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MESENCHYMAL NICHE CONTRIBUTION TO NORMAL AND MALIGNANT HEMATOPOIESIS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To knowledge seekers

ABSTRACT

Normal hematopoiesis is tightly regulated by hematopoietic microenvironment/niche in bone marrow (BM) via direct hematopoietic cell-niche cell interaction and factors secreted by various types of cellular niches. The BM niche consists of cells of mesenchymal cell origin including mesenchymal stem cells (MSCs) and progenitor cells (MPCs). Accumulated evidence suggests the important role of BM mesenchymal cell niche for the maintenance of normal hematopoiesis and leukemogenesis. However, the exact role of different niche elements and the molecular mechanisms in leukemia development remain poorly defined. Knowledge is required for developing new therapeutic strategy to effectively treat the diseases.

This thesis focuses on mesenchymal niche contribution to normal and malignant hematopoiesis, particularly, myeloproliferative neoplasms (MPN) and acute myeloid leukemia (AML). By using mouse models, multi-color flow cytometry, RNA-sequencing, transplantation and lineage tracing techniques, the thesis work has demonstrated the contribution of BM mesenchymal cell niche in the initiation of the myeloproliferative disease and progression of AML. The specific role of BM MSCs and laminin isoforms in AML progression and therapy response were studied. In addition, this thesis reports the features of native skin MSCs and their function in supporting normal hematopoietic and AML stem cells.

In **paper I**, the instructive role of BM niches in MDS/MPN initiation is studied by using signal-induced proliferation associated 1 (*Sipa1*) gene deleted mice (*Sipa1*$^{-/-}$) that develop age-dependent MPN. The loss of *Sipa1* induces BM niche alterations prior to the disease onset, including biased differentiation of *Sipa1*$^{-/-}$ MSCs towards adipocytes and upregulated expression of pro-inflammatory genes (*Il-6* and *TGF-β*). Concomitantly, hematopoiesis maintenance gene (*Cxcl12*, *Angptl1*, *Kitl*) expressions were reduced in *Sipa1*$^{-/-}$ BM MSCs and MPCs. Transplantation of *Sipa1*$^{+/+}$ hematopoietic cells to young *Sipa1*$^{-/-}$ mice resulted in MDS/MPN development, supporting the causative role of *Sipa1* deficient niche for the development of MDS/MPN.

The role of BM MSCs and MPCs during progression of AML is reported in **paper II**. By transplanting MLL-AF9^{+} AML cells to immunocompetent mice, we showed dynamic niche alterations induced by AML cells. During AML development, frequency of BM MSC & MPC were increased while hematopoiesis gene (*Kitl*, *Cxcl12*, *Angptl1*, *Nov* and *Igf1*) expression in BM MPCs were down regulated in correlation to AML engraftment in BM. Moreover, the expression of pro-inflammatory gene (*Il-6*) is elevated following the AML progression. Specifically, BM primitive subset of MSC (Ebf2^{+}) is altered by AML cells to generate more progenies including Ebf2^{-}MSC, MPC and CD44^{+} cells in the leukemic niche. The depletion of Ebf2^{+} cells accelerated AML development, demonstrating the suppressive role of Ebf2^{+} MSCs in AML progression possibly by maintaining normal hematopoiesis.

In this study, upregulation of *laminin 4* (*Lama4*) in both MSC and MPC was observed. To further investigate the functional consequence of *Lama4* during AML development, the *Lama4* (*Lama4*$^{-/-}$) deficient mice were employed in **paper III**.

We firstly studied the role of *Lama4* in hematopoiesis regeneration following irradiation-induced stress and observed impaired recovery of erythropoiesis and megakaryopoiesis in *Lama4*$^{-/-}$ mice. On the contrary, AML progression and relapse were accelerated post transplantation of MLL-AF9^{+} AML cells. Furthermore, the *Lama4*$^{-/-}$ MSCs promoted AML cell growth and confer AML stem cell chemoresistance to cytarabine (Ara-C) via providing more metabolic support to the AML stem cells (LSCs). Taken together, paper III shows critical role of *Lama4* in hematopoiesis recovery following irradiation and during AML development.

Recent study has shown that AML LSCs infiltrate extramedullary organ. Meanwhile, skin has been reported to contain MSC-like population although the characteristics are not well defined. In **paper IV**, we employed *Ebf2-gfp* transgenic mice to prospectively characterize skin MSC phenotypically and functionally at bulk and single cell level. Skin Ebf2^{+} cells represent purified MSC while the Ebf2^{-} fraction contained more differentiated MSCs that can be generated by the Ebf2^{+} cells, revealed by the *in vivo* lineage tracing of Ebf2^{+} MSCs.

Both skin Ebf2^{+} cells and Ebf2^{-}MSC displayed hematopoiesis supportive function, similar to their BM counterpart. Furthermore, co-culture of AML and AML CAFC on skin Ebf2^{+} and Ebf2^{-}MSCs showed that skin MSCs also supported normal HSCs and provided chemoprotection for AML LSCs. In skin tissue of AML mice, infiltration of AML cells was observed and remained in skin tissue after Ara-C treatment, suggesting a possible contribution of skin MSCs to the persistence of AML cells. The skin Ebf2^{+} were found to be reduced in AML mice. However, the functional consequence of the skin MSCs remains to be investigated in the future. Altogether, paper IV reports skin harbors Ebf2^{+} and Ebf2^{-}MSC with similar characteristics to BM MSC. Both skin Ebf2^{+} and Ebf2^{-}MSCs support normal HSC and AML cells. Importantly, skin MSCs provide chemoprotection for AML LSC.

In conclusion, the work in this thesis shows the role of BM niche for the initiation and progression of the myeloid malignancies using several transgenic mouse models. The work also provides evidence for critical role of *Lama4* in hematopoiesis recovery following irradiation and AML progression. Furthermore, the biological features of skin MSCs and their function in supporting normal hematopoietic and AML cells. During Ara-C treatment, skin MSCs also displayed protective role for AML LSCs, indicating skin MSC possible role as a reservoir of chemoresistant AML LSC.

LIST OF SCIENTIFIC PAPERS

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

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

III. Kondo, M.[#], Cai, H.[#], **Sandhow, L.,** Xiao, P., Johansson, A.S., Sasaki, T., Trygvarsson, K., Ekblom, M., Qian H. Critical role of Laminin α4 for hematopoiesis regeneration under irradiation-induced stress and acute myeloid leukemia. *Manuscript.*
 # equal contribution

IV. **Sandhow, L.,** Cai, H., Xiao, P., Kondo, M., Johansson, A.S., Qian, H. Novel characteristics of skin mesenchymal stem cell and its function in normal hematopoiesis and acute myeloid leukemia. *Manuscript.*

RELATED PUBLICATION NOT INCLUDED IN THE THESIS

I. Rudd, S.G., Tsesmetzis, N., Sanjiv, K., Paulin, C.B., **Sandhow, L.,** et al., Ribonucleotide reductase inhibitors suppress SAMHD1 ara-CTPase activity enhancing cytarabine efficacy. EMBO Mol Med, 2020. 12(3): p. e10419.

II. Newton, P.T., Li, L., Zhou, B., Schweingruber, C., Hovorakova, M., Xie, M., Sun, X., **Sandhow, L**., et al., A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. Nature, 2019. 567(7747): p. 234-238.

III. Gao, H., Volat, F., **Sandhow, L**., et al., CD36 Is a Marker of Human Adipocyte Progenitors with Pronounced Adipogenic and Triglyceride Accumulation Potential. Stem Cells, 2017. 35(7): p. 1799-1814.

CONTENTS

LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
BM	Bone Marrow
CAR	Cxcl12-abundant Reticular Cell
CAFC	Cobblestone Area Forming Cell
CFU-C	Colony Forming Unit-Cell
CFU-E	Colony Forming Unit-Erythroid
CFU-F	Colony Forming Unit-Fibroblast
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
CNL	Chronic Neutrophilic Leukaemia
Ebf2	Early B-Cell Factor 2
EC	Endothelial Cell
ECM	Extracellular Matrix
EMCN	Endomucin
FACS	Fluorescence-Activated Cell Sorting
GM	Granulocyte Monocyte
GME	Granulocyte, Monocyte and Erythrocyte
GMP	Granulocyte Monocyte Progenitor
HGB	Hemoglobin
HSC	Hematopoietic Stem Cell
HPC	Hematopoietic Progenitor Cell
HSPC	Hematopoietic Stem and Progenitor Cell
IF	Immunofluorescence
Lama4	Laminin α4 chain
LMPP	Lymphoid Primed Multipotent Progenitor
LT	Long-Term
LSC	Leukemic Stem Cell
MEP	Megakaryocyte Erythroid Progenitor
Mk	Megakaryocyte

MkP	Megakaryocyte Precursor
MLL	Mixed Lymphoid Leukemia
MDS	Myelodysplastic syndromes
MPN	Myeloproliferative neoplasms
MPC	Mesenchymal Progenitor Cell
MSC	Mesenchymal Stem Cell
MSPC	Mesenchymal Stem and Progenitor Cell
OXPHOS	Oxidative Phosphorylation
PLT	Platelet
PMF	Primary Myelofibrosis
PV	Polycythemia Vera
RBC	Red Blood Cell
ROS	Reactive Oxidative Species
Sipa1	Signal-induced proliferation associated 1
SNS	Sympathetic Nervous System
ST	Short Term
TAM	Tamoxifen
WAT	White Adipose Tissue
WBC	White Blood Cell

1 INTRODUCTION

Hematopoiesis is one of the important process in physiological systems that ensures sufficient blood cell production throughout life. After birth, hematopoiesis occurs primarily in the bone marrow (BM) where the hematopoietic stem and progenitor cells (HSPCs) interact with their microenvironment (niche). In BM, HSPCs are organized in well-controlled hematopoietic developmental hierarchy and they are responsible for the generation and the maintenance of hematopoiesis via their balanced self-renewal and differentiation capacity. The processes are tightly regulated by BM niche via direct cell-cell/matrix interactions and factors secreted by the stromal cells as well as intrinsic molecular pathways triggered by the signals from the niche.

The niche is spatial location in BM consisting of heterogeneous niche components, extracellular matrix (ECM) and cytokines that regulates hematopoiesis extrinsically. In steady state hematopoiesis, the loss of niche cells impairs hematopoiesis mostly due to reduction of HSPC in BM (Mendez-Ferrer et al., 2010). Genetic aberration in niche may initiate development of malignant myeloid lineage cells that result in myeloid neoplasm (Hoggatt et al., 2016; Mendez-Ferrer et al., 2020). On the other hand, genetic mutation or rearrangement in hematopoietic cells such as translocation of MLL to AF9 (MLL-AF9) transforms hematopoietic cells intrinsically to clonally proliferated leading to acute myeloid leukemia (AML) at expense of normal hematopoietic cells.

With recent advance in mouse modeling of hematopoiesis, multicolour flow cytometry (FACS) and single cell analysis, different niche cells have been identified based on cell surface markers, allowing further studies on specific niche cell contribution to normal and malignant hematopoiesis. One cellular compartment in BM niche is mesenchymal stem and progenitor cells (MSPCs) which remain heterogenous and consist of a small fraction of mesenchymal stem cells (MSCs) and mesenchymal progenitor cells (MPCs). There is increasing evidence about the role of MSPC in normal and malignant hematopoiesis (Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Raaijmakers et al., 2010; Schepers et al., 2013). However, the niche regulation is complex and dynamic. So far, the exact contribution and molecular mechanism of MSC and MPC during leukemogenesis and leukemia progression remain largely unknown.

The focus of this thesis is to understand the contribution of MSC and MPC to normal and malignant hematopoiesis. By employing several advanced transgenic mouse models, FACS, global gene expression analysis, transplantation and state-of the art co-culture system, the role of BM MSC and MPC during the initiation and progression myeloid malignancy were evaluated. In addition, unlike BM MSC, the MSCs in extramedullary organs such as skin tissue have not been well characterized and their function for hematopoiesis has been ignored. This thesis further extends to study the characteristics of skin MSC as extramedullary AML site and provides evidence for the role of skin MSCs in supporting HSCs and AML-initiating stem cells. The AML infiltration and retention in skin tissue was also observed post chemotherapy.

