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Role of membrane-type 1 matrix metalloproteinase in hematopoietic stem/progenitor cell trafficking

by

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Abstract

Hematopoietic stem/progenitor cells (HSPC) are routinely used for transplantation to restore hematopoietic function; however, the molecular mechanisms governing their trafficking are not completely understood. Soluble matrix metalloproteinase (MMP)-2 and MMP-9 have been shown to mediate HSPC mobilization from the bone marrow (BM) to peripheral blood (PB) and their homing back from PB to the BM niches. However, the role of membrane type (MT)-1 MMP (localized on the leading edge of migrating cells and activates proMMP-2), in these processes is unknown. In this thesis I test the hypothesis that MT1-MMP is involved in HSPC trafficking. In the first part, I investigated the role of MT1-MMP in granulocyte-colony stimulating factor (G-CSF)-induced mobilization and found that G-CSF incorporates MT1-MMP into membrane lipid rafts of hematopoietic cells in a phosphatidylinositol 3-kinase (PI3K)-dependent manner and enhances chemoinvasion of HSPC. Moreover, MT1-MMP expressed by HSPC activates proMMP-2 secreted by fibroblasts under conditions simulating the BM microenvironment. Thus, MT1-MMP contributes to a highly proteolytic BM microenvironment that facilitates egress of HSPC into the circulation. In the second part, I investigated the role of MT1-MMP in the homing-related responses of cord blood (CB) HSPC. Previously, we demonstrated that supernatants (isolated from leukapheresis products of G-CSF-mobilized patients) or their components [complement C3a, fibrinogen, fibronectin, hyaluronic acid (HA) and thrombin] had a priming effect on HSPC homing by enhancing their chemotaxis to stromal cell-derived factor-1 (SDF-1) and stimulating their secretion of

proMMP-2 and proMMP-9. Here, I focused on the effect of HA and thrombin on the modulation of MT1-MMP expression in CB HSPC. HA and thrombin, in a PI3K- and Rac-1 GTPase-dependent manner, increase MT1-MMP expression in CB HSPC which activates proMMP-2 secreted by endothelial cells. Moreover, crosstalk between PI3K and Rac-1 signaling pathways leads to signal amplification and enhanced chemoinvasion of CB HSPC towards a low SDF-1 gradient in a MT1-MMP-dependent manner. Thus, MT1-MMP contributes to the priming of homing-related responses of HSPC. In conclusion, findings from this thesis suggest that modulation of MT1-MMP expression has potential as a target for development of new therapies for faster mobilization, homing and engraftment of HSPC, leading to improved transplantation outcomes.

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BGS	Bovine growth serum
BM	Bone marrow
BSA	Bovine serum albumin
CaR	Calcium sensing receptor
CB	Cord blood
CC	Complement cascade
CFU-F	Colony-forming unit-fibroblasts
CFU-Meg	Colony-forming unit-megakaryocytes
CFU-S	Colony-forming unit-spleen
CG	Cathepsin G
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
CPM	Carboxypeptidase M
CTX	Cholera toxin
DC	Dendritic cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	(-)-Epigallocatechin 3-gallate
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
ERM	Exrin/radixin/moesin
ESC	Embryonic stem cells
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
G-CSF	Granulocyte-colony stimulating factor
FOXO	Forkhead box O
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM1	Monosialotetrahexosylganglioside
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMP	Granulocyte/macrophage progenitors
GPI	Glycosylphosphatidylinositol
GvHD	Graft versus host disease
h	Hour
HA	Hyaluronic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase

HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem/progenitor cells
HPC	Hematopoietic progenitor cells
HUVEC	Human umbilical vein endothelial cells
ICAM	Intra-cellular adhesion molecule
Ig	Immunoglobulin
IGF	Insulin growth factor
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
iPSC	Induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
LT	Long-term
m	Mobilized
MAPK	Mitogen activated protein kinase
MβCD	Methyl-beta-cyclodextrin
MEP	Megakaryocytic/erythroid progenitors
mg	Milligram
min	Minutes
ml	Milliliter
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MPP	Multipotent progenitor cells
MSC	Mesenchymal stem cells
MT-MMP	Membrane type-matrix metalloproteinase
mTOR	Mammalian target of rapamycin
mRNA	Messenger ribonucleic acid
n	Normal
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
NaF	Sodium fluoride
NE	Neutrophil elastase
ng	Nanogram
NH_4Cl	Ammonium chloride
NK	Natural killer
NOD	Non-obese diabetic
Na_2VO_3	Sodium orthovanadate
OPN	Osteopntin
p	Probability
P-	Phospho-
PAK	p21 activated kinase 1 protein
PAR-1	Protease-activated receptor-1

PB	Peripheral blood
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear cells
PMSF	Phenylmethylsulfonyl fluoride
PMV	Platelet-derived microvesicles
PSC	Pluripotent stem cells
Pyk	Proline-rich tyrosine kinase
RANKL	Receptor activator of nuclear factor k-B ligand
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse-transcription polymerase chain reaction
SCF	Stem cell factor
SCID	Severe combined immune deficiency
SDF-1	Stromal cell-derived factor-1
siRNA	Small interfering ribonucleic acid
SLP	Supernatants of leukapheresis product
ST	Short-term
ss	Steady-state
suPAR	Soluble urokinase-type plasminogen activator receptor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
μg	Microgram
μM	Micromolar
uPAR	Urokinase-type plasminogen activator receptor
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VEGF	Vascular endothelial growth factor
VSEL	Very small embryonic-like

Chapter 1 Introduction

1.1 Overview of stem cells

Stem cells are unspecialized cells that have the ability to self-renew through mitotic cell division and differentiate into a diverse range of specialized cell types. The most primitive human stem cell is the totipotent zygote that gives rise to both the embryo and the placenta and subsequently to all types of tissues in the body (Figure 1-1) [1,2]. The trophoblast or the outer wall of the blastocyst adheres to the uterine wall and the inner cell mass contains pluripotent stem cells (PSC), also called embryonic stem cells (ESC), capable of generating all three germ layers: ectoderm, mesoderm and endoderm [2]. PSC expanded in vitro as pluripotent ESC lines were first established from mice [3] and later from humans [4]. PSC further differentiate to give rise to multipotent and monopotent stem cells which are adult or somatic stem cells [1,2]. One example of multipotent stem cells is hematopoietic stem cells (HSC), which differentiate into the myeloid lineage that gives rise to granulocytes, monocytes, megakaryocytes and erythrocytes; and the lymphoid lineage that gives rise to T-, B- and natural killer (NK)-cells [5]. Another example is mesenchymal stem cells (MSC), which differentiate into osteocytes, adipocytes and chondrocytes. Monopotent stem cells are tissue-specific stem cells such as neural, epithelial, liver and pancreatic stem cells [6]. Recently, a very rare population of stem cells that display several features of pluripotent ESC and gives rise to all three germ layers was identified in bone marrow (BM), cord blood (CB) and other tissues and these cells have been termed very small embryonic like (VSEL) stem cells [7-9]. Pluripotency

can be induced in somatic cells by retroviral transfer of specific transcription factors as was demonstrated by the Yamanaka group [10]. These cells are called induced pluripotent stem cells (iPSC) which have been shown to be molecularly and functionally similar to ESC [10,11].

Given their unique regenerative abilities, stem cells offer great potential for treating degenerative disorders [2,6,12]. However, much work remains to be done in the laboratory and in the clinic to understand how to use these cells for therapies to treat various diseases. HSC are currently the best studied among adult stem cells and have been successfully used in clinical transplantation for hematopoietic reconstitution.

1.2 Hematopoietic stem cells

HSC are predominantly located in the BM but can also be found in CB, spleen, fetal liver and, to a lesser extent, in circulating peripheral blood (PB) [13]. The first evidence that HSC exist came from the landmark studies carried out by Till and McCulloch [14]. In a series of experiments, visible nodules were observed in the spleens of irradiated mice following injection of BM cells, and were found to contain cells that were called colony forming units-spleen (CFU-S). These initial studies triggered further work to pursue the identity and function of HSC.

1.2.1 Hematopoietic hierarchy

In the hematopoietic system, HSC give rise to hematopoietic progenitor cells (HPC) and mature blood cells. (Figure 1-2) [5]. There is no single marker

that is exclusively expressed by HSC; rather they express several markers including CD34, CD38, c-Kit receptor tyrosine kinase (CD117) and CD133 whose expression is either gained or lost during differentiation [15].

HSC are lineage-negative (lin^-) for markers found on T- and B-cells, granulocytes, monocyte/macrophages, megakaryocytes and erythrocytes. In the murine system, HSC express the cell surface molecules c-Kit, Sca-1, and Thy-1 [16]. These multipotent c-Kit^+ (K), Thy-1^{lo} (T), lin^- (L), Sca-1^+ (S) HSC are the most immature and have the highest self-renewal capacity. They are also known as long-term (LT)-HSC since they are capable of maintaining hematopoiesis for a lifetime after transplantation in mice. The extensive self-renewal capacity of LT-HSC can be assessed by serial transplantation where HSC capable of extensive self-renewal propagate themselves and their progeny after secondary and tertiary transplants. LT-HSC give rise to multipotent short-term (ST) HSC which have a lower self-renewal capacity of up to six to eight weeks [17].

Lower in the hierarchy of ST-HSC are the multipotent progenitor cells (MPP) that have multipotent lineage capacity but no self-renewal capacity. The hematopoietic hierarchical model suggested by the Weissman group shows that at this stage MPP can form either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP) [15]. CMP can give rise to two oligopotent progenitors: the megakaryocytic/erythroid progenitors (MEP) that differentiate into megakaryocytes/platelets and erythrocytes; and the granulocyte/monocyte progenitors (GMP) that differentiate into granulocytes, macrophages and dendritic

cells (DC) [5,18]. CLP have the potential to differentiate into T-cells, B-cells, NK-cells and DC [5,15].

1.2.2 Human hematopoietic stem cell markers

In the human system, the classification of subpopulations of stem and progenitor cells is less clear. Although the CD34 antigen has been accepted as a marker for human HSC, it is recognized that $CD34^+$ cells are a heterogeneous population of stem and progenitor cells which are thus referred to as hematopoietic stem/progenitor cells (HSPC) [19]. However, some of the $CD34^-$ population do express CD133 (another marker for primitive HSC) [20]. The lineage-restricted progenitors express the myeloid cell surface antigen CD38. The more immature human HSPC are $CD34^+CD38^-$ and are better able to engraft immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) β2-microglobulin-null mice pretreated with high dose chemotherapy [21].

1.3 Hematopoietic stem/progenitor cell transplantation

HSPC transplantation is a procedure in which HSPC capable of reconstituting normal BM function (hematopoiesis) are administered intravenously to a patient who has undergone preparative regimens including chemotherapy and/or irradiation. Approximately 50,000 autologous and allogeneic HSPC transplants are performed annually worldwide for both malignant and non-malignant hematological and non-hematological disorders listed in Table 1-1 [22,23]. The sources of HSPC used for transplantation are BM, PB and CB.

1.3.1 Bone marrow

HSPC reside primarily in the BM and constitute about 1.1% of all hematopoietic cells [24]. For transplantation, BM is harvested by means of 100 to 200 punctures in the posterior iliac crests of the patient/donor under general anesthesia. Side-effects of BM harvest include intense pain, fever, bleeding or local infection [25]. Nevertheless, the procedure is relatively safe, and serious complications are rare (two fatalities in 8000 harvests) [24]. BM transplantation was pioneered in the 1950s by a team led by E. Donnall Thomas whose work showed that BM-derived stem cells infused intravenously could repopulate the recipient BM and reconstitute hematopoiesis [26]. The subsequent discovery of the major histocompatibility complex and the development of human leukocyte antigen (HLA) typing methods made allogeneic transplantation feasible [23]. However, graft-versus-host disease (GvHD) may occur due to differences in minor histocompatibility antigens. While donor T-cells are undesirable as effector cells of GvHD, they have been proven to have a valuable graft-versus-tumor effect [23].

1.3.2 Peripheral blood

During steady-state homeostasis, approximately 0.06% of BM HSPC circulate continuously in the PB [24]. The number of PB HSPC can be increased with the use of chemotherapeutic drugs (e.g. cyclophosphamide) and/or cytokines [e.g. granulocyte colony-stimulating factor (G-CSF)] that ‘mobilize’ BM HSPC into the PB [27]. Mobilized (m) PB is a convenient source of HSPC because it is relatively easy to collect by apheresis in an outpatient setting and because

engraftment after transplantation is faster. Currently mPB HSPC transplantation has almost replaced BM harvest for autologous and most allogeneic transplantations [27,28].

