method marks a significant milestone in the development of a thorough 3D model of the human BM and offers the possibility for initial spatial interrogations.

Results

Paper I

In this paper, we investigated the role of the *EGR1* gene in primary BM MSCs. We found that *EGR1* is highly expressed in specific BM MSC populations and plays a significant role in regulating hematopoietic stroma support function. Here is a summary of the key findings:

EGR1 Expression in BM MSCs: We demonstrated that *EGR1* expression is significantly higher in certain BM MSC populations, particularly in $lin^{-}CD45^{-}CD271^{+}CD140a^{-}$ BMSC, compared to other BM MSC populations and non-colony-forming cells.

Hematopoietic Stroma Support: EGR1 was found to be crucial for the hematopoietic stroma support function of BM MSCs. Knocking down *EGR1* in BM MSCs led to a reduction in their ability to support the expansion and maintenance of $CD34^{+}$ hematopoietic cells. Conversely, overexpression of *EGR1* enhanced these functions.

Soluble Factors and Cell-Cell Interactions: EGR1's effects on hematopoietic support were mediated through both cell-cell interactions and soluble factors. The study showed that *EGR1* overexpressing BM MSCs secreted higher levels of c AGM chemokine ligand 28 (CCL28), a soluble factor that supports hematopoiesis. Additionally, *EGR1* upregulation led to increased expression of VCAM1, a cell-surface molecule that aids in hematopoietic cell attachment.

Gene Expression Changes: An array-based gene expression analysis revealed that *EGR1* overexpression in BM MSCs led to the upregulation of genes associated with hematopoiesis support, including cytokines and cell surface molecules like CCL28 and VCAM1.

Proliferation Regulation: EGR1 played a dual role in regulating BM MSC growth and function. Knocking down *EGR1* promoted BM MSC proliferation, while overexpression inhibited it. This effect was mediated, at least partly, by changes in intracellular ROS levels and the expression of genes involved in cell death and apoptosis regulation.

Other BM MSC Functions Unaffected: Importantly, changes in *EGR1* expression did not affect other functions of BM MSCs, such as their multi-differentiation

capacity (adipogenic, osteogenic, and chondrogenic) or their surface marker expression profile.

In summary, *EGR1* was highly expressed in specific BM MSC populations and played a crucial role in regulating hematopoietic stroma support through both soluble factors and cell-cell interactions. It also had a dual role in regulating BM MSC proliferation. Understanding these mechanisms can have implications for improving HSC transplantation and regenerative medicine.

Paper II

In this paper, we explored the cellular composition of the human BM (BM) stroma using scRNAseq profiling. We aimed to understand the diversity of cell populations within the BM microenvironment. Here is a summary of the key findings:

Cell Sorting Strategy: We sorted non-hematopoietic cells from healthy donors' BM using the $CD45^{low/-}CD235a^{-}$ population. BM stromal stem/progenitor cells are known to have low frequencies in BM, making them challenging to detect. To address this, $CD45^{low/-}CD235a^{-}CD271^{+}$ cells, which are highly enriched for CXCL12-expressing BM stromal stem and progenitor cells, were also sorted. Only the combination of these two datasets allowed for a detailed analysis of the BM stroma.

Dataset Details: The dataset comprised 25,067 cells, with a median of 856 genes and 2937 UMIs per cell. This dataset formed 42 clusters, representing distinct cell types and differentiation stages.

Identification of Hematopoietic Cells: The scRNAseq analysis of $CD45^{low/-}CD235a^{-}$ cells captured various hematopoietic cell types, including a cluster of HSPCs enriched for CD34 expression and other HSPC markers.

Identification of Non-Hematopoietic Cells: Importantly, we identified non-hematopoietic cells, including stromal cells and non-stromal cells such as ECs, basal cell-like clusters, and clusters enriched for neuronal markers.

Identification of Stromal Clusters: After excluding hematopoietic and other non-stromal cell types, we identified nine stromal clusters, which were only detectable after including $CD45^{low/-}CD235a^{-}CD271^{+}$ cells in the analysis. This confirmed the effectiveness of the $CD271^{+}$ cell enrichment strategy.

Cellular Heterogeneity of Stromal Progenitors: The nine stromal progenitor populations shared a unique gene expression profile, distinguishing them from other non-hematopoietic cell types. These stromal clusters were further annotated into six distinct cell types: multipotent stromal stem cells (MSSCs), highly adipocytic gene-

expressing progenitors (HAGEPs), balanced progenitors, pre-osteoblasts, osteochondrogenic progenitors, and pre-fibroblasts.