2 LITERATURE REVIEW

2.1 HEMATOPOIETIC CELL LINEAGE HIERARCHY

The mature blood cells are generated from hematopoietic stem cells (HSCs) through a well-orchestrated developmental hierarchy where the rare primitive HSC population act as the cell source for replenishing all the short-lived mature blood cells. Adult HSCs are quiescent, self-renewal and have capacity to generate different types of blood lineages (Cheshier et al., 1999; Morrison and Weissman, 1994). These cells can be identified based on their unique antigen profile. In mouse hematopoietic system, HSCs do not express mature hematopoietic marker (LIN^-) such as CD3, CD4, CD8, B220, GR-1, NK1.1 and erythroid marker TER119, but express KIT and SCA1 (LSK) (Morrison et al., 1995). HSCs consist of long-term (LT) HSC, short term (ST) and lymphoid primed multipotent progenitor (LMPP). LT-HSC is phenotypically defined as $LSKCD150^+$ while ST-HSC and LMPP are defined as $LSKFLT3^-CD34^+$ and $LSKFLT3^+CD34^+$, respectively (Adolfsson et al., 2001; Christensen and Weissman, 2001; Kiel et al., 2005; Yang et al., 2005). Recent studies using single cell analysis have revealed the heterogeneity of BM HSCs and proposed a revised developmental pathway of HSCs with distinct lineage biased differentiation potency (Carrelha et al., 2018). The generation of almost all mature blood cells follows the hierarchical differentiation process where HSCs can first differentiate to hematopoietic progenitor cells (HPCs) with limited self-renewal capacity, then lineage restricted progenitors and corresponding mature blood lineages (Figure 1).

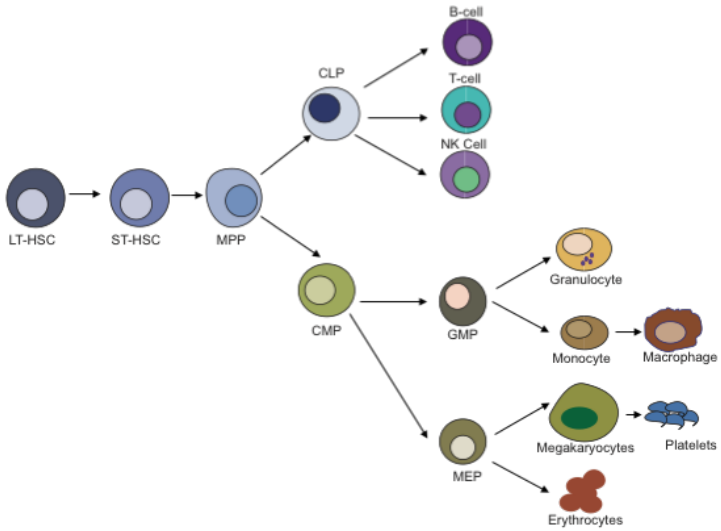

Figure 1. Murine hematopoietic cell hierarchy. All blood lineages are generated from Hematopoietic stem cell (HSCs).

HPCs contain common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CLP is phenotypically defined as $Lin^-IL7R^+Thy1.1^-SCA1^{low}KIT^{low}$ and give rise to lymphoid cells (T, B and NK cells) (Fathman et al., 2011; Kondo et al., 1997). On the other hand, the CMP ($LIN^-SCA1^-KIT^+CD34^+CD15/32^-$) is committed to generate myeloid cell lineage.

In the hematopoietic lineage hierarchy, the CMP give rise to granulocyte monocyte progenitor (GMP, $LIN^-SCA1^-KIT^+CD34^+CD16/32^+$) and megakaryocyte erythroid progenitor (MEP, $LIN^-SCA1^-KIT^+CD34^{low/-}CD16/32^-$) which can be distinguished based on CD16/32 and CD34 expression(Akashi et al., 2000). The GMP is terminally differentiate to granulocyte ($CD11b^+GR\text{-}1^+$) and monocyte ($CD11b^+$) which can further differentiate to macrophage. The MEP is a precursor for erythrocytes ($TER119^+$) and megakaryocyte (Mk, $CD41^+$). Megakaryocytes further undergo maturation to produce platelet. Beside CMP, HSC is also able to generate MEP directly (Pronk et al., 2007).

2.2 BONE MARROW (BM) NICHE

The major anatomical location for hematopoiesis in adult is bone where HSCs reside and function to maintain hematopoiesis. In bone, HSCs preferentially locate at endosteal region with higher potency in comparison to HSPC in central marrow (Grassinger et al., 2010; Haylock et al., 2007). The specialized microenvironment surrounding HSCs is proposed as HSC niche which is not only an anatomical location of the cells but also a functional unit for regulating HSC activities (Scadden, 2006) (Figure 2). There are various cellular niche components and the factors produced by them.

There are two major proposed niche types in bone, initially, the endosteal niche with bone lining osteoblasts as major niche cells and vascular niche with endothelial cells lining in sinusoidal vessels and arterioles as the cellular niche component (Itkin et al., 2016). Furthermore, many other cell types have been demonstrated to act as cellular niche elements for HSCs in mice. This includes MSCs and MPCs as well as adipocytes and osteocytes (Hoggatt et al., 2016; Le et al., 2018). In addition, several types of the cells of hematopoietic origin including megakaryocytes and macrophages were reported to be a cellular niche for HSCs. **Mks** regulates HSC quiescence via TGFb1, CXCL4 and TPO (Bruns et al., 2014; Nakamura-Ishizu et al., 2014; Zhao et al., 2014). Following myelosuppression, Mks migrate to blood vessels and promote HSC expansion through FGF1 to recover the hematopoiesis (Zhao et al., 2014). The **macrophages in BM ($CD169^+$)** promotes retention of HSPC and late erythroid maturation (Chow et al., 2013; Chow et al., 2011).

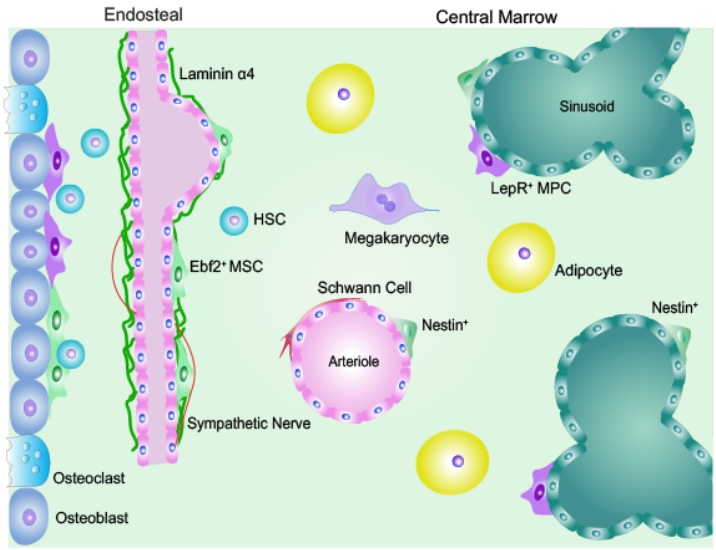

Figure 2 Illustration of mouse BM niche (by Huan Cai, 2020).

This thesis review mainly focuses on the niche cells of mesenchymal cell origin

2.3 CELLULAR COMPARTMENT OF BM MESENCHYMAL NICHE & ROLE IN HEMATOPOIESIS AT STEADY STATE

2.3.1 Mesenchymal Stem Cell (MSCs)

In BM niche, at steady state, MSCs are mostly distributed at endosteal sites. **Phenotypically** mouse BM MSC is defined as population with no expression hematopoietic marker ($CD45^-$ $TER119^-$), EC marker ($CD31^+$) and CD44, but expressing CD51, CD140a/PDGFRA (PαS) and SCA1 (Morikawa et al., 2009; Pinho et al., 2013; Qian et al., 2013; Qian et al., 2012). **Functionally,** MSCs are characterized as cells with ability to form CFU-F and differentiate toward multiple mesenchymal lineages osteogenic, adipogenic and chondrogenic cells at bulk or single cell level (Qian et al., 2013; Sacchetti et al., 2007). Several markers including nestin, leptin receptor, Prx1 were shown to be expressed in MSPCs enriched with MSCs and MPCs.

Some fraction of **$Nestin^+$** cells in BM display MSC characteristics although the CFU-F frequency is as low as 1/130 (Mendez-Ferrer et al., 2010). Depletion of the $Nestin^+$ cells in BM leads to HSC dislocation (Mendez-Ferrer et al., 2010).There are 2 subsets of $Nestin^+$ cells in BM which are $Nestin^{bright}$ at pre-arteriolar and $Nestin^{dim}$ at sinusoidal in central marrow and decorated with sympathetic nerves and schwann cells. The expression of hematopoiesis supportive genes (*Cxcl12*, *Kitl*, *Angptl1*) is higher in $Nestin^{bright}$cells (Kunisaki et al., 2013). The numbers and the function of $Nestin^+$ cells to maintain HSPC in BM are regulated by transcription factor *Snai2* that suppresses *Spp1*, the negative regulator of hematopoiesis (Wei et al., 2020).

Early B-cell Factor 2 (Ebf2) is uniquely expressed in non-hematopoietic and non-endothelial cells and the Ebf2-expressing cells in mouse BM are highly enriched with primitive MSC expressing SCA1, CD51 and CD140a (Qian et al., 2013). There is about 60% of $Ebf2^+$ cells lowly expressing Nestin. In mouse BM, the $Ebf2^+$ cells are in quiescence, located mostly at endosteal sites with MSC characteristics and can generate more differentiated $Ebf2^-$ mesenchymal cells indicating $Ebf2^+$ cells are primitive MSC and contribute to mesenchymal cell turnover. Perturbation of Ebf2 expression reduces HSC in adult mice (Kieslinger et al., 2010).

2.3.2 Mesenchymal Progenitor Cells (MPCs)

Similar to MSC, MPCs are found in non-hematopoietic and endothelial fraction ($CD45^-$ $TER119^-CD31^-$), do not express CD44 but express CD51 and CD140a (Schepers et al., 2013). The phenotypic difference between MSC and MPC is SCA1 expression. While MSCs are $SCA1^+$, the MPC do not express SCA1. MPCs have been studied in different transgenic mouse models that bring different terminology for referring MPC, as discussed below.

CAR (Cxcl12-abundant reticular cells). The terminology comes from study that employs CXCL12-GFP transgenic mice. CAR cells are MPC and distributed in sinusoid area (Sugiyama et al., 2006). CAR highly express *Cxcl12* and *Scf* for maintaining HSPC (Ding and Morrison, 2013; Greenbaum et al., 2013).

This feature and their immunophenotype have been validated by single cell sequencing of the BM stromal cells (Baccin et al., 2020; Baryawno et al., 2019). BM CAR cells have potency to generate both adipocytes and osteoblasts and depletion of the cells abrogates HSPC maintenance and promotes myeloid differentiation (Omatsu et al., 2010). In CAR, transcription factor *Foxc1* is critical for regulating HSPC niche formation since loss of *Foxc1* increases adipogenesis and reduces BM HSPC phenotypically and functionally. In addition, expression of *Cxcl12* and *Scf* are also downregulated when CAR cells lose *Foxc1* (Omatsu et al., 2014). CAR cells in BM express transcription factor early B-cell Factor 3 (*Ebf3*). *Ebf3* expression in CAR controls the osteogenic differentiation, contribute to expression of *Cxcl12* and *Scf* as well as maintaining number of LT-HSC, MEP, GMP and pro-E (Seike et al., 2018).

LepR (Leptin Receptor$^+$) cells are highly overlapping with CAR cells or mainly are SCA1$^-$ MPC (Zhou et al., 2017). Specific loss of *Cxcl12* in LepR$^+$ cells does not alter HSC (Asada et al., 2017). Following irradiation, expression of *Scf* in LepR$^+$ cells plays important role in hematopoiesis recovery in mice (Zhou et al., 2017).

2.3.3 Osteoblast & Adipocyte

Osteoblasts are bone forming cells located at endosteal site. Osteoblasts maintain HSC frequency via Notch signaling by expressing Notch ligand *jagged 1 (Jag1)* for HSC (Calvi et al., 2003). The expression of *Cxcl12* in osteoblast is required to maintain CLPs, but is dispensable for HSC maintenance (Ding and Morrison, 2013). Ablation of osteoblast causes reduction of HSC frequency at quiescence state and impairs HSC self-renew properties as reflected by impaired hematopoiesis reconstitution in secondary transplantation (Bowers et al., 2015). In contrast to osteoblast, **BM adipocytes** is a negative regulator for hematopoiesis maintenance as increased adipocytes reduces frequency and function of HSPC (Naveiras et al., 2009).

2.3.4 Endothelial cell (EC) & Sympathetic nervous system (SNS)

BM EC consists of arteriolar and sinusoidal EC (Itkin et al., 2016; Kusumbe et al., 2014). Arteriolar ECs are distributed in trabecular area, central marrow and close to endosteal area. In central bone (marrow), the large unbranched arteries are wrapped with α-SMA while the arteries close to endosteal area branches to small arteriole with high expression of CD31 and Endomucin (EMCN). The CD31^{high}EMCN^{high} ECs are located closely to endosteal site and mediate growth of bone vessel (Kusumbe et al., 2014). The sinusoids are mostly distributed in central marrow and express low CD31 and EMCN (Kusumbe et al., 2014). Compared to the artery, sinusoid ECs are leaky which is a suitable trafficking site of HSPCs. The arteriolar ECs support quiescence HSC with low ROS level (Itkin et al., 2016). Arteriole in BM is decorated with **sympathetic nervous system (SNS)** that also contribute to hematopoiesis via β3-adrenergic receptor (Mendez-Ferrer et al., 2008).