G-CSF is the most commonly used mobilizing agent in the clinic with regimens using 10μg/kg/day for five days when used alone or 10 to 14 days when used in combination with chemotherapeutic agents [27]. The exact timing of collection is determined by the $CD34^+$ cell count which is monitored in the PB by flow cytometry [27]. Randomized trials with mPB HSPC transplantation have shown that neutrophil and platelet engraftment generally occurs at a median of 9-14 days compared to 21 days with BM HSPC [29]. This has been attributed to the higher numbers of $CD34^+$ cells collected and transplanted. A limitation of mPB HSPC transplantation is that patients' responses to G-CSF vary: 5-10% of allogeneic normal donors mobilize poorly and up to 40% of autologous patients fail to mobilize depending on their disease and the intensity/number of prior chemotherapy regimens [30]. Hence, it is imperative to better understand the molecular mechanisms of HSPC mobilization so that more efficient mobilizing agents and protocols can be used.

1.3.3 Cord blood

An alternative source of HSPC is CB obtained from the umbilical cord at the time of childbirth, after the cord has been detached from the newborn. The first CB transplant was reported by Gluckman in 1989 using CB from an HLA-matched sibling [31]. Since then approximately 20,000 CB transplants have been performed worldwide in pediatric and adult patients [32]. CB has several

advantages over BM or mPB HSPC transplantation. CB contains lower numbers of more immature immunocompetent T-cells, and thus requires less stringent HLA matching; this means that a mismatch at one or two loci can be tolerated without significant increase in GvHD or decrease in overall survival [32,33]. However, the main limitation of CB transplantation is the low $CD34^+$ cell dose available in one CB unit which is generally insufficient to support engraftment in adult patients [33].

Retrospective analyses of outcomes of CB and BM transplantation in adults have reported delayed neutrophil engraftment: 25 days with CB versus 18 days with BM, and platelet engraftment: 59 days with CB versus 27 days with BM [34,35]. However there was no significant difference in leukemia-free survival between CB and BM transplantation outcomes suggesting that CB is a valid alternative source of HSPC for transplantation when a matched BM donor is not available.

Currently, efforts are being made to increase CB cell dose in order to speed up engraftment and hematopoietic recovery. Strategies to use more than one CB unit [36] or to expand CB $CD34^+$ cells ex vivo [37] however, have met with limited success [33]. A more comprehensive knowledge of CB HSPC biology and the mechanisms of their homing could lead to improvements in CB transplantation outcomes.

1.4 Hematopoietic stem cell niche

The stem cell niche as first proposed by Schofield is a three-dimensional, spatially organized structure in which stem cells are housed and maintained where

they self-renew without differentiating [38]. Two types of niches that support the hematopoietic system: the osteoblastic niche and the vascular niche, have been suggested (Figure 1-3) [39-41]. The endosteal/osteoblastic niche close to the bone, is a site of relative hypoxia where immature osteoblasts are in close contact with HSC, and plays a major role in the maintenance of HSC quiescence [39,40]. The vascular niche consisting of sinusoidal vessels provides a microenvironment rich in nutrients, growth factors and oxygen, and plays a role in HSC proliferation and differentiation, and ultimately egress of mature progenitors into the circulation [39,40,42,43]. The relationship between these niches and the HSC populations found in each of them is however controversial [40]; for example HSC expressing SLAM ($CD150^+$ $CD48^-$) markers have been detected on the trabecular bone lining osteoblasts as well as near sinusoidal endothelial cells [44].

Regulation of the stem cell niche requires a balance between self-renewal and differentiation to maintain a reservoir of stem cells while still supplying adequate numbers of mature hematopoietic cells [39,40]. Multiple signaling and adhesion molecules contribute to the stem cell niche interactions including Jagged/Notch, angiopoietin-1/Tie2, stem cell factor (SCF)/c-Kit and the Ca^{2+} sensing receptor (CaR) [45-47]. In addition, cytokines and chemokines such as SCF, osteopontin (OPN), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor-4 and stromal cell-derived factor (SDF)-1 modulate HSC proliferation within the niches [48,41]. The chemokine SDF-1 is secreted by stromal cells and osteoblasts and its receptor CXCR4 is expressed by HSC [49]. SDF-1 maintains the HSC in quiescence which is critical

for sustaining a pool of undifferentiated HSC and also mediates their homing and engraftment to BM niches [39,49].

1.5 Hematopoietic stem/progenitor cell mobilization

The mechanisms of HSPC mobilization are not completely understood but are believed to be a complex interplay between adhesion molecules, the SDF-1−CXCR4 axis and proteases (figure 1-4) [50,51]. During homeostasis, continuous traffic of HSPC between the BM and PB contributes to normal hematopoiesis. The ability to enhance these physiological processes and, for example, enforce egress (mobilization) of HSPC from the BM to circulation has been invaluable in enabling clinical transplantation [27,28]. Several cytokines (G-CSF, GM-CSF, Flt-3 ligand, IL-8 and SCF) and chemokines (SDF-1 and GROβ) can trigger mobilization in varying degrees [52-58]. We recently demonstrated that G-CSF also increases plasma HGF levels in mobilized patients and expression of its receptor c-Met in HSPC and myeloid cells, suggesting that G-CSF-mediated HSPC mobilization occurs in part through the HGF/c-Met axis [59]. Recent studies have demonstrated that thrombolytic agents such as microplasmin, tenecteplase and recombinant tissue plasminogen activator enhance G-CSF-induced mobilization in murine models [60]. Components of innate immunity also participate in G-CSF-induced HSPC mobilization as proposed by the Ratajczak group and us [61-64]. During G-CSF-induced mobilization, the complement cascade (CC) is activated by the classical pathway. However, in the early steps of the CC, C1q, C3 and its cleavage fragments C3a and $_{desArg}$C3a increase retention of HSPC in BM while in the later steps of the CC, C5 and its

cleavage fragments C5a and $_{desArg}$C5a enhance HSPC mobilization and their egress into PB [61-65].

1.5.1 Role of adhesion molecules

Adhesion molecules play an important role in the retention of HSPC in the BM. Very late antigen (VLA)-4 is expressed by HSPC and its ligand, vascular cell adhesion molecule (VCAM)-1, is constitutively expressed by endothelial and stromal cells [51]. It has been demonstrated that functional blocking of either VLA-4 or VCAM-1 by neutralizing antibodies or deletion of α4 integrins leads to HSPC mobilization [66]. Moreover, anti-VLA-4 induced HSPC mobilization is significantly enhanced in β2 integrin-deficient mice indicating that both VLA-4 and β2 integrins contribute to the anchoring of HSPC in the BM [66]. CD44, a polymorphic integral membrane glycoprotein, binds to several extracellular matrix (ECM) components such as hyaluronic acid (HA), fibronectin and collagen. Mice treated with anti-CD44 antibody or lacking CD44 exhibit impaired mobilization in response to G-CSF [67]. Selectins also mediate adhesion of HSPC to the endothelium. P- and E-selectins are expressed by endothelial cells while L-selectin is expressed by HSPC. Mice treated with fucoidan, a sulfated glycan that inhibits P- and L-selectins in vivo, induced a robust HSPC mobilization response [68]. Moreover, fucoidan treatment led to an increase in plasma levels of SDF-1 with a concomitant decrease in levels of BM SDF-1 in mice [69].

1.5.2 Role of proteases

G-CSF induces neutrophil proliferation, activation and degranulation with the subsequent release of the serine proteases neutrophil elastase (NE), cathepsin G (CG), and matrix metalloproteinase (MMP)-9 into the BM, making it a highly proteolytic microenvironment [50,51]. NE and CG have been shown to accumulate in the BM and their levels correlate not only with increased HSPC mobilization but also inversely with the levels of their inhibitors serpina1 and serpina3 [70,71]. These proteases cleave and inactivate a number of molecules involved in the retention of HSPC in the BM. For example, VCAM-1 is a substrate of NE and CG in vitro as well as in vivo and is cleaved during G-CSF induced mobilization resulting in elevated levels of soluble VCAM-1 fragments in the plasma of mobilized patients [72]. However, cleavage of VCAM-1 is not necessary for HSPC mobilization since mice lacking both NE and CG mobilize normally and VCAM-1 expression in their BM stromal cells is unchanged [73].

MMP-9 has been reported to be elevated in plasma after mobilization with G-CSF or IL-8 [70,74]. Murine studies have also revealed that MMP-9 releases soluble kit-ligand and is a potential mechanism for G-CSF induced mobilization; although, other studies have shown that mobilization in MMP-9-deficient mice is normal indicating that MMP-9 is not absolutely necessary for mobilization to occur [73,75]. Mobilization is thought to occur predominantly through the action of neutrophils; however, HSPC themselves contribute to this process by secreting MMP-2 and MMP-9 [76]. While BM HSPC in steady-state do not secrete MMPs, upon stimulation with G-CSF, HSPC secrete both MMP-2 and MMP-9 leading to

their enhanced migration through reconstituted basement membranes (Matrigel) [76]. Other proteases such as carboxypeptidase M (CPM) and CD26 have also been reported to induce HSPC mobilization by disrupting SDF-1−CXCR4 interactions in the BM microenvironment [77,78].

1.5.3 Role of the SDF-1−CXCR4 axis

The SDF-1−CXCR4 chemotactic axis is essential for retention of HSPC in the BM and perturbation of the SDF-1 gradient in the BM as well as a decrease in the responsiveness of HSPC to SDF-1 leads to mobilization [49,50]. The proteases CPM [77] and CD26 [78] cleave the C-terminus of SDF-1 resulting in attenuated chemotactic responses of HSPC; moreover, G-CSF-induced mobilization is impaired in CD26-deficient mice [78]. Desensitization of CXCR4 by the urokinase-mediated plasminogen activation system during G-CSF-induced mobilization has been demonstrated [79]. G-CSF has been shown to increase urokinase-type plasminogen activator receptor (uPAR) expression in myeloid cells and to increase levels of soluble uPAR (suPAR) in serum of G-CSF-mobilized patients. Interestingly, cleaved suPAR (presumably by preoteases activated during mobilization) down-regulated CXCR4 expression in $CD34^+$ cells and impaired their SDF-1-dependent chemotaxis and additionally, chemoattracted BM HSPC through the high-affinity fMet-Leu-Phe receptor [79].

In murine studies, SDF-1 concentration in the BM significantly decreased following G-CSF administration and was significantly correlated with mobilization, suggesting that a physiological drop in SDF-1 level in the BM is a critical step in HSPC mobilization [80,81]. A decrease in BM SDF-1 levels has

been reported to coincide with a peak of proteolytic activity of NE, CG and MMP-9 [50]. Both SDF-1 and CXCR4 are targets of degradation by NE, CG, MMP-9, MMP-2 and membrane type (MT)1-MMP [82-84], and can be inactivated by proteolytic cleavage. Serine protease inhibitors have been reported to attenuate SDF-1 inactivation and HSPC mobilization in response to G-CSF in vivo suggesting that cleavage of SDF-1 is at least partly responsible for mobilization [71]. However, low BM SDF-1 levels are also observed in mice deficient in NE and CG suggesting that protein expression of SDF-1 is not dependent on these proteases and that SDF-1 regulation occurs at the mRNA level due to the suppression of SDF-1-producing osteoblasts [85]; however, the mechanism is still unclear.

Increased osteoclast activity also plays a role during G-CSF-induced mobilization [86]. Mouse osteoclasts stimulated with receptor activator of nuclear factor κ-B ligand (RANKL) (secreted by bone-lining osteoblasts) led to increased secretion of MMP-9 and cathepsin-K [86]. Moreover, RANKL-induced osteoclast activity led to decreased expression of SDF-1, SCF and OPN, and subsequently egress of BM HSPC into circulation [86]. The sympathetic nervous system has been demonstrated to play an indirect role in HSPC egress during G-CSF-induced mobilization via norepinephrine signaling by suppressing osteoblasts and decreasing BM SDF-1 levels [87]. Catecholamines also facilitate HSPC mobilization by inducing expression of MMP-2 and MT1-MMP and by chemoattracting $CD34^+$ cells [88].

