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UNDERSTANDING THE ROLE OF BONE MARROW NICHE IN MYELOID MALIGNANCIES

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Understanding the role of bone marrow niche in myeloid malignancies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents

ABSTRACT

Normal hematopoiesis is generated and maintained by rare hematopoietic stem cells (HSCs) through their capacity to self-renew and differentiate. This process is rigorously controlled, both by HSC-intrinsic molecular programs and extrinsic signals emitted by the local bone marrow (BM) microenvironment, the so-called HSC niche.

The BM niche consists of many cellular elements, including mesenchymal stem cells (MSCs), and soluble factors secreted by the cells. The niche homeostasis is critical for maintenance of normal hematopoiesis, and disruption of this BM niche may lead to malignant hematopoiesis, including leukemia. On the other hand, once malignant hematopoiesis is established, the niche structure and composition can be altered to protect leukemia-initiating stem cell (LSC). The aims of the presented thesis were to investigate the role of the BM niche in development of myeloid malignancies.

In study **I**, we analyzed expression of leukotriene (LT) signaling molecules in LSCs derived from chronic myeloid leukemia (CML) patients, and tested their response to pharmacological inhibition of LT signaling. By using single cell PCR, we found only low expression of *ALOX5* in patient BCR-ABL^{+} LSCs and BCR-ABL^{-} HSCs. Moreover, in contrast to previous observations in mice and in liquid cultures *in vitro*, pharmacological inhibition of *ALOX5* did not result in any significant growth suppression of CML LSCs in long-term culture initiating cell (LTC-IC) assay on a stromal cell layer. Furthermore, although expression of *CYSLT1* was detected in the majority of analyzed LSCs, treatment with its antagonist, montelukast, did not significantly reduce the LTC-IC activity of LSCs. Thus, these results suggest that pharmacological inhibition of the LT pathway might not be sufficient to eradicate LSCs, particularly in the presence of BM stromal cells.

In study **II**, we investigated the role of BM niche in pathogenesis of MDS/MPN by using a *Sipa1*$^{-/-}$ mouse model. We found that *Sipa1* was expressed in BM stromal cells from mice and healthy humans, but was downregulated in these cells from patients with MPN and MDS/MPN. Additionally, *Sipa1* deficiency in mice led to phenotypical and functional alterations in the BM cellular niche prior to disease development, and reciprocal transplantation experiments further confirmed that *Sipa1*$^{-/-}$ BM niche was a prerequisite for MDS/MPN development. Moreover, RNA sequencing analysis showed dysregulated expression of inflammatory cytokines and growth factors in the BM stromal cells from young, disease-free *Sipa1*$^{-/-}$ mice. Altogether, our data suggest that *Sipa1* expression in the BM stromal cells is critical for maintaining BM niche homeostasis, and that *Sipa1* deficiency in BM niche plays an instructive role in development of MDS/MPN in mice.

Finally in study **III**, we prospectively characterized BM stromal cells in newly-diagnosed patients with CML. First of all, we discovered that patient's BM stromal cells share similar immunophenotype as normal BM (NBM) counterparts, but that the CML BM niche composition was changed, showing increased frequency of endothelial cells. Moreover, we found alterations in functional properties of CML-derived MSCs, e.g. an impaired osteo-

chondrogenic differentiation potential, and an enhanced capacity to support NBM hematopoietic stem and progenitor cells *in vitro*. Even though no BCR-ABL fusion gene was detected in CML BM stromal cells, the RNA sequencing revealed cytokine dysregulation, particularly loss of CXCL14 in CML BM niche. Interestingly, restoration of CXCL14 expression in stromal cells suppressed the growth of LSCs in LTC-IC assays, but promoted their differentiation. These results indicate that CXCL14 might help to eradicate LSCs and therefore serve as a new therapeutic candidate for CML treatment.

To conclude, we herein showed that BM niche might contribute to myeloid malignancies in mice and human. Thus, targeting the dysregulated BM niche factors and the abnormal interaction between BM niche and LSCs could be a promising therapeutic approach to treat patients with myeloid malignancies.

LIST OF SCIENTIFIC PAPERS

I. **Leukotriene signaling *via* ALOX5 and cysteinyl leukotriene receptor 1 is dispensable for *in vitro* growth of $CD34^+CD38^-$ stem and progenitor cells in chronic myeloid leukemia.**
Dolinska M., Piccini A., Wong W.M., Gelali E., Johansson A.S., Klang J., Xiao P., Yektaei-Karin E., Strömberg U., Mustjoki S., Stenke L., Ekblom M., Qian H.
Biochem Biophys Res Commun, 2017, 490(2):378-384

II. ***Sipa1* deficiency-induced bone marrow niche alterations lead to the initiation of myeloproliferative neoplasm.**
Xiao P., Dolinska M., Sandhow L., Kondo M., Johansson A.S., Bouderlique T., Zhao Y., Li X., Dimitriou M., Rassidakis G.Z., Hellström-Lindberg E., Minato N., Walfridsson J., Scadden D.T., Sigvardsson M., Qian H.
Blood Adv, 2018, 2(5):534-548

III. **Prospective characterization of bone marrow niche in patients with chronic myeloid leukemia identifies new potential therapeutic targets.**
Dolinska M., Xiao P., Klang J., Bouderlique T., Li X., Kondo M., Sandhow L., Johansson A.S., Deneberg S., Söderlund S., Jädersten M., Östman A., Ungerstedt J., Mustjoki S., Stenke L., Le Blanc K., Hellström-Lindberg E., Olsson-Strömberg U., Ekblom M., Lehmann S., Sigvardsson M., Qian H.
Manuscript.

Related publications not included in the thesis

i. **A novel $Lin^-CD34^+CD38^-$ integrin $\alpha2^-$ bipotential megakaryocyte-erythrocyte progenitor population in the human bone marrow.**
Wong W.M., Dolinska M., Sigvardsson M., Ekblom M., Qian H.
Leukemia, 2016, 30(6):1399-1402.

ii. **Modulation of leukotriene signaling inhibiting cell growth in chronic myeloid leukemia.**
Yektaei-Karin E., Zovko A., Nilsson A., Nasman-Glaser B., Kanter L., Radmark O., Wallvik J., Ekblom M., Dolinska M., Qian H., and Stenke L.
Leuk Lymphoma, 2017, 58(8):1903-1913

iii. **Distinct roles of bone marrow mesenchymal stem and progenitor cells during the development of acute myeloid leukemia in mice.**
Xiao P., Sandhow L., Heshmati Y., Kondo M., Bouderlique T., Dolinska M., Johansson A.S., Sigvardsson M., Ekblom M., Walfridsson J., Qian H.
Blood Adv, 2018, 2(12): 1480-1494

CONTENTS

LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
AML	Acute myeloid leukemia
ANGPTL1	Angiopoietin like 1
ANGPTL4	Angiopoietin like 4
BM	Bone marrow
CAR cells	CXCL12-abundant reticular cells
CFU-C	Colony forming unit-cell
CFU-F	Colony forming unit-fibroblast
CML	Chronic myeloid leukemia
CNL	Chronic neutrophilic leukemia
CXCL12	CXC chemokine ligand 12
CXCL14	CXC chemokine ligand 14
CXCR4	CXC chemokine receptor type 4
FABP4	Fatty acid binding protein 4
HSC	Hematopoietic stem cell
IL	Interleukin
JAK2	Janus kinase 2
JMML	Juvenile myelomonocytic leukemia
KITL	Kit ligand
LSC	Leukemic stem cell
LSK	$Lin^{-}SCA1^{+}cKIT^{+}$
LTC-IC	Long-term culture-initiating cell
MCAM	Melanoma cell adhesion molecule
MDS	Myelodysplastic syndrome
Micro/μCT	Micro-computed tomography
MPN	Myeloproliferative neoplasm
MSC	Mesenchymal stem cell
MPC	Mesenchymal progenitor cell
Ocn	Osteocalcin
Opn/Spp1	Osteopontin

Osx/Sp7	Osterix
PDGF-BB	Platelet-derived growth factor-BB
PDGFRA/B	Platelet-derived growth factor receptor α/β
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule 1
PPARγ	Peroxisome proliferator-activated receptor γ
RANKL	Receptor activator of NFκB ligand
RapGAP	Rap GTPase-activating protein
RARγ	Retinoic acid receptor γ
SCA1	Stem cell antigen 1
SCF	Stem cell factor
SIPA1	Signal-induced proliferation-associated gene-1
TGF-α/β	Transforming growth factor α/β
TGF-β1	Transforming growth factor β1
THPO	Thrombopoietin
TKI	Tyrosine kinase inhibitor
VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

1 INTRODUCTION

1.1 NORMAL HEMATOPOIESIS

Hematopoiesis is a continuous and dynamic process of developing the diverse blood cell repertoire in the bone marrow (BM) throughout whole postnatal life. Every day, the hematopoietic system of a healthy adult produces about one trillion new blood cells with different functions. Among these are white blood cells, involved in immune response, red blood cells, responsible for oxygen and carbon dioxide transport, and platelets, necessary for coagulation (Table 1). After discovering a big variety of cell morphologies within the BM, hematopoiesis was postulated to be organized as a cellular hierarchy derived from a common ancestor - hematopoietic stem cells (HSC) (Maximow, 1909 reviewed in Doulatov et al., 2012). Thereafter, the ability to regenerate the blood system by BM transplantation into patients exposed to irradiation, further suggested the existence of blood-forming cells in the BM (Jacobson et al., 1951). However, it was not until the utilization of animal models that hematopoiesis was proven to depend on the rare cell population of HSCs. For the first time, the existence of HSCs was experimentally demonstrated by Till and McCulloch, by showing that lethally irradiated mice could be rescued by transplantation of BM cells from normal donors (Till and McCulloch, 1961). Following that, the ability of HSCs to self-renew and differentiate was proven by Osawa and colleagues in single cell transplantation experiments (Osawa et al., 1996). These early findings, as well as currently available methods of prospective isolation of HSCs (based on cell surface markers) and a range of functional assays, shaped the view of normal hematopoiesis as a differentiation hierarchy.

Blood cell type	Main function
Lymphocytes	
B cells	Regulation of humoral immunity; production of antibodies
T cells	Regulation of cellular immunity; destruction of virus-infected cells
NK cells	Cytotoxicity against virus-infected cells and selected tumor cells
Dendritic cells	Antigen presentation; link between innate and adaptive immune responses
Monocytes	
Macrophages	Effective phagocytosis of pathogens, dead cells, cell fragments and other debris within tissues
Granulocytes	
Neutrophils	Phagocytic; defense against bacterial infections and fungi; recruits other immune cells to the site of infection
Eosinophils	Phagocytic; defense against larger parasites; participate in inflammatory response of allergic reactions and asthma
Basophils	Promotes inflammation; release histamine and heparin; attract other granulocytes to an infection site
Mast cells	Modulatory role in inflammatory responses, including allergy; contribute to immune tolerance; tissue repair and angiogenesis
Erytrocytes	Transport of oxygen and carbon dioxide between tissues and lungs
Platelets	Hemostasis; blood clotting, release of growth factors for tissue healing

Table 1. The variety of mature blood cells and their main functions.

The multipotent HSCs with self-renewal capacity reside on top of such a hematopoietic "tree", while terminally differentiated mature blood cells can be found at the bottom of the hierarchy. HSCs have the ability to self-renew as well as to differentiate into hematopoietic progenitors. During differentiation, HSCs lose their self-renewal capacity and give rise to multipotent progenitors (MPPs) that retain multi-lineage potential. This classical model of hematopoiesis suggests early specification of lymphoid and myeloid lineages as the next step in developmental hierarchy. However, accumulating evidence from mouse studies indicate presence of myelo-lymphoid progenitors (MLPs), harboring potential to differentiate into both myeloid and lymphoid lineages but not megakaryocytic/erythroid (Mk/Er), suggesting that the first lineage separation occurs already between myeloid/lymphoid and Mk/Er lineages (Kohn et al., 2012; Lai and Kondo, 2006). Moreover, Mk/Er progenitors (MEPs) may bypass the lymphoid primed MPP stage and arise directly from HSCs (Adolfsson et al., 2005; Pronk et al., 2007; Yamamoto et al., 2013). Downstream of bipotent MLPs, myeloid-restricted cells named granulocyte-macrophage progenitors (GMPs) (Görgens et al., 2013) and common lymphoid progenitors (CLPs) can be found. GMPs give rise to granulocytes, monocytes and myeloid dendritic cells (Doulatov et al., 2010; Lee et al., 2015; Manz et al., 2002), while CLPs have the potential to generate B-cells, T-cells, NK cells and dendritic cells (Galy et al., 1995; Karsunky et al., 2008; Kondo et al., 1997). Moreover, recent mouse studies indicate that HSCs remain heterogenous and can be classified as HSCs with distinct lineage differentiation potentials (lineage-bias) without passing through distinct hierarchically organized progenitor populations (Carrelha et al., 2018; Ceredig et al., 2009; Eaves, 2015; Sanjuan-Pla et al., 2013; Velten et al., 2017), further suggesting that lineage commitment in the developmental hierarchy might not be as strict as initially thought (Doulatov et al., 2012; Nestorowa et al., 2016; Yamamoto et al., 2013).

This complex hematopoiesis process requires strict mechanisms regulating HSC fates, including differentiation, self-renewal, proliferation and migration. And indeed, transition in cell fate is regulated by both cell-autonomous (intrinsic) and microenvironment-derived (extrinsic) factors. The intrinsic mechanisms include: transcription factors that control gene expression (Rojas-Sutterlin et al., 2014), epigenetic regulators that control the structure and organization of DNA and chromatin (Karnati et al., 2015; Mahmud et al., 2014), molecular regulators of the cell cycle (Matsumoto and Nakayama, 2013; Pietras et al., 2011), the proteins involved in regulation of cell division (Laurenti et al., 2015; Park et al., 2014) and anti-apoptotic signals (Domen, 2000). In addition, HSCs postnatally receive signals from surrounding cells in the BM through direct cell-cell interactions and various factors secreted by the stromal cells (discussed more in section 1.3.)

Importantly, defects in this complex regulatory network, such as acquisition of genetic mutations and disruptions in important signaling pathways, may cause transformation of hematopoietic cells into malignant cells, leading to blood disorders such as leukemia (Doulatov et al., 2012). Therefore, thorough understanding of the mechanisms that regulate normal HSCs is crucial for understanding the process of leukemogenesis.

1.2 MYELOID MALIGNANCIES

Myeloid malignancies are a heterogenous collection of clonal hematopoietic disorders, disturbing myeloid blood lineages by abnormal cell expansion, abnormal differentiation, or a mixture of both. They arise as a consequence of a multistep process, involving accumulation of genetic and epigenetic lesions in HSCs and myeloid progenitors, which leads to perturbations in key processes, such as abnormal self-renewal, blockage of differentiation and excessive proliferation (Vardiman et al., 2009). The myeloid malignancies consist of acute and chronic myeloid neoplasms, that can be classified based on BM morphology and percentage of immature myeloid cells (blasts) in peripheral blood or BM (Swerdlow et al., 2017).

Acute myeloid leukemia (AML) is the common form of leukemia in adults (accounting for 80% of all acute leukemia in adults) mainly affecting people older than 60 years of age (Thein et al., 2013). It is a clinically and genetically heterogenous disease, arising from hematopoietic stem or progenitor cells that acquired numerous mutations or cytogenetic abnormalities. The treatment strategy consists of intensive induction chemotherapy, however is not very effective, with overall 5 year survival around 20% (Noone AM). AML can be distinguished from chronic myeloid malignancies by BM hypercellularity with accumulation of blasts (over 20% of nucleated cells) in the BM, and frequent anemia and thrombocytopenia (Vardiman et al., 2009).

On the other hand, chronic myeloid neoplasms are subdivided into four subgroups (Table 2): (1) myeloproliferative neoplasms (MPN), (2) myelodysplastic syndromes (MDS), (3) MDS/MPN overlap and (4) myeloid/lymphoid neoplasms with eosinophilia and rearrangements of *PDGFRA*, *PDGFRB*, or *FGFR1*, or with *PMC1-JAK2* (Arber et al., 2016). In addition, mastocytosis is no longer considered a subgroup of the MPN, and is now a separate disease category (Arber et al., 2016).

1.2.1 Myeloproliferative neoplasms

The MPNs comprise a group of clonal HSC disorders (listed in table 2), with combined incidence of six to ten cases per 100 000 people annually, that usually affect adults from 50 to 70 years of age (Vardiman et al., 2009). MPNs are characterized by abnormal proliferation and expansion of one or more myeloid cell lineages (including granulocytic, erythroid and megakaryocytic) and often present with increased blood count and BM cellularity, splenomegaly and fairly effective hematopoietic maturation. Therefore, morphology of the BM cells, except megakaryocytes, is relatively normal and the frequency of the BM blasts in chronic phase is close to normal or slightly increased (below 10%) (Vardiman et al., 2009). MPNs can be distinguished from MDS and MDS/MPN by the absence of morphologic dysplasia (including dyserythropoiesis and dysgranulopoiesis) and the lack of monocytosis (Barbui et al., 2018). Moreover, abnormalities in the BM niche, including increased angiogenesis, fibrosis and thickening of bone trabeculae are often found, suggesting potential contribution of the BM niche to pathophysiology of the diseases (Hoermann et al., 2015). This thesis focuses mainly on BCR-ABL1-positive MPN, described below in section 1.1.2.

	CHRONIC MYELOID NEOPLASMS			
	Myelodysplastic syndromes	Myelodysplastic syndromes/ myeloproliferative neoplasms overlap	Myeloproliferative neoplasms	Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*, or with *PCM1-JAK2*
Peripheral blood or bone marrow morphology	Absence of cytosis Dyserythropoiesis Dysgranulopoiesis Monocytosis Granulocytosis Thrombocytosis Erythrocytosis Eosinophilia			
Diseases	MDS with single lineage dysplasia MDS with ring sideroblasts MDS with multilineage dysplasia MDS with excess blasts MDS with isolated del(5q) MDS, unclassifiable	Chronic myelomonocytic leukemia Atypical chronic myeloid leukemia, *BCR-ABL1* negative Juvenile myelomonocytic leukemia MDS/MPN with ring sideroblasts and thrombocytosis MDS/MPN, unclassifiable	*BCR-AB1* positive: Chronic myeloid leukemia *BCR-AB1* negative: Chronic neutrophilic leukemia Polycythemia vera Primary myelofibrosis Essential thrombocythemia Chronic eosinophilic leukemia, not otherwise specified (NOS) MPN, unclassifiable	Myeloid/lymphoid neoplasms with *PDGFRA* rearrangement Myeloid/lymphoid neoplasms with *PDGFRB* rearrangement Myeloid/lymphoid neoplasms with *FGFR1* rearrangement Myeloid/lymphoid neoplasms with *PCM1-JAK2*

Table 2. Classification of chronic myeloid neoplasms and clinical presentation. Adapted from Tefferi and Pardanani, 2015; Arber et al., 2016; Barbui et al., 2018.