In arteries, there are a subset of Apelin (Apln$^+$) ECs which co-express EMCN, CD31, VE-cadherin, VEGFR2 and VEGFR3. Compared to other EC, Apln$^+$ EC is highly enriched with gene for sprouting angiogenesis and VEGFR signaling. Depletion of Apln$^+$ EC reduces frequencies of CLP, CMP and HSC in BM as well as impairs HSC function to reconstitute hematopoiesis (Chen et al., 2019).

BM EC has been reported to maintain HSC through E-selectin and Jag1. The interaction of vascular E-selectin with HSCs is critical for maintaining HSC quiescence and preserve HSC from 5-FU injection (Winkler et al., 2012). Furthermore, loss of *Jag1* decreases LT-HSC phenotypically and functionally as LT-HSCs become more cycling (Poulos et al., 2013). Recently, single cell mapping of BM niche cells revealed the expression of other Notch ligands (*Dll1* & *Dll4*) in BM ECs. The abrogation of *Dll4* in ECs decreases CLP frequency while the myeloid progenitor increases (Tikhonova et al., 2019). In addition to *Jag1*, BM EC also express *Cxcl12* which has been shown to be important for maintaining BM HSC (Ding and Morrison, 2013).

2.4 EXTRACELLULAR MATRIX (ECM) IN BM NICHE

In HSC niche unit in BM, besides HSCs and the niche cells, extracellular matrix proteins (ECMs) such as fibronectin, collagen type I, III, IV, osteopontin, laminin, nidogen and tenascin-c are a big family of molecular niche components (Balzano et al., 2019; Nakamura-Ishizu et al., 2012; Nilsson et al., 1998). Existence of extracellular matrix (ECM) not only provides integrity, but also regulating hematopoiesis. HSC anchorage to **Collagen type I** via calcium-sensing receptor enhances CXCR4 signal for HSC retention (Adams et al., 2006; Lam et al., 2011). The anchoring of HSPC to **osteopontin** in BM niche via β1 integrin (α9β1 and α4β1) and CD44 play a role in HSC migration and retention (Grassinger et al., 2009). Osteopontin regulates hematopoiesis in a negative manner since it suppresses the HSC expansion (Stier et al., 2005). **Tenascin-C also involves in regulating hematopoiesis**. At steady state, loss of tenascin-C reduces colony forming capacity while the BM cell number and structure are maintained (Nakamura-Ishizu et al., 2012; Ohta et al., 1998). Furthermore, following myelosuppression, tenascin-C expression in $CD31^+$ and MSPC are upregulated and enhance HSPC proliferation cooperatively with fibronectin to reconstitute hematopoiesis (Nakamura-Ishizu et al., 2012).

Laminin

Laminin is a heterotrimeric glycoprotein in basement membranes of endothelial cells, skeletal muscles and developing kidney (Scheele et al., 2007). There are 18 different isoforms of laminins described based on heterotrimeric combination of α, β and γ chain. In BM, the blood vessels express different laminin chains including laminin α4 chain which is bound via integrin α6. The integrin α6 is widely expressed on HSCs and facilitates HSC migration as well as proliferation (Gu et al., 2003; Qian et al., 2006; Susek et al., 2018). **However, the role of Lama4 in malignant hematopoiesis has not been reported.**

2.5 BM NICHE IN MYELOID MALIGNANCY

Myeloid malignancy (neoplasia) is a group of diseases with perturbed myelopoiesis due to gene alterations related to proliferation, differentiation or self-renewal. Based on 2016 revision to the WHO classification (Arber et al., 2016; Barbui et al., 2018) myeloid neoplasm can be categorized into chronic myeloid neoplasms and acute myeloid leukemia (AML).

The chronic myeloid neoplasms include (1) myeloproliferative neoplasms (MPN), (2) myelodysplastic syndromes (MDS), (3) MDS/MPN (CMML, aCML, MDS/MPN with ringsideroblast &thrombocytosis and JMML), and (4) myeloid / lymphoid neoplasms with eusinophilia and rearrangement of PDGFRA, PDGFRBB and FGFR1 or PMC1-JAK2. MPN could be further classified based on presence of Philadelphia chromosome (BCR-ABL1). The BCR-ABL1^{+} MPN is defined as chronic myeloid leukemia (CML) while BCR-ABL1^{-} MPN is further categorized into polycythemia vera (PV), essential thrombocythemia (ET), chronic neutrophilic leukemia (CNL) and primary myelofibrosis (PMF).

The niche contribution to myeloid malignancies have been increasingly recognized. Studies with mouse models have provided evidence for the role of BM niche in the pathogenesis of the diseases (Hoggatt et al., 2016). Mice with deletion of **Signal-induced proliferation-associated gene-1 (*Sipa1*), a negative regulator in G-protein signaling,** is reported to develop age-dependent myeloproliferative disorders resembling human CML at chronic and blast crisis, anemia and MDS (Ishida et al., 2003; Kometani et al., 2006). Furthermore, activation of BCR-ABL1 reduces *Sipa1* expression and increases frequency of LSK in spleen in mice (Kometani et al., 2006). **However, it is not clear whether the disease is initiated by loss of *Sipa1* in hematopoietic cells or in BM niches**. Nevertheless, the long latency of the disease increases the possibility of clonal evolution of hematopoiesis in the *Sipa1*$^{-/-}$ mice, thus, make the mice serve as a good model to study the potential contribution of BM niche to the MPN development.

2.5.1 The instructive role of BM niche myeloid neoplasm

2.5.1.1 Dicer1 perturbation in osteoprogenitor promotes MDS

By using Osx-GFP-Cre^{+}Dicer$^{fl/fl}$ to delete *Dicer1* in osteoprogenitor cells, Raaijmakers *et al.* 2010 demonstrated the role of osteoprogenitor in the development of MDS (Raaijmakers et al., 2010). The osteoprogenitor lacking *Dicer1* reduces osteogenic differentiation as represented by decreased alkaline phosphatase and calcified deposition *in vitro* and declined mature osteoblast expressing osteocalcin *in vivo*. However, loss of *Dicer1* does not reduce bone volume. Besides alteration in osteogenesis, *Dicer1*-deficient osteoprogenitor cells increase HSC proliferation and impair Mk differentiation. The induced MDS is manifested by leukopenia, profound anemia & thrombocytopenia, splenomegaly, increased myeloid frequency and dysplastic megakaryopoiesis. The causative role of *Dicer1* deficient niche in the disease development was confirmed by transplantation of normal hematopoietic cells in the Osx-GFP-Cre^{+}Dicer$^{fl/fl}$ mice. In addition to bone defects, *Dicer1* deletion also results in reduction of Shwachman-Diamond-Bodian syndrome (*Sdbs*), the gene mutated in patients with SDBS and predisposition to leukemia development (Boocock et al., 2003). This study for the first time proposed the instructive role of a specific BM niche cell population in the initiation of myeloid malignancies. However, the mechanism underlying the malignant transformation of hematopoietic cells should be further studied.

2.5.1.2 Mutation of protein tyrosine phosphatase (Ptpn 11) in MSPCs drives MPN

Ptpn11 is gene encoding protein tyrosine phosphatase Shp2. The mutation of *Ptpn11* has been identified in juvenile myelomonocytic leukemia (JMML), a childhood MDS/MPN, and in childhood AML (Tartaglia et al., 2004). A recent study has reported that activating mutation of *Ptpn11* (*Ptpn11*$^{E76K+}$) in MSPCs, not differentiated osteoblasts promotes the development of MPN in mice via abnormal production of pro-inflammatory cytokines (IL-1β & Ccl3) in BM (Dong et al., 2016). The phenotypes are manifested by increased frequency of myeloid cells in BM and spleens with evidence of splenomegaly. The recapitulation of MPN-like phenotype is accompanied by increased apoptotic LT-HSCs (LSKCD150^{+} cells). The high level of CCL3 attracted inflammatory myeloid (CD115^{+}Gr1^{+}) to BM where HSC reside in the *Ptpn11*$^{E76K+}$ mice, subsequently led to HSC activation and displacement from its mesenchymal niche. Administration of CCL3 receptor antagonist could reverse the MPN phenotypes in *Ptpn*$^{11E76K+}$ mice. The findings show evidence for important contribution of BM microenvironment to the pathogenesis of MPN.

In addition, activating β-catenin mutation in BM osteoblasts (Kode et al., 2014) and loss of Notch signaling in BM ECs (Wang et al., 2014) could induce malignant transformation of hematopoietic cells, providing additional evidence for the impact of specific genetic manipulation of osteoblasts and ECs for the development of myeloid malignancies including AML.

2.5.2 Remodeling of BM niche in myeloid malignancies

2.5.2.1 Nestin^{+} cells in Jak2(V617F) mutated MPN mice & Niche remodeling in CML mouse model

It has been shown that the role of BM niche is stage-specific (Lane et al., 2011). Once leukemia is established, the BM niche can be altered by the malignant hematopoietic cells and render the niche more supportive for the malignant cell growth but detrimental for normal hematopoiesis (Behrmann et al., 2018; Hoggatt et al., 2016; Schepers et al., 2013). The induction of MPN by transplanting JAK2-V617F cells into normal recipient mice result in alteration of Nestin^{+} cells, increased fibrosis and bone formation (Arranz et al., 2014). The Nestin^{+} cell frequency and ability to form colony are reduced due to induced apoptosis. Molecular profiling shows that Nestin^{+} cells in MPN mice show reduced expression of genes related to HSC maintenance and MSC characteristic. Nestin^{+} cells have been shown to be important niche component for HSCs (Mendez-Ferrer et al., 2010). In line with this, depletion or reduction of Nestin^{+} cells could cause further reduction of HSCs and accelerates MPN progression. The decreased frequency of Nestin^{+} cells results from schwann cell death induced by activation of JAK2 (V617F) mutation and elevation of IL-1 in mutant hematopoietic cells in MPN BM. Administration of neuroprotective drug inhibits MPN progression by preventing loss of Schwann cells and Nestin^{+} cells.

The niche-remodeling effects were also observed in a CML mouse model (Schepers et al., 2013). CML engraftment in BM results in expansion of MSC and osteoblast progenitor cells in BM and increased level of CCL3, THPO and G-CSF in BM plasma of MPN mice.

The altered niche factors including upregulated inflammatory cytokines and cellular niche composition in turn promote MPN progression in the mice, providing a molecular and cellular evidence for creation of self-reinforcing leukemic niche and new insight into new strategy in therapeutic intervention of CML.

2.5.2.2 BM niche & AML

The niche regulation is disease type specific as shown previously in CML and AML (Krause et al., 2013), making it imperative to study the specific niche contribution to AML development and treatment response. AML is heterogenous myeloid malignancy originated from immature progenitor cells with genetic disorders. AML could develop as *de novo* AML and secondary AML following chronic stage of myeloid neoplasm (Lindsley et al., 2015).

Mixed Lymphoid Leukemia (MLL) rearrangement with translocation partner gene is common genetic disorder in AML. One of translocation partner gene is AF9 (Meyer et al., 2018). Rearrangement of MLL provide proliferative advantage and self-renewal properties in AML cell through enhancement of *HOX* genes (Li et al., 2009). Most common drugs for AML treatment are cytarabine (Ara-C) and danorubicin (Buchner et al., 2012). Depending on phenotypes of AML, targeted therapy can be administered. FLT3 inhibitor (midostaurin) is administered for FLT3 (FLT3-ITD) mutated AML or Enasidenib for IDH2 mutated AML (Wei and Tiong, 2017). Another therapeutic drug is BCL2 inhibitor (venetoclax) which acts on mitochondrial activity of AML cells (Scheffold et al., 2018; Wei and Tiong, 2017).

However, the treatment outcome for AML patients has not been much improved in last decades. Current challenge for treating AML therapy is poor survival of patients, in particularly in elderly patients (> 60 years old) which only has 5%-15% survival within 5 years (Burnett et al. 2011). The short survival is mainly due to AML relapse from residual AML stem cells (LSCs) (Bonnet and Dick, 1997; Shlush et al., 2017). **Therefore, there is urgent need to develop new treatment strategy for AML.**

Similar to the finding in CML blast crisis, the AML induced BM niche alterations including expansion of MPCs with dysregulated functionality have been demonstrated in mice. This might be associated with progressive loss of normal hematopoiesis and AML progression since the critical niche factors for maintaining normal HSCs were dysregulated and reduction of HSPCs were observed (Baryawno et al., 2019; Hanoun et al., 2014)

In AML mice, **BM ECs** are increased but become hypoxic and leaky due to increased nitric oxide production as NOS3 is activated in *Nox4* highly expressed AML EC (Passaro et al., 2017). The endosteal vessels are progressively reduced accompanied by elevated production of anti-angiogenic (CXCL2) and pro-inflammatory TNF (Duarte et al., 2018). Perturbation of sympathetic nervous systems (SNS) in BM promotes AML development (Hanoun et al., 2014).