Efforts are being made in the clinical setting to increase the mobilization efficiencies of HSPC for transplantation and currently new mobilizing agents are being investigated. Plerixafor (AMD3100), a bicyclam molecule that specifically and reversibly inhibits SDF-1 binding to CXCR4, has shown promising results in HSPC mobilization. Recently Phase III clinical trials comparing a combination of plerixafor and G-CSF versus G-CSF alone in patients with non-Hodgkin's lymphoma and multiple myeloma showed that the combination mobilized a significantly higher number of $CD34^+$ cells compared to G-CSF alone [89,90]. Pegfilgrastim (pegylated G-CSF) is a variant of G-CSF with a longer half-life of 33 hours compared to the 4-6 hour half-life of G-CSF. Recent studies have demonstrated that pegfilgrastim alone or in combination with chemotherapy is capable of efficiently mobilizing HSPC although it did not translate into faster hematopoietic recovery [91]. Other agents such as SCF, Groβ and parathyroid hormone are also currently being investigated as ways to increase mobilization efficiencies [92].

1.6 Hematopoietic stem/progenitor cell homing

Interactions between HSPC and their niches that are disrupted during mobilization need to be re-established during their homing to the BM and repopulation [93]. Previously it was believed that mobilization and homing were mirror images of each other, however, emerging evidence now suggest that this is not the case, although both processes involve many of the same adhesive and chemotactic interactions [51,93]. The homing of HSPC to BM is a rapid process occuring within hours after their transplantation, and is a prerequisite for their

repopulation and engraftment in the BM [93]. Homing requires three consecutive steps: extravasation from PB through the BM endothelium; migration through stroma; and lodgement into niches, all of which are directed through the SDF-1−CXCR4 axis [51,93] and are shown in the schema (Figure 1-5) [94].

1.6.1 Role of adhesion molecules

The extravasation of circulating HSPC within the BM requires a set of molecular interactions that mediates the recognition of circulating HSPC by the endothelium of BM sinusoids [93]. The mechanisms of HSPC extravasation are similar to that of leukocytes into inflamed tissues in that they are mediated by adhesion molecules [95]. The BM endothelium constitutively expressing P-selectin, E-selectin and VCAM-1 mediates rolling and tethering of homing cells to the blood vessel wall prior to their extravasation [94]. HSPC expressing their receptors CD44 and VLA-4 interact with their ligands on the endothelial surface. The coordinated action of these adhesion molecules is triggered by SDF-1 presented at the surface of endothelial cells. SDF-1 mediates activation of lymphocyte function-associated antigen-1, VLA-4 and VLA-5, converting the rolling of $CD34^+$ cells into stable arrest on the endothelium [96].

1.6.2 Role of SDF-1−CXCR4 axis

The SDF-1−CXCR4 interaction and signaling is critical in the regulation of HSPC homing, engraftment and retention in the BM. Blockade of its receptor, CXCR4, was shown to inhibit HSPC homing and engraftment in NOD/SCID mice [97]. Overexpression of CXCR4 by CB and mPB $CD34^+$ cells leads to

increased SDF-1 induced in vitro migration and in vivo homing in a NOD/SCID mouse model [97,98]. Moreover, in vitro migration of $CD34^+$ cells towards SDF-1 has been shown to correlate with their in vivo homing potential in NOD/SCID mice [99]. We recently demonstrated that mPB $CD34^+$ cells that had higher responsiveness to SDF-1, had high CXCR4 expression, and could compensate for a lower $CD34^+$ cell dose in achieving faster hematopoietic engraftment after transplantation [100].

1.6.3 SDF-1−CXCR4 signaling and the regulation of HSPC homing

The receptor CXCR4 belongs to the group of seven transmembrane receptors that are linked to G proteins [101]. SDF-1 binding to CXCR4 activates phosphatidylinositol 3-kinase (PI3K), the phospholipase C-γ (PLC- γ)/protein kinase C (PKC) cascade and p44/42 mitogen activated protein kinase (MAPK) [102]. SDF-1−CXCR4 signaling also activates the atypical PKC subtype PKCζ which mediates cell polarization, adhesion, MMP-9 secretion and chemotaxis of $CD34^+$ cells [103]. Moreover, PKCζ phosphorylation and kinase activity has been shown to be dependent on PI3K, Pyk-2 and MAPK, and in vivo studies revealed that engraftment but not homing of human $CD34^+$ cells was dependent on PKCζ activity [103]. Interactions mediated by the Rho family GTPases (Rac, Rho and Cdc42) have also been implicated in HSPC homing and engraftment [104]. Following engraftment, deletion of Rac-1 and Rac-2 GTPases led to massive mobilization of HSPC from BM. Knocking out Rac-1 significantly reduced migration towards SDF-1 and attenuated the homing of murine HSC to the endosteum, which is essential for LT-HSC repopulation [105]. We previously

demonstrated that Rac-1 co-localization with CXCR4 in membrane lipid rafts of HSPC promotes their in vivo homing in a murine model [106]. Moreover, in vitro priming of HSPC by modulating the SDF-1−CXCR4 axis using supernatants of leukapheresis products (SLP) or their components such as C3a and platelet derived microvesicles (PMV) can speed up in vivo homing [106-108].

1.6.4 Role of proteases

Proteases regulate HSPC migration and tissue localization and have been shown to play important roles in HSPC homing [93]. MMPs degrade various ECM molecules and facilitate HSPC trans-migration across basement membrane barriers. SDF-1 and other growth factors induce the BM HSPC secretion of MMP-2 and MMP-9, thus facilitating in vitro chemotaxis of HSPC towards SDF-1 [76]. Incubation of HSPC with SCF induces MMP-2 and MMP-9 secretion and homing in NOD/SCID mice [109]. Inhibition of CD26 enhances in vitro migration of $CD34^+$ cells towards SDF-1 and their in vivo homing and engraftment in a murine model [110]. Previously, we demonstrated that MT1-MMP mediates migration of CB $CD34^+$ cells and megakaryocytic progenitors towards SDF-1 [111]. Recently, another group of investigators reported that homing of MT1-$MMP^{-/-}$ c-Kit^+ cells was lower than that of wild type cells in a chimeric mouse model and inhibition of MT1-MMP by monoclonal antibody attenuated homing of human HSPC in a NOD/SCID mouse model [112].

1.7 Matrix metalloproteinases

MMPs belong to a family of Zn^{2+}-binding, Ca^{2+}-dependent endopeptidases whose essential function is proteolysis of the ECM, a process that is required in

several cellular processes [113]. Currently, 25 MMPs have been identified in mammals that have structural similarities but vary in their expression profiles and substrates. MMPs are classified based on their substrate specificity into stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7, MMP-26), gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1, MMP-8, MMP-13 and MMP-14) [113]. Apart from ECM molecules, MMPs act on a whole array of substrates including other proteinases and MMPs, proteinase inhibitors, growth factors, cytokines, chemokines, cell surface receptors and cell adhesion molecules as listed in Table 1-2 [114-116]. Thus, MMPs regulate many processes such as cell migration, proliferation, apoptosis, angiogenesis, tumor expansion and metastasis [113,116].

MMPs' expression and functions are regulated at different levels. Generally expressed at low levels, they are upregulated during tissue remodeling, inflammation, wound healing, and cancer progression [113,116,117]. They are synthesized as latent enzymes that are either secreted or membrane-anchored known as MT-MMPs (Figure 1-6) [118-120]. Six MT-MMPs have been identified so far, of which four, MT1-/MMP-14, MT2-/MMP-15, MT3-/MMP-16, and MT5-/MMP-24, have a transmembrane domain while the other two, MT4/MMP-17 and MT6/MMP-25, have a glycosylphosphatidylinositol domain (Figure 1-6) [113,118-120]. Their membrane anchoring allows them to carry out pericellular proteolysis. MMPs are activated by the proteolytic release of the N-terminal propeptide domain. Once active, they can be inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs), the reversion-inducing cysteine-rich

protein with Kazal motifs (RECK) and tissue factor pathway inhibitor-2 as well as by plasma inhibitor (α2-macroglobulin) [113,118]. Therefore, a balance between MMPs and their inhibitors is important to ECM remodeling in the tissue.

Among the MMPs, the gelatinases MMP-2 and MMP-9 have been extensively studied in cancer and other diseases [121]. MMP-2 is secreted by fibroblasts, endothelial cells, epithelial cells and transformed cells whereas MMP-9 is secreted predominantly by leukocytes [121]. MMP-2 and MMP-9 play important roles in various physiological and pathological conditions. They are required for physiological ECM remodeling, during growth and development, inflammation, wound healing, angiogenensis, and leukocyte mobilization [113,118]. These MMPs are also involved in pathological processes such as cancer, inflammation, and neural and vascular degenerative diseases [113,116]. Although MMP-2 and MMP-9 are secreted by cells in the developing embryo, mice deficient in these gelatinases are viable. However, mice deficient in MMP-9 do exhibit minor developmental defects such as delayed vascularization and ossification resulting in moderate skeletal abnormalities while mice deficient in MMP-2 exhibit defects in developmental angiogenesis [121].

MMP-2 and MMP-9 are similar in many respects but differ in their glycosylation pattern, activation and substrate specificity. The 92 kDa MMP-9 has two glycosylation sites in the prodomain and the catalytic domain whereas the 72 kDa MMP-2 is a non-glycosylated protein [122]. MMP-2 activation is a cell surface event mediated by the formation of a ternary complex containing MT1-MMP, TIMP-2 and proMMP-2 (Figure 1-7) [123]. The N-terminal domain of

TIMP-2 binds and inhibits MT1-MMP whereas the C-terminal domain of TIMP-2 binds the hemopexin domain of proMMP-2. An adjacent MT1-MMP free of TIMP-2 subsequently activates proMMP-2 by cleaving its propeptide domain [123]. MMP-9 activation is mediated by a proteolytic cascade involving MMP-3, MMP-2 and MMP-13 [124]. MMP-3 is activated by plasmin generated from plasminogen by uPA bound to its receptor on the cell surface [125]. Similarly, MMP-2 and MT1-MMP both activate proMMP-13 which then activates proMMP-9 [126].

MMP-2 and MMP-9 share similar proteolytic activities and degrade a number of ECM molecules such as gelatin, collagens type IV, V, and XI, and laminin. In addition MMP-2 also degrades collagens type I, II, and III similar to collagenases. These gelatinases also cleave several non-ECM molecules such as SDF-1, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, plasminogen and uPA, among many others (Table 1-2) [113-116].

1.8 MT1-MMP

Since MT1-MMP was identified in the 1990s, extensive studies have provided evidence that it plays an important role in both physiological and pathophysiological processes. Mice deficient in MT1-MMP exhibit craniofacial dysmorphism, dwarfism, osteopenia, fibrosis of soft tissues, arthritis and premature death, emphasizing the important role of MT1-MMP in ECM degradation during development [127]. On the other hand, elevated MT1-MMP expression has been observed in a wide variety of cancers including lung, breast, cervical, brain, liver, head and neck, indicating MT1-MMP's role in tumor

progression and metastasis [119]. In addition, it has been implicated in angiogenesis, bone development, atherosclerosis, inflammation and wound healing [116].

MT1-MMP degrades several ECM macromolecules including collagens, laminins, fibronectin, aggregan and fibronectin, and activates proMMP-2 and proMMP-13 [113,116,128]. Among the cell surface adhesion molecules, MT1-MMP cleaves CD44, processes α_v integrin to its mature form, and degrades tissue transglutaminase (Table 1-2), thus modifying the immediate cell environment and affecting cellular functions in a variety of ways [123,128].

1.8.1 MT1-MMP structure

Like all MMPs, MT1-MMP has a common domain structure composed of a propeptide region, catalytic domain, hinge region, hemopexin-like domain and, in addition, a type I transmembrane domain with a cytoplasmic tail at the C-terminus which anchors it to the cell surface (Figure 1-6) [120]. MT1-MMP is also expressed in its latent form and cleavage of the propeptide region by furin or related protein convertases renders it active [128-130]. Activation of MT1-MMP takes place in the trans-Golgi network complex during secretion and the enzyme is expressed in its active form at the cell surface [129,130].

1.8.2 MT1-MMP regulation

Active MT1-MMP is inhibited by RECK, TIMP-2, TIMP-3 and TIMP-4 but not by TIMP-1. However, TIMP-2 has a dual role. On the one hand it inhibits MT1-MMP and on the other promotes activation of proMMP-2 [129,130]. At the

cell surface the 60 kDa active MT1-MMP molecule undergoes autodegradation or self-proteolysis by removal of its catalytic domain which results in an inactive 44 kDa species [131]. The proteolytic processing of MT1-MMP is considered to be an indicator of how active it is at the cell surface. A high level of truncated MT1-MMP coincides with high proMMP-2 activation. In some cells, the whole ectodomain has been shown to be shed [131].