1.2.2 Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a MPN characterized by uncontrolled expansion and accumulation of myeloid cells in the BM (Figure 1), blood and other organs like the spleen. The disease accounts for overall incidence of one to two cases per 100 000 and constitutes around 15% of adult leukemia (Rohrbacher and Hasford, 2009). Clinically, the course of the disease includes three phases, characterized by increasing frequency of the blasts in blood or BM: chronic (<10%), accelerated (10-19%) and acute leukemia-like blast crisis (≥ 20%). The vast majority of the patients are diagnosed in the chronic phase, when white blood cell count is increased. At that time, patients can present with fatigue, weight loss, night sweats, palpable splenomegaly and anemia, or shown no noticeable symptoms. However a small group of the patients are diagnosed during the accelerated phase or acute leukemia-like blast crisis (Swerdlow et al., 2017).

CML is caused by a reciprocal translocation in HSCs, between the Abelson (ABL) gene on chromosome 9 (region q34) and the Breakpoint Cluster Region (BCR) on chromosome 22 (region q11). This t(9;22) translocation (also known as the Philadelphia chromosome) results in the *BCR-ABL1* oncogene, encoding a constitutively active tyrosine kinase, and causes the transformation of HSCs into leukemic stem cells (LSCs) (Deininger et al., 2000). Given that tyrosine kinase ABL plays an important role in controlling the cell cycle, apoptosis and interactions of hematopoietic cells with the BM niche, the fusion gene *BCR-ABL1* deregulates proliferation, apoptosis, differentiation and adhesion of LSCs. Moreover, the fusion tyrosine kinase activity modulates response to DNA damage and extends activation of cell cycle checkpoints when exposed to chemotherapeutic drugs, prolonging time for DNA repair. *BCR-ABL1* expression can also facilitate genomic instability, possibly by generation of reactive oxygen species (ROS) and enhanced spontaneous DNA damage in tumor cells (Penserga and Skorski, 2006). The genomic instability and accumulation of new genetic aberrations might in turn lead to acquired drug resistance and malignant progression (Goldman and Melo, 2008).

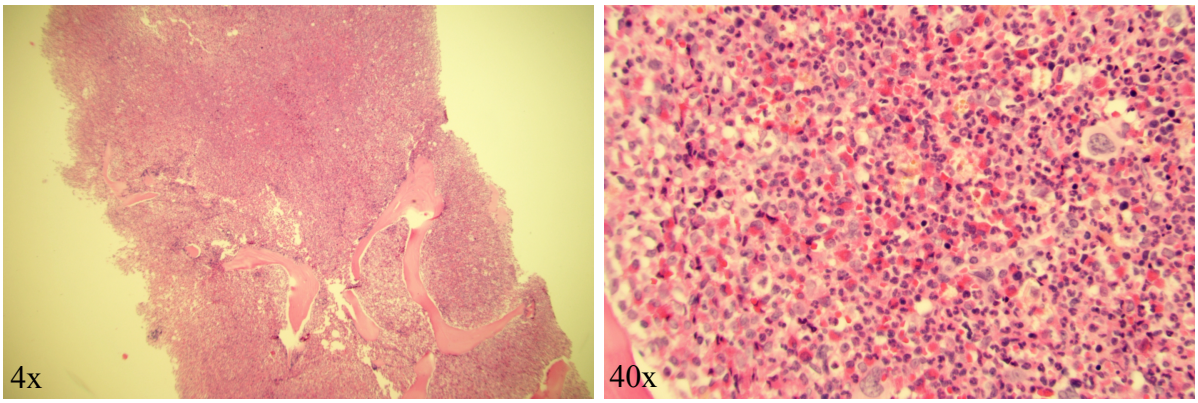

Figure 1. The BM biopsy of CML patient at diagnosis (chronic phase) showing hypercellularity with increased granulopoiesis, eosinophilia and dysplastic megakaryocytes. Pictures courtesy of Monika Klimkowska.

Development of imatinib – a tyrosine kinase inhibitor (TKI) – revolutionized CML treatment, as the majority of treated patients achieve clinical remission and dramatic reduction in *BCR-*

ABL1 transcript level (also referred to as molecular remission) (Druker et al., 2001; Hughes et al., 2003). Imatinib effectively targets cycling BCR-ABL1$^+$ leukemic cells, but not quiescent LSCs with leukemia-initiating capacity (Corbin et al., 2011). As a result, BCR-ABL1$^+$ LSCs persist in the BM of patients who are in deep remission and may contribute to molecular relapse after treatment cessation (Chomel et al., 2016; Chu et al., 2011). Thus, most of the CML patients in the chronic phase are not cured and require a life-long TKI therapy (Saussele et al., 2018). To achieve treatment-free remission, it is important to better understand and target additional pathways responsible for LSC retention.

Studies have shown that survival of CML LSCs does not depend on BCR-ABL1 kinase activity, which suggests that other mechanisms might contribute to LSC persistence (summarized in Figure 2) (Hamilton et al., 2012).

One of the pathways related to LSC resistance is PI3K/AKT/FOXO, as BCR-ABL1 upregulates PI3K/AKT signaling and leads to AKT-mediated phosphorylation of FOXO transcription factors, which results in their cytoplasmic localization and inactivation. Consequently, inhibition of BCR-ABL1 activity by TKI downregulates PI3K/AKT signaling in LSCs, and leads to re-localization of FOXO to the nucleus, where it can actively regulate transcription of BCL6. This is important, as the aberrant upregulation of BCL6 confers protective, pro-survival effect to CML cells by repressing the tumor suppressor p53 and the cell cycle inhibitor CDKNA2A (also known as ARF) (Hurtz et al., 2011; Pellicano et al., 2014). In addition, TGF-β-FOXO axis has been shown to maintain LSCs, whereas blocking of the TGF-β pathway seems to reverse the nuclear localization of FOXO and inhibits clonogenic activity of LSCs (Naka et al., 2010; Pellicano and Holyoake, 2011).

The Hedgehog pathway, specifically, activation of smoothened (SMO), a G-Protein-Coupled Receptor, has also been implicated in regulation of LSC self-renewal and proliferation. SMO activation causes activation of another transcription factor – GLI1, which in turn leads to degradation of TP53. Repression of p53 targets results in reduced apoptotic responses and possibly cell-cycle arrest in CML (Abraham et al., 2016). In line with that, deletion of SMO, as well as its pharmacological inhibition in a CML mouse model, resulted in loss of LSCs (Dierks et al., 2008; Irvine et al., 2016; Zhao et al., 2009).

Several lines of evidence indicates the JAK/STAT pathway as an important player in the regulation of LSC survival (Traer et al., 2012). It has been shown that pharmacological inhibition of JAK2 results in a loss of LSC *in vitro* and *in vivo* (Gallipoli et al., 2014). Additionally, a heterozygous mutation in STAT5a attenuates the CML-like disease in mouse models (Ye et al., 2006), while RNA inhibition of STAT5 impairs BCR-ABL1$^+$ colony formation (Scherr et al., 2006). Activation of STAT3 has also been implicated to have a protective effect on CML cells after exposure to TKI (Bewry et al., 2008), and it has been shown that combined inhibition of BCR-ABL1 and STAT3 leads to the loss of otherwise resistant LSCs (Eiring et al., 2015).

Moreover, Wnt signaling and its key mediator – β-catenin, are involved in LSC survival. TKI therapy-related upregulation of CD27 signaling has been shown to result in re-localization of β-catenin to the nucleus and activation of NOTCH and c-MYC genes (Riether et al., 2015; Schürch et al., 2012). In agreement, loss of β-catenin in a CML mouse model causes inhibition of LSC self-renewal and impairs development of the disease (Zhao et al., 2007). Similarly, pharmacological inhibition of β-catenin combined with TKI treatment leads to the loss of LSCs (Heidel et al., 2012). In addition, β-catenin levels can be regulated by enzymes involved in fatty acid metabolism, namely arachidonate 5- and 15-lipoxygenase (5-LO and 15-LO encoded by *Alox5* and *Alox15,* respectively), which have been found to be upregulated in LSCs (Chen et al., 2009). Deficiency of *Alox5* or *Alox15*, as well as their pharmacological inhibition, affects division and apoptosis of LSCs, and attenuates CML onset in mice (Chen et al., 2009). Moreover, combining TKI and a 15-LO inhibitor seems to have synergistic effect on human LSCs in liquid culture *in vitro*, possibly mediated through the P-selectin SELP, which is otherwise repressed to promote LSC survival (Chen et al., 2014). However, treatment with the 5-LO inhibitor, zileuton, seems to have only minor effect on survival of human LSCs in presence of stromal cells *in vitro* (Dolinska et al., 2017), suggesting a BM niche-mediated mechanism of the drug resistance.

Although abovementioned mechanisms were initially thought to be cell-autonomous, there is an increasing evidence suggesting an important role of BM niche in the pathogenesis of myeloid neoplasms, as well as its contribution to drug resistance (as described in section 1.4).

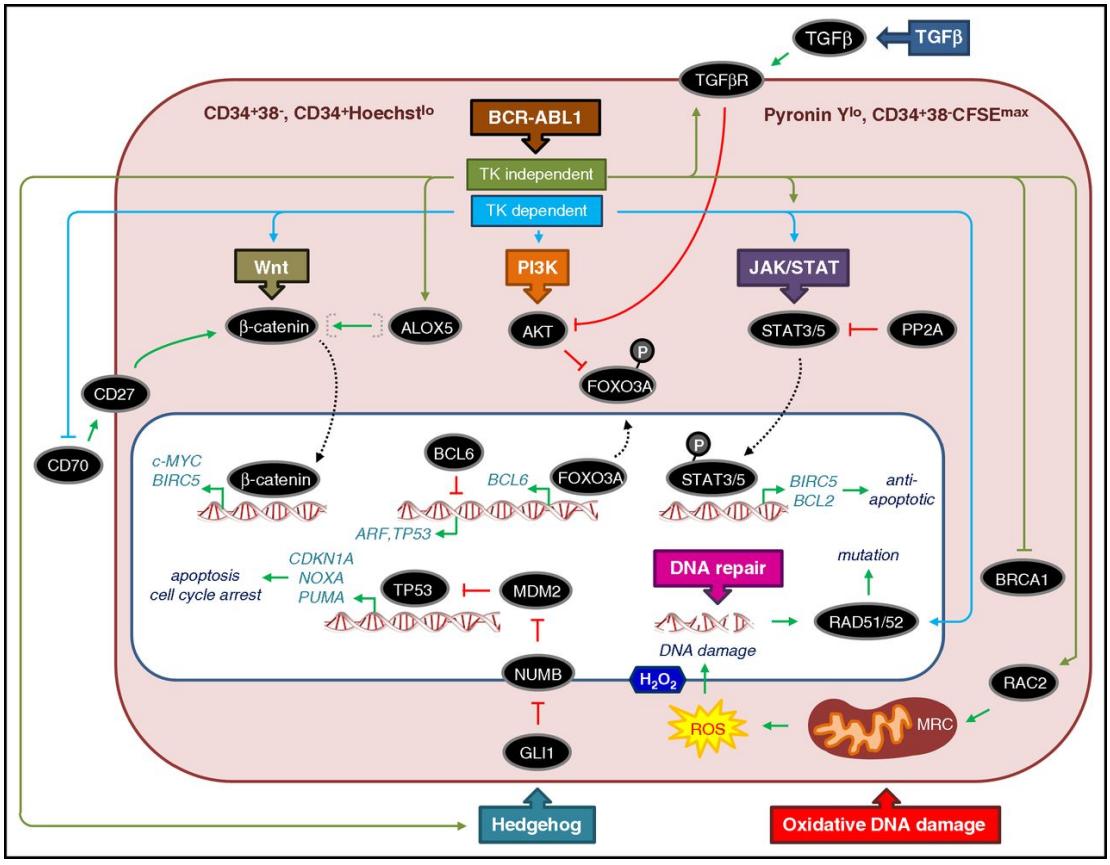

Figure 2. Critical pathways that contribute to CML LSCs persistence. Originally published by Holyoake and Vetrie, 2017, and reprinted with permission from American Society of Hematology.

1.3 THE BM MICROENVIRONMENT IN NORMAL HEMATOPOIESIS

As mentioned before, hematopoiesis is tightly regulated by autonomous (cell-intrinsic) mechanisms, but also, and to large extent by non-autonomous (extrinsic) signals derived from the BM microenvironment, also called the BM niche (Zon, 2008). The niche signals can in turn trigger cell intrinsic mechanisms that may lead to alterations of HSC fates. The concept of HSC niche was first suggested by Schofield, who defined it as a specialized microenvironment within bone cavity that supports HSCs and maintains their functions (Schofield, 1978). Accumulated evidence from mouse studies demonstrates that the BM niche is critical for regulation of HSC fates, including differentiation, self-renewal, quiescence and migration by production of soluble factors and adhesive interaction (Morrison and Scadden, 2014; Yu and Scadden, 2016). Up to date, many cellular components of the BM niche have been identified, such as mesenchymal stem cells, osteoblasts, endothelial cells, adipocytes, sympathetic nervous system, megakaryocytes, macrophages and more (Figure 3) (Morrison and Scadden, 2014; Park et al., 2012b). In addition to extracellular matrix proteins, numerous cytokines and growth factors, as well as factors such as oxygen and mineral concentrations, have been indicated to play an important role in the interaction between HSCs and other cells present within the BM niche (Camacho et al., 2017; Hoggatt, 2016). The BM HSC niche has been categorized based on the respective cellular niche component, as described below.

1.3.1 Hypoxia

The physiological condition of the BM niche is generally considered to be hypoxic (Spencer et al., 2014), raising the possibility that low oxygen tension is important for maintaining HSCs (Eliasson and Jönsson, 2010). Several studies show that low oxygenic-niche derived BM hematopoietic cells have higher repopulation capacity (Parmar et al., 2007; Winkler et al., 2010a) and that pre-incubation of HSCs and progenitor cells in hypoxia has favorable effect on the proliferation of mouse BM-derived colony-forming cells (Eliasson et al., 2010), providing evidence for the important role of oxygen tension for HSCs activity. Moreover, it has been shown that hypoxia regulates HSCs through stabilization of hypoxia-inducible factor 1α (HIF-1α) (Semenza, 2014), which acts as transcriptional regulator of numerous genes important for hematopoiesis. HIF-1α regulates proliferation and survival (Lin et al., 2006), homing (through the CXCL12-CXCR4 axis) (Ceradini et al., 2004), secretion of angiogenic factors, like VEGF (Levy et al., 1995), and protection of hematopoietic cells from damage caused by accumulation of ROS (Jang and Sharkis, 2007; Kirito et al., 2009). In a mouse model, conditional deletion of *Hif1α* leads to progressive loss of long term quiescent HSCs, which is associated with loss of HSCs under various stress conditions (Takubo et al., 2010). Similarly, *Hif1α*$^{-/-}$ mice show a selective loss of quiescent endosteal HSCs expressing GRP78 (Miharada et al., 2011). Similarly, deficiency of aryl hydrocarbon receptor nuclear translocator (ARNT), an important regulator of hypoxia-related factors, leads to HSCs apoptosis (Krock et al., 2015). Taken together, these studies suggest that hypoxia might play an important role in maintaining HSCs features. However, to improve the therapeutic expansion of HSCs, we need to learn more about the molecular mechanisms of hypoxia-mediated regulation.

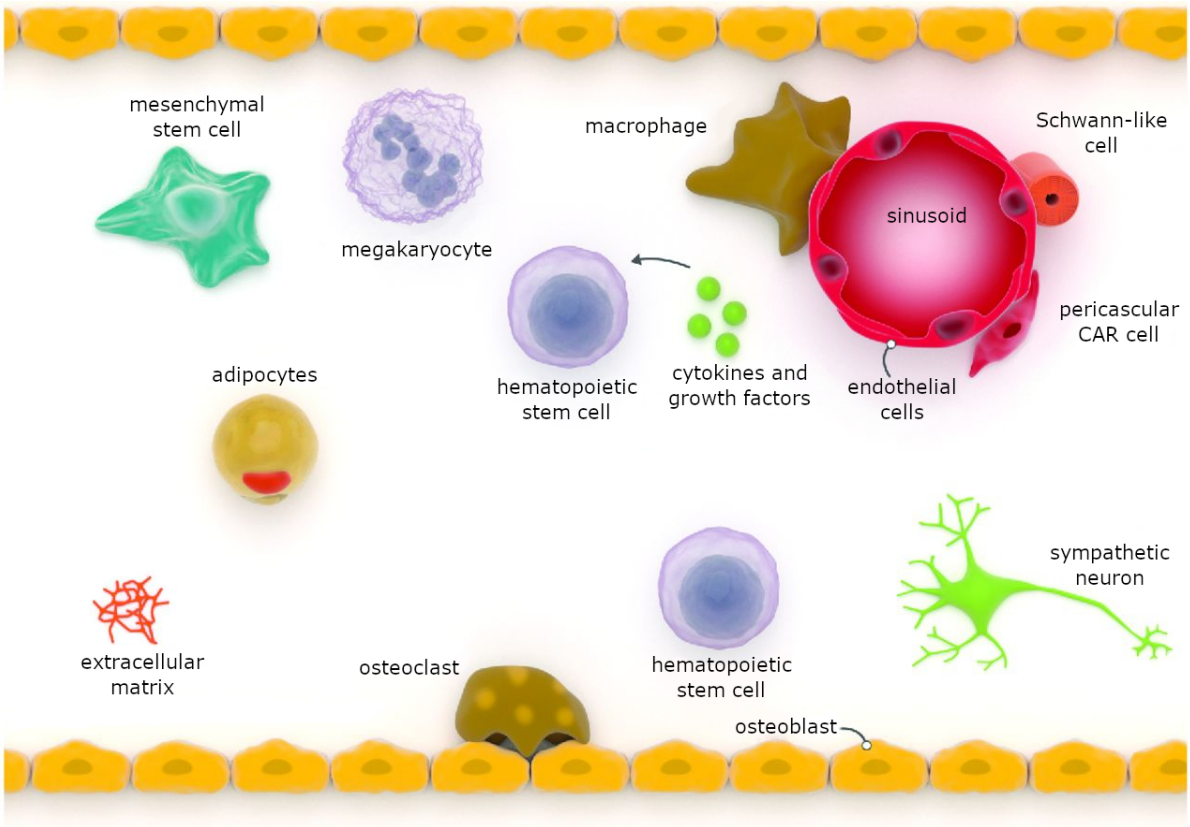

Figure 3. The BM niche is composed of multiple different cell types that contribute to the regulation of hematopoiesis. Modified, originally published by Krause and Scadden, 2015, and reprinted with permission from Hematologica; copyright Ferrata Storti Foundation.