Besides the vascular alterations, the **mature osteoblasts** and bone volume of AML mice are reduced (Duarte et al., 2018; Hanoun et al., 2014), resulting in loss of LT-HSCs. On the other hand, constitutively activating β-catenin signal in osteoblast leads to genetic mutation in LT-HSC and AML development (Kode et al., 2014). Perturbation of *FoxO1* in osteoblast with constitutive β1-catenin signal prevents the AML development (Kode et al., 2016). Impaired hematopoiesis supportive function also occurs in other cell population in hematopoiesis niche.

2.5.3 BM niche in chemoprotective function of leukemic cells

2.5.3.1 Cell adhesive interactions of leukemic cells with their niche in chemoprotection

In AML mice, the BM $CD31^+$ cells upregulate E-selectin while AML cells increase binding to E-selectin (Barbier et al., 2020). The E-selectin binding facilitates survival of AML cells through AKT and NF-κB signaling pathway. Blocking E-selectin binding in combination with chemotherapy (Ara-C + Doxorubicin) prolongs survival of AML mice.

AML cells have been reported to express integrin such as α4β1 integrin (VLA-4) while BM niche consists of ligands for the integrin, such as fibronectin. Such interaction during danorubicin or Ara-C administration to AML cells increases AML cell survival (Matsunaga et al., 2003). The VLA-4 mediated interactions with EC also provides survival advantage for AML cells (Poulos et al., 2014).

2.5.3.2 BM niche & metabolic preference of AML LSCs

Similar immunophenotypes and quiescence characteristics of HSC and LSC create a challenge to target LSC specifically. The metabolic preference, on the other hand, is different between HSC and LSC (Farge et al., 2017; Simsek et al., 2010). For maintaining self-renewal, HSCs depend on anaerobic glycolysis while oxidative phosphorylation (OXPHOS) in mitochondria is repressed (Simsek et al., 2010). In contrary, AML LSCs rely on OXPHOS for survival and during chemotherapy (Farge et al., 2017). Along with OXPHOS activity, elevation of fatty acid metabolism, fatty acid receptor (CD36) expression and reactive oxidative species (ROS) production are also detected in AML LSCs. Impaired OXPHOS in AML LSC prolongs survival of AML mice due to increased oxidative stress leading to apoptosis (Baccelli et al., 2019). Recent study reports BM $Nestin^+$ cells mediate the elevation of OXPHOS in AML cells via mitochondria transfer that also enhances AML antioxidant defense (Forte et al., 2020).

2.5.4 Extramedullary site provides chemoprotection for LSCs

The presence of $CD36^+$ CML LSCs have been reported in gonadal white adipose tissue (WAT) in mice (Ye et al., 2016). In blast crisis CML that resembles AML mouse model (Ye et al., 2016), the infiltrated AMLs in gonadal WAT use fatty acid metabolism and remain in quiescence. The $CD36^+$ LSCs could escape therapeutic agents and are preferentially retained in gonadal WAT.

2.6 MESENCHYMAL NICHE IN EXTRAMEDULLARY ORGAN

The infiltration of AML LSCs in gonadal WAT indicates leukemic niche may exist at extramedullary sites in addition to BM and spleen. Furthermore, presence of mesenchymal-like population such as early adipocyte progenitor cells with similar characteristics to BM MSC (Ambrosi et al., 2017; Festa et al., 2011; Rivera-Gonzalez et al., 2016; Rodeheffer et al., 2008) suggests AML LSCs might receive hematopoiesis support from MSC-like population at extramedullary sites.

Skin is other extramedullary organ containing dermal adipose tissue and mesenchymal-like population. Skin-derived precursor cells are multipotent adult stem cell population in skin with Nestin expression and able to form sphere-forming cells in cultivation with EGF and FGF (Toma et al., 2001; Toma et al., 2005). Although the existence of MSC-like population is reported in skin tissue (Kimlin and Virador, 2013; Vaculik et al., 2012), the true identity and biological properties of skin MSC remain unknown since the cultivation method for selecting the mesenchymal stromal cells alters the native characteristics of the cells (Jones and Schafer, 2015; Qian et al., 2012). In addition, lack of knowledge of skin MSC natural phenotype hinders further study to evaluate the role of mesenchymal niche in skin tissue for the growth of the infiltrated leukemic cells and their response to chemotherapy.

3 RESEARCH AIMS

The present study aims to investigate mesenchymal niche contribution to normal and malignant hematopoiesis with focuses on myeloid malignancy including MDS/MPN and AML

Paper I

To evaluate the role of BM niche in the initiation of MDS/MPN using *Sipa1*$^{-/-}$ mice

Paper II

To investigate BM niche-remodeling dynamics in AML and the specific contribution of BM MSC to AML progression.

Paper III

To evaluate the role of Laminin α4 chain in hematopoiesis regeneration during hematopoietic stress and chemotherapy response

Paper IV

To prospectively characterize skin MSCs and explore their function in normal hematopoiesis and AML.

4 MATERIALS AND METHODS

To address the research questions, we have employed a list of transgenic mouse models and advanced technologies including sequencing, lineage-tracing, confocal imaging, *in vivo* and *in vitro* stem cell assays. In this section, advantages and limitations of selected methodological approaches are discussed. The detailed descriptions of the methods are presented in the original papers (Paper I – IV).

4.1 MOUSE-MODELLING OF HEMATOPOIESIS

Mouse modelling has been widely used in the field of normal and malignant hematopoiesis due to both biological similarities between mice and human and ethical reason. Our studies have mainly used mice with C57BL/6J background since most of the previous studies on hematopoiesis have been done on this mouse strain, which makes it possible for us to apply and compare previous findings with our results. However, the *Ebf2-Egfp* transgenic reporter mice (in **paper II** and **paper IV**) with FVB/N genetic background were used for studying the role of Ebf2-expressing MSCs for AML development and characterizing skin MSCs. Here, to avoid graft-versus host/leukemia effects, the AML cells used for transplantation were generated on the same background. In addition, we were aware of strain-specific difference in immunophenotype (such as CD34 and CD90) of the mesenchymal stroma cells (Peister et al., 2004), thus, we have chosen the MSC-markers that are expressed in MSCs from mice of different mouse background.

FVB/N and C57BL/6J genetic background have different haplotype of Ly-6 locus which are $Ly6^a$ (Ly6E.1) and $Ly6^b$ (Ly6A.2), respectively (Jurecic et al., 1993; Spangrude and Brooks, 1993). The frequency of $SCA1^+$ in BM LIN^- is lower in $Ly6^a$ mouse than $Ly6^b$ (Spangrude and Brooks, 1993). Furthermore, the BM mesenchymal stroma of FVB/N mice have higher SCA1 expression (Peister et al., 2004). In order to circumvent result bias due to the difference of genetic background, the present study was designed to allocate mouse strain with same genetic background between compared experiment.

In paper II and IV, to analyze the *in vivo* function of Ebf2-expressing MSCs to AML niche formation and AML development, the *Ebf2-gfp-*Cre^{ERT2}*-Tomato mice and Ebf2-*Cre^{ERT2}*-DTA* mice with mixed genetic background of FVB/N and C57BL/6J were backcrossed to be on C57BL/6J background.

In **paper I and III**, the mice with germline deletion of *Sipa1* and *Lama4* (*Sipa1*$^{-/-}$ and *Lama*$^{-/-}$) were used, respectively. To specifically study the niche impact on hematopoiesis, the **paper I** took advantage of reciprocal transplantation following irradiation. The irradiation induced effect on the host niche and radiosensitivity of the mice that might interfere experiment results were considered.

4.2 INDUCIBLE CRE/LOXP SYSTEM & TAMOXIFEN EFFECT ON MOUSE HEMATOPOIETIC SYSTEM

The inducible Cre/Lox P system driven by *Ebf2* promoter enabled us to track or deplete Ebf2^{+} cells in *Ebf2-gfp-Cre^{ERT2}-Tomato* and *Ebf2-Cre^{ERT2}-DTA* mice in a spatial and temporal manner. By employing Cre^{ER} variant (Feil et al., 1996), Cre recombinase is activated by tamoxifen (TAM) as TAM binds to the Estrogen receptor fused with Cre and translocates Cre to nucleus. The Cre recombinase subsequently recognizes the l*oxP* sites that flanks STOP codon, resulting in activation of Tomato or DTA.

To avoid high toxicity in hematopoietic cells, the present study employed lower dose (3mg – 150mg/kg for 3 days) than reported toxic dose (175mg/kg for 5 days) of TAM (Higashi et al., 2009). Furthermore, as 5mg tamoxifen hematological effects are reversible within 2 weeks (Uhmann et al., 2009), the subsequent experiments were performed approximately 4 weeks after the last tamoxifen injection in order to excluding the unspecific effects induced by TAM. In addition, for testing effect of MSC deletion on AML development, we also treated our control mice with TAM.

4.3 PROSPECTIVELY NICHE CELL ISOLATION WITH MULTICOLOUR FLUORESCENCE ACTIVATED CELL SORTING (FACS)

Since niche cell isolation based on *in vitro* culturing results in cell alteration phenotypically and molecularly (Jones and Schafer, 2015; Qian et al., 2012), the niche cells were prospectively isolated by FACS after enzyme dissociation. The niche cells isolated in present study were (1) MSC, (2) MPC and (3) EC (CD31^{+}) cells. The BM MSCs and MPCs were sorted by excluding hematopoietic and endothelial cell (CD45^{-}TER119^{-}CD31^{-}) prior selecting CD44^{-} population. Further sub-fractionation was done based on SCA1 and CD51 expression. The **BM MSC was phenotypically defined as CD45^{-}TER119^{-}CD31^{-}CD44^{-}SCA1^{+}CD51^{+}** while **MPC was defined as CD45^{-}TER119^{-}CD31^{-}CD44^{-}CD51^{+}SCA1^{-}**. For isolating MPN patient niche cells in paper I, the human MSC was sorted based on CD45-CD235^{-}CD31^{-}CD44^{-} phenotypes while CD31^{+} cells were sorted based on CD45^{-}CD235^{-}CD31^{+} phenotypes. The MSC identity were usually confirmed by their capacity in forming CFU-Fs and *in vitro* expansion. The procedure is tedious since niche cells are rare in frequency. However, this will allow us to reveal the biological properties and true molecular profile of the cells.

4.4 PROSPECTIVE CHARACTERIZATION OF MSC

MSC was characterized based on **(1) phenotypic analysis** and **(2) functional assays**. The phenotypic analysis was performed based on phenotypically defined MSC (CD45^{-}TER119^{-}CD31^{-}CD44^{-}CD140a^{+}CD51^{+}SCA1^{+}) by FACS. The MSC functional assays are:

4.4.1 Colony Forming Unit-Fibroblast (CFU-F) assay

CFU-F assay was used to functionally evaluate MSCs by platting sorted MSC at single cell or low density *in vitro*. One CFU-F was defined if the colony consisted of 50 cells with fibroblastic morphology. It is important to note that each MSC should be able to form a CFU-F, however, not all the CFU-Fs are MSCs unless it is can be serially re-plated *in vitro* and transplanted *in vivo*. The CFU-F assays and *in vitro* expansion of MSC were performed in hypoxic condition which preserves multilineage properties and inhibits senescence of MSC (Tsai et al., 2011).

4.4.2 *In vitro* multilineage differentiation assay

MSCs in BM have potency to differentiate toward adipocyte, osteoblast and chondrocytes (Qian et al., 2013; Sacchetti et al., 2007). Thus, *in vitro* osteogenic, adipogenic and chondrogenic differentiation were performed to evaluate MSC function. In paper IV, chondrogenic differentiation was performed in both monolayer and micromass culture to validate the chondrogenic potencies of skin MSC. The chondrogenic differentiation was evaluated based on toluidine blue staining which visualizes the deposition of proteoglycan. For evaluating osteogenic differentiation, the formed mineralization was detected by alizarin red staining. The positive adipogenic differentiation was evaluated based on formation of lipid droplets that were stained by Oil-Red-O dye.

4.5 MSC CHARACTERIZATION AT SINGLE CELL LEVEL

In **paper IV**, in order to estimate self-renewal ability of skin MSC, single cell sorting of $Ebf2^+$ and $Ebf2^-$PαS cell were performed. Number of CFU-F generated from single cell sorting was counted to calculate frequency of MSC in skin $Ebf2^+$ and $Ebf2^-$PαS fraction. The $Ebf2^+$ and $Ebf2^-$PαS clones generated in the single cell CFU-F assays were sampled for *in vitro* serial re-plating and multilineage differentiation assays. The frequency of the cell clone with multilineage potency and serial re-plating ability were considered to functionally define MSCs.