In migrating tumor cells, MT1-MMP localizes predominantly in the lamellipodia through its interaction with CD44 [132]. It has been reported that this interaction and the consequent shedding of CD44 stimulates cell motility [133]. The hemopexin domain of MT1-MMP interacts with the stem region of CD44 which interacts with F-actin through its cytoplasmic domain mediated by the exrin/radixin/moesin (ERM) proteins. Binding of MT1-MMP with CD44 indirectly links the proteinase to the cytoskeleton and thereby enables its localization to the lamellipodia [134].

MT1-MMP has been shown to be internalized by either clathrin-dependent or caveolae-dependent pathways and, interestingly, this internalization process is essential for the enzyme to promote cell migration [134,135]. It has also been demonstrated that the internalized MT1-MMP is also recycled back to the surface [134]. Accumulating evidence suggests that this internalization and redistribution of MT1-MMP to sites of degradation such as the lamellipodia of endothelial cells and the invadipodia of tumor cells is highly complex and involves a dynamic interplay between endocytic and exocytic trafficking [136]. After its synthesis in the trans-Golgi network complex, MT1-MMP is translocated to the cell surface

rapidly via Rab8 and VAMP7 resulting in the polarized localization of the proteinase to invasive structures [137,138]. However, in most cell types surface expression of MT1-MMP is low due to rapid endocytosis resulting in intracellular accumulation of the proteinase in the early and late endosomes. This dynamic regulation suggests that the spatiotemporal recruitment of MT1-MMP to specialized domains plays a critical role in its invasive properties [139].

1.8.3 MT1-MMP signal transduction

The role of MT1-MMP in cell migration has been extensively studied in tumor and endothelial cells. Various growth factors, chemokines and inflammatory mediators have been reported to modulate MT1-MMP expression in different cell types. In the fibrosarcoma cell line HT1080, Rac-1 modulation of MT1-MMP and its processing to its 44 KDa form correlated with proMMP-2 activation [140]. Furthermore, Rac-1 has been demonstrated to promote hemophilic complex formation of MT1-MMP and recruitment to the lamellipodia-rich cell surface and subsequent proMMP-2 activation [141]. In Lewis lung carcinoma cell line, type 1 insulin-growth factor (IGF-1) increases invasiveness of these cells through increased MT1-MMP expression [142]. Furthermore, IGF-1 increases MT1-MMP synthesis through the PI3K/AKT/mTOR pathway but not through the MAPK/ERK1/2 pathway [142,143]. The SDF-1−CXCR4 axis promotes melanoma cell invasion and metastasis by upregulating MT1-MMP through Rac-1 and RhoA-GTPases [144]. Moreover, through activation of Rac-1 and its downstream effector ERK1/2, SDF-1 upregulates MT1-MMP intracellularly; however, when these cells were in

contact with Matrigel, a PI3K-dependent transient redistribution of MT1-MMP to the cell surface was observed [145].

In endothelial cells, VEGF mediated upregulation of MT1-MMP at the mRNA level occurs through the MAPK/JNK pathway whereas protein expression is regulated by PI3K [146,147]. MT1-MMP clustering on the cell surface is dependent on cortical actin polymerization which is regulated by PI3K, and this clustering of MT1-MMP has been suggested to be more important than its internalization [135]. In MSC, we reported that MT1-MMP mediates chemotactic migration of MSC towards SDF-1 and HGF [148]. Increased chemoinvasion of MSC through upregulation of MT1-MMP by cytokines TGF-β, TNF-α and IL-1 was also reported [149]. In hematopoietic cells, MT1-MMP has been demonstrated to mediate trans-endothelial migration of monocytes [150]. Interaction of monocytes with fibronectin, and endothelial ligands such as VCAM-1 and intracellular cell adhesion molecule (ICAM)-1, increased MT1-MMP clustering and localization into membrane protrusions or lamellipodia.

1.9 References

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Table 1-1. Diseases commonly treated with hematopoietic stem cell transplantation [23].

Autologous transplantation	**Allogeneic transplantation**
Cancers Multiple myeloma Non-Hodgkin's lymphoma Hodgkin's disease Acute myeloid leukemia Neuroblastoma Ovarian cancer Germ-cell tumors	**Cancers** Acute myeloid leukemia Acute lymphoblastic leukemia Chronic myeloid leukemia Myelodysplastic syndromes Myeloproliferative disorders Non-Hodgkin's lymphoma Hodgkin's disease Chronic lymphoblastic leukemia Multiple myeloma Juvenile chronic myeloid leukemia
Other diseases Autoimmune disorders Amyloidosis	**Other diseases** Aplastic anemia Paroxysmal nocturnal hemoglobinuria Fanconi's anemia Blackfan-Diamond anemia Thalassemia major Sickle cell anemia Severe combined immunodeficiency Wiscott-Aldrich syndrome Inborn errors of metabolism

Table 1-2. MMPs and their substrates [114-116].

MMP	Alternative Name	ECM Substrates	Non-ECM Substrates
MMP-1	Interstitial Collagenase-1	Collagen (II, III, VII, VIII, X), gelatin, aggregan, casein, nidogen, serpins, perclan, tenascin-C	α_1-antichymotrypsin, α_1-antitrypsin, IGFBP-3, IGFBP-5, IL-1β, L-selectin, proTNF-α, SDF-1, IL-8, MCP-1, MCP-3
MMP-8	Collagenase-2	Collagen (I, II, III, V, VII, X), gelatin, aggregan, laminin, nidogen	α_1-antiplasmin, proMMP-8, ITAC
MMP-13	Collagenase-3	Collagen (I, II, III, IV,V, IX, XI), gelatin, aggregan, fibronectin, laminin, perlecan, tenascin	Plasminogen activator 2, proMMP-9, proMMP-13, SDF-1, IL-8, MCP-3
MMP-3	Stromelysin-1	Collagen (II, IV, IX, X), gelatin, aggregan, casein, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan	α_1-antichymotrypsin, antithrombin III, E-cadherin, fibrinogen, IGF-BP3, L-selectin, proHB-EGF, proTNF-α, SDF-1, MCP-1, MCP-3
MMP-10	Stromelysin-2	Collagen (III, IV, V), gelatin, fibronectin, laminin, nidogen	proMMP-1, proMMP-8, proMMP-10
MMP-11	Stromelysin-3	Laminin	α_1-antitrypsin, α_1-proteinase inhibitor, IGF BP1
MMP-2	Gelatinase-A	Collagen (I, IV, V, VII, X, XI, XIV), gelatin, aggregan, elastin, fibronectin, laminin, nidogen	FGF RI, IGF-BP3, IGF-BP5, IL-1β, proTNF-α, TGF-β, SDF-1, uPA
MMP-9	Gelatinase-B	Collagen (IV, V, VII, X, XI, XIV), gelatin, aggrecan, cleastin, fibronectin, laminin, nidogen, proteoglycan link protein	CXCL5, IL-1β, IL-2R, uPA, plasminogen, proTNF-α, SDF-1, TGF-β, ITAC
MMP-7	Matrilysin-1, neutrophil collagenase	Collagen (I, II, III, IV, V, X), Aggregan, casein, elastin, enactin, laminin	β_4 integrin, decorin, E-cadherin, Fas-L, plasminogen, proMMP-2, proMMP-7, proTNF-α, transferring, syndecan, ITAC
MMP-12	Macrophage elastase	Elastin	Plasminogen, Gro-β, IL-8, ITAC, MCP-3
MMP-19	RASI-1	Collagen (I, IV), gelatin, aggrecan, casein, fibronectin, laminin, nidogen, tenascin	
MMP-20	Enamelysin	Aggregan, amelogenin	

Table 1-2. MMPs and their substrates continued [114-116].

MMP	Alternative Name	ECM Substrates	Non-ECM Substrates
MMP-14	MT1-MMP	Collagen (I, II, III, VIII), gelatin, aggrecan, dermatan sulfate, proteoglycan, fibrinogen, fibrin, fibronectin, nidogen, laminin, perlecan, tenascin, vitronectin	$\alpha_v\beta_3$ integrin, syndecan, CD44, proMP-2, proMMP-13, proTNF-α, SDF-1, IL-8, MCP-3, transglutaminase, HB-EGF, apolipoprotein, factor XII, MCP-3
MMP-15	MT2-MMP	Collagen (I, II, III), gelatin, aggrecan, fibronectin, laminin, nidogen, perlecan, tenascin, vitronectin	proMMP-2, proMMP-13
MMP-16	MT3-MMP	Collagen (I, III), gelatin, aggrecan, casein, fibronectin, laminin, perlecan, vitronectin	
MMP-17	MT4-MMP	Gelatin, fibrin, fibronectin,	
MMP-24	MT5-MMP	Gelatin, chondrotin sulfate, dermatin sulfate, fibronectin	proMMP-2, proMMP-13
MMP-25	MT6-MMP	Collagen (IV), gelatin	Fibrin, fibronectin, proMMP-2

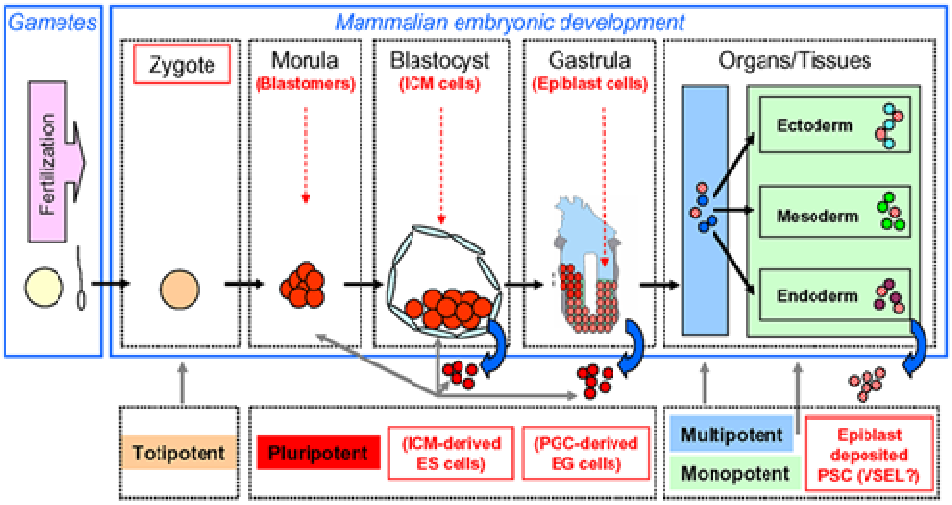

Figure 1-1. Developmental hierarchy of the stem cell compartment. The most primitive stem cell is a totipotent zygote or the first blastomere that derives from the first division of the zygote. The totipotent stem cell divides to form both embryo and placenta. Pluripotent stem cells derived from the inner cell mass of a blastocyst give rise to an established embryonic stem cell line. Adult or somatic stem cells can be multipotent or monopotent stem cells which are already committed to particular organs/tissues [1].