Functional Annotations: Each stromal cluster had distinct gene expression patterns associated with their differentiation capacities. For example, cluster 3 (MSSCs) displayed multi-lineage differentiation capacity with both adipogenic and osteogenic markers. Cluster 5 (HAGEPs) expressed adipogenic markers at a higher level compared to other clusters, while cluster 16 (balanced progenitors) showed similar expression levels of adipogenic and osteogenic markers. Cluster 38 (pre-osteoblasts) expressed more mature osteogenic markers.

Hematopoiesis-Related Markers: Some stromal clusters (clusters 6, 37, and 8) expressed both adipogenic and osteogenic markers as well as hematopoiesis-related markers. Additionally, the expression of S100 genes and murine fibroblast markers (S100A4 and S100 A6) was observed in these clusters, leading to their annotation as pre-fibroblasts.

In silico prediction of cell-cell interactions: We used computational analysis to predict interactions between stromal cells and hematopoietic cells in the human BM. Different stromal cell groups showed diverse interactions with various hematopoietic cell types. CXCL12 played a significant role, especially for subsets A and C in perivascular locations, while subset B had predominant interactions mediated by SPP1 in endosteal localization. This highlights location-specific and stromal subset-specific regulations in hematopoiesis. Endothelial cells also interacted broadly with hematopoietic cells but through different mechanisms compared to stromal cells.

In summary, the paper utilizes scRNAseq profiling to identify and characterize distinct cell populations within the human BM stroma, including stromal stem and progenitor cells with different differentiation capacities. Our findings provide valuable insights into the structural and developmental organization of the BM stroma at a high resolution.

Paper III

In this paper, we aimed to establish multicolor IF staining and 3D analysis of the human BM architecture. Here is a summary of the key points in the paper:

Experimental Setup: The study used the Olympus VS120 slide scanner, an automated fluorescence microscope capable of scanning up to six slides simultaneously using five different IF channels. A panel of at least four surface markers along with a nuclear stain was developed to describe the spatial relations of various BM stromal and hematopoietic elements. The selected markers included DAPI, CD34, CD45, CD271, and CD31, which allowed the identification of

different cell types within the BM. Due to limitations in using certain channels (Cy3 and Cy7) because of background interference and insufficient signal brightness, we adopted a sequential staining approach.

Sequential Staining Approach: The sequential staining method involved introducing bleaching steps to eliminate fluorescence before re-staining and re-scanning the same specimen. This approach allowed the successful combination of four IF antibodies (CD34, CD31, CD45, and CD271) with DAPI as a nuclear stain. Bleaching effectively removed the fluorescence signal from the previous staining round, ensuring that only the targeted epitopes were stained in the next round.

3D Reconstitution of BM Samples: The study utilized the Arivis Vision 4D software to generate 3D stacks of approximately 50-60 µm from sequentially cut slides. Landmarks such as MKs or larger structures spanning multiple slides were used to align the stacks. Specific regions of interest (endosteal and vascular niches) were cropped from the full z-stack to focus on relevant structures in subsequent surface reconstructions.

Surface Reconstruction and BM Architecture: Analysis pipelines in Arivis Vision 4D were used to define objects in the 3D stacks based on marker expression, size, and morphology. Different cell types, including MSCs, MKs, HSPCs, and vessels, were identified and represented in 3D surface reconstructions. Trabecular bone structures were manually added layer by layer into appropriate empty spaces within the stack to create an accurate representation.

MPN-Specific BM Changes: The study compared age and gender-matched control BM samples with those from patients with different types of MPNs, including ET, PV, PMF, and pre-PMF. Structural and morphological changes specific to MPNs were observed, such as variations in cellularity, adipocyte levels, and the appearance of MKs. Volumetric data from segmented objects were used to analyze cellularity, MK volume, and vessel volume in different BM regions.

Spatial Analysis Based on 3D Volumes: The spatial distribution of hematopoietic cells in relation to stromal elements, vessels, and bone was examined. Distances between annotated cells and important niche compartments like bone and vessels were calculated using the Blender software. The study found significant differences in distances between MKs and vessels in PV samples compared to controls.

Fibrosis Grade Correlation: An increase in $CD271^+$ cells, particularly in the vascular niche, was observed in MPN BMs, especially in PMF, which is known for extensive BM fibrosis. $CD271^+$ cell volumes varied among different MPN samples, with differences in both the endosteal and vascular regions.

In summary, this paper presents a comprehensive approach to studying the human BM architecture using multicolor IF staining, 3D analysis, and sequential staining techniques. It also highlights the specific structural changes associated with different types of MPNs and their correlation with CD271 expression.