4.6 *IN VIVO* LINEAGE TRACING OF $EBF2^+$ CELLS

Lineage tracing was conducted to trace $Ebf2^+$ MSC and their role in the formation of leukemic niche in BM (**paper II**) and skin tissue homeostasis (**paper IV**). For tracing $Ebf2^+$ cells, the *Ebf2-gfp-Cre^{ERT2}-Tomato* mice were employed. Upon *Cre* activation by TAM injection at desired time point, Tomato became permanently expressed in the $Ebf2^+$ cells and the cells generated from them. The $GFP^+Tomato^+$ cells long term post TAM injection should represent the $Ebf2^+$ MSCs that were activated and maintained by being in quiescent state or self-renewed. The progeny of $Ebf2^+$ lost the Ebf2 expression would be $GFP^-Tomato^+$.

4.7 PHENOTYIC ANALYSIS WITH FACS

FACS was utilized to phenotypically analyze the cells and sort the cells based on their cell surface markers for subsequent functional and molecular assays. The live cells were defined based on Propidium Iodide (PI^-) or $7AAD^-$. The frequency of niche cells within total live cells, non-hematopoietic ($CD45^-TER119^-$) and non-endothelial ($CD31^-$) stromal cells were quantify. FACS was also used to analyze cell cycle state, HSPC phenotypic analysis and AML engraftments (**Paper II – IV**). In **paper II& IV**, FACS was also employed to obtain frequency of $Ebf2^+(GFP^+)Tomato^+$ cells and $GFP^-Tomato^+$ frequency in lineage tracing experiment.

To achieve high quality results by FACS, several aspects should be considered: **(1)** For making the most of limited cell number, the multicolour FACS was performed by firstly creating the antibody panels based on FACS optical configuration and selected fluorochromes for allowing optimal fluorochrome signal separation. **(2)** Except cell cycle analysis, the phenotypic analyses were performed on freshly isolated mononuclear cells. For maintaining a good viability, cells were re-suspended in 5% - 10% serum. **(3)** During acquisition for sorting or analysis, Fluorescence Minus One (FMO) for each fluorochrome signal was used to define the positive or negative gating.

4.8 IMMUNOFLUORESCENCE (IF) STAINING AND CONFOCAL IMAGING

To localize cell distribution in tissue, IF and confocal imaging were performed. In **paper III and IV**, confocal imaging was employed to evaluate AML cell infiltration in BM and spleen. In **paper IV**, staining of vascular marker (CD31) was performed on dorsal skin tissue prior confocal imaging to distinguish circulating AML cells and infiltrated AML cells in dorsal skin tissue.

For *in vivo* lineage tracing of skin $Ebf2^+$ (**paper IV**), IF & confocal imaging were employed to define location and identity of skin $Ebf2^+$ cells as well as their progenies ($GFP^-Tomato^+$). Thus, whole mount IF staining of vascular (CD31 or MECA32) and perivascular markers (α-SMA, NG2 and Nestin) were performed. For evaluating anti-GFP antibody specificity, skin tissues from WT littermate were used. The setup of confocal microscopy configuration was done by using skin tissue from single transgenic mouse and secondary antibody staining control. Image processing and analysis were performed with NIS-element AR-analysis ver 5.20.00 64-bit software (Nikon).

4.9 SYNGENIC TRANSPLANTATION FOR ASSAYING HSC AND LEUKEMIA DEVELOPMENT

Prior to transplanting BM cells, the recipient mice were either sub-lethally irradiated (6 Gy) or lethally irradiated to allow donor cells engraft in BM. The sub-lethally irradiation was performed in **paper I** with aim to preserve host microenvironment while enabling sufficient donor cell engraftment. The lethal irradiation impaired the function of niche cells although niche cells were anatomically present (Abbuehl et al., 2017; Friedenstein et al., 1981; Greenberger et al., 1996; Naparstek et al., 1986; Severe et al., 2019)

In **paper I**, transplantation of BM hematopoietic cells was performed to evaluate whether *Sipa1* acted intrinsically or extrinsically in the development of MDS / MPN. To evaluate the extrinsic mechanism, BM $CD45^+$ from $Sipa1^{+/+}$ (WT) were injected to $Sipa1^{-/-}$ recipient mice while intrinsic mechanism was evaluated in reciprocal transplantation setting.

In **paper III**, the lethally irradiation was performed on the $Lama4^{+/+}$ and $Lama4^{-/-}$ mice that developed AML to eliminate AML and allow for maximal homing of the donor cells to mimic clinical therapeutic settings where patients undergo total body irradiation prior to hematopoietic cell transplantation.

4.10 AML CELL TRANSPLANTATION TO GENERATE AML MOUSE MODEL

To generate AML mouse model, MLL-$AF9^+$ AML cells (Somervaille and Cleary, 2006) were intravenously injected to immunocompetent (non-irradiated) recipient mice. The recipient mice did not need to be irradiated due to the aggressiveness of MLL-$AF9^+$ AML cells. The advantage of this setting is to maintain host niche integrity, providing an ideal model to study niche-remodeling by AML. This method was employed in **paper II, III and paper IV**. For visualizing AML cells in skin tissue (**paper IV**), bone marrow and spleens (**paper III and IV**), the MLL-$AF9^+$ AML cells with β-actin-dsRed expression were transplanted after sublethal irradiation. The irradiation was necessary since the β-actin-dsRed MLL-$AF9^+$ AML cells were originally generated by retroviral transduction to GMP cells (Hartwell et al., 2013) while the non-dsred MLL-$AF9^+$ AML cells were generated in BM KIT^+ cells (Somervaille and Cleary, 2006).

4.11 CO-CULTURE BETWEEN HEMATOPOIETIC CELLS AND MSPCS

For evaluating interaction between HSPC or MLL-$AF9^+$ AML cells and specific mesenchymal niche cells, co-culture was performed in normoxic condition and followed up by *in vitro* hematopoiesis assay. For co-culture MSC with HSPC (**Paper I & IV**) or MLL-$AF9^+$ AML cells (**Paper III & IV**), MSCs were pre-plated to form feeder layer and allow for complete coverage of well plate.

Since the purpose of co-culture was to evaluate mesenchymal niche function to support hematopoietic cells, the co-culture was performed in serum-free medium with limited growth factors (M5300, supplemented with 10^{-6}M hydrocortisone and penicillin streptomycin) to prevent growth competition between MSCs or MPCs and hematopoietic cells. In co-culture between MLL-$AF9^+$ AML cells and MSC, 1ng/mL IL-3 was added to maintain MLL-$AF9^+$ AML growth while 5-10ng/mL IL-3 was used when MSC was not applied. After the co-culture, Colony Forming Unit-Cell (CFU-C) or FACS analysis was performed to evaluate HSPC or AML proliferation and differentiation.

4.12 COLONY FORMING UNIT CELL (CFU-C) ASSAY

CFU-C assays was performed in semisolid medium (methocult^{TM} GF M3434) allowing spatial isolation of a single HSPC cell (**Paper I & IV**) or LT-HSC (**Paper IV**) to clonally expand and form different myeloid colonies. The M3434 medium contains IL-3, IL-6, EPO, and SCF to promote generation of CFU-Granulocyte Monocyte (CFU-GM) and Granulocyte, Monocyte and Erythrocyte (CFU-GME). For visualizing ´erythroid colonies, staining of haemoglobin with 2,7 diaminofluorene (DAF) was performed (McGuckin et al., 2003)

4.13 COBBLESTONE AREA FORMING CELL (CAFC) ASSAY

CAFC is an assay used to evaluate frequency of HSPC based on number of CAFC formation beneath stroma cells *in vitro* (Phloemacher 1989). The assay was performed by platting total MSC or different subtype of MSCs (Ebf2^{+} and Ebf2^{-}PαS) to cover well surface. Once all surface area was covered, small number of hematopoietic cells were plated (150 – 250 LSK or 300 MLL-AF9^{+} cells). The CAFC was usually performed at 32-35°C for 14 to 21 days. Each CAFC is defined as a colony consists of at least 3 cells beneath stromal cells. In **paper III and IV**, CAFC assays were performed to evaluate MSC function for supporting MLL-AF9^{+} AML cell growth as well as providing chemoprotection. it is worth mentioning that CAFC from AML cells should be counted no later than 10 days since AML cells grow much faster and aggressively. On the contrary, the CAFC from HSPCs takes longer time to form, thus should be counted at later time points, up to 2-3 weeks.

Differ from original methods where heterogenous stromal cell population was seeded, we here have used FACS-isolated MSCs in order to specifically evaluate the role of MSC in supporting normal HSPC or AML cells. Moreover, by combining prospectively isolated different MSC population (a highly enriched MSC (Ebf2^{+}) and Ebf2^{-}MSC (PαS)) and CAFC method, this assay could be employed as high-fidelity tool for evaluating interactions between normal and malignant stem cells with specific MSC fraction, and the underlying molecular mechanisms.

4.14 MITOCHONDRIA TRANSFER

To evaluate *Lama4*$^{-/-}$ MSC chemoprotective function, mitochondria transfer assay was performed in **paper III**. Firstly, the MSCs were labeled with MitoTracker^{TM}Green. In order to remove unbound probe, the MSCs were washed and incubated for 3-4 hours prior to seeding the MLL-AF9^{+} cells. The frequency of MLL-AF9^{+} AML cell receiving mitochondria from MSC was detected by FACS while the localization of mitochondria in AML cells was observed with confocal microscopy.

4.15 MOLECULAR PROFILING & GENE EXPRESSION ANALYSIS OF RARE CELL POPULATIONS

The molecular profiling was evaluated on freshly sorted niche cells including MSCs and MPCs. The cells were sorted directly into lysis buffer and kept in -80°C prior RNA extraction which was performed prior cDNA synthesis by RT-PCR. The cDNA was then used for qPCR or subjected to library preparation prior RNA-sequencing (RNAseq).

4.15.1 RNAseq

The purpose of RNAseq in the present study was to provide transcriptomic profile among different niche cell populations. The RNAseq procedure consisted of (1) RNA extraction and library preparation where cDNA fragments were tagged with adaptors, (2) sequencing with a high throughput platform, (3) data analysis. During RNAseq data analysis, the generated sequences were mapped and counted. After normalizing the mapped counts, the differentially expressed genes (DEGs) were defined. For Gene Set Enrichment Analysis (GSEA), GSEA (v4.0.3) platform were used to identify enriched genes in tested cell population based on multiple molecular gene sets included in gene ontology, hallmark and KEGG databases (**Paper III & IV**). When P value < 0.05 and FDR < 0.25, the gene sets were considered significantly enriched.

4.15.2 qPCR with limited materials

qPCR was performed in paper I-IV to validate finding from RNA sequencing and evaluate relative expression of genes related to MSC characteristics, hematopoiesis and inflammatory. The qPCR reaction captures the FAM signals generated from amplified gene during exponential phase in real time. The house keeping gene (*Hprt*) of each cell population was included in every qPCR experiment. To calculate relative expression of target gene to *Hprt*, the ΔCT method ($2^{(CT^{target\ gene} - CT^{Hprt})}$) was used.

4.16 STATISTICAL ANALYSIS

Justification of statistical test was based on generated hypothesis prior the experiment. Following data generation, the data distributions were tested prior to selecting statistical interference (parametric or non-parametric test). All tests for data distribution and statistical interference was performed with GraphPad Prism. P-value < 0.05 is considered significant.

4.17 ETHICAL CONSIDERATION

All experiments were performed in compliance to approved ethical permit (animal study: S40-14 and 1869, and for human materials: 2012/4:10, 2013/3:1 and 2013/1248-31/4). For animal studies, the 3R (Replacement, Reduction and Refinement) was always considered. In compliance to **Replacement**, *in vitro* assay had been put as first choice in pilot study whenever it was possible prior moving to *in vivo* animal study. To reduce the use of animal (**Reduction**), the present study allocated the total use of animal efficiently and effectively.

One way to achieve it was by thoroughly discussing each experiment protocol within all group members to prevent unnecessary pitfalls that may impact in increased animal number. To comply with **Refinement**, each procedure related to animal handling was performed only by trained persons while animal husbandry was taken care by experienced and trained staff at KI animal facility. To reduce suffering of the mice, we always terminated the mice that were in severe health conditions defined by local ethical committee and institution in conformity with our ethical approvals. Animals were maintained at *ad libitium* state and monitored regularly. Prior conducting experiment procedure at KI animal facility, the experiment protocols were presented to the animal facility staff who provided feedback and technical expertise for minimizing harm to the animal. For experiment where patient samples were included (**Paper I & II**), donor confidentiality was maintained.

5 RESULTS

5.1 PAPER I: *SIPA1* DEFICIENCY–INDUCED BONE MARROW NICHE ALTERATIONS LEAD TO THE INITIATION OF MYELOPROLIFERATIVE NEOPLASM

Sipa1 gene deleted mice (*Sipa1*$^{-/-}$) develop CML-like disease in an age-dependent manner (Ishida et al., 2003; Kometani et al., 2006). These findings suggest an important role of *Sipa1* in the development of MPN. However, it remained unclear whether the MDS/MPN development in the aged *Sipa1*$^{-/-}$ mice was due to the intrinsic loss of *Sipa1* in hematopoietic cells or in BM niche via non-autonomous manner. In paper I, this question is addressed.