1.3.2 Mesenchymal Stem Cells

After the discovery of HSCs, the adult BM was shown to contain another population of multipotent cells. In the series of studies, Friedenstein et al. demonstrated that rodent BM contains plastic-adherent cells with the ability to form fibroblastoid colonies, differentiate into bones and reconstitute the hematopoietic niche *in vivo* after subcutaneous transplantation (Friedenstein et al., 1968). Subsequently, these adherent BM cells were shown to support hematopoietic cells in long term cultures *in vitro* (Dexter et al., 1977). Similar cells, with the capacity to form fibroblast-like colonies (CFU-F) (Castro-Malaspina et al., 1980) and differentiate into osteogenic, adipogenic and chondrogenic lineages *in vitro*, were also detected in human BM aspirates (Gartner and Kaplan, 1980). However, due to their undefined features, these cells were not termed as Mesenchymal Stem Cells (MSCs) until 1991 (Caplan, 1991). Later on, the *in vivo* differentiation, but not self-renewal, of human BM-derived MSCs, was formally demonstrated by transplantation of single colony-derived BM MSCs into immunodeficient mice where they formed bones (Kuznetsov et al., 1997). Nevertheless, the stem cell identity of the retrospectively defined mesenchymal cells with CFU-F capacity remains very poorly defined. Therefore, the International Society for Cellular Therapy (ISCT) has recommended the term *Multipotent Mesenchymal Stromal Cells* to describe plastic-adherent, CFU-F forming cells, thereby keeping the acronym MSC in use (Horwitz et al., 2005). The ISCT has also introduced minimal criteria to define human MSCs, including: 1) plastic adherence under standard conditions; 2) ability to differentiate into osteoblasts,

adipocytes and chondrocytes; 3) expression of CD105, CD73 and CD90 surface markers as well as 4) lack of expression of hematopoietic markers such as CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR (Dominici et al., 2006). However, these criteria have not been sufficient to fully understand functions of different MSC populations *in vivo*, particularly because *in vitro* cultivation alters both, the immunophenotype and the global gene expression profile of freshly isolated, native MSCs (Qian et al., 2012; Tormin et al., 2011; Churchman et al., 2012; Quirici et al., 2002; Harichandan et al., 2013). Therefore, the prospectively isolated and characterized cells might better represent native MSCs *in vivo*.

Prospective isolation of human MSCs using cell surface markers

Different markers have been described to label human MSCs in the BM. These markers can be used either alone, or in combination with others, to prospectively isolate MSCs.

CD271, also known as The Low Affinity Nerve Growth Factor Receptor (LNGFR), is expressed by BM stromal cells adjacent to sinusoids (Cattoretti et al., 1993) and therefore it is used to identify MSCs. $CD271^+$ cells are capable to form CFU-F (Quirici et al., 2002) and to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Jones et al., 2006). Moreover, freshly isolated $CD271^+$ cells express *CXCL12*, *ANGPTL1*, *SP7* and *FABP4*, suggesting their multilineage potency and hematopoiesis supportive properties *in vivo* (Churchman et al., 2012).

CD146, known as Melanoma Cell Adhesion Molecule (MCAM), is also expressed by BM MSCs that form CFU-F, self-renew and provide support for hematopoietic cells when transplanted as heterotopic ossicles into immunocompromised mice (Sacchetti et al., 2007; Tormin et al., 2011).

CD44 Within the non-hematopoietic and non-endothelial population of adult BM, primitive and highly clonogenic MSCs can be distinguished based on lack of the CD44 expression, while more mature cells, which are unable to form CFU-F, are $CD44^+$ (Qian et al., 2012).

Others In addition to the markers described above, other studies have suggested CD90 and CD106 to be expressed by human BM MSCs (Mabuchi et al., 2013). Moreover, co-expression of CD146 and CD105 (Isern et al., 2013), or CD140A, CD51 and Nestin (Pinho et al., 2013), marks MSCs with clonogenic capacity, multilineage differentiation potential and the capacity to support HSC expansion *in vitro*. However, the immunophenotype of MSCs might be developmentally regulated. For example, the $CD140A^+CD51^+$ seems to mark MSCs in fetal BM, whereas the $CD140A^-$ stromal cells are enriched for CFU-F in adult BM (Li et al., 2014).

Prospective isolation of murine MSCs using cell surface markers

There are several markers suggested to label MSCs in mouse BM, which can be used to prospectively isolate MSCs and mesenchymal progenitors (Figure 4).

CD140A and SCA-1 First reports show that, within the non-hematopoietic population, $CD140A^+SCA\text{-}1^+$ cells (PαS) have MSC characteristics, including CFU-F activity and

multilineage differentiation potential *in vitro*, as well as self-renewal and differentiation capacity *in vivo*. Additionally, PαS cells show high expression of genes important for HSC regulation, including *Cxcl12* and *Angptl1*, further supporting their role in the BM niche. Interestingly, the single-positive $SCA1^+$ or double-negative ($CD140A^-SCA\text{-}1^-$) populations seem to consist of more committed progenitor cells with restricted differentiation capacity (Morikawa et al., 2009a; Morikawa et al., 2009b).

CD44 Just like in human, native MSCs in murine BM can be identified based on lack of CD44 expression. The $CD44^-$ MSCs show high clonogenic activity, trilineage differentiation potential *in vitro* and expression of genes involved in regulation of HSC fate, such as *Angptl1*, *Nestin*, *Col1a1*, *Fmod* and *Nov* (Qian et al., 2012).

CD105 Moreover, CD105 expression labels a population with self-renewal potential and the ability to transfer the BM niche after transplantation under kidney capsule of adult mice (Chan et al., 2013). However, these cells have an osteo-lineage restricted differentiation potential (Chan et al., 2013) that overlaps with $Nestin^+$ cells (Isern et al., 2013).

CD140A and CD51 In addition to these markers, Pinho et al. suggests a combination of CD140A and CD51 expression to identify murine MSCs. The majority of $CD140A^+CD51^+$ cells, which are $Nestin^+$ but $SCA1^-$ (Mendez-Ferrer et al., 2010), have self-renewal and trilineage differentiation capacity *in vitro*. Furthermore, these MSCs have high expression of HSC niche-associated genes, including *Cxcl12*, *Scf*, *Angptl1*, *Vcam1*, suggesting their hematopoiesis supportive properties (Pinho et al., 2013).

Characterization of MSCs and progenitors using genetically engineered mouse models

Similarly to studies on hematopoiesis, much of our understanding of MSC functions has been attained from mouse models. Genetically modified mice are crucial tools, allowing gene-specific labeling of MSCs (Figure 4), to subsequently investigate their properties, localization and role in the BM niche *in vivo*.

Nestin *Nestin*-GFP model was the first one suggested for labeling MSC population within non-hematopoietic BM cells (Mendez-Ferrer et al., 2010). Perivascular $Nestin^+$ cells have CFU-F activity, the potential to differentiate into three lineages *in vitro* and can self-renew *in vivo*. In addition, $Nestin^+$ MSCs express high levels of *Kitl*, *Cxcl12* and *Angptl1* – the genes involved in HSC maintenance. Moreover, the depletion of $Nestin^+$ cells compromises the frequency of HSCs and hematopoietic progenitor cells in the BM, and mobilizes hematopoietic cells to the spleen, suggesting an important role of the $Nestin^+$ cells in maintaining normal hematopoiesis (Mendez-Ferrer et al., 2010). However, the impact the cytokines derived from these mesenchymal progenitor cells may have on hematopoiesis remains controversial. The specific deletion of *Cxcl12* (Ding and Morrison, 2013b) or *Scf* (Ding et al., 2012) from $Nestin^+$ cells does not affect HSC frequency or function in the BM, but leads to reduced frequency of HSCs and hematopoietic progenitor cells in the spleen. These studies suggest that CXCL12 and SCF produced by $Nestin^+$ cells might not be necessary for normal hematopoiesis in the BM, but SCF may be important for HSC migration or extramedullary hematopoiesis in the

spleen. However, the exact effect of *Nestin*$^{+}$ cell depletion on HSC functionality and heterogeneity in the BM requires further characterization at the single cell level.

LepR Other studies use Leptin Receptor (*LepR*) as a marker of primitive MSCs in the BM of adult mice (Zhou et al., 2014). Multipotent *LepR*$^{+}$ cells represent a rare cell population (approximately 0.3%) with the capacity to form CFU-F and to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* and *in vivo* (Zhou et al., 2014). Moreover, the majority of BM stromal cells expressing SCF or CXCL12, co-express *LepR*. Studies have shown that specific deletion of *Scf* (Ding et al., 2012) and *Cxcl12* (Ding and Morrison, 2013b) from the *LepR*$^{+}$ cells leads to a depletion of HSCs and certain progenitor cells and their release to the circulation, indicating that these MSCs are an important player in HSC regulation. Moreover, *in vivo* fate-mapping shows that *LepR*$^{+}$ MSCs give rise to most of the bones and adipocytes in the BM, and contribute to post-injury recovery (Zhou et al., 2014). During differentiation into mature osteoblasts, *LepR*$^{+}$ cells lose LepR expression (Mizoguchi et al., 2014). In line with that, conditional deletion of *Foxc1*, a transcription factor important for development and maintenance of the mesenchymal cells, from *LepR*$^{+}$ cells diminishes HSC pool in the BM, providing additional evidence that *LepR*$^{+}$ MSCs are a key component of the HSC niche (Omatsu et al., 2014).

Prx1 Similarly to *LepR*$^{+}$ MSCs, Paired related homeobox gene 1 (Prx1)$^{+}$ cells show MSC characteristics. *Prx1*$^{+}$ MSCs are highly enriched in CFU-F activity, have the ability to differentiate into osteoblasts and adipocytes *in vitro* and can secrete CXCL12 (Greenbaum et al., 2013). Notably, depletion of *Cxcl12* from *Prx1*$^{+}$ MSCs results in the loss of HSCs with long-term repopulation capacity, as well as lymphoid progenitors in the BM (Ding and Morrison, 2013b; Greenbaum et al., 2013). Interestingly, deletion of LepR from *Prx1*$^{+}$ MSCs leads to increased bone formation at the expense of adipogenic differentiation (Yue et al., 2016), indicating the role of LepR in BM MSCs in tissue homeostasis.

Ebf2 Furthermore, Early B cell factor 2 (*Ebf2*), a transcription factor from the Ebf family but not expressed in hematopoietic cells, marks the MSC population in mouse BM (Qian et al., 2013). *Ebf2*$^{+}$ MSCs have high self-renewal (reflected in CFU-F frequency) and multilineage differentiation capacity, both *in vitro* and *in vivo*. Moreover, genes important for HSC maintenance, including *Angptl1, Fmod, Nestin, Nov, N-cadherin* and *Runx2,* are highly enriched in *Ebf2*$^{+}$ MSCs, suggesting the hematopoiesis supportive properties of the cells (Qian et al., 2013). *In vitro* co-culture experiments show that *Ebf2*-deficient stromal cells have detrimental effects on normal HSCs and, in line with that, *Ebf2*$^{-/-}$ mice are described with decreased numbers of HSCs and progenitor cells, as well as declined lymphopoiesis. Importantly, transplantation experiments indicate that BM niche-dependent mechanisms lead to such abnormalities in hematopoiesis (Kieslinger et al., 2010).

CAR Another stromal cell population, localized in close proximity to HSCs and therefore suggested as an important component of the BM niche, is composed of CXC12-abundant reticular cells (CAR cells). The term CAR cells refers to a heterogeneous population of BM stromal cells with high expression of CXCL12 (Sugiyama et al., 2006) and SCF (Omatsu et

al., 2010). In fact, deletion of the CXCL12 receptor, CXCR4, results in a reduction of HSC frequency and higher sensitivity to myelotoxic drugs (Sugiyama et al., 2006). Consequently, ablation of CAR cells impairs production of SCF and CXCL12, and leads to a reduction of quiescent HSCs and an increased expression of early myeloid genes (Omatsu et al., 2010). Nevertheless, freshly isolated CAR cells do not have CFU-F capacity, but they express genes involved in osteogenic and adipogenic differentiation (Omatsu et al., 2010), suggesting they are a population consisting of adipo-osteogenic progenitors and mature stromal cells.

Others More studies suggest that other populations, such as Myxovirus resistance-1 (*Mx1*) (Park et al., 2012a) and bone morphogenetic protein (BMP) antagonist Gremlin 1 (*Grem1*) expressing cells (Worthley et al., 2015), contribute to the formation of the mesenchymal niche. Non-hematopoietic *Mx1*$^+$ cells can form CFU-Fs, however with significantly lower frequency than abovementioned *LepR*$^+$ or *Prx1*$^+$ MSCs. They also exhibit the capacity to differentiate into adipocytes, osteoblasts and chondrocytes *in vitro*, thereby fulfilling criteria for MSCs. Nevertheless, *in vivo*, *Mx1*$^+$ cells seems to have restricted differentiation capacity to osteoblasts only, and therefore they are considered to be an osteoprogenitor population (Park et al., 2012a). Likewise, *Grem1*$^+$ cells have been shown to have self-renewal capacity (measured by CFU-F assays), and to be able to differentiate *in vitro* towards osteoblasts and chondrocytes, but not adipocytes. Comparably to *Mx1*$^+$, *Grem1*$^+$ cells merely contribute to bone formation and healing *in vivo*, and for that reason they are also considered to be an osteo-restricted progenitor population (Worthley et al., 2015).

Taken together, these studies provide strong evidence that MSCs and mesenchymal progenitors are components of the BM niche and may be the key players in regulation of normal HSCs and hematopoietic progenitor cells. However, it is still not fully understood how the described MSC populations (labeled by different markers) relate to one another, and more studies are needed to clarify reported differences in marker expressions and properties.

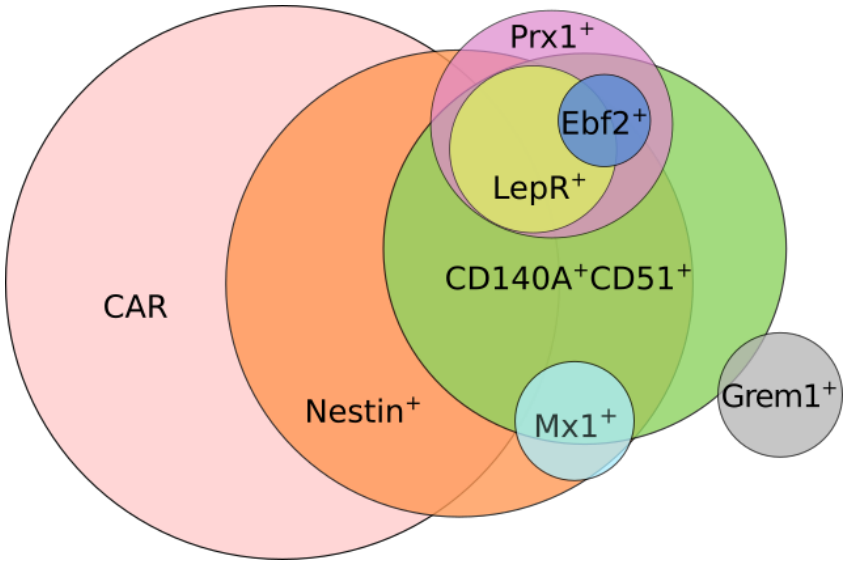

Figure 4. Relationship between various BM MSC and mesenchymal progenitor cell populations, defined by different marker expression.

1.3.3 Osteoblastic niche

Early mouse studies have shown that many HSCs localize close to the inner bone surface – the endosteum (Gong, 1978; Lord et al., 1975). Given that osteolineage cells line the endosteal region, they are the first cell population proposed as BM cellular niche. Demonstration of osteoblasts ability to support growth of human HSCs *in vitro* by producing cytokines, further highlighted the role of the endosteal niche in hematopoiesis regulation (Taichman et al., 1996; Taichman, 1994). However, transgenic mouse models were needed to prove that hypothesis and to provide the first evidence *in vivo*.

Two hallmark studies, performed at the beginning of the previous decade, have demonstrated the essential role of osteoblastic cells in HSC expansion and maintenance in mice. The first of them shows that constitutive activation of parathyroid hormone receptor (PTHR) in osteolineage cells leads to the activation of Notch signaling and an increase in numbers of osteoblastic cells, as well as HSCs and progenitor cells (Calvi et al., 2003). In line with these findings, activation of Notch signaling results in increased number of HSCs (Stier et al., 2002). Another study presents a similar, parallel increase in the numbers of osteoblastic cells and HSCs after the deletion of BMP receptor 1A from *Mx1*$^{+}$ osteoprogenitors, thus suggesting that BMP signaling in osteoblasts is another important player in the regulation of HSCs (Zhang et al., 2003). Since then, more studies using different transgenic mouse models have demonstrated osteoblast-mediated regulation of HSCs, and suggest the underlying mechanisms and signaling pathways. One of the examples is the conditional ablation of Col2.3-labeled osteoblast lineage, which results in reversible BM hypocellularity, a decreased number of HSCs and increased extramedullary hematopoiesis (Visnjic et al., 2004). Similarly, preventing osteolineage differentiation by depletion of *Osterix*$^{+}$ cells, leads to loss of hematopoiesis in the BM of adult mice (Zhou et al., 2010). Moreover, overexpression of Wnt signaling inhibitors (Dkk1 or Wif1) in *Col2.3*-labeled osteoblasts leads to the increased cycling of HSCs (Fleming, 2008; Schaniel et al., 2011). A more recent study also shows that depletion of *Col2.3*-expressing osteoblast leads to reduced HSC quiescence, long-term engraftment and secondary repopulating capacity, and points to a potential role of osteoblast-derived Jagged-1 in hematopoiesis regulation (Bowers et al., 2015). In addition, several other molecules produced by osteoblasts contribute to HSC fates regulation. One of them is osteopontin (OPN), which binds to integrin αvβ1/CD51 and CD44 on the surface of HSCs, and plays an important role in cell adhesive interaction as well as proliferation and mobilization of HSCs (Nilsson et al., 2005). Consistent with this report, deletion of *Opn* in mice causes an increase in number of HSCs, showing that OPN is a negative regulator of HSCs (Stier et al., 2005). Osteoblasts can also express agrin, a regulator of neuromuscular and immunologic synapses, that binds to the α-dystroglycan receptor on hematopoietic progenitor cells, and thereby modulates their survival and proliferation. Lack of that direct cell-cell interaction in *Argin*$^{-/-}$ mice leads to apoptosis of HSCs and disturbs hematopoiesis (Mazzon et al., 2011). Furthermore, it has been also shown that the endosteal niche retain HSCs within the BM by high concentration of calcium, as HSCs deficient in calcium-sensing receptors were found mainly in the circulation (Adams et al., 2006).