Paper IV

In this paper, we analyzed the BM architecture in patients with ALL and PMF compared to hematologically normal control samples with multi-color IF staining and fluorescent in situ mRNA probes. Here are the key results:

Experimental Setup: We used a panel of five markers (DAPI, CD34, CD45, CD271, and CD31) for sequential multicolor IF staining of BM biopsies. These markers allowed the identification of various cell types, including HSPCs, MKs, MSCs, ECs, adipocytes, and $CD45^+$ hematopoietic cells. Trabecular bones were identified as non-fluorescent areas with few DAPI-positive nuclei.

Comparison of BM Samples: Compared to healthy controls, both ALL and PMF samples showed increased MKs, higher cellularity, increased MSC density (particularly around vessels), and fewer adipocytes. The ALL samples exhibited even higher cellularity and a very low number of adipocytes. Leukemic blasts expressing typical surface markers (CD45, CD31, and CD271) were predominant in ALL samples.

Fibrosis Grade and CD271 Expression: Fibrosis grade correlated with CD271 expression. $CD271^+$ MSCs were increased in fibrotic ALL and PMF samples compared to controls or non-fibrotic ALL samples. CD271 expression in fibrotic regions resembled classic fibrosis staining and was particularly dense near vessels. MSC cell volume was increased in fibrotic ALL samples and PMF samples compared to controls.

In Situ RNA Staining for TGFB1 and PDGFA1: We used in situ RNA staining for *TGFB1* and *PDGFA1* mRNA in combination with IF staining to analyze spatial expression. *TGFB1* and *PDGFA1* expressions were similar in PMF samples compared to controls. ALL samples tended to show increased overall expression of *TGFB1* and *PDGFA1* compared to PMF and control samples. *TGFB1* and *PDGFA1* expression was notably higher in MKs in the ALL samples compared to PMF and control samples.

Spatial Analysis of TGFB1 and PDGFA mRNA: We further investigated the spatial distribution of *TGFB1* and *PDGFA1* expression in regions with and without MKs. The expression of *PDGFA1* was relatively similar in MK regions and non-MK regions in fibrotic ALL, PMF, and control samples, but significantly higher in the MK region of the ALL sample with fibrosis degree 2. *TGFB1* tended to have higher expression in the MK region of ALL samples compared to non-MK regions, especially in the sample with fibrosis degree 2, while the opposite trend was observed in PMF and control samples. Non-MK regions in the ALL samples tended to show higher expression of *TGFB1* and *PDGFA1* compared to both control and PMF regions.

In summary, the results suggest that there are significant differences in the BM architecture and cytokine expression patterns in patients with ALL and PMF compared to healthy controls. These differences include increased MKs, higher cellularity, altered MSC density, and changes in cytokine expression, especially in MKs, which may have implications for understanding disease pathogenesis and progression.

General discussion and future perspectives

The work presented in this thesis paves the way for a more in-depth understanding of human HME in health and disease. Historically the hematopoietic niche is much more in-depth unraveled in the mouse, here the concept of endosteal and vascular niche was postulated (Acar, Kocherlakota et al. 2015). Transgenic strains, full bone preparations, and intra-vital imaging methods are only possible in mice. However, how applicable the found implications in mice are to the human setting is still eluding and sparked our desire to explore postulated hematopoietic interactions and organizations in the human context.

EGR1 in human primary BM MSCs

BM MSCs are vital for BM stroma turnover, skeletal tissue maintenance, and hematopoietic support (Omatsu, Seike et al. 2014; Seike, Omatsu et al. 2018). Yet, the mechanisms governing these functions are poorly understood. *EGR1* emerges as a key regulator in human BM MSCs, showing higher expression in steady-state adult BM MSCs compared to fetal or regenerating marrow (Guillot, Gotherstrom et al. 2007; Abbuehl, Tatarova et al. 2017). In paper one we showed that *EGR1* overexpression enhances BM MSC hematopoietic stroma support and promotes efficient generation of transplantable HSCs. Modulating *EGR1* levels affects BMSC proliferation and supports ex vivo HSC expansion. CCL28, a potent niche-secreted soluble growth factor (Karlsson, Baudet et al. 2013), contributes to the *EGR1*-mediated ex vivo expansion of CB CD34+ cells. Several approaches to expanding HSC have been pursued, including enhancing positive signals and inhibiting negative signals for HSC self-renewal (Guo, Huang et al. 2018; Mehrnaz Safaee, Agatheeswaran et al. 2018). *EGR1* modification represents a novel approach for optimizing the BMSC microenvironment to support HSC self-renewal and growth, holding promise for improving HSC expansion conditions. Strategies to enhance *EGR1* expression *in vivo* could enhance hematopoietic stromal support function, benefiting transplantation patients and those with insufficient hematopoiesis. *EGR1*'s influence on cell proliferation varies with the biological context (Adamson and Mercola 2002; Thiel and Cibelli 2002). High *EGR1* expression inhibits the

proliferation and colony formation of human BM MSCs, while reduced levels have the opposite effect. Proteomic analysis of *EGR1* knockdown cells highlighted down-regulated proteins associated with oxidative reduction processes, leading to increased ROS levels. Elevated ROS levels, known to promote cell proliferation, differentiation, and survival, signify the role of *EGR1* in these processes. These findings significantly advance our understanding of BMSC regulation, presenting potential therapeutic strategies to optimize hematopoietic stroma support and tissue repair.