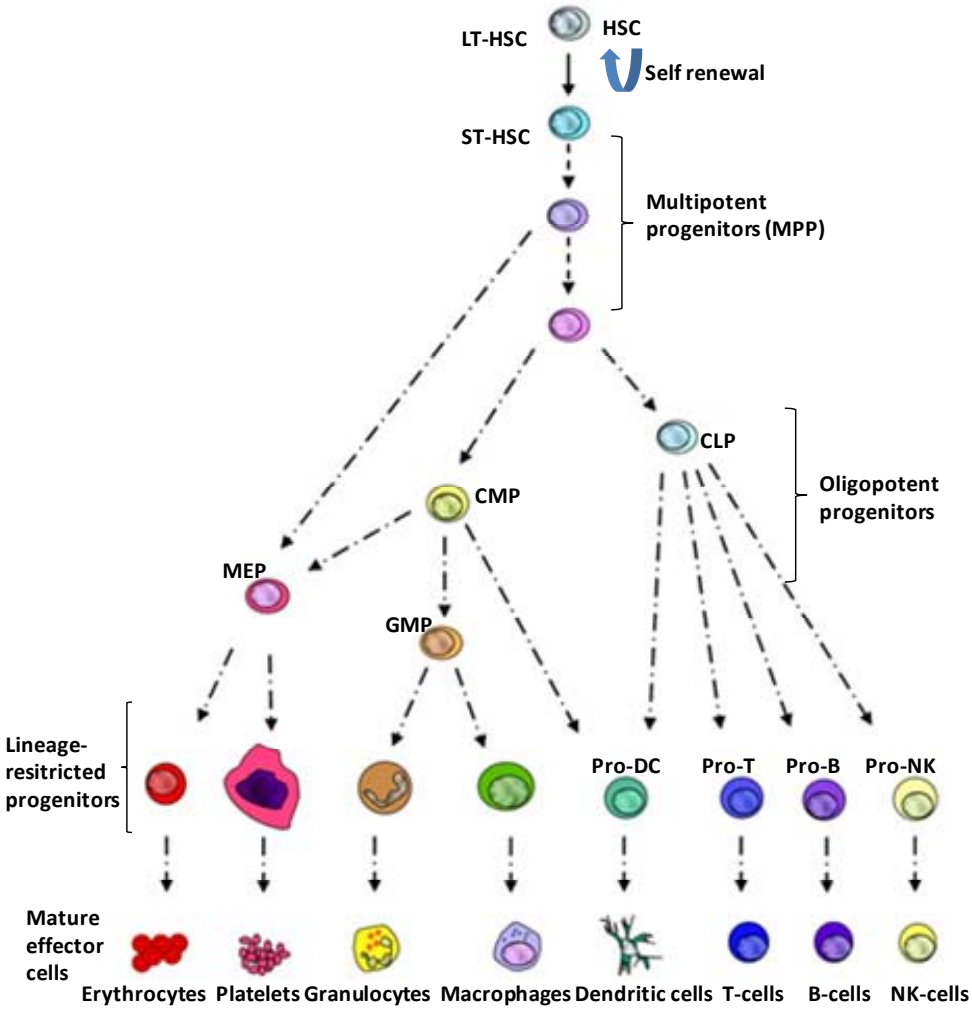

Figure 1-2. Model of the hematopoietic developmental hierarchy. Self-renewing HSC reside at the top of the hierarchy and give rise to a number of multipotent progenitors. Multipotent progenitors give rise to oligopotent progenitors including the common lymphoid progenitor (CLP), which gives rise to mature B lymphocytes, T lymphocytes, and natural killer (NK) cells. The common myeloid progenitor (CMP) gives rise to granulocyte-macrophage progenitors which differentiate into monocytes/macrophages and granulocytes, and megakaryocyte/erythrocyte progenitors which differentiate into megakaryocytes/platelets and erythrocytes. Both CMP and CLP have been proposed to give rise to dendritic cells (modified from [5]).

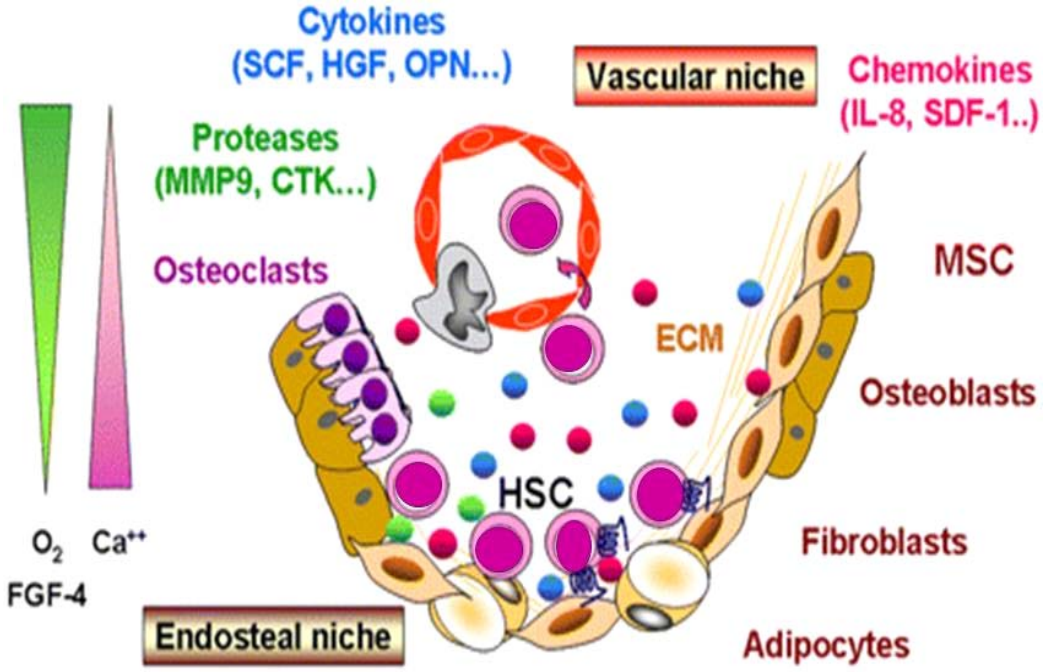

Figure 1-3. Model of HSC niches. HSC are engaged in a constant crosstalk within specific niches at the proximity of the endosteal surface (osteoblastic niche) and of the perivascular space (vascular niche). Regulatory signals emanating from these different niches include cell-intrinsic regulatory mechanisms, adhesion molecule interactions, ECM, and environmental components: calcium (Ca^{++}), oxygen (O_2) concentration; proteases: MMP-9, cathepsin K (CTK); humoral factors including cytokines: SCF, HGF, OPN and chemokines: IL-8, SDF-1 (modified from [41])**.**

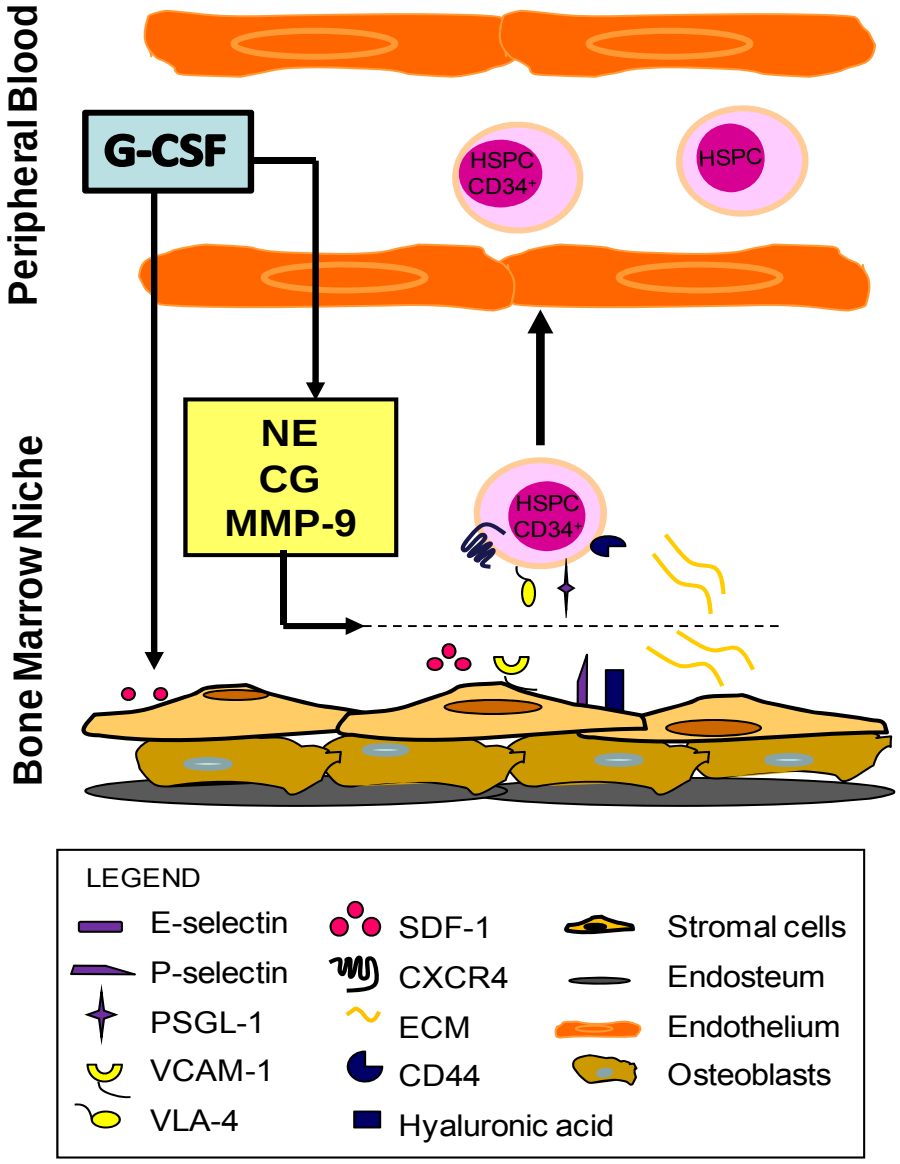

Figure 1-4. Schematic representation of HSPC mobilization. During steady-state HSPC adhere to BM stroma through interactions between adhesion molecules on HSPC and their cognate ligands expressed on BM stroma and osteoblasts. G-CSF: (A) through neutrophil activation, induces the release of proteases (CG, NE, MMP-9) resulting in proteolytic cleavage of key adhesion molecules; and (B) decreases SDF-1 mRNA through suppression of SDF-1-producing osteoblasts leading to reduced retention of HSPC in the BM niche. (Modified from [27]).

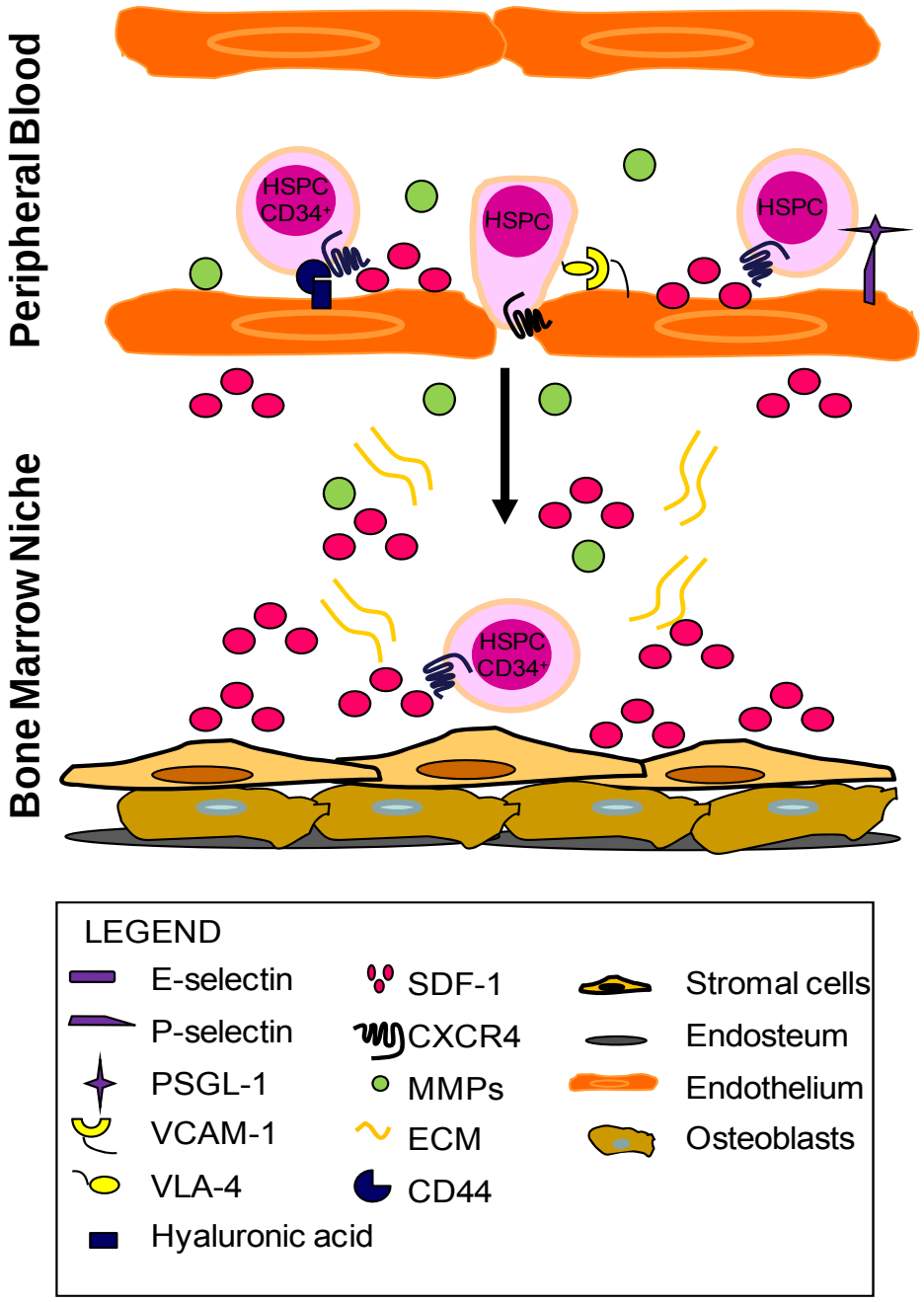

Figure 1-5. Schematic representation of HSPC homing. After intravenous infusion HSPC enter the BM sinusoidal vessels. Cell adhesion molecules on the HSPC cell surface bind to their respective ligands on the endothelial surface, allowing rolling and firm adhesion. Extravasation and trans-migration through the sinusoidal endothelial cells towards the SDF-1 gradient in the BM is facilitated by MMPs (Modified from [94]).