Sipa1 is reported to be expressed in HSPCs and lymphocytes (Ishida et al., 2003; Kurachi et al., 1997) while the expression of *Sipa1* in BM marrow niche cells was unknown. To explore the impact of *Sipa1* loss on BM niche, we firstly examined *Sipa1* expression in BM mesenchymal niche including MSC and MPC. The freshly sorted BM MSC and MPC from mice and human expressed *Sipa1* as well as CD31^{+} endothelial cells. Interestingly, *SIPA1* expression in BM MSCs and ECs was decreased in patients with MPN (CML and CNL). Similar but not identical to previous findings, most of the aged *Sipa1*$^{-/-}$ mice (>16 months old) showed MDS/MPN phenotypes as manifested by increased white blood cell (WBC), granulocytes (CD11B^{+}GR1^{+}) and neutrophils in peripheral blood, anemia, thrombocytopenia, splenomegaly and Mk dysplasia in BM. The BM niche composition of the aged *Sipa1*$^{-/-}$ mice was altered with reduced frequency of BM MSC but increased MPCs compared to age-matched *Sipa1*$^{+/+}$ mice. Functionally, the ability of *Sipa1*$^{-/-}$ BM MSCs to differentiate toward osteogenic *in vitro* was impaired in aged *Sipa1*$^{-/-}$ mice. These data showed alterations of the BM mesenchymal niche in MDS/MPN mice. **However, it remained to be investigated whether the disease was caused by the niche alteration or was induced by the malignant hematopoietic cells**.

To address this, we analyzed the BM niche in young adult *Sipa1*$^{-/-}$ mice (2-3 months old), prior to the onset of MDS/MPN. We found the frequency of BM MSC and MPC were increased in young *Sipa1*$^{-/-}$ mice. Furthermore, BM MSCs from young *Sipa1*$^{-/-}$ mice displayed impaired osteogenic but enhanced adipogenic differentiation potency. In line with reduced osteogenic potency *in vitro*, CT scan indicated reduced femoral bone volume in young *Sipa1*$^{-/-}$ mice. Functionally, the young *Sipa1*$^{-/-}$ BM MSC and MPC displayed higher potency to support HSPC growth and promoted them to form CFU-GM, but less in CFU-GME formation *in-vitro*. Molecularly, RNA sequencing revealed upregulation of inflammatory related genes such as *Il-6* and *TGF-β* in *Sipa1*$^{-/-}$ BM MSCs and downregulation of genes critical for the maintenance of HSCs (*Cxcl12*, *Kitl* and *Angptl1*) in *Sipa1*$^{-/-}$ BM MSCs and MPCs, providing molecular basis of the altered supportive function for HSPCs. Surprisingly, transplantation of young *Sipa1*$^{-/-}$ BM CD45^{+} hematopoietic cells to lethally irradiated *Sipa1*$^{+/+}$ recipient mice failed to recapitulate the hematological disorders observed in the aged *Sipa1*$^{-/-}$ mice. These findings indicated that the development of MDS/MPN in the *Sipa1*$^{-/-}$ mice was not due to the loss of *Sipa1* in hematopoietic cells.

To test whether the development of the disease was caused by *Sipa1* loss-induced BM niche alterations, the BM CD45$^+$ cell isolated from young *Sipa1*$^{+/+}$ was transplanted to either sub-lethally or lethally irradiated young *Sipa1*$^{-/-}$ mice. We found that normal *Sipa1*$^{+/+}$ BM CD45$^+$ cells from young adult mice were transformed into malignant cells in the *Sipa1*$^{-/-}$ recipients, resulting in dyserythropoiesis and increased donor-derived HSPC, GMP and MEP count in BM which resembled the MDS/MPN phenotype based on Bethseda proposal (Kogan et al., 2002). The higher frequency of MEPs was also observed in BM and spleen of lethally irradiated young *Sipa1*$^{-/-}$ recipient mice. The transformation of the *Sipa1*$^{+/+}$ CD45$^+$ cells was confirmed by secondary transplantation where the recipient mice (*Sipa1*$^{+/+}$) showed MDS-like phenotype. Altogether, paper I shows ***Sipa1* deficient BM niche is an instigator of the MDS/MPN development.**

5.2 PAPER II: DISTINCT ROLES OF MESENCHYMAL STEM AND PROGENITOR CELLS DURING THE DEVELOPMENT OF ACUTE MYELOID LEUKEMIA IN MICE

Paper II shows that *Sipa1*$^{-/-}$ niche transforms normal CD45$^+$ cells into malignant myeloid resulting in MDS/MPN, suggesting an instructive role of the BM niche during development of myeloid malignancies. However, the interplay between mesenchymal niche and malignant hematopoietic cells in BM is dynamic and disease stage-specific. Once the malignancy is established, the role of BM niche may become permissive or even supportive for the disease. It has been reported that AML cells alter BM niche to confer AML cell proliferation and suppress host hematopoiesis (Baryawno et al., 2019; Hanoun et al., 2014). Thus, understanding how BM niche is modulated during myeloid malignancy is necessary to identify potential target to disrupt the supportive signals from niche for AML cell growth.

In paper II, we investigated specifically how BM niche is remodeled progressively by AML cells. At the end stage of AML, the BM niche was altered significantly. The frequency of BM MSC, MPC and CD31$^+$ (including CD31$^+$SCA1$^+$ arteriolar EC) were increased in mice engrafted with MLL-AF9$^+$ AML. Similar niche alterations were observed in AML xenograft mice post transplantation of patient derived AML cells. Furthermore, the differentiation potential of BM MSCs toward adipogenic and osteogenic lineages *in vitro* was enhanced in AML mice compared to healthy control mice.

Molecularly, qPCR on freshly sorted BM MSCs, MPCs and CD31$^+$ cells at different time points post AML transplantation revealed dysregulated gene expression in these cellular niches. At the end stage of AML, we detected reduced expression of hematopoiesis supportive genes in both MSCs and MPCs including *Cxcl12*, *Kitl* and *Angptl1*. On the other hand, *Spp1* which is negative regulator of hematopoiesis was increased. Increased *Il-6* expression level indicated an inflammatory BM niche at end stage of AML. *Sipa1* expression was also reduced as AML developed. Elevated expression of *Lama4* and *Lama5* in AML BM MSCs and MPCs were observed, meriting future studies on the role of ECM proteins for AML progression.

Notably, similar to the frequency of BM niche cells that increased in an AML burden dependent manner, the alteration of hematopoiesis-supportive genes in BM MPC also correlated with AML burden/progression, emphasizing the remodeling effects of AML cells on the niche composition and its subsequent impact on the AML progression. **Taken together, these data provide evidence for the dynamic molecular interplay between AML cells and their BM niche although the functional consequences remain to be validated.**

Our previous study reports that BM MSCs contain a more primitive MSC, marked by *Ebf2* expression. In order to investigate the specific role of BM MSCs during AML development, we first analyzed the Ebf2$^+$ cells in the mice post injection of MLL-AF9$^+$ AML into immunocompetent *Ebf2-Egfp* transgenic reporter mouse model. Similar to finding from total BM MSC, frequency of the Ebf2$^+$ cell was increased at the end stage of AML. However, the Ebf2$^+$ cells were altered to become more differentiated toward MPCs. The role of BM Ebf2$^+$ MSC in the formation of leukemic mice was then assessed by lineage tracing strategy using Ebf2 triple transgenic mouse (*Ebf2-Egfp x Ebf2-Cre^{ERT2} x Rosa26^{loxp}Stop^{loxp}Tomato*). In this setup, Ebf2$^+$ cells were reported as GFP$^+$Tomato$^+$ and their progeny will be permanently labelled by Tomato expression (GFP$^-$Tomato$^+$) post Cre activation by tamoxifen injection. The contribution of Ebf2$^+$ cells to the leukemia niche formation was assessed by the presence of the progeny of Ebf2$^+$ cells (GFP$^-$Tomato$^+$) post AML cell injection.

The progeny of BM Ebf2$^+$ cells that have lost *Ebf2* expression (GFP$^-$Tomato$^+$) were found to constitute a major part of the mesenchymal niche cells including Ebf2$^-$MSCs, MPCs and CD44$^+$ cells. The frequency of the GFP$^-$Tomato$^+$ cell was increased at end stage of AML. Majority of the GFP$^-$Tomato$^+$ cells were Ebf2$^-$MSCs and MPCs, which, in consistent with earlier observation, were increased post symptomatic onset of AML. This finding revealed that the Ebf2$^+$ cells contributed to the formation of leukemic niche in the BM by generating different BM mesenchymal niche cells during AML development.

To further evaluate the functional contribution of BM Ebf2$^+$ cells to AML development, the *Ebf2-Cre^{ERT2}X Rosa26-DTA* transgenic mice were employed to allow selective Ebf2$^+$ cell depletion before or after the MLL-AF9$^+$ AML injection. Surprisingly, loss of Ebf2$^+$ cell did not result in the delayed AML onset. On the contrary, the AML development in Ebf2$^+$ depleted mice was accelerated. Since host hematopoiesis is known to be impaired along with AML progression due to a massive growth advantage of AML cells, we then tested whether the deletion of Ebf2$^+$ cells resulted in a greater loss of host HSPCs, which is directly linked to AML progression. Indeed, frequencies of LT-HSC, LSK, Pre-MegE, pre-CFU-E, MkP, GMP and preGM were drastically reduced in BM of the Ebf2$^+$ cell depleted mice. Concomitantly, the frequencies of AML and AML LSC (KIT$^+$) cells were increased.

In summary, the dynamic interplay between AML cells and BM mesenchymal niche resulted in alterations of BM MSC and MPC composition and functions. The AML cells educated BM Ebf2$^+$ MSCs to form leukemic niches which in turn promoted AML growth while suppressing normal hematopoiesis.

5.3 PAPER III: CRITICAL ROLE OF LAMININ α4 FOR HEMATOPOIESIS REGENERATION UNDER IRRADIATION-INDUCED STRESS AND ACUTE MYELOID LEUKEMIA

Although our studies in Paper II and others (Hoggatt et al., 2016) demonstrated the remodeling effects on BM niche by AML cells, the molecular pathways mediating the effects of the altered niche on AML cells and normal hematopoiesis maintenance remain largely unexplored. To this end, we have **in paper III explored the role of ECM protein laminin α4 (Lama4) in hematopoiesis regeneration during myelosuppression and AML progression including AML cell response to drug treatment.**

Paper II showed that *Laminin α4* (*Lama4*) expression was elevated in BM MSCs and MPCs from mice with AML. This prompted us to study the functional consequence of the Lama4 upregulation during AML development. Data mining from AML patient in *Prognoscan* (Raponi *et al.* 2007) suggested an inverse correlation of LAMA4 expression with patient survival. Thus, we hypothesized that *Lama4* could play a role in AML development either by maintaining normal hematopoiesis or inhibiting AML proliferation or both. This hypothesis was tested by using *Lama4*$^{-/-}$ mice (Tyboll et al. 2002) and MLL-AF9 AML mouse model established in Paper II.

At steady state, the impact of *Lama4* on blood lineage production and early hematopoiesis was mild. However, the hematopoiesis regeneration was significantly impaired in *Lama4*$^{-/-}$ mice post irradiation-induced myelosuppression. Compared to *Lama4*$^{+/+}$ mice, the regeneration of erythropoiesis, myelopoiesis and megakaryopoiesis were impaired and delayed in *Lama4*$^{-/-}$ mice post-irradiation.

Since *Lama4* is distributed in the basement membrane of endothelial cells (Tyboll et al. 2002) and megakaryopoiesis is regulated through direct interactions between Mks and ECs (Dhanjal et al. 2007; Junt et al., 2007; Rafii et al., 1995), we further evaluated distribution of Mks at the vascular site of the BM. Six weeks after irradiation, *Lama4*$^{-/-}$ mice had reduced number of Mk adjacent to sinusoidal vessels in BM. Additionally, the vessel density was also reduced in *Lama4*$^{-/-}$ mouse BM. These results provided possible mechanisms underlying the impaired hematopoiesis recovery, including potential impaired Mk transendothelial protrusion and maturation into platelet after irradiation, which in turn could lead to reduced platelet production.

To further explore the cellular mechanisms behind the impaired hematopoiesis, we characterized BM mesenchymal niche in *Lama4*$^{-/-}$ mice. At steady state, the frequency of *Lama4*$^{-/-}$ MPC was reduced in BM while the MSC frequency was not changed. The MPCs were then sorted from mouse *Lama4*$^{+/+}$ and *Lama4*$^{-/-}$ BM post-irradiation for RNA sequencing. Besides upregulated inflammatory related genes *(Tgfb, Il18, Il7d) in the Lama4*$^{-/-}$ MPCs, GSEA analysis revealed enriched genes associated with OXPHOS (oxidative phosphorylation) and activated ROS in the *Lama4*$^{-/-}$ MPCs.