Identification of distinct stromal niche populations in the human bonemarrow

The BM microenvironment plays a critical role in hematopoiesis, but understanding its human stromal cell populations and their functions has been a challenge (Baryawno, Przybylski et al. 2019; Tikhonova, Dolgalev et al. 2019; Baccin, Al-Sabah et al. 2020). In paper two we utilized scRNAseq, to offer insights into the stromal components of the human BM, shedding light on their developmental roles in hematopoiesis. To address the low frequency of stromal stem/progenitor cells in the BM, a sorting strategy incorporating $CD45^{low}$ $CD271^{+}$ cells enriched for stromal stem/progenitor cells (Tormin, Li et al. 2011) was employed. This significantly enhanced stromal cell resolution. After excluding hematopoietic clusters, nine stromal clusters were identified, representing six distinct stromal cell types. Interestingly, we revealed phenotypically and functionally distinct stromal subsets organized hierarchically through RNA velocity analysis, aligning with published murine and human stromal stem cell studies (Zhou, Yue et al. 2014; Baryawno, Przybylski et al. 2019; Wolock, Krishnan et al. 2019). Intriguingly, we phenotypically defined MSSCs and were able to identify them as the most primitive stromal population. Our findings open avenues for future mechanistic studies focusing on cellular state transitions and fate choices. While murine models have aided in understanding stromal cell functions in hematopoiesis, human studies have been limited to in-vitro models. We, therefore, explored cellular interactions based on *in silico* predicted ligand-receptor interactions, revealing a complex crosstalk network and the potential for stromal cells to support hematopoiesis which is consistent with our previously published in-vitro coculture data (Li, Ghazanfari et al. 2014; Li, Lim et al. 2020). Different stromal cell groups showed diverse interactions with various hematopoietic cell types. CXCL12 played a significant role, especially for subsets A and C in perivascular locations, while subset B had predominant interactions mediated by SPP1 in endosteal localization suggesting specialized niches for hematopoietic cells in different locations (Tormin, Li et al. 2011; Pinho, Lacombe et al. 2013). Although using aspiration samples may have limitations, the findings pave the way for further studies, potentially utilizing fresh

BM biopsy samples to enhance our understanding of the human BM microenvironment and its implications.

Three-dimensional human hematopoietic microenvironment in health and disease

Hematopoietic malignancies can result from HME alterations (Méndez-Ferrer, Bonnet et al. 2020). Understanding the interplay between malignant HSCs and abnormal HME cells is essential for comprehending disease progression. In paper three we focused on HSPCs, MKs, ECs, and MSCs. A five-marker panel (DAPI, CD34, CD45, CD271, and CD31) was utilized to distinguish these cell types and investigate their relationships in human BM. Our method employed sequential staining and bleaching for multi-color analysis of standard human BM paraffin slides, facilitating a 3D analysis through automated scanning microscopy. Arivis Vision 4D software enabled the creation of a comprehensive 3D IF reconstruction of human BM, a significant advancement considering that complex BM representations have mainly been reported for murine femoral bones (Holzwarth, Köhler et al. 2018). We aimed to demonstrate the applicability of this method in investigating normal BM anatomy and identifying BM changes in MPNs (Swerdlow, Campo et al. 2017). MPNs originate from mutant clonal HSCs, affecting both hematopoietic cells and the BM stroma. Pathological features of MPNs were successfully replicated in representative samples, showcasing significant morphological differences compared to controls. Notably, PMF samples exhibited distinctive abnormalities and the highest levels of CD271 expression, indicating characteristics of myelofibrosis (Zetterberg, Vannucchi et al. 2007; Kumar, Abbas et al. 2017; Barbui, Thiele et al. 2018). We, further, also measured distances between key cell types and bone or vessel objects in 3D surfaces, revealing significant changes in PV samples compared to matched controls, shedding light on potential MSC and HSPC interactions in disease progression. In summary, we provided a robust technique for analyzing hematopoiesis and HME anatomy in control and MPN BM. The 3D reconstruction technique highlighted spatial abnormalities in MPN patients, presenting a promising avenue for further research and understanding of disease mechanisms.