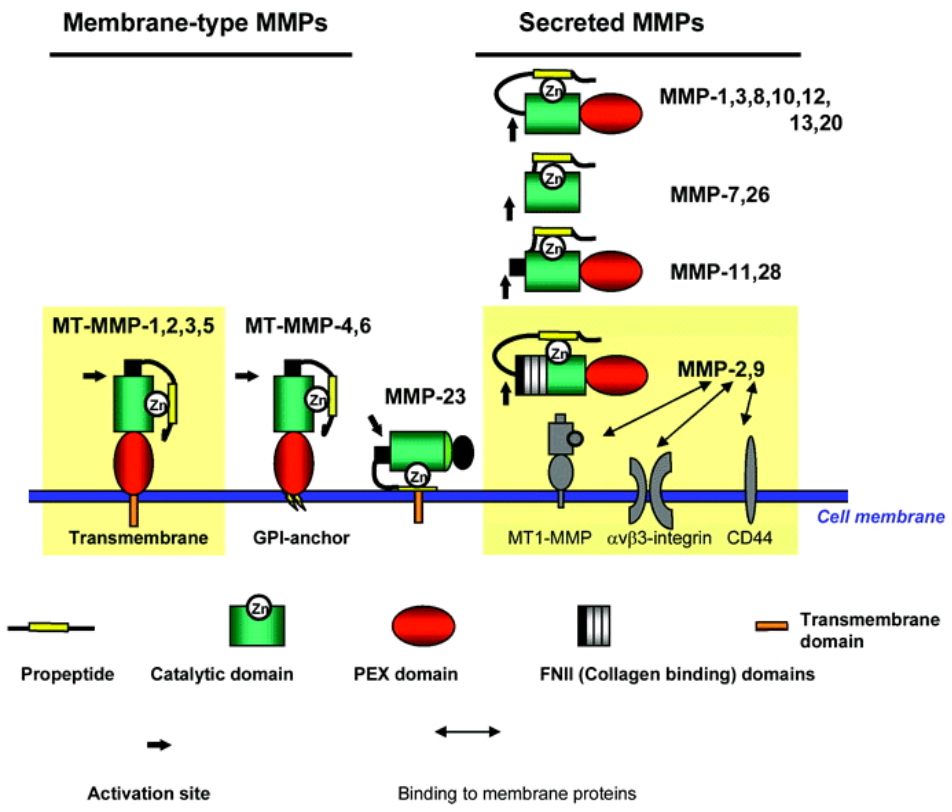

Figure 1-6. Structure of MMPs. MMPs have a specific structure bearing several homologous domains such as the propeptide domain; catalytic domain; and hemopexin domain. MT1-, 2-, 3-, and 5-MMPs have a transmembrane domain and MT4-, and 6-MMPs have a GPI domain that anchors them to the cell surface [120].

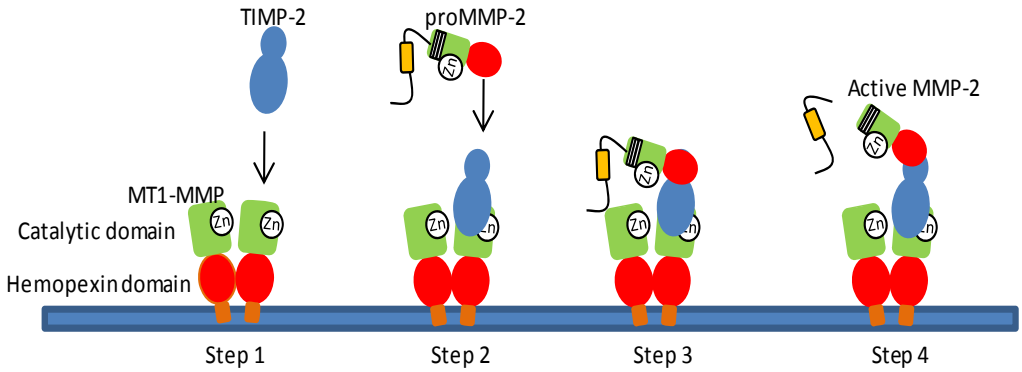

Figure 1-7. Activation of proMMP-2. MT1-MMP forms a homodimer complex through the hemopexin domain (Step 1). One of the MT1-MMPs through its catalytic site binds to TIMP-2 at its inhibitory site (Step 2). ProMMP-2 through its hemopexin domain then binds to the C-terminal domain of TIMP-2 thus forming a ternery complex (Step 3). The TIMP-2-free MT1-MMP then cleaves the propeptide domain of proMMP-2 and renders it active (Step 4) (Modified from [123]).

Chapter 2 Aims, hypotheses and experimental design

Aims

The aim of this thesis was to investigate the role of MT1-MMP in HSPC trafficking to gain more insights into the mechanism of HSPC mobilization and homing. This could have future implications on the design of novel therapeutic strategies improving HSPC transplantation outcomes.

Hypotheses

1. MT1-MMP mediates migration of HSPC during G-CSF-mobilization.
2. MT1-MMP mediates hyaluronic acid- and thrombin-primed homing-related responses of HSPC towards SDF-1.

Experimental design

1. Evaluate MT1-MMP expression in HSPC at the mRNA and protein level using RT-PCR, flow cytometry and Westen blotting.
2. Determine the role of MT1-MMP in migration of HSPC, using the in vitro trans-Matrigel migration assay. MT1-MMP is inhibited by siRNA or other inhibitors, and migration across reconstituted basement membranes (Matrigel) is conducted in modified Boyden chambers.
3. Determine the role of MT1-MMP in the activation proMMP-2, using $CD34^+$ cells co-cultured with BM stromal cells to simulate the bone marrow microenvironment in vitro. The media conditioned by these co-

cultures are evaluated by zymography to determine the pro and active forms of MMP-2.

4. Determine the subcellular localization of MT1-MMP during cell migration, using confocal microcsopy to assess co-localization of MT1-MMP at the cell surface with F-actin.

5. Determine the intracellular signaling pathways regulating MT1-MMP expression and function in HSPC during migration, using Western blotting to evaluate PI3K, MAPK and Rac-1 GTPase signaling pathaways.

Chapter 3 MT1-MMP association with membrane lipid rafts facilitates G-CSF-induced hematopoietic stem/progenitor cell mobilization[1]

3.1 Abstract

Soluble matrix metalloproteinases (MMPs) are known to facilitate egress of hematopoietic stem/progenitor cells (HSPC) from the bone marrow (BM) during granulocyte-colony stimulating factor (G-CSF)-induced mobilization. Membrane-type (MT)1-MMP, which is localized on the leading edge of migrating cells and activates the latent forms of soluble MMPs, also influences this process through mechanisms that are poorly understood. Here we demonstrate that mobilizing agent G-CSF increases: (i) MT1-MMP transcription and protein synthesis in hematopoietic cells; (ii) proMMP-2 activation in co-cultures of HSPC with BM fibroblasts; (iii) chemoinvasion across reconstituted basement membrane Matrigel towards an SDF-1 gradient which is reduced by siRNA silencing of MT1-MMP; and (iv) localization of MT1-MMP to membrane lipid rafts through a mechanism that is regulated by the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Moreover, disruption of raft formation (by the cholesterol sequestering agent methyl-β-cyclodextrin) abrogates PI3K phosphorylation and MT1-MMP incorporation into lipid rafts resulting in reduced proMMP-2 activation and HSPC migration. In conclusion, G-CSF-induced upregulation of MT1-MMP in hematopoietic cells and its incorporation into membrane lipid rafts contributes to the activation of proMMP-2 and to the generation of a highly proteolytic microenvironment in BM, which facilitates egress of HSPC into circulation.

[1]A version of this chapter has been submitted to Experimental Hematology 2010

3.2 Introduction

Mobilized peripheral blood (mPB) stem cells are routinely collected for transplantation because of their faster engraftment and greater convenience compared to bone marrow (BM) harvests [1]; however, molecular mechanisms governing hematopoietic stem/progenitor cell (HSPC) mobilization are not completely understood [2]. Some key molecules and interactions that regulate mobilization have been identified including the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, adhesion molecules (VCAM-1, CD44), and proteases (neutrophil elastase, cathepsin G, matrix metalloproteinases (MMPs)), among others [3-6]. Previously we suggested that soluble MMP-2 and MMP-9 play a role in the release of HSPC from the BM into circulation [7]. However, mice deficient in MMP-9 were found to have unimpaired mobilization, suggesting that other proteases may be involved [8].

MMPs belong to a family of Zn^{2+}-binding, Ca^{2+}-dependent endopeptidases whose substrates include extracellular matrix (ECM) molecules, adhesion molecules, growth factors, chemokines and cytokines [9,10]. Soluble MMPs are secreted as proenzymes and are activated by membrane-type (MT)-MMPs that are anchored on the cell surface [9]. MT1-MMP is the best characterized among all the MT-MMPs and is known to activate proMMP-2. Cell surface MT1-MMP forms a ternary complex with tissue-inhibitor of matrix metalloproteinase (TIMP)-2 and proMMP-2; another MT1-MMP molecule, free of TIMP-2, then activates pro-MMP-2 [11]. MT1-MMP degrades ECM macromolecules (collagens, gelatin, laminins, fibronectin); cytokines and chemokines (TNF-α,

SDF-1); and adhesion molecules (CD44, integrin $\alpha_v\beta_3$) in the pericellular space [14,15] and mediates tumor cell migration, metastasis and angiogenesis [11-13]. We previously showed that mesenchymal stem cells, $CD34^+$ cells and megakaryocytic progenitors express MT1-MMP which mediates their migration [16-18] and others have found MT1-MMP to mediate migration of endothelial cells and monocytes [19,20].

During cell migration, MT1-MMP interacts with CD44, which in turn associates with F-actin, interactions that are critical for their localization in the lamellipodia at the migration front of cells [21,22]. MT1-MMP undergoes endocytosis through association with clathrin-coated pits and with cholesterol-enriched cell membrane microdomains called lipid rafts or caveolae [23]. In endothelial and tumor cells, MT1-MMP is enriched in caveolar domains where it associates with caveolin-1 and promotes cell migration, suggesting that the caveolar association of MT1-MMP regulates its function at the cell surface [24-26].

In hematopoietic cell lines, in which caveolae have not been detected, signal transduction occurs in non-caveolar cholesterol-rich lipid rafts that function as platforms for the assembly of multi-component signal transduction complexes, influencing cytoskeletal re-organization as well as protein and lipid trafficking [27-29]. Lipid rafts play an important role in hematopoietic cell proliferation [30] and, T- and B-lymphocyte activation and signaling [31,32]. Recently we showed that during HSPC migration SDF-1–CXCR4 signaling takes place within lipid rafts where CXCR4 and Rac-1 assemble allowing an optimal chemotactic

response to SDF-1 and, subsequently, homing [33]. However, the role of membrane-bound proteases in the lipid rafts of hematopoietic cells, especially HSPC, during their trafficking has not been established.

Here we focus on characterizing the role of MT1-MMP and its regulation and subcellular localization in HSPC during mobilization. We hypothesize that G-CSF increases expression of MT1-MMP in the lipid raft domains leading to increased ECM degradation, activation of secreted proMMP-2 in the BM microenvironment and enhanced cell egress from the BM.