However, several lines of evidence question the direct effect of osteolineage cells on HSC regulation. It has been shown that osteoblastic N-cadherin is not required for HSC regulation under steady state (Bromberg et al., 2012; Kiel et al., 2007). Moreover, the specific deletion of CXCL12 from *Osterix*$^{+}$ osteoprogenitors (Greenbaum et al., 2013) and *Col2.3*-expressing osteoblasts (Ding and Morrison, 2013b) had little or no effect on HSC proliferation or mobilization. Similarly, osteoblast-specific deletion of SCF does not affect HSC frequency or function (Ding et al., 2012). There is also an evidence suggesting that osteoblasts support lymphopoiesis rather than HSCs. Depletion of *Col2.3*-expressing osteoblasts reduces number of B-cell precursors (Zhu et al., 2007), while deletion of CXCL12 from the same osteoblasts leads to loss of lymphoid progenitors but does not affect HSC number (Ding and Morrison, 2013b). Osteoblasts are also associated with regulation of erythropoiesis (Rankin et al., 2012) and megakaryopoiesis (Xiao et al., 2017). These findings indicate that osteoblasts might be more involved in the regulation of more committed hematopoietic progenitors, rather than of the HSCs. The discrepancy between abovementioned reports might be due to specificity of the conditional, gene-modified mouse models used in the studies, as well as difficulties to distinguish osteoblasts and osteoprogenitors from more primitive mesenchymal progenitors and MSCs, as markers for these populations have overlapping expression pattern (Nakamura et al., 2010).

Other cells found in the endosteal niche, including bone-embedded osteocytes and bone-degrading osteoclasts, also affect hematopoiesis. Osteocytes, which are terminally differentiated osteoblasts, can regulate myelopoiesis through Gsα, subunit of G proteins (Fulzele et al., 2013). It has been shown that mice lacking Gsα in osteocytes display dramatic increase of myeloid cells in the BM, peripheral blood and spleen. Moreover, conditioned media from Gsα-deficient osteocytes stimulates myeloid colonies *in vitro*, while treatment with anti-G-CSF neutralizing antibody blocks that effect (Fulzele et al., 2013). Furthermore, G-CSF-mediated mobilization of HSCs and hematopoietic progenitors to the circulation is impaired in osteocyte-ablated mice (Asada et al., 2013). In contrast, osteoclasts promote egress of HSCs and progenitor cells from the BM (Kollet, 2006). Besides that, pharmacological inhibition of osteoclast activity is associated with a reduced number of HSCs, as well as delayed hematopoiesis reconstitution in irradiated recipients (Lymperi et al., 2011).

1.3.4 Vascular niche

Despite the fact that HSCs can be found in the endosteum region, increasing evidence suggest that vascular niche plays an important role in regulation of hematopoiesis and HSCs. The vascular niche is located closer to the central zone of the BM, and is composed of endothelial cells (ECs), which line the lumen of blood vessels (Potente et al., 2011; Morrison and Scadden, 2014). Anatomic proximity of HSCs (Kiel et al., 2005), including the majority of quiescent HSCs with long-term repopulating capacity (Acar et al., 2015), to sinusoidal vessels suggests an important role of vascular niche in regulation of hematopoiesis. However, around 10% of quiescent HSCs are more closely associated with arterioles (Kunisaki et al., 2013). These perisinusoidal and periarteriolar vascular niches differ in terms of local oxygen concentration,

permeability of the blood vessels, and the capacity of hematopoietic cells to migrate through the walls. More leaky sinusoids represent an exclusive site for hematopoietic cells trafficking between the BM and circulation. Because of that, HSCs might be more exposed to blood plasma, and thus, to an elevated level of ROS (Itkin et al., 2016). Measurements of oxygen tension within the BM of live animals show the perisinusoidal niche is also the most hypoxic region in the BM (Spencer et al., 2014). On the other hand, small arterioles seem to be more resistant to irradiation-induced damage compared to the sinusoids, suggesting that periarteriolar vessels might play an important role in vasculature regeneration, as well as hematopoiesis recovery after irradiation (Kusumbe et al., 2014). Moreover, arterioles are ensheathed by a population of pericytes expressing neural/gial antigen 2 (NG2), which seems to maintain HSCs quiescence, since depletion of these $NG2^+$ cells leads to reduction of HSCs with long-term repopulating capacity and increase of cycling HSCs (Kunisaki et al., 2013). It has been shown that distinct mechanisms are involved in the regulation of HSCs by the perivascular cells. Deletion of CXCL12 from $NG2^+$ pericytes induces mobilization of quiescent HSCs to the circulation, and therefore results in a reduction of HSC numbers in the BM (Kunisaki et al., 2013). On the other hand, deletion of SCF from $NG2^+$ cells is not associated with reduced numbers of HSCs (Asada et al., 2017).

Regardless of the type of blood vessels, ECs are an indispensable part of the BM niche. The *in vivo* functional contribution of ECs to HSC regulation was first demonstrated by use of a *Tie2-Cre* mouse model with conditional deletion of *gp130* cytokine receptor, mediating signals from IL-6 family. Wild-type recipient mice displayed normal hematopoiesis after transplantation of *gp130*-deficient BM, but not the other way around, suggesting a role of EC-mediated IL6 signaling in hematopoiesis (Yao et al., 2005). Moreover, inhibition of Tie2-angiopoietin signaling in the vascular niche affects vascular regeneration and delays hematopoiesis recovery after myelosuppression, indicating an important role of ECs in hematopoiesis recovery *in vivo* (Kopp et al., 2005). Later studies by Hooper et al. also demonstrated that conditional deletion of vascular endothelial growth factor receptor 2 (*Vegfr2*) in adult mice impairs regeneration of sinusoidal ECs and HSC reconstitution after myelosuppression (Hooper et al., 2009). Similarly, inhibition of VEGFR2 using blocking antibodies also leads to impaired HSC engraftment after lethal irradiation (Hooper et al., 2009). Moreover, treatment with anti-VE cadherin neutralizing antibodies inhibits vessel formation in mice after irradiation, and interferes with hematopoietic recovery (Avecilla et al., 2003; Salter et al., 2009). In line with that, *Tie2*-specific deletion of *Bak1* and *Bax*, genes promoting apoptosis, protects ECs from irradiation-mediated damage and at the same time promotes hematopoietic regeneration (Doan et al., 2013b). Moreover, BM hematopoiesis recovery after irradiation can be improved by infusion of endothelial progenitor cells (Li et al., 2010; Salter et al., 2009) Additionally, infusion of young ECs improves engraftment of aged HSCs, suggesting an important role of EC also during aging processes (Poulos et al., 2017). These findings together demonstrate that ECs are crucial for hematopoiesis regenerations.

Many other studies show molecular mechanisms underlying the EC-mediated regulation of HSC expansion and differentiation. It has been reported that ECs could induce HSC expansion

in vitro, and long-term repopulation *in vivo*, by expression of angiocrine factors, e.g. Notch ligands (Butler et al., 2010). Activation of AKT signaling in ECs regulates HSC self-renewal through expression of CXCL12 and SCF. It also leads to activation of MAPK signaling in ECs, which in turn supports HSC differentiation *in vivo* (Kobayashi et al., 2010). Furthermore, EC-specific deletion of Notch ligand, Jagged-1, leads to reduction in HSC number and inhibits hematopoietic regeneration after irradiation (Poulos et al., 2013), whereas expression of Jagged-2 in ECs contributes to hematopoietic recovery after myelosuppression (Guo et al., 2017). In line with that, activation of endothelial Notch signaling in aged mice restores the number of ECs, and increases functionality of HSCs and progenitor cells (Kusumbe et al., 2016). Another signaling pathway important for HSC regulation within BM vascular niche is NF-κB. EC-specific inhibition of NF-κB results in increased self-renewal and regenerative capacities of HSCs. Consistently, injecting ECs with inhibited NF-κB signaling into irradiated mice reduces the radiotherapy-related damage to the hematopoietic system (Poulos et al., 2016).

Studies using EC-specific cytokine deficient mice demonstrated that BM ECs contribute to hematopoiesis through production of key HSC niche factors and angiocrine factors (Yu and Scadden, 2016). EC-specific deletion of SCF (Ding et al., 2012) or CXCL12 (Ding and Morrison, 2013a; Greenbaum et al., 2013) results in HSC reduction and decreases their long-term repopulating activity. What is more, conditional deletion of *Il7* in ECs affects B-lymphopoiesis, suggesting that endothelial niche can regulate HSC function and fate (Cordeiro Gomes et al., 2016). Moreover, BM ECs can support hematopoiesis *via* expression of specific surface markers, including E-selectins, P-selectin, VCAM-1 and ICAM1. Deficiency in E-selectins induces quiescence of HSCs, and improves their survival after irradiation or chemotherapy, proving that E-selectins promote HSC proliferation (Winkler et al., 2012b). Likewise, EC-derived heparin-binding growth factor, pleiotropin (PTN), and epidermal growth factor (EGF) are important players in the regeneration of HSCs following myeloablation. It has been shown that *PTN* deficiency or administration of anti-PTN antibodies might lead to impaired HSC maintenance and hematopoietic regeneration following myelosuppression (Himburg et al., 2012). Consistent with this finding, systemic treatment of irradiated mice with PTN supports expansion of HSCs *in vivo* (Himburg et al., 2010). Likewise, administration of EGF promotes recovery of HSCs and improves survival of mice after irradiation. By contrast, mice lacking the EGF Receptor in hematopoietic cells present with increased HSC apoptosis and delayed BM recovery compared to wild type mice (Doan et al., 2013a).

Taken together, ECs contribute to hematopoiesis maintenance and regulate HSC function through direct cell-cell contact, cytokines and angiocrine factors, as well as through indirect pathways. Moreover, the endothelial niche is crucial for hematopoietic system recovery after hematopoietic insult.

1.3.5 Adipocytes

Adipocytes, also called the yellow marrow, reside in the bone cavity (mainly in the diaphysis area) and gradually replace hematopoietic cells in human BM during aging (Tuljapurkar et al.,

2011). Initially it was suggested that adipocytes are simply occupying space within BM without any regulatory function (Gimble, 1990; Gimble et al., 1996). However, the negative correlation between yellow marrow abundance and decreasing functionality of HSCs, as well as several *in vitro* studies, all indicate that adipocytes are negative regulators of hematopoiesis (Hoggatt, 2016; Naveiras et al., 2009; Omatsu et al., 2010). In addition to reduced numbers of HSCs and progenitors in adipocyte-rich marrow in mice, it has been shown that inhibition of adipogenesis, by treatment with peroxisome proliferator-activated receptor gamma (PPARγ) inhibitor, leads to better HSC engraftment in the BM (Naveiras et al., 2009), and improves recovery after chemotherapy (Zhu et al., 2013). Moreover, "fatless" mice, which are genetically incapable of forming adipocytes, show higher engraftment of HSCs in the BM compared to wild-type controls (Naveiras et al., 2009). Recent study suggested that adipocytes suppress hematopoiesis and BM regeneration by excessive expression of dipeptidyl peptidase 4 (DPP4/CD26), which is involved in processes of apoptosis and immune response (Ambrosi et al., 2017). However, enhanced adipogenesis by treatment with triglitazone has no effect on the frequency of HSCs in the BM under homeostatic conditions, suggesting that adipocytes might not be a part of the functional BM niche during homeostasis (Spindler et al., 2013). In line with that, Zhou et al. has shown that adipocytes produce SCF, which has no effect on HSCs under steady-state, but it is required for HSC engraftment after transplantation. Consequently, "fatless" mice in this study display normal HSC number under normal conditions, but impaired recovery after BM transplantation. These findings suggest that adipocytes might be a pro-regenerative component of the BM niche. It is important to note that the Adipoq-Cre/ER$^+$ mouse model used in this study cannot distinguish whether the observed effects are mediated by mature adipocytes or immature (LepR$^+$) progenitors (Zhou et al., 2017) in this study. Further studies are needed to better understand the roles of adipocytes in hematopoiesis regulation.

1.3.6 Sympathetic nervous system (SNS)

BM is highly innervated with sympathetic nerves that can regulate HSCs (Hanoun et al., 2015; Katayama et al., 2006; Kunisaki et al., 2013). The SNS is composed of non-myelinating Schwann cells that present regulatory BM niche functions. They have been reported to produce cytokines and express cytokine receptors, and thus affecting the microenvironment where they are located (Gordon, 2014; Ozaki et al., 2008; Höke et al., 2006). As a source of active TGF-β, the Schwann cells can maintain HSC quiescence and regulate their activation (Yamazaki et al., 2009). Consequently, BM denervation leads to decreased TGF-β signaling and causes loss in the quiescent HSC pool (Yamazaki et al., 2011).

Sympathetic signals are involved in HSC mobilization mediated by G-CSF, regulation of CXCL12 expression in the BM, and HSC retention through circadian oscillations (Casanova-Acebes et al., 2013; Katayama et al., 2006; Lucas et al., 2008; Méndez-Ferrer et al., 2008). Even though sympathectomy, caused by treatment with neurotoxin, does not affect HSC number, it impairs their mobilization from the BM (Katayama et al., 2006; Lucas et al., 2008; Afan et al., 1997). Moreover, chemotherapy-induced sensory neuropathy inhibits hematopoiesis and BM regeneration. Consequently, protection of SNS and its regeneration

from chemotherapy-related injury improves hematopoietic recovery (Lucas et al., 2013). As HSCs express catecholaminergic receptors, they can directly receive and respond to sympathetic catecholamine signals (Spiegel et al., 2007). It has been shown that treatment of HSCs with dopamine agonists and norepinephrine can increase colony formation *in vitro* and improve engraftment *in vivo* (Kalinkovich et al., 2009; Spiegel et al., 2007). Moreover, loss of SNS nerves in the BM of young mice leads to premature aging of HSCs, while administration of a symphathomimetic drug to old mice rejuvenates functions of aged HSCs *in vivo*, suggesting an important role of the SNS in HSC aging (Maryanovich et al., 2018). However, the underlying molecular mechanisms remain poorly understood.

1.3.7 Megakaryocytes

Megakaryocytes (MKs) are hematopoietic cells adjacent to the BM sinusoids. They co-localize with HSCs and regulate their fate under steady-state as well as in response to stress (Bruns et al., 2009; Bruns et al., 2014; Heazlewood et al., 2013; Zhao et al., 2014). *In vivo* ablation of MK cells leads to activation of quiescent HSCs and reduced levels of TGF-β1 in the BM. Interestingly, conditional deletion of *Tgfb1* from MKs similarly affects activation and proliferation of HSCs, while treatment with TGF-β1 restores HSC quiescence in MK-depleted mice, suggesting that MKs regulate HSC fate through TGF-β signaling under steady-state (Zhao et al., 2014). In response to stress, MK-derived fibroblast growth factor 1 (FGF1) prevails over TGF-β signals and stimulates HSC expansion. Hence, ablation of MKs under stress condition may inhibit HSC expansion. (Zhao et al., 2014). Additionally, MKs can regulate the HSC pool size *via* secretion of CXCL4 (also named platelet factor 4/PF4) as *Cxcl4*$^{-/-}$ mice present an increased number of HSCs and that injection of CXCL4 into mice leads to a reduction in HSC number as a consequence of their increased quiescence (Bruns et al., 2014). Notably, conditional ablation of CXCL4-expressing MKs leads to reduced levels of thrombopoietin (THPO) in the BM – another cytokine important for HSC maintenance (Qian et al., 2007) – and egress of HSCs from the BM. This study shows that perturbed cell cycle activity of HSCs, caused by MK depletion, can be restored by THPO administration, suggesting its important role in MK-mediated regulation of HSC quiescence (Nakamura-Ishizu et al., 2014). Under stress condition, MKs are required for both, hematopoietic regeneration and osteogenesis. After radioablation, MKs are recruited to the endosteum, where they produce platelet-derived growth factor-β (PDGF4) and stimulate osteoblast expansion to promote hematopoietic recovery (Olson et al., 2013). Blocking the THPO receptor (MPL) reduces MK migration to the endosteum, and subsequenty HSC engraftment (Olson et al., 2013). Overall, these observations confirm that MKs contribute to the BM niche and indirectly regulate HSC quiescence and function through production of multiple factors.

1.3.8 Macrophages

The BM contains various macrophages (MΦs) with different functions. They line the endosteal surface and participate in bone mineralization, thus, they are often termed as osteomacs (Alexander et al., 2011; Chang et al., 2008; Pettit et al., 2008). While studying HSC mobilization to peripheral blood using G-CSF, Winkler et al. observed reduced number of

MΦs, indicating they play a role in HSC trafficking (Winkler et al., 2010b). Following that, depletion of $CD169^+$ MΦs was associated with HSC mobilization and reduction in erythroblasts numbers in the BM under steady-state conditions, as well as after myeloablation and acute blood loss in a hemolytic anemia model (Chow et al., 2011). Later findings suggest that $CD169^+$ MΦs secrete oncostatin M (OSM), which stimulates stromal cells to express CXCL12, which interacts with CXCR4 on HSC and therefore keeps them in the BM niche (Albiero et al., 2015). In line with these findings, treatment of diabetic mice with CXCR4 antagonist (Chow et al., 2011) or OSM blocking antibodies (Albiero et al., 2015) improves G-CSF-mediated mobilization of HSC to the circulation. Taken together, these studies indicate that BM MΦs can support erythropoiesis, regulate HSC retention and influence other BM niche cells *via* production of soluble factors.

1.3.9 Complex cross-talk between cellular components of the BM niche

As described above, the BM niche consists of different, partially redundant, cellular niche components that interact with HSCs and control their fate (Hoggatt, 2016). However, BM niche cells can also regulate and depend on one another.