In situ spatial mRNA analysis of cytokine expression in fibrotic pediatric ALL and PMF bone marrow

In paper four, we delved into variations in cellular composition and spatial expression of the fibrotic drivers, *TGFB1* and *PDGFA1*, in the BM of patients with fibrotic ALL and PMF. Both conditions are characterized by distinct hematological abnormalities and fibrosis progression. Our findings shed light on the potential roles of these drivers in fibrosis initiation, progression, and treatment response. BM fibrosis in ALL is linked to elevated levels of therapy-resistant leukemia cells and a poorer prognosis (Norén-Nyström, Roos et al. 2008). Although the impact of fibrosis on ALL prognosis is not universally confirmed, its prevalence in a substantial proportion of B-cell ALL cases underscores its significance (Bharos, Jong et al. 2010). Utilizing a five-color panel for BM scans of standard pathology samples, we recapitulated known pathological features. PMF samples displayed atypical MK features and high levels of CD271 expression, both characteristic of myelofibrosis (Kumar, Abbas et al. 2017; Barbui, Thiele et al. 2018). ALL samples exhibited a high frequency of blast cells and a reduced number of MKs (Qian and Wen-jun 2013; Swerdlow, Campo et al. 2017). We examined the density of MSCs identified by CD271 expression, finding an increase in $CD271^+$ MSCs in both PMF and fibrotic ALL samples, particularly dense near blood vessels. This implies a potential association between MSC density and fibrosis progression, supported by literature suggesting leukemic cells can produce stromal growth factors that contribute to fibrosis progression and MSC differentiation into myofibroblasts (Tsai, White et al. 1994; Bieker, Padró et al. 2003; Schneider, Mullally et al. 2017). We investigated the spatial expression of *TGFB1* and *PDGFA1*, critical fibrotic drivers, in the BM microenvironment of ALL and PMF patients. Elevated expression of *TGFB1* and *PDGFA1* in MKs within the BM of ALL patients with varying degrees of fibrosis suggests their potential role in fibrosis initiation and progression in ALL. Understanding these fibrotic drivers could pave the way for anti-fibrotic strategies, potentially overcoming the negative effects of fibrosis on treatment outcomes. This insight could guide the development of innovative therapeutic strategies targeting both the hematological disease and its microenvironment, ultimately improving treatment outcomes and patient well-being.

Of mice and men

In our first paper, we focused on stromal cell regulation in the BM niche, and in particular how BM MSCs are regulated. The primary roles of BM MSCs are to uphold the turnover of both the BM stroma and skeletal tissues, while also supplying

essential support for hematopoiesis. We showed that *EGR1* was highly expressed in specific BMSC populations and played a crucial role in regulating hematopoietic stroma support through both soluble factors and cell-cell interactions. Here we utilized a mouse model to test human $CD34^+$ CB stem cell engraftment after co-culturing them with human BM MSCs. Even though this hints towards a similar mechanism in the human BM, we are still using immune incompetent mice as models to show engraftment of CB stem and progenitor cells. In human immunocompetent BM, the effect could be different.

The exploration of the hematopoietic system heavily relies on animal models. To evolve away from less informative mouse models a trend in recent years was the generation of ectopic human bone organoids – ossicles. These miniature BMs are implanted in mice and show the ability to host a functional human HSC niche that supports hematopoiesis. Ossicles have generated significant interest as an advanced model for investigating human-specific molecular and cellular events. The advent of more precisely defined xenotransplantation models, *in vitro* culture systems, and advanced immunophenotyping has refined our understanding of human hematopoiesis, illuminating the differences between species. Although many aspects of hematopoiesis are shared between mice and humans (Sykes and Scadden 2013), it's essential to consider several differences before applying insights gained from mice to humans (Doulatov, Notta et al. 2012).

Ossicles are a way of developing more predictive preclinical models, they are predominantly generated by injecting BM MSCs in a suitable scaffolding matrix containing more or fewer differentiation factors subcutaneously into an immune incompetent mouse (Dupard, Grigoryan et al. 2020). BM MSCs were initially suggested as the exclusive cells capable of producing functional humanized ossicles due to their distinct epigenetic endochondral signature (Reinisch, Etchart et al. 2015). However, subsequent studies have shown the endochondral formation of ectopic BM niches using different MSC sources, such as adipose tissue (Osinga, Di Maggio et al. 2016) and cord blood (Pievani, Sacchetti et al. 2017). After implantation, the maturing ossicle undergoes further humanization through the transplantation of healthy or malignant hematopoietic cells, either locally into the ossicle (intra-ossicle) or systemically by intravenous injection (tail vein or retro-orbital). Studies have consistently shown that intra-ossicle transplantation notably accelerates engraftment of both healthy (achieving up to 90% hCD45 chimerism) and malignant hematopoietic cells. More recently, this transplantation approach has been extended to include samples from conditions like PMF, which traditionally face challenges in engrafting within the mouse BM (Reinisch, Thomas et al. 2016).