3.3 Material and methods

3.3.1 Cells and cultures

Samples from unrelated BM donors, normal volunteers and G-CSF-mobilized patients (diagnosed with malignancies without BM involvement) were collected with donors' informed consent and in accordance with the guidelines approved by the University of Alberta Health Research Ethics Board. Briefly, mononuclear cells (MNC) from BM and mPB samples were separated using a 60% Percoll gradient (GE Healthcare, Baie D'Urfe, PQ) and enriched for $CD34^+$ cells using the Miltenyi MACS Technology (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions and as described previously [7]. The purity of the isolated $CD34^+$ cells was greater than 95%, as determined by fluorescence activated cell sorter (FACS) analysis. Polymorphonuclear cells (PMN) were isolated by centrifugation using Lympholyte-poly (Cedarlane, Burlington, ON). BM leukocytes were prepared by lysing red blood cells with lysis buffer (150 mM NH_4Cl, 1 mM EDTA, 10 mM $NaHCO_3$, all from Sigma, St Louis, MO) for 10

min at room temperature and stimulated or not (control) with 100 ng/ml G-CSF (R&D Systems, Minneapolis, MN) in RMPI 1640 (Invitrogen, Burlington, ON) supplemented with 5% bovine growth serum (BGS, Hyclone, ThermoFisher Scientific, Nepean, ON) for 48 h at 37ºC.

BM colony forming unit-fibroblast (CFU-F) cultures were established from normal BM buffy coat cells as described [34]. Human umbilical vein endothelial cells (HUVEC, a gift from Dr. Allan Murray, Department of Medicine) were isolated from several umbilical cords, pooled, and cultured on gelatin-coated flasks in media consisting of M199, 10 mM L-glutamine, 250 IU/ml penicillin-streptomycin, 20% FCS (all from Invitrogen) and endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA). CFU-F and HUVEC were grown to sub-confluence, trypsinized and washed in serum-free IMDM; 1 x 10^5 cells/ml were plated in 24-well cell culture plates. For co-culture experiments, BM or mPB $CD34^+$ cells (suspended in serum-free IMDM at 2 x 10^6 cells/ml) were seeded onto the stromal monolayers and were either unstimulated (control) or stimulated with 100 ng/ml G-CSF. In some experiments, co-cultures of normal (n)PB MNC with BM stromal cells were preincubated with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY290042 (30 μM) or with the p44/p42 mitogen activated protein kinase (MAPK) inhibitor PD98059 (50 μM) (both from Calbiochem, La Jolla, CA) for 1 h before adding G-CSF. Supernatants were obtained after 48 h incubation at 37ºC in 5% CO_2 and analyzed by zymography. The myelomonocytic THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD), grown in RPMI

media supplemented with 10% BGS and used for phosphorylation experiments (see below).

3.3.2 RT-PCR analysis

Expression of MT1-MMP mRNA was evaluated using RT-PCR in nPB, mPB, BM leukocytes, MNC and $CD34^+$ cells. Cell pellets were used for isolation of total RNA which was performed using TRIZOL (Invitrogen) according to the manufacturer's instructions. Concentrations were determined by measuring the absorbance at 260 nm in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). PCR reactions were carried out in an Eppendorf Mastercycler (Westbury, NY) as described [16]. Sequences of MT1-MMP and GAPDH were obtained from GenBank (Los Alamos, NM) and used to design primer pairs. PCR products were run on 2% agarose gels containing 100 μg/ml ethidium bromide (Sigma), visualized under UV light and photographed using the FluorChem Imaging System (Alpha Innotech, San Leandro, CA). Semi-quantitative evaluation by densitometric analysis of the bands in each sample was carried out using AlphaEaseFC Software (Alpha Innotech). The relative level of target mRNA was regarded as the ratio between the intensities of the target primer and the GAPDH bands.

3.3.3 FACS analysis

Normal steady-state BM leukocytes stimulated with G-CSF or not (control), BM and mPB $CD34^+$ cells and nPB MNC were stained with rabbit anti-MT1-MMP (Millipore, Billerica, MA) for 45 min at 4^oC followed by goat anti-rabbit IgG-555

(Molecular Probes/Invitrogen) as the secondary antibody. The cells were then fixed in 1% paraformaldehyde and analyzed by the FACS Calibur flow cytometer (Becton Dickinson, San José, CA) and FCS Express V3 (DeNOVO software, Los Angeles, CA).

3.3.4 Zymography

In order to evaluate MMP-2 and MMP-9 activities, zymography assays were performed using cell-conditioned media as described previously [7]. Briefly, cells were incubated in serum-free IMDM at a concentration of 2 x 10^6 cells/ml for 48 h (37^{o}C, 5% CO_2); the conditioned media were collected after centrifugation and applied onto a 12% SDS-PAGE gel containing 1.5 mg/ml gelatin (Sigma). Clear bands at 92 kDa and 72 kDa against a Coomassie blue background indicated the presence of latent forms of MMP-9 and MMP-2, respectively. The intensity of the bands was analyzed by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

3.3.5 Western blotting

For MT1-MMP total protein analysis, the cells were lysed for 30 min in lysing buffer (1% Triton, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, all from Sigma) containing protease inhibitors (1 mM PMSF and 2 mM protease inhibitor cocktail, Sigma), followed by sonication and centrifugation at 14000 rpm for 10 min at 4^{o}C. The protein concentrations in lysates were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The cell lysate was separated under denaturing conditions in a 10% polyacrylamide gel

and transferred to a nitrocellulose membrane (BioRad), followed by blocking with 5% fat-free dried milk in Tris-buffered saline and 0.05% Tween 20. MT1-MMP was detected with a rabbit anti-MT1-MMP antibody (Abcam, Cambridge, MA) and a secondary antibody (goat anti-rabbit, HRP-conjugated IgG, Pierce Biotechnology, Rockford, IL). Chemiluminescence detection was performed using the Super Signal West Pico system (Pierce Biotechnology) and exposed on X-Ray film. The intensities of the bands were analyzed (using Commassie blue as loading control as described [35]) by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

3.3.6 SiRNA electroporation

SiRNA was targeted against 21-nucleotide sequences of MT1-MMP (Dharmacon, Lafayette, CO) as described [36]. A control siRNA sequence was generated from a scrambled MT1-MMP sequence. MNC and $CD34^+$ cells (5×10^6) were electroporated with siRNA oligonucleotides (5 μg) using a nucleofector kit (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions, and incubated for 24 h in RPMI + 10 % FCS at 37^oC and 5% CO_2. Viability of the transfected cells, determined by the trypan blue exclusion test was > 80%. The efficiency of MT1-MMP knockdown was determined by RT-PCR.

3.3.7 Trans-Matrigel migration assay

Cell migration was determined using the trans-Matrigel migration assay as described by us [7]. Briefly polycarbonate filters were coated with Matrigel (BD, Franklin Lakes, NJ). The lower compartments of Boyden chambers (Neuro Probe

Inc, Gaithersburg, MD) were filled with serum-free media with (or without) SDF-1α (100 ng/ml). $CD34^+$ cells pre-incubated with or without G-CSF (100 ng/ml) for 2 h were loaded in the upper chambers (2 x 10^5 cells/chamber) and incubated (37^oC and 5% CO_2) for 3 h. Percentage migration was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded. In some experiments mPB MNC or $CD34^+$ cells were pre-incubated with 50 μM (-)-epigallocatechin 3-gallate (EGCG, Sigma) or 10 μg/ml MT1-MMP mouse monoclonal antibody (Millipore); LY290042 (30 μM) or PD98059 (50 μM) for 1 h, before G-CSF treatment.

3.3.8 Isolation of lipid rafts

Lipid rafts were isolated by centrifugation on a sucrose density gradient as we described previously [37]. Briefly, 50 x 10^6 mPB MNC were kept in RPMI + 0.5% BSA overnight at 37^oC and 5% CO_2 to render the cells quiescent. Cells were then stimulated with 100 ng/ml G-CSF for 2 h. In some experiments, cells were treated with 10 mM methyl-β-cyclodextrin (MβCD, Sigma) for 2 h at 37^oC and 5% CO_2. Cell pellets were then washed with PBS and lysed in 450 μl buffer containing 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors (1 mM PMSF and 2 mM protease inhibitor cocktail) for 90 min at 4^oC. The lysate was subjected to Dounce homogenization using a loose-fitting pestle and clarified by centrifugation at 2000 rpm for 5 min. The supernatant was adjusted to 40% sucrose and overlaid with 30% and 5% sucrose. Buoyant-density centrifugation was performed at 45,000 rpm for 18 h at 4^oC in a

Beckman SW60Ti rotor. Eight fractions were collected from the top of the gradient. Proteins from fractions 2-8 were precipitated and analyzed by Western blotting as described above. Lipid raft fractions were detected using rabbit anti-flotillin-1 antibody (Abcam) and goat anti-rabbit IgG-HRP (Pierce) as secondary antibody. The blots were then stripped and reprobed with rabbit anti-MT1-MMP (Abcam).

3.3.9 Confocal analysis

BM $CD34^+$ cells or nPB MNC stimulated with G-CSF or not (control) were plated on coverslips coated with fibronectin or poly-L-lysine (Sigma) for 2 h, fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked with 1% BSA in PBS and incubated with the primary antibodies polyclonal rabbit or monoclonal mouse anti-MT1-MMP antibody. Goat anti-rabbit AlexaFluor-555 IgG or goat anti-mouse AlexaFluor-488 (Molecular Probes) were used as secondary antibodies. F-actin was detected using AlexaFluor 564-phalloidin (Molecular Probes). For visualization of lipid rafts the cells were stained with GM1-CTX-488 (Molecular Probes). Finally they were mounted in ProLong anti-fade reagent (Invitrogen) and examined using an LSM510 laser scanning confocal microscope (Carl Zeiss, Germany).

3.3.10 Phosphorylation of intracellular pathway proteins

Phosphorylation of intracellular kinases was detected by Western blot analysis as described previously [38]. THP-1 cells were kept in RPMI + 0.5% BSA overnight at 37^oC and 5% CO_2 to render the cells quiescent. Cells were then stimulated with

100 ng/ml G-CSF for 0, 2 and 5 min at 37°C and lysed as described above. Phosphorylation of 44/42 MAPK and AKT proteins was detected by protein immunoblotting using rabbit anti-Phospho-p44/42 MAPK and rabbit anti-Phospho-AKT antibodies (Cell Signaling Technology, Danvers, MA) followed by goat anti-rabbit, HRP-conjugated IgG secondary antibody. The blots were then stripped and reprobed with total rabbit anti-p44/42 MAPK and total rabbit anti-AKT (Cell Signaling Technology) to ensure equal loading.

3.3.11 Statistical analysis

Arithmetic means and standard deviations were calculated and statistical significance was defined as $p < 0.05$ using Student's *t* test.

3.4 Results

3.4.1 Normal steady-state BM leukocytes and CD34$^+$ cells express MT1-MMP at a low level and G-CSF upregulates it

First we examined and compared the expression of MT1-MMP in different hematopoietic cell populations from steady-state (ss) BM and mobilized (m) PB. Using RT-PCR we found that ss BM leukocytes, MNC, and CD34$^+$ cells express MT1-MMP mRNA which was significantly lower ($p < 0.05$) than their G-CSF mobilized counterparts isolated from leukapheresis products (Figure 3-1A) suggesting that G-CSF stimulates MT1-MMP transcription. Consistent with this we observed that MT1-MMP protein expression is higher in mPB leukocytes and CD34$^+$ cells than in ssBM cells and PB leukocytes (Figure 3-1B). Furthermore, MT1-MMP was expressed on 67.75% ± 17.02 of mPB monocytes and 87.5% ±

4.51 of mPMN, which is significantly higher ($p < 0.005$) than non-mobilized nPB monocytes (5.5% ± 0.5) and nPMN (10.9% ± 2.1) (Figure 3-1C).

To confirm that G-CSF enhanced MT1-MMP expression, ssBM leukocytes were incubated with 100 ng/ml G-CSF for 48 h. Both mRNA and surface MT1-MMP levels increased as determined by RT-PCR (up to two-fold) and flow cytometry analysis (Figures 3-2A and 3-2B). Moreover, Western blot analysis revealed that G-CSF stimulation significantly increases ($p < 0.05$) MT1-MMP protein expression on ssBM MNC, ssBM and mPB $CD34^+$ cells (Figure 3-2C).

3.4.2 MT1-MMP activates proMMP-2 in co-cultures of G-CSF-stimulated BM $CD34^+$ cells with stroma and mediates migration of $CD34^+$ cells and MNC across Matrigel

To simulate cell interactions occurring in a BM environment we co-cultured mPB MNC or $CD34^+$ cells with BM fibroblasts. Using zymography we found that active MMP-2 is released into the media of these co-cultures (Figure 3-3A). Interestingly, active MMP-2 although not detected when ssBM $CD34^+$ cells were similarly co-cultured, was detected only after G-CSF stimulation (Figure 3-3A). Treatment of this cell-conditioned media with the potent MT1-MMP inhibitor EGCG resulted in inhibition of proMMP-2 activation in a dose-dependent manner (Figure 3-3B). Moreover, G-CSF increased the trans-Matrigel migration of $CD34^+$ cells towards an SDF-1 gradient up to two-fold (Figuer 3-3C) and specific anti-MT1-MMP monoclonal antibody or EGCG inhibited it by 40% and 52%, respectively ($p < 0.05$) (Figure 3-3C).