Leukemia represents a severe hematopoietic insult where normal hematopioiesis is progressively lost while leukemic cells are massively proliferated. Both processes are closely linked, as illustrated by exponential growth of AML cells in paper II. The critical role of *Lama4* for AML progression was studied by transplanting MLL-AF9 cells into *Lama4*$^{-/-}$ mice. The development of AML in *Lama4*$^{-/-}$ mice was faster compared to *Lama4*$^{+/+}$ mice, demonstrated by the reduced survival time of the *Lama4*$^{-/-}$ mice and the higher frequency of AML LSC in BM and PB of *Lama4*$^{-/-}$ mice. Concomitantly, the platelets (PLTs) were further reduced in *Lama4*$^{-/-}$ mice. In order to evaluate *Lama4* role in AML relapse, we treated the *Lama4*$^{+/+}$ and *Lama4*$^{-/-}$ AML mice with BM transplantation following lethal irradiation to eliminated AML cells. The AML relapsed faster in *Lama4*$^{-/-}$ mice which was associated with reduced survival of *Lama4*$^{-/-}$ mice.

The chemoprotective function of the *Lama4*$^{-/-}$ niche for AML LSCs was evaluated by CAFC assay *in vitro* and *in vivo* treatment of AML by chemotherapy Ara-C. The higher frequency of CAFC reflecting AML LSCs from AML co-cultured with *Lama4*$^{-/-}$ MSC showed an enhanced supportive function of *Lama4*$^{-/-}$ MSC for AML LSCs. The increased number of residual CAFCs and AML LSCs (KIT^{+}) from AML cells cultured with *Lama4*$^{-/-}$ MSCs post Ara-C indicated a higher chemoprotective function of the *Lama4*$^{-/-}$ MSCs. Consistent with *in vitro* data, 1 day after Ara-C last injection, chemoresistant AML cells were found to be higher in spleen and BM of *Lama4*$^{-/-}$ mice. In addition, the AML cells in *Lama4*$^{-/-}$ mice had lower ROS compared to that in *Lama4*$^{+/+}$ mice, suggesting a possible enhanced metabolic support from *Lama4*$^{-/-}$ niche for AML cells to detoxify oxidative stress induced by massive AML cell proliferation and the sublethal irradiation.

Mitochondria transfer has been reported as mechanism provided by niche for acute lymphoblastic leukemia chemoprotection (*Burt et al. 2019)*. We then evaluated the mitochondria transfer from pre-labeled *Lama4*$^{-/-}$ MSC to AML cells *in vitro*. Both *Lama4*$^{+/+}$ and *Lama4*$^{-/-}$ MSC did transfer their mitochondria to AML, however, the transfer from *Lama4*$^{-/-}$ MSC into the AML was more pronounced during Ara-C treatment. Taken together, **the findings in paper III shows (1) role of *Lama4* in hematopoiesis regeneration after irradiation stress, in particular, in erythropoiesis and megakaryopoiesis. (2) The *Lama4*$^{-/-}$ mesenchymal niche promotes AML cell proliferation and provides enhanced chemoprotection for AML LSCs by transferring mitochondria to AML cells. Nevertheless, more work is required to investigate the specific effect of the transferred mitochondria in AML cells for their survival during chemotherapy by using inhibitors to block mitochondrial transfer. Altogether, these findings from this study suggest that activation of LAMA4-receptor signaling pathway could potentially reduce niche chemoprotection for AML cells and sensitize their response to chemotherapy.**

5.4 PAPER IV : NOVEL CHARACTERISTICS OF SKIN MESENCHYMAL STEM CELL AND ITS FUNCTION IN NORMAL HEMATOPOIESIS AND ACUTE MYELOID LEUKEMIA

Besides BM, it has been reported that extramedullary organ acts as reservoir of chemoresistant leukemic cells (Ye et al., 2016). However, whether the mesenchymal niche in extramedullary organ contributes to AML survival is not known. The existence of mesenchymal-like population has been reported in skin tissue (Toma et al. 2001; Vanculik et al. 2012). The cells were defined based on retrospective characterization, which entails further questions whether skin MSCs are physiologically similar to BM MSCs in supporting normal hematopoietic cells and AML cells.

One of the important findings in paper IV is the characteristic of skin MSC *in vivo*. By employing *Ebf2-Egfp* transgenic mice, the presence of skin MSC was shown. Similar to the BM, skin Ebf2^{+} cells were highly enriched with MSCs, demonstrated by single cell colony assay, multilineage differentiation and serial replating *in vitro*. Different from BM MSC, skin Ebf2^{+} cells have little potency to differentiate towards chondrogenic *in vitro*, which was supported by lower expression of the genes associated with chondrogenesis, revealed by RNA sequencing. Nevertheless, ability to undergo positive osteogenic and adipogenic differentiation (bilineage) could be recapitulated. A small fraction of skin MSCs did not express Ebf2, but positive for CD140A and SCA1 (Ebf2^{-}MSC (PαS)). The skin Ebf2^{+} cells were located at perivascular site with a high expression of pericyte markers αSMA, NG2 and PDGFRb.

Our lineage tracing experiments using Ebf2 triple transgenic mice (*Ebf2-Egfp x Ebf2-Cre^{ERT2} x Rosa26^{loxp}Stop^{loxp}-Tomato*) revealed the hierarchical relationship between skin Ebf2^{+} cells and Ebf2^{-}PαS MSCs. The Ebf2^{-}PαS MSCs were generated from skin Ebf2^{+} cells, but not *vice versa*. Besides, the Ebf2^{+} cells also generated MPC lacking SCA1 expression although major fraction of skin Ebf2^{+} cell progeny was Ebf2^{-}PαS MSCs. While the frequency of skin Ebf2^{+} progeny (Tomato^{+} cells) was increased, Ebf2^{+} frequency remained constant for up to 12 months, providing evidence for the self-renewal properties of skin Ebf2^{+} cells. Taken together, the lineage tracing demonstrated that skin Ebf2^{+} cells were more primitive during MSC developmental hierarchy and that generated downstream mesenchymal cells in skin, as reported in BM (paper II).

The molecular profiles of skin MSC subsets were then determined by RNA sequencing and compared with their BM counterparts. Both skin MSCs (Ebf2^{+} and Ebf2^{-}PαS) expressed hematopoiesis supportive niche genes (*Angptl1*, *Cxcl14* and *Cxcl12*) as similar as that in BM MSCs, indicating skin MSCs may also support hematopoiesis and leukemia. Formation of similar numbers of CAFC from HSPC co-cultured with skin MSCs to that with BM MSCs corroborated robust hematopoiesis supportive function of skin Ebf2^{+} and Ebf2^{-}PαS *in vitro*. The potential role of skin MSCs for supporting AML cells was suggested by massive MLL-AF9^{+} AML cells that infiltrated into the dorsal skin tissue and altered numbers of the skin Ebf2^{+} cells in the skin tissue from the mice that developed AML.

In consistent with BM, the frequency of skin arteriole ($CD31^+SCA1^+$) increased suggesting AML cells also modulated skin mesenchymal niche. Skin MSC supportive function for AML LSC was demonstrated by similar number of CAFCs derived from AML cells co-cultured with skin MSC subsets as observed in BM MSCs in CAFC assays.

To test whether skin MSC also have chemoprotective function for AML LSCs, we firstly performed AML CAFC assays in the presence of Ara-C. In contrast to the efficient killing of AML cells in culture without MSCs, similar numbers of remaining CAFCs from AML were observed in the cocultures with skin MSC subsets and BM MSCs. Additionally, frequency of AML LSC (KIT^+) was maintained equally by both skin MSC subsets, indicating skin MSC could provide chemoprotection for resistant AML LSCs. In order to evaluate the *in vivo* role of skin as extramedullary organ with a chemoprotective function, we employed AML-promoting *Lama4*$^{-/-}$ mice as a model to study AML chemoresistance. Consistent with *in vitro* findings, dorsal skin of *Lama4*$^{-/-}$ mice harbored more residual AML cells compared to *Lama4*$^{+/+}$ mice. Interestingly, the frequency of phenotypically defined chemoresistant AML LSC ($CD36^+$) was found to be higher in *Lama4*$^{-/-}$ dorsal skin post Ara-C treatment, emphasizing the role of dorsal skin as a potential reservoir of residual AML cells.

In summary, paper IV reports (1) the skin MSC compartment consists of $Ebf2^+$ and $Ebf2^-$PαS MSCs with similar features as their BM MSC counterparts. The skin $Ebf2^+$ cells are more primitive MSCs and can generate more differentiated $Ebf2^-$PαS (MSCs). (2) Skin MSCs display similar HSC- and AML-supportive function, as BM MSCs *in vitro.* (3) Skin mesenchymal niche may act as extramedullary reservoir of AML cells and protect them from chemotherapy.

Taken together the findings from Paper I-IV, the present study shows the role of the BM niche for the initiation and progression of myeloid malignancies in mice. During this AML progression, LAMA4 is required for maintaining BM niche homeostasis and suppressing AML progression during development and drug treatment. In addition, the mesenchymal cell niche in skin tissue may contribute to AML maintenance during chemotherapy. However, more work is required to explore the mechanism and validate the result in patient.

6 DISCUSSION

6.1 INSTRUCTIVE ROLE OF BM NICHE IN THE INITIATION OF MYELOID MALIGNANCIES (MDS/MPN)

In paper I, we have studied the role of the BM niche in MDS/MPN development using *Sipa1*$^{-/-}$ mice that develop age-dependent MPN. The majority of the aged (16month old) *Sipa1*$^{-/-}$ mice showed a MDS/MPN phenotype. The disease phenotype did not completely resemble previous report (Ishida et al., 2003; Kometani et al., 2006) where majority of the mice developed CML-like MPN at the age of 12months and beyond. This discrepancy might be due to different genetic background and housing environment. By prospectively characterizing BM niche in the *Sipa1*$^{-/-}$ mice at different time points, we have showed that deletion of *Sipa1* resulted in phenotypic and molecular alterations of BM cellular niches including MSCs and MPCs prior to the onset of the disease, suggesting possible involvement of the altered *Sipa1*$^{-/-}$ niche in the initiation of the MDS/MPN. This possibility was validated by the development of MDS/MPN after transplantation of normal hematopoietic cells into the *Sipa1*$^{-/-}$ host mice. It is necessary to note that no hematopoiesis defects were detected in reciprocal transplantation of *Sipa1* deficient hematopoietic cells into wild type mice, showing *Sipa1* deficiency in hematopoietic cells did not lead to malignant transformation.

Proinflammatory niche and impaired hematopoiesis supportive functions are hallmarks of myeloid neoplasms (Arranz et al., 2014; Baryawno et al., 2019; Dong et al., 2016; Schepers et al., 2013). In line with this, the BM MSCs from young *Sipa1*$^{-/-}$ mice had elevated genes related to inflammatory cytokines (*Il6, Tgfb1*). Additionally, the finding of elevated *Thpo* in young *Sipa1*$^{-/-}$ MPCs was consistent with previous report (Schepers et al., 2013) showing increased THPO in BM plasma of BCR-ABL1 MPN mice. Furthermore, a higher *Thpo* expression might contribute to the increased granulopoiesis, Mk dysplasia and enhanced MEPs in *Sipa1*$^{-/-}$ mice. The impaired hematopoiesis supportive function of *Sipa1*$^{-/-}$ MSCs and MPCs were demonstrated by reduced ability of the niche cells to support HSPCs (LSK cells) to generate CFU-GME meanwhile enhanced capacity to form myeloid colonies (CFU-GM). The functional alterations were supported by the downregulation of the key HSC regulators such as *Cxcl12*, *Angptl1* and *Kitl* in both MSC and MPC. Altogether, paper I provides new evidence for abnormal niche-driven MDS/MPN.

These findings together with the *SIPA1* downregulation in BM MSC and CD31^{+} cells isolated from MPN patients meriting future study to investigate the prognostic value of *SIPA1* expression in BM niche in MPN patients who undergo allogenic HSC transplantation. Furthermore, delineating exact niche cell type or its anatomical distribution in *Sipa1* deficient mice that specifically contribute to MDS/MPN development would facilitate to identify the specific niche cells as potential target in an attempt to prevent MDS/MPN development.