With the ability to utilize patient-specific or genetically altered MSCs for ossicle generation, this approach shows promising possibilities to probe HME interactions with healthy and malignant HSCs in a humanized environment. Ossicles are already coming closer to the human setting since MSC-derived stroma and injected HSCs are both of human origin. However, the microenvironment is deficient in immune

components and both the nervous system as well as the vasculature originate from mice (Bourgine, Fritsch et al. 2019).

There is a strong interest in identifying alternatives to animal testing. It is not only costly, time-consuming, and ethically challenging, but it often yields results that do not accurately predict outcomes in human clinical trials (Seok, Warren et al. 2013; Golding, Khurana et al. 2018). Organ-on-a-chip microfluidic culture devices represent a recent breakthrough in the quest for *in vitro* human microphysiological systems that replicate organ-level and even organism-level functions. These microfluidic microphysiological systems come in various dimensions and configurations. However, they all comprise hollow channels lined by living cells and tissues cultured under dynamic fluid flow. Certain devices mimic organ-level structures (e.g., tissue–tissue interfaces) and offer mechanical stimuli (e.g., breathing and peristalsis-like motions) essential for accurately modeling organ physiology and disease conditions. By connecting two or more organ chips through fluidic channels, human multi-organ systems can be developed to simulate whole-body physiology as well as drug distribution and metabolism. Progress in stem cell technology, including induced pluripotent stem cells and organoids, has facilitated the procurement of patient-specific stem cells. These stem cells can now be incorporated and specialized within organ chips, establishing patient-specific preclinical models (Ingber 2022).

Recently, several approaches to generating functional bone-marrow-on-a-chip could be seen. A 3D coculture model utilizing a hydroxyapatite-coated zirconium oxide scaffold incorporating MSCs and HSPCs derived from cord blood allowed for the successful culture of HSPCs for up to 28 days within a microfluidic multiorgan chip (Sieber, Wirth et al. 2018). Another BM chip was engineered by integrating human $CD34^+$ hematopoietic cells along with stromal cells from the BM embedded within a 3D ECM gel, isolated from another organ chip lined with ECs. The chip successfully simulated the differentiation and maturation of various blood cell types for one month in culture. It also replicated myeloerythroid toxicities triggered by exposure to clinically significant doses of the cancer drug 5-fluorouracil and γ-radiation (Chou, Frismantas et al. 2020). Lastly, a 3D *in vitro* model of human BM was introduced, encompassing both the perivascular and endosteal niches and featuring dynamic, perfusable vascular networks. The study demonstrated the model's ability to emulate *in vivo* BM function including maintenance and differentiation of $CD34^+$ HPSCs, the egress of neutrophils, and niche-specific responses to doxorubicin and granulocyte-colony-stimulating factor (Glaser, Curtis et al. 2022). These groundbreaking studies showcase the potential of human BM chips to address diverse questions in human HME biology and pathology. They outperform previous culture systems and mouse models, closely mimicking human pathophysiology and predicting clinical responses to therapeutics. Additionally, they enable the use of patient-specific cells, a stride toward personalized medicine, enabling very specific inquiries.

The mentioned methods advance a physiologically accurate study of the human BM microenvironment. They reduce reliance on mouse studies and enhance findings by focusing on specific interactions. These efforts are pivotal, promising more meaningful and clinically applicable studies in the future. The insights gained will expedite treatment discovery, accelerating clinical studies from the bench to the bedside.

A new frontier – the **human** bone marrow microenvironment

Our understanding of the spatial structure and compartmentalization of the BM niche primarily arises from investigations in diverse model organisms and animals. Due to challenges in obtaining human BM biopsies and the limitations in utilizing knockout or lineage tracing models, research often relies on readily accessible mouse models. However, investigations of the human BM niche would offer valuable insights into numerous human diseases, hematological disorders, and malignancies, in which an altered microenvironment is implicated.