In line with that, deletion of *Angpt1* from $LepR^+$ stromal and hematopoietic cells increases vascular leakiness (Zhou et al., 2015). Moreover, decline of $Endomucin^{high}$ ECs is associated with a reduction in osteoprogenitor cells in aged mice. By contrast, expansion of ECs leads to increased bone formation, indicating the coupling of angiogenesis and osteogenesis (Kusumbe et al., 2014). Furthermore, mice with disrupted Notch signaling in ECs present with decreased bone mass and defective release of Noggin, an antagonist of bone morphogenetic proteins, from ECs. Interestingly, administration of Noggin restores number of osteoprogenitors and bone growth in mutant mice, pointing out that EC-specific Notch-mediated mechanisms regulate both blood vessel and bone formation (Ramasamy et al., 2014). Paracrine cross-talk between osteolineage cells and ECs has been shown to be important under stress condition too, for example after hematopoietic injury. Under steady state, Osx^+ cells produce Dkk1 protein, a Wnt inhibitor, which induces secretion of EGF by ECs to promote BM regeneration after irradiation (Himburg et al., 2016). Moreover, osteoblasts support megakaryopoiesis by secreting IL-9 as shown by mouse models where IL-9 administration stimulates platelets production and accelerates platelet recovery after chemotherapy (Xiao et al., 2017). On the other hand, norepinephrine produced by the SNS regulates CXCL12 release by acting on β3 adrenergic receptors on stromal cells (Méndez-Ferrer et al., 2008). Sympathetic nerves in turn receive physical support from arterioles in the BM (Kunisaki et al., 2013). Moreover, neutrophils seem to be indirectly involved in the regulation of HSC trafficking. After entering the BM, aged neutrophils are phagocytosed by MΦs, that interact with the stromal cells leading to downregulation of CXCL12, and thus, egress of HSCs to the circulation (Casanova-Acebes et al., 2013). Depletion of $CD169^+$ MΦs was also accompanied with decreased osteoblasts numbers (Chow et al., 2011). Similar studies confirmed that MΦs support osteolineage cell function through production of soluble factors (Christopher et al., 2011).

Taken together, these studies indicate evidence that various BM niche cells act together to provide an interactive unit for hematopoiesis regulation. Therefore, ablation of one population or removal of soluble factors might affect other cellular components of the BM niche and overall tissue integrity.

1.4 THE BM MICROENVIRONMENT IN MYELOID MALIGNANCIES

Similarly to normal HSCs, LSCs also reside in a specific microenvironment that regulates their functions. Recently, our understanding of the BM niche modifications and their contribution to abnormal hematopoiesis has advanced thanks to studies using transgenic mouse models. The strong evidence that BM niche plays an important role in myeloid malignancies revived the "seed and soil" hypothesis, proposed by Stephen Paget over hundred years ago to describe the relationship between environment (soil) and tumor cells (seeds) in metastasis (Goulard et al., 2018).

1.4.1 Interaction between the BM niche and malignant cells

Interaction with BM niche is crucial for leukemic cell growth (summarized in Figure 5), as it can mediate their resistance to treatment (Jacamo and Andreeff, 2015). It has been implicated by *in vitro* studies, where treatment with either JAK2 inhibitor, or ALOX5 inhibitor, reduces the growth of malignant cells only in the absence of stromal cells (Manshouri et al., 2011; Yektaei-Karin et al., 2017), but does not show the same effect when primary CML LSCs are co-cultured with stromal cells (Dolinska et al., 2017).

One of the mechanisms controlling LSC survival is the adhesive interaction with the BM niche (Schepers et al., 2015). Studies have shown that expression of cell surface glycoprotein CD44, which binds to extracellular matrix proteins and osteopontin, is increased in CML cells and mediates their homing and engraftment. Moreover, this study shows that treatment with an antibody against CD44 inhibits CML development in wild-type recipients (Krause et al., 2006). Similarly, high expression of CD44 is important for AML induction or relapse in murine model (Quéré et al., 2010), and is required for homing of human AML leukemic cells in immunodeficient mice (Jin et al., 2006b). Different studies have shown that E-selectins, which are crucial for engraftment of CML cells (Krause et al., 2014), are also highly expressed on ECs in AML BM and promote chemoresistance in the vascular niche (Winkler et al., 2012a). Another family of cell adhesion receptors – integrins – also regulate leukemic cell functions *in vitro* and *in vivo*. It has been shown that integrin β1 can regulate proliferation of CML cells through adhesion to BM stromal cells and components of the extracellular matrix, such as fibronectin, (Bhatia et al., 1996; Bhatia et al., 1994; Lundell et al., 1997). Furthermore, interaction of a leukemic cell line with fibronectin mediates downregulation of proapoptotic factor *Bim1*, and leads to treatment resistance of LSCs (Hazlehurst et al., 2007). Similarly, integrin β3 is involved in AML development (Miller et al., 2013) and enhancement of Wnt signaling in AML cells (Yi et al., 2016). Moreover, integrin α4β1, also called very late antigen-4 (VLA4) can bind to vascular cell adhesion molecule 1 (VCAM1) (Jacamo et al., 2014) or

fibronectin (Matsunaga et al., 2003), and thus, induce chemoresistance of AML cells. In addition, glycoprotein CD98, which binds to integrins, enhances the interaction between AML and BM stromal cells, and thereby promotes maintenance and proliferation of leukemic cells (Bajaj et al., 2016). Notably, CD98 deficiency increases survival of the AML mice, whereas treatment with the anti-CD98 antibody inhibits growth of human AML cells in xenograft models (Bajaj et al., 2016). Similarly, CD82 mediates adhesion of human AML leukemic stem/progenitor cells to the extracellular matrix, and anti-CD82 treatment in combination with chemotherapy prolongs survival of mice transplanted with human AML cells (Nishioka et al., 2015). In addition, studies show that interaction with the BM stromal cells induces expression of Galectin-3 (Gal-3) in CML cell lines, promotes leukemic cell proliferation and subsequently leads to TKI resistance *in vitro*. In line with these findings, Gal-3 overexpression *in vivo* promotes lodgement of CML cells in the BM niche (Yamamoto-Sugitani et al., 2011). Similarly, AML cells induce expression of growth arrest-specific gene (Gas6) in the BM niche to further promote their own proliferation (Janning et al., 2015). The same regulatory axis has been shown to support the self-renewal of CML LSCs (Jin et al., 2017). Another adhesive molecule, N-cadherin, mediates adhesion of CML cells to the BM niche, and might induce TKI insensitivity of leukemic cells by activation of β-catenin and increased Wnt signaling (Zhang et al., 2013).

Moreover, the BM niche can promote disease progression through abnormal cytokines and chemokines production (Figure 5) (Camacho et al., 2017). For example, CXCL12-CXCR4 signaling between the BM niche and AML cells can promote leukemic cell survival, as shown by decreased leukemia burden and prolonged survival of AML mice after treatment with CXCR4 antagonists (Nervi et al., 2009; Tavor et al., 2004; Zeng et al., 2009). In CML, CXCR4 upregulation increases survival of quiescent LSCs *in vitro* by stimulating migration of leukemic cells towards BM stromal cells (Tabe et al., 2011), and thus, inhibition of CXCL12-CXCR4 restores sensitivity of LSCs to TKI *in vitro* (Vianello et al., 2010). Furthermore, it has been shown that CML LSCs have higher expression of IL-1 receptor, suggesting that IL-1 signaling contributes to their maintenance (Zhang et al., 2016). In line with that, treatment with IL-1 receptor antagonists suppresses the growth of CML LSCs, and displays an additive effect when combined with TKI treatment (Zhang et al., 2016). Similarly, LSCs from AML patients overexpress CXCR2, a receptor for proinflammatory cytokine IL-8, which has been shown to promote their proliferation (Schinke et al., 2015). Also, tumor necrosis factor α (TNFα), an inflammatory cytokine often increased in myeloid malignancies, enhances proliferation and survival of malignant cells (Gallipoli et al., 2013).

Additionally, a mitochondrial transfer from the BM stromal cells to AML cells, but not normal $CD34^+$ hematopoietic stem/progenitor cells, leads to increased chemoresistance (Moschoi et al., 2016). Inhibition of NADPH oxidase 2 (NOX2)–derived superoxide prevents this mitochondrial transfer, and leads to increased apoptosis of leukemic cells and prolonged survival of mice transplanted with patient-derived AML cells (Marlein et al., 2017)

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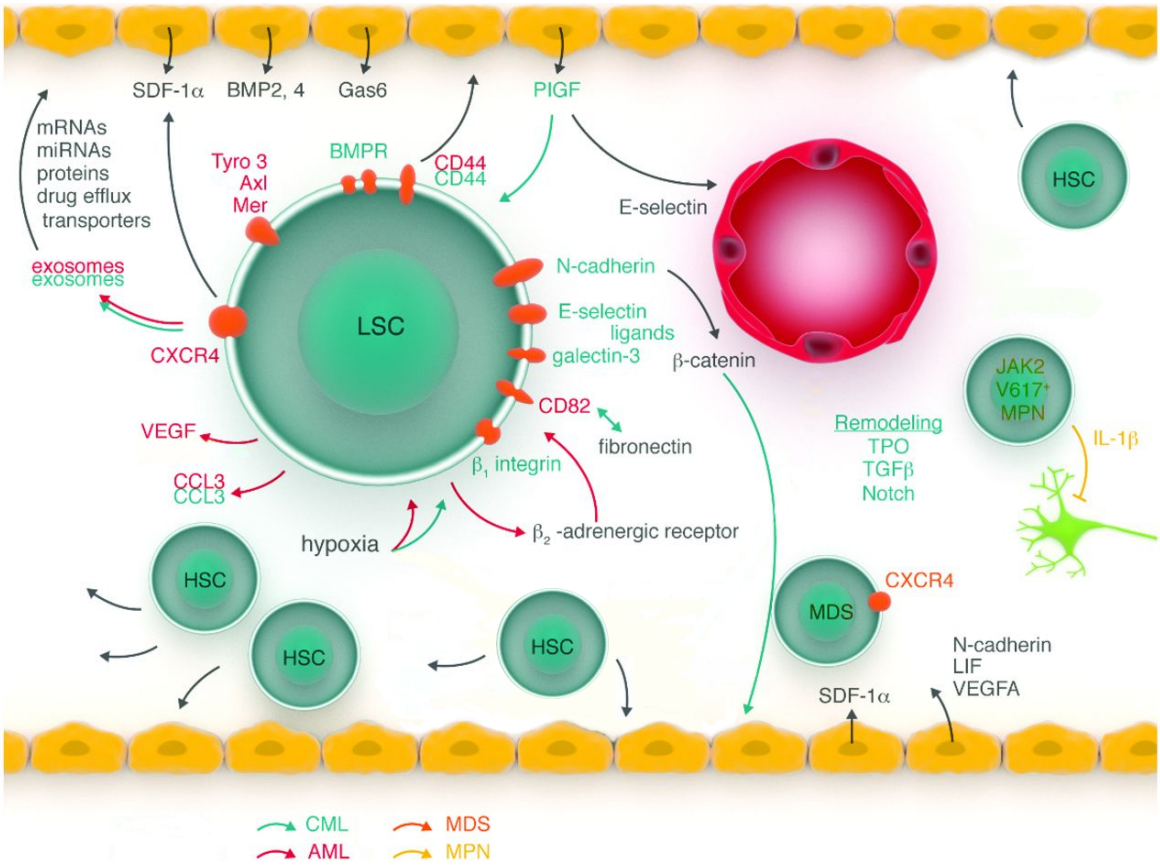

Figure 5. Malignant cells interact with their BM niche through multiple pathways. Modified, originally published by Krause and Scadden, 2015, and reprinted with permission from Hematologica; copyright Ferrata Storti Foundation.

1.4.2 The BM niche-remodeling by malignant cells

LSCs are known to share many biological properties with normal HSCs (Kreso and Dick, 2014). Given that normal hematopoiesis is impaired in patients with hematological malignancies, it has been postulated that LSCs can outcompete HSCs and remodel the BM niche into a self-reinforcing, malignant microenvironment (Hanahan and Coussens, 2012; Tabe and Konopleva, 2017). Therefore, the BM niche might be responsible for leukemic relapse and disease progression (Ishikawa et al., 2007).

1.4.2.1 The BM niche remodeling in CML

In a transgenic CML mouse model, the abnormal BM microenvironment produces significantly more cytokines that selectively support malignant cell growth over healthy HSCs (Zhang et al., 2012). CML cells produce higher levels of G-CSF, which in turn causes decreased expression of CXCL12 by the BM niche cells. Moreover, eradication of leukemic cells, using imatinib treatment, partially corrects the cytokine expression pattern, suggesting that BM niche remodeling was induced by leukemia (Zhang et al., 2012). Similarly, increased levels of GM-CSF, IL-6 and IL-1α can be found in the serum of CML mice (Welner et al., 2015). Moreover, exposing normal HSCs to CML cells leads to increased myeloid differentiation capacity and

reduced self-renewal potential of the HSCs, as well as altered gene expression patterns in hematopoietic progenitor cells. Interestingly, treatment with anti-IL-6 blocking antibody attenuates CML onset, suggesting that BCR-ABL1$^+$ leukemic cells promote the disease and affect normal hematopoiesis through IL-6 signaling. It is important to note here that patient-derived CML cells impose similar changes on normal HSCs and hematopoietic progenitors in culture, which further confirms that both mouse and human LSCs have the capacity to modulate surrounding cells in the BM niche (Welner et al., 2015). Moreover, CML cells promote secretion of PIGF by BM stromal cells during co-culture (Schmidt et al., 2011). In this study, increased PIGF level stimulates angiogenesis and proliferation of the leukemic cells. Consequently, treatment with PIGF antagonist prolongs the survival of CML mice and has an additive therapeutic effect when combined with imatinib, indicating the capacity of CML leukemic cells to reprogram the BM niche to promote progression of the disease (Schmidt et al., 2011). Using a similar CML mouse model, Schepers et al. shows that CML cells can remodel the BM endosteal niche, which consists of increased fraction of osteoblasts with altered gene expression pattern and compromised capacity to support normal HSCs (Schepers et al., 2013). Also, the ablation of osteoblasts has been shown to significantly accelerate development of leukemia, and reduces survival of CML mice. In this study, the CML BM niche affects cycling of hematopoietic cells through higher expression of a Notch ligand, Jagged-1 (Bowers et al., 2015).

1.4.2.2 The BM niche remodeling in AML

We have recently shown the dynamics of BM niche remodeling by the AML leukemic cells (Xiao et al., 2018b). The AML-induced BM cellular and molecular alterations are strongly correlated with leukemia burden. Changes in the expression of important hematopoiesis-supportive niche factors appear 14 days post transplantation, but before massive expansion of leukemic cells. Moreover, *in vivo* fate-mapping experiments reveal that the *Ebf2*$^+$ MSC population is an important player in leukemic niche formation, however, depletion of *Ebf2*$^+$ cells before AML onset enhances leukemia engraftment and accelerates disease progression. This data suggests that native BM MSCs may initially have a leukemia-suppressive role, and could later be reprogrammed by AML cells to promote the disease (Xiao et al., 2018b). Moreover, MLL-AF9 leukemic cells can trigger MKs to express higher levels of *Tgfb1* upon a co-culture *in vitro*. Interestingly, an increased level of TGF-β1 can be found in the AML BM, where it seems to inhibit proliferation of normal HSCs, and leads to defective hematopoiesis *in vivo* (Gong et al., 2018). AML cells can also inhibit the activity of osteoblastic cells (as measured by decreased levels of osteocalcin) and cause a decrease in mineralized bone volume. Increased CCL3 levels, found in AML cells from mice and patients, might be involved in that phenotype (Frisch et al., 2012). In line with these finding, osteoblast ablation inhibits normal hematopoiesis, and increases MLL-AF9 leukemia burden. Accordingly, restoration of the osteoblast population, by pharmacological treatment, reduces the leukemic burden and prolongs survival of leukemic mice (Krevvata et al., 2014). Furthermore, MLL-AF9-induced AML mice have decreased numbers of pericytic NG2$^+$ cells and lower density of sympathetic nerves (Hanoun et al., 2014). This in turn causes expansion of Nestin-GFP$^+$ MSCs with

increased commitment towards osteolineage, however, they are unable to differentiate into mature osteoblasts. Moreover, decreased expression of the genes regulating HSC maintenance and retention in the BM niche cells leads to reduced repopulation ability of HSCs, and their mobilization out to peripheral blood and spleen. These data indicate that AML cells modulate the BM niche to promote leukemia at the expense of normal hematopoiesis *via* disruption of SNS and expansion of immature mesenchymal progenitors (Hanoun et al., 2014).

1.4.2.3 The BM niche remodeling in MPN

MPN cells are also able to reinforce the disease by altering the BM niche composition. In contrast to AML, $JAK2^{V627F}$ MPN mice show a decrease of *Nestin*$^+$ MSCs and Schwann cells (Arranz et al., 2014). In this model, mutated hematopoietic cells produce IL-1β, which triggers BM neural damage and compromises MSC survival. In line with these findings, depletion of *Nestin*$^+$ MSCs results in expansion of malignant cells and accelerates MPN progression, whereas treatment with neuroprotective drugs inhibits the growth of mutated cells. The restoration of sympathetic regulation in the BM niche can both prevent the loss of *Nestin*$^+$ cells and block MPN progression, alltogether indicating the critical role of the BM niche damage in disease development (Arranz et al., 2014). A study by Mead et al. demonstrates that hematopoietic cells with the Flt3/ITD activating mutation can modulate the BM niche, as competitive transplantation into healthy recipients leads to a decrease of ECs and MSCs, coincided with the loss of normal hematopoietic cells and eventually Flt3-ITD-driven myeloproliferation in the recipient mice (Mead et al., 2017). Notably, this study shows that TNF, a cell-extrinsic negative regulator of HSCs, is overexpressed in the BM niche cells from Flt3-ITD knock-in mice, and that anti-TNF treatment partially rescues the progressive loss of HSCs (Mead et al., 2017).

1.4.2.4 The BM niche remodeling by exosomes

Moreover, malignant cells can reprogram the BM niche through release of exosomes (Cheng et al., 2018). Exosomes are cell membrane–derived vesicles carrying protein and RNA that might act as mediators in cell–cell communication (Boyiadzis and Whiteside, 2015). Proof-of-concept *in vitro* studies show that primary AML cells release exosomes that can enter neighboring cells in co-culture. The uptake of such exosomes causes changes in secretion profile, and alters proliferation and migration capacity of the BM stromal cells (Huan et al., 2013). In addition, the CML blast crisis cell line transfers exosomes containing microRNA miR-126 into ECs during co-culture, which leads to reduced VCAM-1 and CXCL12 expression in ECs, and reduced adhesion capacity of the CML cell line (Taverna et al., 2014). Thereafter, it has been shown that injection of AML-derived exosomes increases the numbers of MSCs with blocked osteoblast differentiation in the BM niche. Interestingly, wild-type recipients transplanted with AML cells with a knockdown of Rab27a, a protein involved in exosome release, show significantly prolonged survival compared to control mice (Kumar et al., 2017).

Taken together, these studies indicate that malignant cells can create a self-reinforcing niche by conferring signals to surrounding BM cells. The remodeled BM niche becomes detrimental for normal HSCs, but supportive for leukemic cells, and therefore accelerating the malignancy.

1.4.3 Instructive role of the BM niche in pathogenesis of myeloid malignancies

Not only "malignancy-induced microenvironment" but also a "microenvironment-induced oncogenesis" model has been proposed (Shiozawa and Taichman, 2010). Several studies have provided the evidence that alterations in the BM niche could be sufficient to trigger myeloproliferative disease in mice.