6.2 NICHE-REMODELING BY LEUKEMIA AND BM NICHE CONTRIBUTION TO AML PROGRESSION

In paper II, we report BM niche remodeling by MLL-AF9$^+$ AML cells and the cellular and molecular mechanisms involved in the leukemic niche for AML progression in mice. The frequency of BM MSC, MPC, CD31$^+$ were increased in AML burden dependent manner. The BM MSCs from AML mice had enhanced the osteogenic and adipogenic potency. The dynamic remodeling also occurred molecularly, in particular, in MPCs where expression of hematopoiesis niche genes such as *Cxcl12*, *Angptl*, *Nov*, and *Igf1* (Arai et al., 2004; Greenbaum et al., 2013; Gupta et al., 2007; Omatsu et al., 2010; Thoren et al., 2008) were reduced as AML burden increased. On the other hand, the expression of inflammatory gene (*Il6*) in BM MSC and MPC were enhanced in correlation to AML burden. While loss of hematopoiesis function and high pro-inflammatory cytokines have been reported (Baryawno et al., 2019; Hanoun et al., 2014), our findings provide additional evidence for molecular alterations in BM niche from AML mice. The functional consequence of the alterations should be studied in the future.

Our previous study has shown BM Ebf2$^+$ cells mark primitive MSC populations and generate Ebf2$^-$ cells *in vitro* (Qian et al., 2013). In paper II, we examined the specific role of BM Ebf2$^+$ cells during AML development. By performing *in vivo* lineage-tracing of Ebf2$^+$ cells, we demonstrated the contribution of BM Ebf2$^+$ cells in the formation of AML niche. The BM Ebf2$^+$ cells generated different progenies that lost *Ebf2* expression and consisted of less primitive Ebf2$^-$MSCs, MPCs and CD44$^+$ mature stromal cells in the AML niche as AML progressed. The Ebf2$^+$ cells were also altered by AML cells to undergo lineage bias toward more differentiated MPC, which resulted in the imbalance of primitive MSC and more differentiated MPC in the BM. Thus, depletion of Ebf2$^+$ cells may amplify this lineage imbalance resulting in even more dramatic niche reconstruction and greater loss of host HSPC facilitated the faster AML development.

During AML development, *Sipa1* expression in BM MSCs and MPCs was also downregulated at end stage of AML. This is particularly interesting since we showed in paper I that loss of *Sipa1* in BM niche drove MDS/MPN development. In line with this finding the reduced *Sipa1* expression in BM niche may contribute to AML progression. In addition, we here report the increased *Lama4* and *Lama5* expression in BM MSCs and MPCs in AML mice, suggesting BM ECM was also remodeled by AML cells. The LAMA4 and LAMA5 are active chains of several laminin isoforms (Laminin-411/421, laminin-511/521), respectively to form cell-binding domains. The binding between laminin and HSC has been reported to play a critical role in HSC homing and engraftment (Qian et al., 2007; Qian et al., 2006). Moreover, in a mouse study where CML cell proliferation was promoted, *Lama4* expression was elevated. Altogether it warranted further study of *Lama4* role in AML.

6.3 THE ROLE OF *LAMA4* IN THE BM NICHE FOR HEMATOPOIESIS REGENERATION AND AML PROGRESSION

In paper III, we studied the role of *Lama4* gene encoding one of BM ECM (Scheele et al., 2007; Susek et al., 2018) in hematopoiesis recovery under hematopoiesis insults including irradiation and AML. Following irradiation, *Lama4*$^{-/-}$ mice displayed impaired recovery of RBC, HGB and PLTs in PB. Furthermore, the *Lama4*$^{-/-}$ mice had rapid regeneration of MEP while GMP and MkP were slowly recover in BM, showing the important role of *Lama4* in erythropoiesis and megakaryopoiesis. Following AML-induced hematopoiesis insult, PLT impairment was more pronounced and host hematopoiesis disruption became more severe, emphasizing the important role of *Lama4* for hematopoiesis maintenance and AML progression.

Mechanistically, *Lama4*$^{-/-}$ mice had lower frequency of ECs and MPCs in the BM. Moreover, the *Lama4*$^{-/-}$ MPC displayed reduced expression of *Angpt1, Cxcl10, Fn1, Nid1,* and upregulation of negative regulators of hematopoiesis, such as Osteopontin (*Spp1*) (Nilson et al. 2015, Stier et al. 2005), other inflammatory genes (*Tgfb, Il17d and Il18*) and genes related to OXPHOS activity. These molecular alterations could further cause hematopoiesis regeneration defects under stress following irradiation.

6.4 *LAMA4* IN THE BM NICHE INHIBITS AML DEVELOPMENT AND LOSS OF *LAMA4* PROMOTES CHEMOPROTECTIVE FUNCTION OF BM NICHE FOR AML LSC VIA INCREASED MITOCHONDRIA TRANSFER

In paper II we have shown that *Lama4* was upregulated in BM MSCs and MPCs in AML mice. However, the functional impact of *Lama4* in BM niche for AML progression was not known. To this end, in paper III we evaluated the role of *Lama4* during AML development. The *Lama4*$^{-/-}$ mice developed AML faster and maintained higher frequency of AML LSC in BM, compared to *Lama4*$^{+/+}$ mice. CAFC assay further demonstrated the enhanced ability of *Lama4*$^{-/-}$ MSCs to maintain AML LSCs, pointing to the inhibitory role of *Lama4* in AML development.

BM MSPCs have been reported to provide chemoprotection for AML LSCs via mitochondria transfer (Forte et al., 2020). Our *in vitro* AML CAFC assays demonstrated similar finding as MSC transferred mitochondria to AML cells. In addition to that, we showed increased mitochondria transfer from *Lama4*$^{-/-}$ MSCs to AML in presence of Ara-C. Future studies to profile the mitochondria activity in *Lama4*$^{-/-}$ MSC will be necessary to elucidate the critical factors in *Lama4*$^{-/-}$ MSC mitochondria that contribute to AML LSC salvage. Altogether, paper III reports that loss of *Lama4* enhances niche chemoprotection for AML LSCs via mitochondria transfer, thus, *Lama4* in BM niche may inhibit AML development by maintaining niche homeostasis.

6.5 CHARACTERISTICS OF MSC IN EXTRAMEDULLARY SKIN TISSUE AND THEIR FUNCTION FOR SUPPORTING NORMAL HEMATOPOIETIC AND AML STEM CELLS

Extramedullary niche for leukemia development and as reservoir of chemoresistant LSC has been recognized (Ye et al., 2016). However, the exact contribution of the extramedullary niches for hematopoiesis and leukemia have not been well defined due to lack of knowledge of the niche cell identity and the secreted factors from the niches. In paper IV, we firstly characterized MSC in dorsal skin tissue and evaluated their contribution to AML LSCs.

We have previously shown that BM Ebf2$^+$ cells are primitive MSCs (Qian et al., 2013) and contribute to AML development (Paper II). Similar to BM Ebf2$^+$ cells, skin Ebf2$^+$ cells were also highly enriched with MSCs. In dorsal skin, the *in vivo* lineage tracing data showed skin MSCs had a same mesenchymal hierarchy as BM MSCs in which skin Ebf2$^+$ cells seemed to reside at the top of the MSC developmental hierarchy and could generate the less primitive MSCs (Ebf2$^-$ PαS (MSC)) and MPCs. Different from BM MSCs, skin MSCs were enriched with genes related to skin regeneration and expressed lower level of genes related to bone regeneration, indicating tissue-specific phenotype of MSC from BM and skin. This study demonstrates novel characteristics of skin MSCs and provides techniques to prospectively isolate native skin MSCs.

Another novelty in paper IV is the findings of skin MSC hematopoiesis supportive function and its chemoprotection on AML LSCs. Molecularly, both skin Ebf2$^+$ and Ebf2$^-$ PαS (MSC) expressed hematopoiesis supportive genes such as *Angptl1*, *Cxcl12*, *Kitl*, *Cxcl14*. *Lama4* was also expressed in skin Ebf2$^+$ and Ebf2$^-$ PαS (MSC). The *in vitro* hematopoiesis functional assay supported the molecular profiling as skin Ebf2$^+$ and Ebf2$^-$ PαS (MSC) maintained LT-HSC. Although the assay was *in vitro* based, the numbers of CAFC and CFU-C showed ability of skin MSC to maintain LT-HSC with ability to generate myeloid lineage. Furthermore, CAFC number has been shown to correlate with the number of injected HSC in transplantation assay (Ploemacher et al., 1989), highlighting high fidelity of our CAFC assay for assessing LSC growth *in vitro*. Nevertheless, the selected *in vitro* assays limited the evaluation of LT-HSC potency to generate lymphoid lineage.

Studies in BM have shown the AML cells receive various supports from MSPC, but whether MSPCs in the extramedullary organ display similar function is not known. Here, we reported that skin MSC (Ebf2$^+$ and Ebf2$^-$ PαS (MSC)) maintained AML cells similarly to BM MSCs. Nevertheless, the alteration of skin Ebf2$^+$ cells during AML development was found to be different to BM Ebf2$^+$ cells. While BM Ebf2$^+$ cells were increased, the frequency of skin Ebf2$^+$ cells were reduced, indicating mesenchymal niche alteration might be unique for AML extramedullary mesenchymal niche. Besides maintaining AML cells, the skin Ebf2$^+$ and Ebf2$^-$ PαS (MSC) also provided chemoprotection for AML cells as did their BM counterpart.

Using *Lama4*$^{-/-}$ mice which in paper III was shown to promote chemoresistance of AML LSC, we demonstrated dorsal skin acted as extramedullary site where massive AML infiltrated and chemoresistant AML LSCs were retained.

The possibility of clonal selection within retained chemoresistant AML LSCs in skin needs to be further studied. Additionally, the study on interplays between skin MSC and AML cells would be beneficial to investigate mechanism underlying skin MSC chemoprotective function.

7 CONCLUSIONS

The thesis work shows the contribution of mesenchymal cell niche to normal hematopoiesis maintenance and the development of myeloid malignancies in mice. **In paper I**, we have demonstrated that the loss of *Sipa1* induces BM niche alterations which drives the MDS/MPN development in *Sipa1*$^{-/-}$ mice. **In paper II**, our data show niche-remodeling effect in a transplantation-induced AML mouse model and the suppressive role of BM MSCs in AML niche formation and the progression of AML. Specifically, the primitive BM MSC (Ebf2^{+} cells) are instructed to generate higher frequency of progenies including more differentiated Ebf2^{-}MSCs, MPCs and CD44^{+} cells. During AML development, depletion of Ebf2^{+} cells accelerates AML onset, showing a suppressive role of the Ebf2 expressing MSCs. The AML cells also increased *Lama4* expression in BM MSCs and MPCs. However, deletion of *Lama4* in mice accelerates AML progression, shown in **paper III**. Mechanistically, loss of *Lama4* in MSCs promotes AML LSC proliferation and provide chemoprotection via mitochondria transfer and modulating antioxidant activity in the AML cells. Further investigation in **paper IV** suggests that increased number of AML cells infiltrated in skin tissue in the *lama4* deficient mice during AML development and post Ara-C therapy. The skin MSCs share similar immunophenotype and stem cell properties, and also display supportive function for normal HSCs and chemoresistant AML LSCs, highlighting the previous unrecognized role of extramedullary MSC in AML development and chemotherapy response.

8 POINT OF PERSPECTIVE

Although BM niche contribution to hematopoiesis has been acknowledged, the dynamic roles and mechanisms of the niche in leukemia remain poorly understood. The findings in this thesis support a notion that the niche function is disease-specific. In paper I, the MDS/MPN development in germline deleted *Sipa1* mice is driven by the altered BM niches. In this paper, the BM niches act as instigator for the development of MDS/MPN, as shown by the transplantation of *Sipa1*$^{+/+}$ mice to *Sipa1*$^{-/-}$ recipient mice whose BM niche were already altered due to *Sipa1* loss. Meanwhile, in paper II, the findings showed BM niche can be remodeled by AML cells. Since AML mouse model was generated by injection of MLL-AF9^{+} AML cells to healthy and immunocompetent mice, the remodeling effects of AML cells on BM niche was illustrated by the dynamic alteration of BM niche following AML injection. The altered niche become more supportive for AML cells but suppressive for host hematopoietic cells. Future work should be focusing on identification of the molecular pathways mediating the niche-remodeling effects.

The findings of the role of MSCs and *Lama4* during normal hematopoiesis and myeloid malignancy provide new insights into future study on the role of these factors in AML in patients. Patient derived xenografted mouse model could be a useful tool for the study. Restoration of BM niche by modulating MSC proliferation and/or by administering LAMA4 or activator for *Lama4* pathway might be a good strategy to explore the therapeutic possibility to treat the leukemia.

Findings of AML infiltration in skin tissue provide evidence that skin is AML extramedullary reservoir. Furthermore, skin MSCs supported and protected AML from Ara-C which triggers further research questions on skin MSC contribution to AML relapse. In line with it, another follows up study would be evaluating the clinical relevance of our finding in AML mouse which could be carried out by investigating AML infiltration in patient skin tissue.

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"The real voyage of discovery consists not in seeking new landscapes,

But in having new eyes"

a paraphrase from Remembrance of Things Past – Marcel Proust

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