Our present comprehension of the human BM microenvironment primarily relies on BM aspirations and standard pathology-prepared trephine biopsies, often stained for a maximum of two markers. While aspirations coupled with FACS and scRNAseq have revealed valuable insights into non-hematopoietic stromal populations and their alterations during malignancy (de Jong, Kellermayer et al. 2021; Triana, Vonficht et al. 2021), they lack a crucial aspect: spatial organization. In paper two, we could infer interactions between stromal and hematopoietic cells within the BM by combining scRNAseq and *in silico* interaction prediction. However, without functional studies and thorough spatial investigations, these interactions remain predictions.

IF staining of human trephine biopsies provides a means to investigate spatial interactions at a cellular level. However, the standard thin 5 µm cut slides limit volumetric analysis, and the restricted availability of host species for IF primary antibodies hinders the potential for multiplexing, crucial in the complex cellular milieu of the BM. Currently, 3D BM analysis using IF has been reported exclusively in mice, such as the work by Gomariz et al., utilizing clearing medium, confocal imaging, and genetic cell labeling (Gomariz, Helbling et al. 2018). In paper 3 we devised a method to do multiplex IF with HME markers, employing sequential staining and bleaching. Combined with scanning IF microscopy and advanced imaging analysis and stacking, this approach enables 3D reconstitutions and utilization of Blender for analyzing cell distributions and spatial relationships in three dimensions. While this approach offers the possibilities of 3D spatial analysis in readily available BM samples it is very labor and time intensive. There are

alternative methods for creating 3D BM volumes, such as deep penetrating confocal microscopy or light sheet microscopy. Confocal microscopy offers precise z-stepping and can produce an undisturbed 3D volume with similar thickness (~50µm), but it involves longer scanning times and necessitates adjustments in sample preparation for thicker slides. On the other hand, light sheet microscopy enables the scanning of larger 3D volumes quickly. However, it presents technical challenges in terms of clearing the sample before imaging and re-implementing staining and bleaching steps, especially with our sequential method.

ScRNAseq provides mRNA expression data at a cellular level, while IF stainings offer insights into (surface) marker expression, giving a glimpse into proteomics. In paper 4, we integrated both types of information by using multiplexed IF images with RNAscope probes that were also IF labeled. This allowed us to identify stromal cellular identity and mRNA expression of fibrosis-related genes simultaneously. With higher multiplexing and in combination with our 3D volume generation technique we could generate highly relevant spatial data in healthy and diseased BM and shed light on cellular interactions in disease initiation and progression.

Exploratory approaches utilizing comprehensive probe panel methods like Xenium from 10X or the Nanostring nCounter platform can provide valuable insights beyond targeted approaches like RNAscope. *In situ* sequencing methods like Visium from 10X or the non-commercial Slide-seqV2 (Stickels, Murray et al. 2021) offer an unbiased approach, albeit at the expense of resolution. A recent pre-publication on bioRxiv showcased a combination of Xenium, Visium, and scRNAseq from the same FFPE block, resulting in high-resolution mapping of the tumor environment in breast cell cancer. This integration of technologies offers in-depth molecular signatures crucial for understanding microenvironment heterogeneity, paving the way for advancements in oncology research, diagnostics, and therapeutics (Janesick, Shelansky et al. 2022).

Using bone samples for on-slide biochemical assays like Visium presents challenges related to tissue detachment and low RNA content, especially in human bone samples. This can lead to spatially deficient, less complex, and less sensitive data, making bone a challenging tissue for analysis according to 10X. Decalcification is necessary for histological, immunological, and molecular studies on bone tissue, but it results in RNA fragmentation. EDTA-based decalcification methods are preferred for preserving nucleic acid quality over acid-based methods like formic, hydrochloric, and nitric acids (Singh, Salunga et al. 2013; Zheng, Lin et al. 2016).

Advanced spatial high-plex methods, particularly in three dimensions, are predestined to provide potent insights into the HME and its interplay with HSCs in the future. These insights hold the potential to drive innovative treatments that target the microenvironment and its role in disease initiation and progression.

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I heard people often say their Ph.D. studies were the best time of their lives, but I am not sure I can agree. Even though I liked the new challenges and the elevated scientific environment I was coming to in Sweden, the actual work turned out to be harder and often more frustrating than I had hoped. I am happy that I survived the process and for the most part, kept my sanity. Of course, I would have never managed this enormous feat alone, and that is why it is time for a long list of acknowledgments. As the social animal that I am friends, colleagues, peers, and family were always a very important aspect of my life and made my time in Sweden if not the best time at least a very good and memorable time of my life.