The first evidence of niche-driven disease appeared in 2007, when two simultaneously published studies showed that mice deficient for retinoic acid receptor gamma (*Rarγ*) (Walkley, 2007a) and retinoblastoma (*Rb*) gene (Walkley, 2007b) develop myeloproliferative MPN-like disorder. *Rarγ* is a nuclear hormone receptor acting as transcriptional regulator of gene expression (Balmer and Blomhoff, 2002), whereas *Rb* is a key regulator of the cell cycle. (Weinberg, 1995). The former study shows that transplantation of wild-type hematopoietic cells into *Rarγ* knockout recipient mice, but not *vice versa*, leads to MPN, suggesting that disease is not intrinsic to hematopoietic cells, but rather triggered by genetic alterations in the BM niche (Walkley et al., 2007a). In the latter study, myeloproliferation and loss of HSCs in mice appears only when *Rb* deficiency occurs in both hematopoietic cells and the BM niche at the same time. This finding demonstrates that *Rb* regulates HSCs extrinsically by maintaining the capacity of the BM niche to support normal hematopoiesis, and hence indicates the crucial role of interactions between both cell populations (Walkley et al., 2007b). In addition, inactivation of *Mib1* gene, encoding an enzyme necessary for processing Notch ligands, leads to myeloproliferative disease. Similar to *Rarγ* knockout, only *Mib1*$^{-/-}$ recipients transplanted with wild-type cells develop the disease. Moreover, introducing a constitutively active domain of Notch1 into the knockout BM niche can reverse the effects and suppress progression of the disease. This findings show that disruption of Notch signaling in the BM niche has an instructive role in disease pathogenesis (Kim et al., 2008).

More recently we and others have observed that deletion of *Sipa1*, a gene involved in regulation of cell proliferation, cell adhesion and cell survival *via* Rap1 signaling, leads to age-dependent MPN in mice (Ishida et al., 2003; Xiao et al., 2018a). Moreover, mutation in *SIPA1* can be found in the mononuclear cells from patients with juvenile myelomonocytic leukemia (JMML) (Yoshida et al., 2008). Interestingly, cellular abnormalities in the BM niche of *Sipa1*$^{-/-}$ mice, including expansion of MSCs and mesenchymal progenitors, occur before onset of the disease. Importantly, *Sipa1* knockout recipients transplanted with wild-type hematopoietic cells develop MDS/MPN, while *Sipa1*-deficient hematopoietic cells fail to initiate the disease in wild-type recipient mice. These findings clearly demonstrate that it is the *Sipa1*-deficient BM niche that causes the neoplasia (Xiao et al., 2018a). Similarly, deficiency of intracellular

adhesion molecule (*Icam1*) in the BM niche, but not HSCs, leads to expansion of HSCs with defective repopulation capacity, and increased myelopoiesis in mice (Liu et al., 2018). In line with the results from mouse model, neutralization of ICAM-1 increases proliferation of human HSCs when co-cultured with the BM stromal cells. Worth noting, culture-selected BM stromal cells from CML and AML patients show decreased expression of *ICAM1* (Liu et al., 2018). These results indicate that alterations in the BM niche can lead to perturbed hematopoiesis and myeloid malignancies in mice.

To identify which cellular components of the BM niche contribute to the hyperproliferation of myeloid cells, many studies use genetically modified reporter mice targeting various stromal cell populations. One of them has demonstrated that conditional deletion of microRNA-processing gene *Dicer1* from immature Osx^{+} osteoprogenitors, but not mature Ocn^{+} osteoblasts, results in impaired osteoblast differentiation and leads to MDS and sporadic transformation to AML. These results suggest that *Osx*-expressing osteoblast progenitor cells contribute to disease initiation. Moreover, deletion of *Dicer1* leads to reduced expression of *Schwachman-Diamond-Bodian syndrome (Sbds)* gene in the osteoprogenitors (Raaijmakers et al., 2010). Interestingly, decreased expressions of *DICER1* and *SBDS*, can be also detected in MSC-derived osteoprogenitors from MDS patients (Santamaria et al., 2012). In another mouse model, a $Ctnnb1^{CAosb}$ mutation in the Wnt pathway results in the constitutive activation of β-catenin in *Col1*-expressing osteoblastic cells, and leads to AML development by inducing aberrant Notch signaling in HSCs and progenitor cells. It is important to note that similar observations were reported in 38% of analyzed MDS and AML patients, who had increased β-catenin signaling in the osteoblasts and increased activation of Notch signaling in the hematopoietic cells (Kode et al., 2014; Kode et al., 2015). A recent study has shown that an activating mutation in protein tyrosine phosphatase SHP2, $Ptpn11^{E76K}$, in the BM $Prx1^{+}$, $LepR^{+}$, $Nestin^{+}$ MSCs and $Osx1^{+}$ osteoprogenitors, but not in Ocn^{+} mature osteoblasts or *VE-Cadherin*$^{+}$ ECs, promotes leukemogenesis (Dong et al., 2016). Moreover, the $Ptpn11^{E76K}$ recipient mice develop donor cell-derived MPN after transplantation of wild-type BM cells. Altogether, these findings provide an evidence that mutated mesenchymal progenitors, with altered expression of CCL3 and other proinflammatory cytokines, have the instructive role in the pathogenesis of MPN in mice (Dong et al., 2016). Importantly, the same mutation is found in the mononuclear cells of approximately half of the Noonan Syndrome patients, who have increased risk of developing leukemia and MPN at young age (Dong et al., 2016). Similarly, the prospectively isolated MSCs from low-risk MDS patients show increased expression of genes encoding inflammatory factors (Chen et al., 2016), and elevated levels of damage-associated molecular pattern (DAMP) molecules S100A8/9, which are shown to induce genotoxic stress and support leukemic evolution in pre-leukemic mice (Zambetti et al., 2016). Moreover, transcriptional activation of S100A8/9 axis in mesenchymal niche can be used as predictive factor of disease progression in human MDS (Zambetti et al., 2016).

Alterations in the BM ECs might also be a predisposing factor in the development of myeloid malignancies. For example, loss of Notch signaling in ECs (using *Tie2-RBPJ*$^{-/-}$ mouse model) leads to a lethal MPN-like disease (Wang et al., 2014). Specifically, the loss of Notch/RBPJ

signaling upregulates microRNA miR-155 in BM ECs, leading to activation of NF-κB and increased production of proinflammatory cytokines. Notably, deletion of miR-155 from stromal cells of *RBPJ*$^{-/-}$ mice prevents disease development, suggesting that activation of miR-155/NF-κB in the BM niche drives myeloproliferation. Interestingly, increased expression of miR-155 can also be found in the BM from MPN patients (Wang et al., 2014). Another study shows that the BM vascular niche with a JAK2^{V617F} mutation contributes to expansion of malignant cells (Lin et al., 2016). First, the JAK2^{V617F} ECs increase proliferation of JAK2^{V617F} HSCs and hematopoietic progenitors compared to JAK2^{WT} counterparts *in vitro* (Lin et al., 2016). The competitive BM transplantation experiments further confirm that the JAK2^{V617F} vascular niche promotes growth of JAK2^{V617F} malignant cells over JAK2^{WT} HSCs and progenitors. In contrast, there are no differences between wild-type recipients transplanted with either mutant or normal hematopoietic cells, which indicates a crucial role of altered ECs in MPN development (Zhan et al., 2017). The relevance of these findings increases as the same mutation can be found in ECs from MPN patients (Rosti et al., 2013; Sozer et al., 2009; Teofili et al., 2011), suggesting their potential involvement in disease pathogenesis in patients.

These studies present strong evidence that mutations in the BM niche can have a causative role in malignant transformation in mice. However, it is still unclear whether a single alteration in the BM niche can initiate leukemia in humans.

1.4.4 Disease- and stage-specific BM niche regulation of leukemogenesis

The BM niche regulation of hematopoiesis and leukemogenesis is stage and disease specific (Krause et al., 2013; Lane et al., 2011). For instance, osteoblastic cell-specific activation of the PTH receptor has an opposite effect on BCR-ABL^{+} CML and MLL-AF9^{+} AML cells. PTH signaling attenuates CML-like MPN development, through the suppressive effect of TGF-β1 derived from the remodeling bone, but it promotes AML progression in a mouse transplantation model (Krause et al., 2013). Moreover, the BM niche can regulate AML cells in distinct ways during different stages of leukemia progression. It has been shown that the BM niche is required in pre-leukemic stage, however it becomes more permissive once leukemia is established (Lane et al., 2011).

These studies illustrate distinct niche requirements for different types of leukemia, and suggest that the outcome of niche modulation can be very specific to the disease. Therefore, it is of great importance to understand the mechanisms regulating the BM niche interaction with hematopoietic cells in patients, as well as their contribution to the pathogenesis of myeloid malignancies.

2 AIM OF THE THESIS

The overall aim of this thesis was to investigate the role of the BM niche in development of myeloproliferative neoplasms, including CML.

Specific aims were:

Study I

To analyze expression of leukotriene signaling molecules and to study the effect of leukotriene inhibitors on survival of human CML leukemic stem and progenitor cells.

Study II

To determine the contribution and the underlying mechanisms of BM microenvironment to the pathogenesis of MPN in *Sipa1* deficient mouse model.

Study III

To prospectively characterize human BM niche in patients with CML and identify the disease-associated niche factors.

3 METHODOLOGICAL APPROACHES

All projects included in this thesis were approved by Ethical Review Board in Stockholm and Linköping (2012/4:10, 2013/3:1, S40-14).

The detailed descriptions of laboratory techniques used in this thesis can be found in study I-III. Therefore, only the main methods and considerations are listed below.

3.1 MSC ISOLATION AND ANALYSIS

When first described, MSC isolation was based on their plastic adherence in culture, the ability to form CFU-F, and further selection by subsequent passaging of the cells *in vitro*. Although the method is easy to perform, and generates large number of cells, it has some disadvantages. First of all, culture-selected population is very heterogeneous and includes adherent stromal cells, which do not have MSC characteristics (Horwitz and Keating, 2000). Moreover, even after several passages there can be some residual hematopoietic cells in the culture. Finally and notably, *in vitro* cultivation alters the immunophenotype of native MSCs (by distorting the expression of cell surface markers, including CD44), as well as changes the global gene expression profile (Qian et al., 2012). Therefore, in study **II** and **III**, we used flow cytometry-based protocols, established by Qian et al. (Qian et al., 2012), to prospectively isolate native MSCs from mouse and human tissues.

Fluorescence-activated cell sorting (FACS) is a technique that allows to analyze and sort defined cell populations based on their fluorescent characteristics, one cell at a time as they pass through the beam of a laser (Julius et al., 1972). The cells stained by different fluorescence-conjugated antibodies can be separated by creating distinct gates and sorted into up to four subpopulations simultaneously. In the experiments where multicolor flow cytometry panels are used, it is important to include fluorescence minus one (FMO) controls in order to distinguish positive from negative populations, and place the gates correctly (Baumgarth and Roederer, 2000; Roederer, 2001).

3.1.1 Mouse MSC isolation

The isolation procedure consists of bone crushing, collagenase II treatment and trypsinization. All abovementioned steps are needed to dissociate MSCs and mesenchymal progenitor cells, including the cells located at bone surface (the endosteum). To obtain an optimal cell number, bone fragments should be mixed well during both enzymatic treatments. In addition, it is important to use high (10-20%) serum concentration during isolation procedure, and to keep the cells on ice or at 4°C whenever possible to ensure good viability. Prior to FACS, the majority of BM hematopoietic cells were depleted using Dynabeads® magnetic separation technology. However, before incubation with the BM cells, the beads should be washed, in order to remove preservative and biocidal substance present in the product, which might be harmful for the cells (Jones et al., 1980). After separation, the remaining hematopoietic cells were visualized during FACS, by staining with CD45 and TER119, and omitted when gating. The ECs were distinguished from the other stromal cells based on CD31 expression, while the

non-hematopoietic and non-endothelial population was further subdivided into mesenchymal stem, progenitor and mature cells depend on CD44, CD51 and SCA1 markers expression. All the procedures, including FACS, should be performed as quickly as possible to assure good viability of freshly sorted stromal cells.

3.1.2 Human MSC isolation

For primary MSC isolation, the BM was collected into EDTA-coated or heparin-containing tubes to prevent coagulation. Mononuclear cells (MNCs) were isolated by density gradient centrifugation. Diluted BM was layered on top of Lymphoprep slowly and very carefully in order to avoid mixing of these two layers. Prepared tubes were centrifuged with slow acceleration and deceleration to enable gentle separation of different BM components: plasma, buffy coat (upper layer), buffy coat (intermediate layer containing MNCs), the Lymphoprep layer (containing some of the granulocytes and MSCs), and red blood cells (bottom layer). To increase the number and to include all the MSCs, the buffy coat and underneath Lymphoprep layer were collected and further washed in 2mM EDTA solution, to hinder a cell-cell adhesion. Thereafter, we used CD45 and CD235A magnetic beads to deplete the majority of hematopoietic cells prior to FACS, and to enrich stromal cells. The leftover hematopoietic cells were excluded during sorting by staining with CD45, CD235A antibodies, while the ECs were detected by CD31 marker expression. Within non-hematopoietic and non-endothelial population, we identified MSCs based on the lack of CD44 expression, and co-expression of CD146 and CD271. Similarly to murine MSCs, it is important to process fresh BM without any delay, to ensure good quality of the sorted cells.

3.2 CFU-F ASSAY

CFU-F is an assay used to asses functional MSCs and quantify their number *in vitro* as each colony originates from a single cell (Castro-Malaspina et al., 1980; Friedenstein et al., 1970). We performed CFU-F assays on total, unfractionated BM cells to investigate whether frequencies of MSCs in *Sipa1*$^{-/-}$ mice (study **II**) and CML patient BM (study **III**) were changed compared to normal counterparts (wild-type mice and healthy volunteers, respectively). Additionally, we established CFU-F assays on FACS purified MSCs, to compare the clonogenic activity between cells derived from *Sipa1*$^{-/-}$ and wild-type mice (study **II**), as well as CML patients and healthy age-matched donors (study **III**). Sorted CD44^{-} MSCs from mouse and human BM were cultured for 10-12 days or 12-14 days (respectively), under hypoxic conditions, to prevent senescence and facilitate cell expansion (Tsai et al., 2011). The clusters of more than 50 cells were counted as one colony (Qian et al., 2012).

3.3 IN VITRO MULTILINEAGE DIFFERENTIATION ASSAY

Multilineage differentiation potential towards osteoblasts (bone), chondrocytes (cartilage) and adipocytes (fat) is one of the MSC characteristics. Although *in vitro* expanded cell cultures might not reflect the multipotency *in vivo* (reviewed in Bianco et al., 2008), it is still commonly used assay to predict differentiation potential of MSCs. It is important to note that high input cell density and cell confluence not lower than 80% are crucial for successful osteoblasts

differentiation (reflected by calcium deposition stained with Alizarin Red) as recently reported (Abo-Aziza and A A, 2017). It is also challenging to induce chondrogenic differentiation in monolayer, however optimized culture conditions, including low concentration of serum, and hypoxia, allowed us to differentiate MSCs into chondrocytes *in vitro* as indicated by Toluidine Blue staining of proteoglycans.

We used multilineage differentiation assay in study **II** and **III**, to investigate how myeloproliferative disorders affect MSC functions. Hence, we sorted MSCs from *Sipa1*$^{-/-}$ and wild-type mice (study **II**), and compared their potential to differentiate. Similarly, we used this approach to study multilineage differentiation capacity of the MSCs derived from CML patients and healthy donors (study **III**). In addition, we compared *in vitro* results with gene expression in freshly isolated MSCs, to predict *in vivo* functions of the cells.

3.4 IN VITRO CO-CULTURES AND HEMATOPOIETIC COLONY ASSAYS

As described in the previous sections, the BM niche, including MSCs, regulates and maintains HSCs by providing various signals, soluble factors and through cell-cell interaction. To study such hematopoiesis supportive function of BM MSCs *in vitro*, a co-culture system is required. The effects of MSCs on hematopoietic stem/progenitor cells after co-culture, can be assessed by applying well described and commonly used HSC assays, namely colony-forming unit cell (CFU-C) and long-term culture initiating cell (LTC-IC).

The CFU-C assay is a rapid, *in vitro* method used to measure the frequency of hematopoietic progenitors and their ability to proliferate and differentiate in response to cytokines (Bradley and Metcalf, 1966). CFU-C assays are set by seeding single-cell suspension into semi-solid media containing selected cytokines that are important for HSCs and progenitors growth. The proliferation and differentiation capacity of each plated hematopoietic cell is measured by their ability to form hematopoietic colonies. The viscosity of the media prevents cell migration, and thus, ensures that clonal progeny remain in close proximity (within one colony). This, together with a low density of plated cells, guarantee that each colony derives from one progenitor cell. The differentiation potential of more mature hematopoietic progenitors is restricted to a specific lineage, therefore they give rise to morphologically distinct colonies composed of one type of the cells, e.g. erythrocytes (CFU-E/BFU-E), granulocytes (CFU-G) or monocytes (CFU-M). The more immature, bi- or multipotent HSCs and hematopoietic progenitors usually form bigger colonies which contains a mixture of different cell types, e.g. granulocutes and monocytes (CFU-GM), as well as erythrocytes (CFU-GME) (Purton and Scadden, 2007; van Os et al., 2008). The colony classification and counting is based on morphological and phenotypic criteria, therefore, it requires the experience. CFU-E or BFU-E colonies derived from mouse cells are difficult to count, because they do not appear red. In this case, to confirm the identity of erythroid colonies, the DAF staining of hemoglobin is needed (Worthington et al., 1987). As a result, CFU-E and BFU-E colonies turn blue, whereas GM colonies remain unstained.

In order to identify and quantify even more primitive progenitors, it is necessary to use LTC-IC assay. LTC-IC requires more extensive self-renewal capacity than CFU-C, and therefore, it is used to represent the most primitive hematopoietic cells that can be functionally assayed *in vitro* (Ploemacher et al., 1991; Ploemacher et al., 1989; Sutherland et al., 1989; Sutherland et al., 1990). The assay is performed by culturing hematopoietic cells on a feeder (supportive) layer of the BM stromal cells for five to six weeks followed by CFU-C assay (Sutherland et al., 1991; Sutherland et al., 1993; Zandstra et al., 1998). The feeder layer used can be either the primary BM stromal cells or a cell line. Well established cell lines used for standard LTC-IC assay are murine M2-10B4 and Sl/Sl, both engineered to express human cytokines (IL-3, G-CSF and SCF) (Hogge et al., 1996). During such a long culture time, only the most primitive HSCs can survive and maintain their functional properties, including CFU-C potential. By performing LTC-IC assays at limiting dilution, it is also possible to quantify the numbers of HSCs and hematopoietic progenitors in the tested population (Miller and Eaves, 2002; Szilvassy et al., 1990). Although these culture does not measure the true functional potential of HSCs to repopulate a marrow, and variations in the procedures or stromal cell layers can affect the results, the LTC-IC assay is regarded as a surrogate measure of multipotent stem cells in human, especially when long-term transplantation experiments are impractical. Moreover, the LTC-IC assay is useful for screening the effect of a certain treatment, when the culture conditions are the same and the only variable is the presence of tested agent. It can be used to identify cytokines and other compounds that promote or inhibit hematopoiesis. Since transplantation in humans for research purpose is unethical, and patient-derived xenotransplantation (PDX) models are challenging (due to low engraftment of human cells, particularly primary leukemic cells), the LTC-IC assay is also a valid method to test the hematopoiesis supportive function, and interaction of human MSCs with HSCs and progenitors.

In study **I**, we used limiting dilution LTC-IC assays to test functional response of human primitive LSCs to treatment with leukotriene inhibitors (zileuton or montelukast) alone and in combination with TKI (imatinib). As a feeder layer, we used irradiated M2-10B4 cell line, to prevent their over-proliferation, while still allowing the support for hematopoietic progenitors. In this study, we used only one cell line, to avoid the over-growth of LSCs. FACS purified $CD34^+CD38^-$ cells from CML patients were plated directly on top of supportive cell layer, and then exposed to tested drugs or DMSO. After 6 weeks followed by CFU-C assays, we scored wells as positive (with hematopoietic colonies) or negative (without colonies) and calculated frequency of LTC-IC using L-Calc™ software. We achieved high frequency of LTC-IC from both, NBM and CML $CD34^+CD38^-$ cells while testing the drug response.

In study **II**, we co-cultured normal hematopoietic stem/progenitor cells (LSK) with freshly sorted BM stromal cells from young, disease-free *Sipa1*$^{-/-}$ mice, to evaluate the hematopoiesis supportive function of the BM stromal cells. The cells were cultured in direct contact for 2.5 days, to allow cell-cell interaction (similar to that in bone marrow niche) and avoid the negative

effect of long time culture on HSCs and hematopoietic progenitors. Thereafter, we used CFU-C assay to assess the clonogenic capacity of the cells.

In study **III**, we performed LTC-IC assays to investigate hematopoiesis supportive function of CML MSCs. Hence, we sorted normal HSCs and progenitor cells ($CD34^+$) and culture them in serum-free media, on top of non-irradiated MSCs from either CML patients, or normal donors. At the same time we wanted to study whether normal BM niche can "correct" the phenotype of leukemic cells, or whether NBM MSCs can support non-leukemic ($BCR\text{-}ABL^-$) cells better than leukemic cells ($BCR\text{-}ABL^+$). Therefore, we plated $CD34^+$ from CML patients on top of normal MSCs and CML MSCs for 6 weeks. Thereafter, we subjected the $CD34^+$ cells for CFU-C assay and compared their clonogenic potential.

Additionally, we utilized LTC-IC assay, to understand the effect of CXCL14 on the growth of CML LSCs. For that purpose, we FACS purified $CD34^+CD38^-$ cells from CML patients and co-cultured them with murine fibroblast cell line overexpressing human CXCL14 (NIH3-CXCL14), or control (NIH3-CTRL) for 7 days. After that, CML cells were transferred to LTC-IC assay (established as described above, in study **I**), to evaluate the remaining CML LSCs after the co-culture. After 6 weeks, the colony forming potential of the CML LSCs was measured by CFU-C assay.

3.5 TRANSPLANTATION EXPERIMENTS

The BM transplantation is a gold standard stem cell assay *in vivo*, used to functionally evaluate donor-derived HSCs for their self-renewal and differentiation capacity, by determining the blood cell reconstitution after transplantation. However, the outcome of the assay very much depends on the normal differentiation capacity of tested cells. Therefore, it is important to ensure that genetic alterations in HSCs, if any, does not impair their potential to differentiate towards all blood lineages. Otherwise, it might be impossible to observe lineage reconstitution in a long term. Moreover, transplantation assay usually requires myeloablative pretreatment, e.g. total-body irradiation, to eradicate the endogenous cells and create space for the donor cells to engraft (Green and Rubin, 2014). The irradiation doses can differ depends on the scientific question and the mouse strain. Nevertheless, it should be noted that such treatment might cause tissue damage (e.g. vasculature in BM niche), and elicits inflammatory response, therefore it does not fully recapitulate normal conditions.

In study **II,** we performed BM transplantation experiments, to investigate whether deficiency of *Sipa1* in the hematopoietic cells, or in the BM niche led to MDS/MPN development. To address that question, we applied two different doses of total-body irradiation before transplantation. The sublethal irradiation (6Gy) of $Sipa1^{-/-}$ mice provided enough space in the BM for the donor cells engraftment, but still maintained physiological functions of the BM niche. This allowed us to study contribution of the $Sipa1^{-/-}$ BM niche to MPN development. On the other hand, the lethal irradiation (9.5Gy) of wild-type mice led to eradication of endogenous cells to the greatest degree, and gave us the possibility to test the role of $Sipa1^{-/-}$ hematopoietic cells in MDS/MPN onset. Thanks to this experimental setting, we could observe whether

abnormalities in the hematopoietic cells caused MDS/MPN, or alterations in the BM niche resulted in the disease. To monitor transplantation outcome, we analyzed donor cell reconstitution in the peripheral blood of recipient mice monthly, using automated cell counter for blood components, flow cytometry, and histological staining of blood smears and cytospins.

3.6 QUANTITATIVE AND SINGLE CELL RT-PCR

The gene expression can be measured by amount of messenger RNA (mRNA) transcript copies of that gene. However, in order to detect and quantify low abundance mRNA, it is necessary to amplify the transcripts using RT-PCR (Bustin, 2000). It is rapid and sensitive technique, but its accuracy and reliability depends on preceding preparation steps, including quantity and quality of nucleic acid and reverse transcriptase efficiency. Therefore, it is very important to use the same RNA extraction method, and to normalize the amount of input RNA, to be able to compare gene expression between different samples. Moreover, for direct comparisons of RT-qPCR data, it is crucial to include a reference (housekeeping) gene, which expression level is stable among the samples and not regulated by specific experimental settings. That enables the normalization of expression level of target gene. In addition, the specificity of the assay relies on the correct design of primers, and selected probes used for transcript amplification, which should have an optimal length (90-150bp) and sufficient GC content (20-80%). The RNA templates have to be also free from contaminating DNA, which could affect the amplification signal (Bustin et al., 2009).

We here used RT-qPCR to measure mRNA levels of chosen genes, and to validate gene expression data obtained from RNA sequencing. In study **II** and **III**, we used RNeasy MicroKit® (Qiagen) designed for RNA purification from the small number of cells, and SuperScript™ III Reverse Transcriptase (ThermoFisher) that offers higher cDNA yields and lengths. To normalize the expression data, we chose *Hprt*/*HPRT* as a housekeeping gene, due to its consistent expression level in the BM stromal cells. In order to avoid detection of non-specific, double-stranded DNA, we used TaqMan® fluorogenic probes, which require specific hybridization between the probes and the targets, to generate fluorescent signal. Therefore, RT-qPCR gives low background and detects only the specific amplification product, as it accumulates during PCR. The reactions without RNA template were included as a negative control, to ensure lack of contamination in tested samples.

In study **I**, single $CD34^{+}CD38^{-}$ CML cells were sorted into 96-well plates containing lysis buffer, and deposited at -80°C. Cell lysates were reverse-transcribed using multiple pairs of gene-specific primers, and subjected to the first-round of PCR. The aliquots of the products were further amplified using fully nested, gene-specific primers. The second-round PCR products were applied to gel electrophoresis, and visualized by GelRed™ staining (Invitrogen) (Hu et al., 1997; Qian et al., 2013). An absence of housekeeping gene (*HPRT)* product could arise from technical errors in sorting, such as failure in cell deposition or deposition of a dead cell. Therefore, only the wells with amplification of *HPRT*, or at least two other gene products (if negative for *HPRT*) were considered informative. Empty wells were used as a negative control and never showed signals for tested genes.

3.7 RNA SEQUENCING AND DATA ANALYSIS

RNA sequencing (RNAseq) is a high-throughput technique, used to study the presence and quantity of mRNA in the sample at a given time (Wang et al., 2009). It provides information on the global gene expression pattern, and therefore can be used to identify dysregulated molecules and signaling pathways in the disease (McGettigan, 2013). In brief, the experimental work flow includes four steps: RNA extraction, cDNA synthesis, fragmentation and library synthesis. The first step is the purification of mRNA from total RNA content. As ribosomal RNA (rRNA) represents vast majority of total RNA, it is important to use a good extraction protocol and measure mRNA quality (e.g. using bioanalyzer). The next step is the reverse transcription of mRNA to more stable cDNA, which is further fragmented into sequences of appropriate size (200-300bp) to reduce the influence of primer binding site. Fragmentation is followed by ligation of specific synthetic adaptor sequences at the end of the cDNA fragments, and thereafter, the libraries are amplified to assure sufficient signal intensity during next generation sequencing (NGS). The sequencing starts with adding fluorescent probes to the nucleotides in the flow cell. Once the probes are attached, machine scans the fluorescent signals and translate them into a nucleotides. After that, the probes are washed off and can bind to the next base in the fragment. Such cycle repeats until the machine determines each sequence of nucleotides generating millions of reads at desired length. That raw data have to be first filtered, in order to remove the low quality base calls (with dim fluorescent signal), the artifact reads (e.g. adapter sequences) and the PCR duplicates. Thereafter, the reads are aligned to the reference genome and mapped to the specific genes. Number of reads can differ between sequenced samples (as it depends on the library quality and sample concentration), hence it is important to normalize the raw data, e.g. by calculating reads/fragments per kilobase per million (RPKM/FPKM) or trimmed mean of M-values (TMM). Genes with RPKM values greater than 0.1 are considered to be actively transcribed, and they are used for Differential Gene Expression (DGE) analysis. In addition, TMM normalized data are used to perform Gene Set Enrichment Analysis (GSEA).

We performed RNAseq of freshly sorted stromal cell populations from the BM of *Sipa1*$^{-/-}$ mice (paper **II**) and CML patients (paper **III**), in order to compare the gene expression with healthy counterparts, and tereby, to identify molecular mechanisms involved in the pathogenesis of myeloproliferation.

4 RESULTS AND DISCUSSIONS

4.1 Paper I

Although TKI treatment has been very effective in inducing deep molecular remission in most of the CML patients, it fails to eradicate more quiescent BCR-ABL1$^+$ LSCs. These remaining LSCs cause disease persistence and may contribute to relapse after treatment discontinuation. Therefore, it is important to identify and target additional pathways to selectively eliminate the residual disease. In this study, we tested the effect of leukotriene pathway inhibition on survival of human CML leukemic stem and progenitor cells.

4.1.1 Leukotriene signaling

Leukotrienes (LT) are lipid mediators involved in inflammatory processes and indicated in malignant diseases (Peters-Golden and Henderson, 2007). LT synthesis can be activated by different stimuli (e.g. antigens, microbes, complement, cytokines etc), and it depends on the amount of free arachidonate released from cell-membrane phospholipids. Arachidonic acid is converted into leukotriene A_4 (LTA_4) by 5-lipoxygenase (5-LO) accompanied by its activating protein FLAP (Peters-Golden and Brock, 2003). Then, LTA_4 can be hydrolyzed to LTB_4 or converted to cysteinyl-containing LTC_4, which is further metabolized to cysteinyl LTD_4 and LTE_4. Both products are transported from the cell by specific carrier proteins where they interact with their receptors – B leukotriene receptor 1 (BLT1) and 2 (BLT2) or cysteinyl leukotriene receptor 1 (CYSLT1) and 2 (CYSLT2). Once ligated, all of the mentioned LT receptors activate the Gq or Gi class of G-proteins, thereby causing an increase in intracellular calcium or decrease in cycling AMP (Figure 6). These signals, in turn, lead to activation of kinase cascades and various biological responses (Peters-Golden and Henderson, 2007).

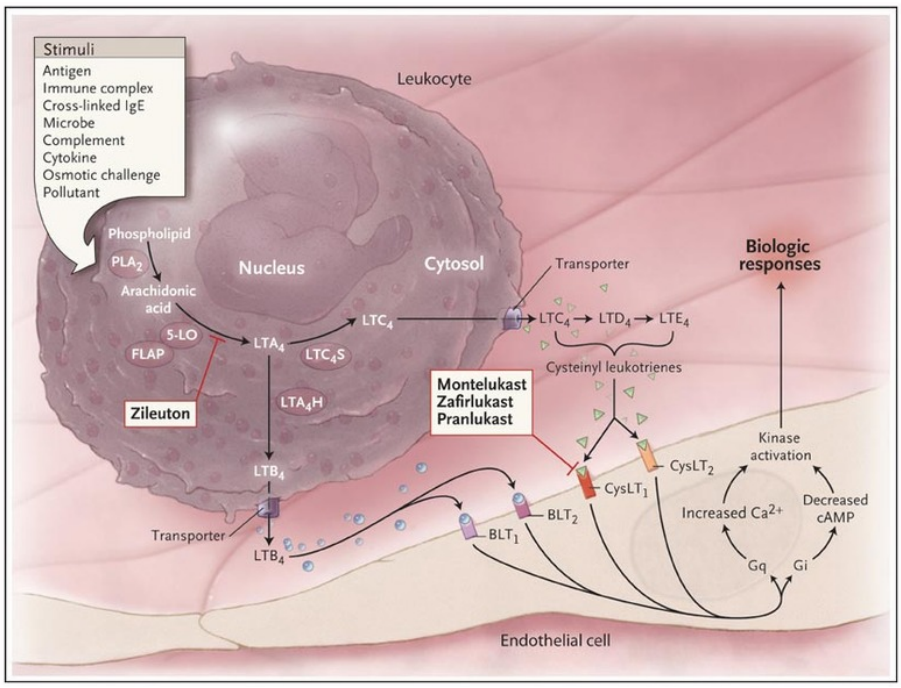

Figure 6. Leukotriene signaling, synthesis and receptors; reproduced with permission from Peters-Golden and Henderson, 2007, copyright Massachusetts Medical Society.

In the murine model of CML, *Alox5* (the gene encoding 5-LO) was overexpressed in HSCs transfected with BCR-ABL (Chen et al., 2009), and the deletion of *Alox5* led to impaired development of leukemia. Similarly, treatment with 5-LO inhibitor, zileuton, resulted in leukemia inhibition without affecting the growth of non-leukemic HSCs (Chen et al., 2009). Therefore, zileuton was suggested as a new therapeutic agent with potential to eradicate also human LSCs and improve treatment outcome of CML patients (O'Hare et al., 2012). However, only low expression level of *ALOX5* was detected in hematopoietic progenitor cells ($CD34^+$) from CML patients at diagnosis, when compared to normal counterparts (Lucas et al., 2014). On the other hand, higher expression and activity of LTC_4 synthase was found in myeloid cells from CML patients (Roos et al., 2008; Sjölinder et al., 2000). Moreover, expression of CYSLT receptors was reported in the CML cell lines, and treatment with CYSLT1 antagonist, montelukast, was shown to suppress the growth of the CML blast crisis cell line (Yektaei-Karin et al., 2017).

To better understand the role of LT pathway in regulation of LSCs and progenitors from CML patients, we analyzed the expression of LT signaling molecules in BM $CD34^+CD38^-$ cells, and tested the functional response of these cells to zileuton and montelukast treatment *in vitro*.

4.1.2 Key findings

1. Multiplex single cell PCR analysis of $CD34^+CD38^-$ cells from CML patients at diagnosis revealed low frequency of *ALOX5*-expressing leukemic $BCR\text{-}ABL^+$ cells (median 10,6%).
2. Majority of CML $CD34^+CD38^-$ express *CYSLT1* and *CYSLT2* receptors (64,5% and 57,3%, respectively) as deceted by PCR at single cell level.
3. However, functional *in vitro* assays (LTC-IC and CFU-C) demontrated no significant differences in the CML $CD34^+CD38^-$ activity after treatment with neither zileuton, nor montelukast alone, indicating that LT inhibition did not suppress the growth of LSCs.
4. Moreover, no significant reduction in the LTC-IC activity of CML $CD34^+CD38^-$ was found after combine treatment of LT inhibitors and imatinib, suggesting that there is no additive effect of the drugs on LSC survival *in vitro*.
5. Expectedly, no significant changes in the frequencies of LTC-ICs were seen in the normal BM $CD34^+CD38^-$ cells cultured with imanitib, zileuton or montelukast.
6. FISH analysis after culture with imatinib and LT inhibitors revealed that there are remaining leukemic $BCR\text{-}ABL^+$ cells after all types of treatments, indicating that even when the total frequencies of LTC-ICs were reduced by imatinib, the LSCs were not eradicated.

4.1.3 Discussion

In contrast to previously published mouse studies (Chen et al., 2009), primitive $CD34^+CD38^-$ cells, sorted from the BM of CML patients at diagnosis, show low expression of *ALOX5*, what indicates a distinct regulation of 5-LO/ALOX5 in mouse and human. In addition, zileuton did not suppress the LTC-IC activity of primary CML $CD34^+CD38^-$ cells in functional stem and

progenitor cell assays *in vitro*. These results suggest that targeting the 5-LO to eradicate imatinib-resistant LSCs, may not be as promising therapeutic approach as expected based on the results from mouse studies. Moreover, in contrast to the results obtained from short term liquid cultures (Yektaei-Karin et al., 2017), we did not observe any significant growth suppression of primitive CML $CD34^{+}CD38^{-}$ cells in response to montelukast in the presence of BM stromal cells. These results suggest that pharmacological inhibition of CYSLT1 might not be a sufficient strategy to eliminate persistent LSCs, and to treat CML patients.

Taken together, our data show that treatment with zileuton, or montelukast has only minor effect on the survival of human LSCs, when cultured in the presence of BM stromal cells *in vitro.* The inconsistency with the previous studies using liquid culture systems, might be attributed to the BM niche-mediated mechanism of drug resistance.

4.2 PAPER II

Mutations or abnormal expression of signal-induced proliferation-associated gene 1 (*SIPA1*) was reported in the patients with solid cancer and hematopoietic malignancies (Minato and Hattori, 2009). In mice, *Sipa1* deficiency leads to the development of age-dependent myeloid malignancies (Ishida et al., 2003; Xiao et al., 2018a). Since an increasing evidence suggest that the BM niche can play an instructive role in development of myeloid malignancies, in this study we investigated the role of *Sipa1*-deficient BM niche in disease initiation.