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The microenvironment for my Ph.D. was B12 here I got a lot of cues, not just for senescence, but also to proliferate the fun. Before the great calamity, we had a lot of parties for example midsummer celebrations in **Giulia**'s house, where we had tons of fun and drinks. I also happily think back to parties in my dorm room in

Smålands nation, the place where I pierced **Maria**'s and **Serena**'s ears. The usual guests were **Serena**, **Roberto**, **Niklas**, **Mikael**, **Rebecca**, **Magda**, **Nicola**, **Gladys**, **Parasha**, **Hugo**, **Sveta,** and **Maria**. I had the best ASH with **Maria**, **Mikael**, **Rebecca**, **Mojgan**, and **Aurelie** in Orlando, I am still thinking back on our glorious water ride idea at Universal Studios and our good times with Limp Dick. I was always delighted about **Magda**'s cheerful and uplifting spirit and glad to have tasted all her amazing cakes. I am looking forward to going dirt-biking with **Mikael** again. I am very happy to not have lost contact with **Maria** and **Rasmus** even when they moved to York, and I am looking forward to having them closer again soon. I am still amazed at **Parasha**'s beer-drinking pace and hope he will get Nygen running well enough without him so we can have a beer again. After the long social-distancing break it felt like social life on B12 died down a bit, that's why I am even more delighted to have a new social group established on the floor. Having a whisky evening brought us together again and sometimes even **Anna K.** joined. The first time I joined the esteemed 750 work-out group was the last time **Jonas** was leading it, now **Johanna** has taken over and I am very happy she keeps me motivated to keep my body fat at a reasonable level. The constant comparison of smartwatch values with **Mo** keeps the competition alive, and a sauna beer can also never hurt. All the love to the gym group: **Johanna**, **Sara**, **Juliane**, **Elena**, **Mo**, **Ganna**, **Anna**, **Roger**, **Marija**, **Stefan L,** and **Els**. I loved our floor-wide Mario Kart tournament, it brought out the best in people, especially in **Oscar** and **Rasmus**. I am glad about all the recent help on several occasions in cleaning out the contents of my bar where **Johanna** and **Sara** were of great help, they also together with **Juliane**, **Elena**, **Rasmus**, **Oscar**, **Niklas**, **Iran**, and a lot of other people helped to turn my probably last Stem Cell Center retreat into a great party. Generally, B12 was a very nice environment: **Shamit** helped to keep the spirit high at the lunch table with great anecdotes. **Anna F** was always there for all questions especially if I had to do my scarce FACS runs. **Göran** was maybe not the best co-supervisor but I got the biggest amount of toilet paper in my life from him. **Elena** might give me the best cheese at some point and was the best cat sitter one could ask for. **Dang** was always good for a sarcastic comment and a kind word, being understanding of what a Ph.D. student has to go through. **Mina** always had kind words for me and taught me why not to buy bread from Mormors. **Mojgan** always believed that I would manage to write this book and now it looks like I actually managed. Everyone else contributed to a good floor climate and a good time at Christmas parties and Friday breakfasts.

Outside of B12 in the greater BMC, I also met a lot of dear friends and wonderful peers in Ph.D. courses, seminars, Stem Cell Center retreats, and sometimes just random bars. **Juliane** was something of a social hub after the Corona years, through her I met lovely people like **Martino**, **Marija**, **Fabio**, **Evelyn**, and **Iran** at dinner parties and birthdays. Her body doubling while writing this thesis was crucial and I am very happy that she agreed to be my mistress of toast. **Franzi**s' new lab brought new blood invading our lab space offering opportunities for chatting and welcome distraction. **Anna N** and **Magareta** were always a light in a dark lab day. **Claire**

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I was one of those Ph.D. students who managed to have a social life outside of the BMC. When I came here I already knew from my internship that the best people in Lund live at Smålands, so naturally, I moved there. I had a great time with **Teres**, **Axel**, **Viktor**, **Mia**, **Kim**, **Victoria**, **Alex**, **Pontus**, **Tim**, and a lot of others. **Teres** is the coolest person and double mother I know. **Mia** and **Viktor** were always great to hang out, hike, and drink lots of beers. With **Victoria**, I still have to build a raft for the Malmö canals. Smålands is also where I got to know my best dumpster diving buddies, **Milton** and **Julia**, I am glad to have them as neighbors in Knästorp now and the possibility to brunch often and long. Speaking of the famous place, in Knästorp I also had **Clemens** and **Anna** as neighbors, fellow Germans with the best skin Fernet. Now they moved to the woods but are always welcoming at their new house and I am looking forward to fixing my motor bike with the help of **Clemens**' TIG welder. Bike Kitchen is a place where I spend a lot of free time volunteering to help people fix their bikes and also started a lot of own bike projects. I got to know many of my fellow bike-fixing friends including **Clemens** and most recently **Frederik** there. At some point I also tried to learn Swedish, I gave up quickly, but I got to know **Wietze** there, and we had a lot of fun while not learning Swedish.

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