4.2.1 Sipa1 in Rap G protein signaling

Rap proteins are ones of the small G proteins, which belong to Ras superfamily and control various cellular processes, e.g. cell adhesion, cell proliferation and gene activation. Importantly, dysregulated Ras activation can lead to senescence and apoptosis in the normal cells.

Rap proteins function as molecular switches with inactive GDP-bound and active GTP-bound confirmation, and are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Figure 7). GEFs convert Rap-GDP into active form, whereas GAPs interact with GTP-loaded Rap and mediate its hydrolysis and inactivation. *Sipa1* encodes one of RapGAPs. Iniatially *Sipa1* was reported to be predominantly expressed in lymphocytes and hematopoietic progenitor cells (Hattori et al., 1995), where it regulates expression of many growth factors and cytokines, e.g. IL-3, GM-CSF and CXCL12 (Hattori and Minato, 2003; Jin et al., 2006a).

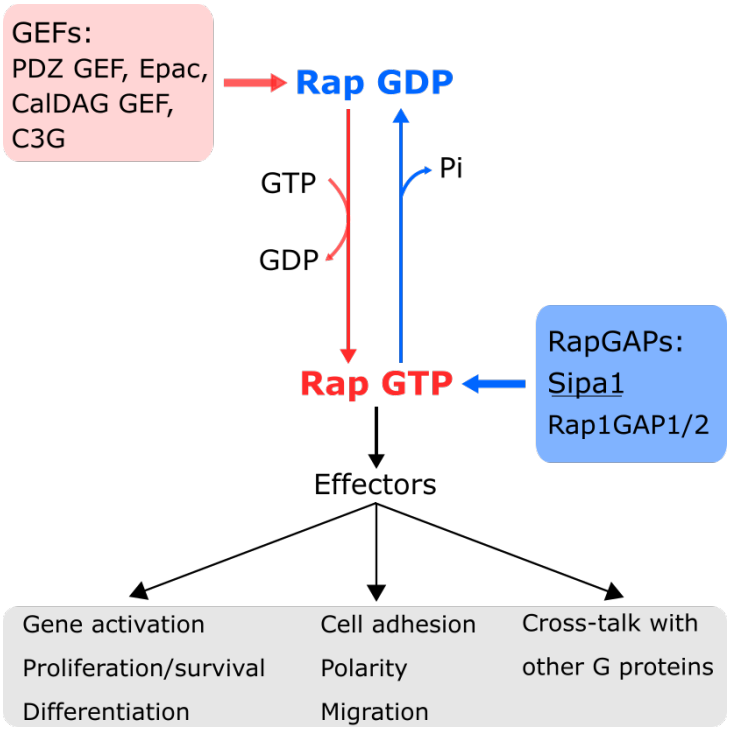

Figure 7. The role of Sipa1 in regulation of Rap signaling. Adapted from Minato and Hattori, 2009.

4.2.2 Key findings

1. *Sipa1* was expressed not only in hematopoietic cells, but also in different BM stromal cells (including ECs, MSCs, mesenchymal progenitors and more mature stromal cells), suggesting that the BM niche might be involved in the development of MPN in *Sipa1*$^{-/-}$ mice.
2. Importantly, *SIPA1* was expressed in normal BM stromal cells from healthy donors, and was downregulated in the BM stromal cells from MPN patients, indicating a that altered *SIPA1* expression might contribute to the disease pathogenesis in patients too.
3. FACS analysis and multilineage differentiation assay revealed a decreased frequency of MSCs, but increased frequency of MPC population, and impaired osteogenic differentiation potential *in vitro*, demonstrating the presence of phenotypic and functional alterations of the BM niche in aged *Sipa1*$^{-/-}$ mice after MDS/MPN onset.
4. However, transplantation of *Sipa1*$^{-/-}$ BM hematopoietic cells into the wild-type recipients did not lead to MPN development, indicating that the loss of *Sipa1* in hematopoietic cells only was not sufficient to initate the disease.
5. Interestingly, significant expansion of MSCs and MPCs was found in the young *Sipa1*$^{-/-}$ mice, prior to MDS/MPN onset. In addition, these MSCs and MPCs displayed: enhanced capacity to support myeloid cell differentiation *in vitro*, reduced osteogenic differentiation potential *in vitro*, and reduced femoral volume *in vivo*, altogether suggesting that dramatic alerations in the BM niche composition and functions occurred before the abnormal hematopoiesis.
6. Moreover, transplantation of normal (*Sipa1*$^{+/+}$) BM hematopoietic cells into sublethally irradiated *Sipa1*$^{-/-}$ recipients resulted in the ineffective hematopoiesis, dysplastic erythropoiesis and significant increase in donor-derived HSCs and progenitors. Even more severe MDS/MPN phenotype (with reduced survival, myeloproliferation, and increased frequency of MEPs and MK hyperplasia) was observed when normal BM hematopoietic cells were transplanted into lethally irradiated *Sipa1*$^{-/-}$ recipients, indicating that normal BM hematopoietic cells transformed into neoplastic cells after transplantation into the *Sipa1*-deficient BM niche.
7. RNA sequencing of FACS sorted, primary BM MSCs and ECs from young *Sipa1*$^{-/-}$ mice revealed elevated expression of genes involved in G-protein signaling in young *Sipa1*$^{-/-}$ mice. Moreover, upregulated IL-6/JAK2/STAT3, TGFβ and TNFα signaling were found in *Sipa1*$^{-/-}$ MSCs and ECs, suggesting inflammation-mediated disease mechanisms. In addition, increased expression of *Thpo* and *Epo* in *Sipa1*$^{-/-}$ MPCs and ECs (respectively) might be involved in MEP overproduction and MK dysplasia.
8. Furthermore, QPCR analysis revealed that genes critical for normal HSC maintanance (including *Cxcl12*, *Kitl* and *Angptl1*), as well as lymphogenesis (*Il7*) were downregulated in *Sipa1*$^{-/-}$ MSCs and MPCs, indicating impaired hematopoiesis supportive function of the *Sipa1*-deficient BM niche.

4.2.3 Discussion

Although recent studies suggest that alterations in the BM niche may have an instructive role in development of myeloid malignancies, the underlying mechanisms are poorly understood. In this study, we discovered that the *Sipa1*$^{-/-}$ BM niche is absolutely required for MDS/MPN development in mice. First of all, alterations in BM niche were found in young, disease-free *Sipa1*$^{-/-}$ mice, what suggests that these changes were attributed to an intrinsic loss of *Sipa1* in the BM niche, rather than remodeling by malignant hematopoietic cells. Furthermore, young *Sipa1*$^{-/-}$ BM hematopoietic cells failed to induce the disease after transplantation into wild-type recipients with normal BM niche. However, reciprocal transplantation of the wild-type BM hematopoietic cells into irradiated *Sipa1*$^{-/-}$ recipient mice led to development of MDS/MPN with a phenotype very similar to that observed in the aged *Sipa1*$^{-/-}$ mice. It indicates that MDS/MPN in aged *Sipa1*$^{-/-}$ mice is actually driven by altered BM niche. Moreover, we observed reoccurrence of the disease in secondary wild-type recipients after transplantation of the spleen cells from primary *Sipa1*$^{-/-}$ recipients that developed MDS/MPN. These results suggest that *Sipa1*-deficient BM niche might lead to the neoplastic transformation of normal hematopoietic cells. To better understand the molecular mechanisms underlying the niche-induced MDS/MPN, we performed RNA sequencing. Similarly, to previously reported data (Kleppe et al., 2015; Reynaud et al., 2011; Welner et al., 2015), we found increased expression of proinflammatory cytokines (TGFβ and TNFα) and IL-6/JAK2/STAT3 signaling in the BM stromal cells from young *Sipa1*$^{-/-}$ mice. This elevated inflammatory signaling might contribute to the myeloproliferation by altering HSC fate decisions, as suggested before (Čokić et al., 2015; Mirantes et al., 2014). Moreover, upregulation of *Thpo* and *Epo* in the *Sipa1*$^{-/-}$ BM stromal cells might be associated with enhanced granulopoiesis, increased MEP production, and MK hyperplasia. Increased number of MK might, in turn, promote disease progression through the secretion of CXCL4, as previously shown (Zhan et al., 2016). Furthermore, reduced expression of *Il7* might further contribute to the myeloproliferation by perturbing lymphopoiesis (Tsapogas et al., 2011), whereas decreased expression of *Cxcl12*, *Kitl* and *Angptl1* in the *Sipa1*$^{-/-}$ BM stromal cells may affect quiescence of normal HSCs and, eventually, promote myelopoiesis.

Taken together, we have shown that *Sipa1* deficiency in the BM stromal cells induces alterations in the BM niche, which might lead to MDS/MPN development in mice. Moreover, downregulated expression of *SIPA1* in the BM stromal cells from MPN and MDS/MPN patients indicates that alterations in *SIPA1* might also contribute to disease pathogenesis in humans.

4.3 PAPER III

Despite the effective treatment with TKI, the LSCs in CML patients cannot be fully eradicated (Holyoake and Vetrie, 2017). These remaining LSCs might contribute to the minimal residual disease, and relapse after treatment discontinuation. Recent reports suggest that the remodeled BM niche might protect the LSCs, and thereby contribute to disease persistence (Hoggatt, 2016; Krause and Scadden, 2015). However, little is known about the BM niche alterations in CML patients, and the potential effect of abnormal BM niche on the disease progression. In this study, we have characterized the BM stromal cells from newly diagnosed CML patients in chronic phase, and studied the impact of dysregulated niche factors, particularly CXCL14, on the LSC survival.

4.3.1 CXCL14

CXCL14 is a chemokine initially identified in breast and kidney cells, and therefore named the breast and kidney-expressed chemokine (BRAK) (Hromas et al., 1999). Up to date, the identity of CXCL14 receptor, and thus intracellular signaling pathway, remains poorly understood (Benarafa and Wolf, 2015). Nevertheless, CXCL14 has been suggested to act as a potent inhibitor of angiogenesis (Shellenberger et al., 2004). Moreover, CXCL14 has been shown to execute antimicrobial immunity, and to regulate the migration of immune cells (Lu et al., 2016). Therefore, the loss of CXCL14 in tumor site may lead to a low recruitment, and decreased number of tumor-infiltrating dendritic cells. That in turn, might inhibit the ability of dendritic cells to recognize the tumor, and to initiate specific antitumor immune responses, thereby creating a tumor escaping system (Lu et al., 2016). CXCL14 expression is relatively high in normal tissues, but lost in various types of malignancies (Hromas et al., 1999; Frederick et al., 2000; Ozawa et al., 2006). Restoration of CXCL14 expression has been shown to suppress the growth of prostate cancer, lung cancer and squamous cell carcinoma (Hata et al., 2015; Ozawa et al., 2009a; Ozawa et al., 2009b; Tessema et al., 2010). On the other hand, a number of studies show that elevated expression of CXCL14 is involved in increased invasion and tumor metastasis (Allinen et al., 2004; Augsten et al., 2009; Lu et al., 2015), together indicating that the role of CXCL14 in cancer may be cell-type specific.

4.3.2 Key findings

1. We demonstrated that BM MSCs from CML patients share similar imunophenotype with normal BM MSCs, and that the frequency of CML MSCs was comparable to that in the normal BM.
2. However, CML MSCs displayed skewed multilineage differentiation capacity *in vitro* with increased adipogenic, but impaired osteogenic and chondrogenic differentiation potential. In addition, we observed that genes associated with osteoblasts and chondrocytes differentiation, were downregulated in freshly sorted CML MSCs.
3. Moreover, normal hematopoietic stem/progenitor cells ($CD34^+$ cells) generated more LTC-IC after co-culture with CML MSCs, compared to the cells cultured with NBM MSCs. The LTC-IC activity of normal $CD34^+$ after co-culture with CML MSCs was

similar to that of CML $CD34^+$ cells, altogether suggesting an enhanced hematopoiesis supportive function of CML MSCs *in vitro.*

4. In contast, the LTC-IC activity of CML $CD34^+$ cells after co-culture with normal BM MSCs and CML MSCs did not differ significantly, suggesting that normal BM MSCs cannot control the growth of leukemic $CD34^+$ cells *in vitro.*
5. FISH analysis of freshly sorted BM stromal cells from CML patients did not detect *BCR-ABL1* fusion in ECs, MSCs or mature stromal cells, indicating that CML BM stromal cells are not part of malignant clone.
6. Nevertheless, RNA sequencing of FACS sorted, native BM MSCs revealed downregulation of inflammatory cytokines, growth factors and genes involved in HSC maintenance, but upregulation of the genes involved in DNA packaging, indicating that CML stromal cells have altered gene expression profile.
7. Moreover, loss of *CXCL14* expression (as shown by both RNA sequencing and QPCR analysis) was detected in the BM niche (including ECs, MSCs and mature stromal cells) from CML patients
8. Most importantly, co-culture of CML LSCs ($CD34^+CD38^-$) in the presence of CXCL14 resulted in the increase numbers of CML cells, but decreased CFU-C and LTC-IC activity of these cells. In line with that, FACS analysis of CML cells after co-culture showed a reduced frequency of $CD34^+CD38^-$ LSCs, but an increased fraction of $CD15^+CD66B^+$ myeloid cells, indicating that CXCL14 promoted differentiation of CML LSCs and supressed their growth *in vitro*.

4.3.3 Discussion

The BM niche-mediated protection of CML LSCs, through the cell-cell interactions or secretion of soluble factors, has been suggested as a potential mechanism involved in disease persistence (Tabe and Konopleva, 2017). Therefore, it is of a great importance to understand how the leukemic BM niche promotes growth of the LSCs. In our study, we prospectively characterized BM niches in CML patients, and we identified dysregulated factors that might serve as a new therapeutic targets. The prospective isolation of the BM cellular components, especially MSCs, is very important, because culture-selected stromal cells consist of very heterogenous population with altered gene expression and cell secretome, compared to freshly isolated MSCs. We found an increased frequency of ECs and decreased fraction of mature stromal cells in the BM of CML patients, which might indicate altered BM cellular niche composition. Although the number of MSCs did not differ between CML and normal BM, CML MSCs displayed altered multilineage differentiation potential, which is important *in vivo* for maintaining the structure and composition of normal HSC niche. These *in vitro* observations were further supported by decreased expression of the genes involved in osteogenic and chondrogenic differentiation in the freshly sorted MSCs, shown by RNA sequencing. Moreover, CML MSCs exhibited a better capacity to support normal hematopoietic stem/progenitor cell activity *in vitro*. On the other hand, the activity of CML leukemic stem/progenitor cells was not affected by culture with normal BM MSCs, suggesting that leukemic cells became independent from normal BM niche *in vitro*. Furthermore, it has

been controversial whether the CML BM niche harbors the same genetic lesions as the leukemic cells, and whether that can be linked to disease pathogenesis. The discrepancy between previously published results might be due to the selection of BM stromal cells in culture, or because of leukemic cell contamination. Therefore, we used freshly isolated, unmanipulated ECs, MSCs and mature stromal cells from CML BM, and performed the FISH analysis. We found that CML BM niche cells did not carry *BCR-ABL1* fusion gene, and therefore they are not part of the malignant clone. To further investigate the molecular characteristics, underlying the abnormal function of CML MSCs, we performed RNA sequencing, and analyzed the global gene expression pattern. The GSEA analysis indicated downregulation of proinflammatory cytokine pathways, chemokine signaling, and decreased expression of the genes important for HSC regulation. On the other hand, CML-derived MSCs and ECs showed a dramatic upregulation of the genes involved in DNA packaging, and increase in epigenetic factors that suppress gene transcription. These results create a possibility that decreased expression of the cytokines and growth factors signaling might be associated with the epigenetic alterations, however, more work is required to elucidate the mechanisms behind molecular alterations in the CML niche. Among downregulated transcripts, we found dramatically reduced expression of *CXCL14* in all analyzed CML BM stromal cells. Since CXCL14 is undetectable in the hematopoietic cells, the stromal cells are the main source of CXCL14 in normal BM. Moreover, enforced expression of CXCL14 in culture led to the loss of CML LSCs and their increased differentiation. Therefore, similarly to the studies on solid tumors (e.g. prostate or lung cancer) (Hata et al., 2015; Ozawa et al., 2009a; Ozawa et al., 2006; Ozawa et al., 2009b; Schwarze et al., 2005; Tessema et al., 2010), our data suggest that CXCL14 suppresses the growth of cancer cells.

Taken together, our study revealed molecular and functional characteristics of the CML BM niche, including loss of *CXCL14* expression, which might contribute to the leukemic cell growth. Restoration of CXCL14 has a suppressive effect on the CML LSCs, and thereby, it might be a new niche-based therapeutic candidate for complementary treatment of CML patients.

5 CONCLUDING REMARKS AND OUTLOOK

The advances in our knowledge over past 10 years led to increased recognition of BM niche and its crucial role in the regulation of normal hematopoiesis. Moreover, continuously emerging studies suggest an important role of BM niche in development of myeloid malignancies. The cross-talk between BM niche and malignant hematopoietic cells, through different pathways, may support LSCs survival as well as protect them from treatment. In addition, LSCs can confer signals that remodel different BM stromal cells into self-reinforcing malignant BM niche. On the other hand, there is an increasing evidence suggesting that BM niche might have an instructive role in initiation of myeloid malignancies. However, it remains unclear whether BM niche alterations facilitate acquisition of mutations in hematopoietic cells or provide a competitive advantage to already mutated clones. Nevertheless, the fact that abnormal BM niche can provide a fertile "soil" for LSCs indicates that targeting the BM niche-derived signals might be a promising therapeutic approach to eradicate minimal residual disease and prevent disease relapse.

Further investigations of the BM niche components and mechanisms regulating hematopoiesis are fundamental to dissect cellular and molecular pathways contributing to hematological disorders. One of the aspects that we need to understand better is the architecture and the cellular interactions in human BM niche. Is there any specific BM niche for LSCs? Or are there any cell subsets that sustain LSCs? Moreover, more studies are required to identify the key BM niche alteration that can lead to leukemia initiation. What are the alterations in the BM niche that contribute to leukemic transformation? Or what kind of abnormalities in the BM niche confer LSCs the competitive advantage over normal HSCs? Are there any undiscovered growth factors that regulate normal and/or malignant hematopoiesis? Furthermore, it is important to elucidate how extramedullary hematopoiesis is regulated and what type of cells and mechanisms in the spleen support LSCs. Finally, we need to recognize the differences between BM niche in mouse and human. Although mechanisms identified in mouse models have given us an important knowledge, they might not fully recapitulate interactions in human. Therefore, we need to continue developing new humanized preclinical models.

Availability of advanced imaging technologies, single cell RNA sequencing as well as genome editing technologies open new possibilities for modeling BM complexity and will hopefully help to answer some of the remaining questions.

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