Next we used siRNA to silence the gene expression of MT1-MMP in mPB MNC and $CD34^+$ cells. In these transfected cells expression of MT1-MMP was 50% compared to that in cells transfected with scrambled siRNA (control) and in non-transfected cells (Figure 3-3D). We found that in supernatants obtained from co-cultures of HUVEC with mPB MNC or mPB $CD34^+$ cells transfected with MT1-MMP siRNA, active MMP-2 was attenuated, indicating that high expression of MT1-MMP on hematopoietic cells is necessary for activation of proMMP-2 (Figure 3-3E). Moreover, we found a 60% reduction in the trans-Matrigel migration index of the MT1-MMP siRNA-transfected mPB MNC and mPB $CD34^+$ cells compared to controls (Figure 3-3F).

3.4.3 G-CSF increases MT1-MMP incorporation into lipid rafts

We recently reported that incorporation of CXCR4 into lipid rafts is required for trafficking of HSPC [33]. To determine whether MT1-MMP is present in lipid rafts we isolated low-density detergent-insoluble membranes from mPB MNC by discontinuous sucrose gradient fractionation and found that MT1-MMP was localized in fractions containing the lipid raft marker flotillin-1 (Figure 3-4A). Stimulation of mPB MNC with G-CSF increased MT1-MMP incorporation into these fractions; furthermore, when mPB MNC cells were treated with MβCD, which destabilizes lipid raft formation, MT1-MMP incorporation into lipid rafts did not occur (Figure 3-4B). Using confocal microscopy we confirmed that MT1-MMP co-localized with the lipid raft marker ganglioside GM1 in BM $CD34^+$ cells stimulated in vitro with G-CSF (Figure 3-4C) and in G-CSF-moblized PB $CD34^+$ cells and MNC (Figure 3-4C). Treatment

with MβCD reduced trans-Matrigel migration of mPB MNC and $CD34^+$ cells towards SDF-1 (Figure 3-4D), suggesting that MT1-MMP localization in lipid rafts is necessary for proteolytic degradation of the reconstituted basement membrane. Moreover, treatment with MβCD of co-cultures of BM $CD34^+$ cells with BM fibroblasts reduced activation of proMMP-2 (Figure 3-4E), indicating that MT1-MMP incorporation into lipid rafts is required for proMMP-2 activation.

3.4.4 G-CSF-induced MT1-MMP upregulation in lipid rafts of hematopoietic cells is PI3K-dependent

Next we examined intracellular signaling pathways activated by G-CSF and found that, G-CSF induced phosphorylation of AKT and p44/42 MAPK in myelomonocytic THP-1 cells (Figures 3-5A and 3-5B). Interestingly, treatment with MβCD reduced the phosphorylation of AKT but had no effect on the phosphorylation of MAPK (Figures 3-5A and 3-5B). To determine whether MT1-MMP is regulated by these signaling pathways we pre-incubated nPB MNC with the PI3K inhibitor LY290042 and the p44/p42 MAPK inhibitor PD98059 for 1 h prior to G-CSF treatment. We found that upregulation of MT1-MMP by G-CSF was abrogated by LY290042 whereas PD98059 had no effect (Figure 3-5C), indicating that G-CSF-induced upregulation of MT1-MMP is PI3K-dependent. Moreover, zymographic analysis of supernatants from co-cultures of nPB MNC and BM $CD34^+$ cells with BM fibroblasts pretreated with LY290042 before G-CSF stimulation showed reduced activation of proMMP-2 (Figure 3-5D), indicating that activation of proMMP-2 is also PI3K-dependent. To further

determine the role of PI3K-AKT signaling in G-CSF-induced MT1-MMP upregulation in lipid rafts, we pretreated mPB MNC with LY290042 for 1 h before stimulating with G-CSF and isolating the lipid rafts from the cell lysates. We found that pretreatment with LY290042 abrogated the G-CSF-induced increase of MT1-MMP incorporation into membrane lipid rafts whereas PD98059 had no effect (Figure 3-6A). This effect could also be observed in mPB $CD34^+$ cells as revealed by confocal microscopy (Figure 3-6B).

MT1-MMP has been shown to associate with F-actin, which is necessary for localization of MT1-MMP to the migration front [11,12]. To determine the subcellular localization of MT1-MMP, PB MNC stimulated with G-CSF were evaluated using confocal microscopy. We found that the MT1-MMP co-localized with F-actin in the lamellipodia; moreover, this co-localization was attenuated by LY290042 but unaffected by PD98059 (Figure 3-6C). PI3K inhibition by LY290042 also resulted in reduced trans-Matrigel migration of mPB MNC (Figure 3-6D, top panel) and mPB $CD34^+$ cells (Figure 3-6D, bottom panel).

3.5 Discussion

We previously reported that MT1-MMP is expressed on HSPC [17,18], and recently it was postulated that MT1-MMP expression in HSPC is regulated by the endogenous inhibitor reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and that high MT1-MMP and low RECK levels in HSPC resulted the egress of BM progenitors into circulation [39]. Here we provide evidence that MT1-MMP expression on the surface of HSPC is regulated by its incorporation

into membrane lipid rafts, and that both MT1-MMP expression and proMMP-2 activation are PI3K-dependent.

First, we demonstrated that ssBM leukocytes and $CD34^+$ cells have low MT1-MMP expression both at the mRNA and protein levels in contrast to high expression in their G-CSF-mobilized counterparts. Second, we showed that G-CSF stimulation in vitro upregulated MT1-MMP protein expression of $CD34^+$ cells and BM leukocytes, although it had no effect on the MT1-MMP gene and protein expression in stromal cells (data not shown). Earlier we proposed that exogenously administered mobilizing factors (e.g., G-CSF, SCF and GM-CSF) could facilitate HSPC egress from the BM by stimulating the secretion of proMMP-2 and proMMP-9 [7]. As only active MMP-2 degrades ECM components, the activation of proMMP-2 by MT1-MMP is considered to be a critical step in this process. We found that in co-cultures of BM fibroblasts with BM $CD34^+$ cells, proMMP-2 is not activated. However, pre-incubation with G-CSF of ssBM $CD34^+$ cells, highly upregulated MT1-MMP, which only then was able to activate proMMP-2 in similar co-cultures. Interestingly, co-culturing stromal cells with mPB $CD34^+$ cells or mPB MNC led to proMMP-2 activation without additional pre-incubation with G-CSF, indicating that proMMP-2 could be activated by highly upregulated MT1-MMP on the surface of in vivo-mobilized cells. Consistent with this, in co-cultures of stromal cells with mPB $CD34^+$ cells that had been transfected with MT1-MMP siRNA, active MMP-2 was not detectable. The MT1-MMP activation of proMMP-2 in the BM microenvironment is important because active MMP-2 not only initiates a cascade

of activation of other MMPs, but also inactivates SDF-1 and CXCR4, adhesion molecules, and plays an active role in matrix remodeling [13]. This could facilitate egress of HSPC from BM niches across the ECM and subendothelial membranes.

Moreover, MT1-MMP promotes cell motility by pericellular ECM degradation [11,12]. In this respect we demonstrated that MT1-MMP promotes migration across reconstituted basement membrane of mPB $CD34^+$ cells and MNC since specific inhibition of MT1-MMP by siRNA significantly abrogated it. In addition to matrix remodeling, MT1-MMP cleaves adhesion molecules such as CD44 and integrins [10], and chemokines such as SDF-1. MT1-MMP cleavage of SDF-1 results in loss of binding to CXCR4 and reduced chemoattraction for $CD34^+$ cells [40]. Therefore, during G-CSF-induced mobilization, upregulation of MT1-MMP expression in HSPC has consequences that could lead to their reduced retention in the BM and egress into PB. The definitive proof of this would involve studies using MT1-$MMP^{-/-}$ mice but such mice have severe developmental abnormalities resulting in their early death [14]; hence in vivo mobilization experiments are not possible. However, in a recent study using a chimeric mouse model, mobilization of MT1-$MMP^{-/-}$ c-kit^+ cells was low compared to wild-type cells and, furthermore, administration of anti-MT1-MMP antibody in NOD/SCID mice abrogated mobilization of human HSPC [39].

The initial cell response to cytokine or chemokine stimulation is the re-organization of the actin cytoskeleton during which several signaling pathways are activated including PI3K, MAPK, and Rho family GTPases [41]. MT1-MMP

has been shown to be modulated by each of these pathways depending on the cell type and stimulant [36,42,43]. Here we demonstrated that, in hematopoietic cells upon G-CSF stimulation, although both PI3K and MAPK are activated; MT1-MMP expression and proMMP-2 activation are only PI3K-dependent. Furthermore, inhibition of the PI3K-AKT axis by LY290042 also inhibits cell polarization, co-localization of MT1-MMP with F-actin and trans-Matrigel migration of mPB $CD34^+$ cells. Our results are consistent with recent studies in which mice treated with rapamycin, an inhibitor of the PI3K-AKT downstream effector mTOR, decreased MT1-MMP expression in HSPC and impaired G-CSF mobilization compared to untreated controls [39].

Murine HSPC studies have shown that cytokine-mediated lipid raft clustering activated the AKT-FOXO signaling pathway, which is essential for entry into cell cycle, and inhibition of lipid raft formation by MβCD led to repression of this pathway and hibernation-like state of HSPC [44]. The recruitment of the regulatory PI3K subunit p85 to lipid rafts after cytokine stimulation is required for activation of the downstream effectors of the PI3K-AKT axis, and is dependent on lipid raft integrity [45]. Consistent with these findings we show here, that G-CSF-mediated activation of the PI3K-AKT pathway in hematopoietic cells is indeed lipid raft-sensitive. Moreover, G-CSF-mediated PI3K signaling results in incorporation of MT1-MMP into membrane lipid rafts. Such incorporation enables HSPC migration, proMMP-2 activation, and ECM degradation because disrupting raft formation by MβCD inhibited these processes. This is consistent with previous studies where was shown that in tumor

and endothelial cells, caveolar expression of MT1-MMP is required for MT1-MMP processing and proMMP-2 activation [24,25].

Surface expression of MT1-MMP in resting cells is low, with most of the protein residing intracellularly. However, in response to physiological and pathological signals intracellular MT1-MMP is mobilized to specialized cell membrane domains when focal proteolysis is required [46]. Consistent with this we demonstrated that within 2 h of G-CSF stimulation, MT1-MMP incorporates into lipid rafts and, furthermore, this incorporation is PI3K-dependent. PI3K activity has also been shown to play an important role in protein trafficking, regulated exocytosis, and redistribution of surface protein [47]. Whether PI3K-dependent MT1-MMP cell surface expression occurs due to increased exocytosis or reduced internalization requires further investigation. Nevertheless, G-CSF-induced MT1-MMP upregulation and redistribution into lipid rafts is a mechanism that contributes to a highly proteolytic microenvironment in the BM and a greater invasiveness of hematopoietic cells, including HSPC.

In summary, G-CSF-induced PI3K activation in lipid rafts leads to reorganization of the actin cytoskeleton, recruitment of MT1-MMP into lipid rafts, and proMMP-2 activation which increases the degradation of basement membrane and interstitial matrix in the BM microenvironment and eventually the egress of HSPC into circulation as shown in the schema (Figure 3-7). We therefore propose that localization of MT1-MMP in lipid rafts serves as a mechanism that regulates its expression on the HSPC cell surface where it can elicit several functions. This mechanism could also serve as a means for

accumulation of other membrane-anchored proteases within lipid rafts and to localize proteolysis at the migration front of HSPC. For example, membrane proteases such as CD26 [48] and carboxypeptidase M [49], which have been shown to play important roles in G-CSF-induced mobilization, are anchored to the cell surface by their glycosylphosphatidylinositol (GPI) domains and are constitutively present in lipid rafts [50]. Further investigations will be required to elucidate the role of lipid raft-induced signaling in HSPC mobilization. A better understanding of the process by which HSPC leave the BM could result in improved clinical mobilization protocols, particularly for patients who mobilize poorly.

3.6 References

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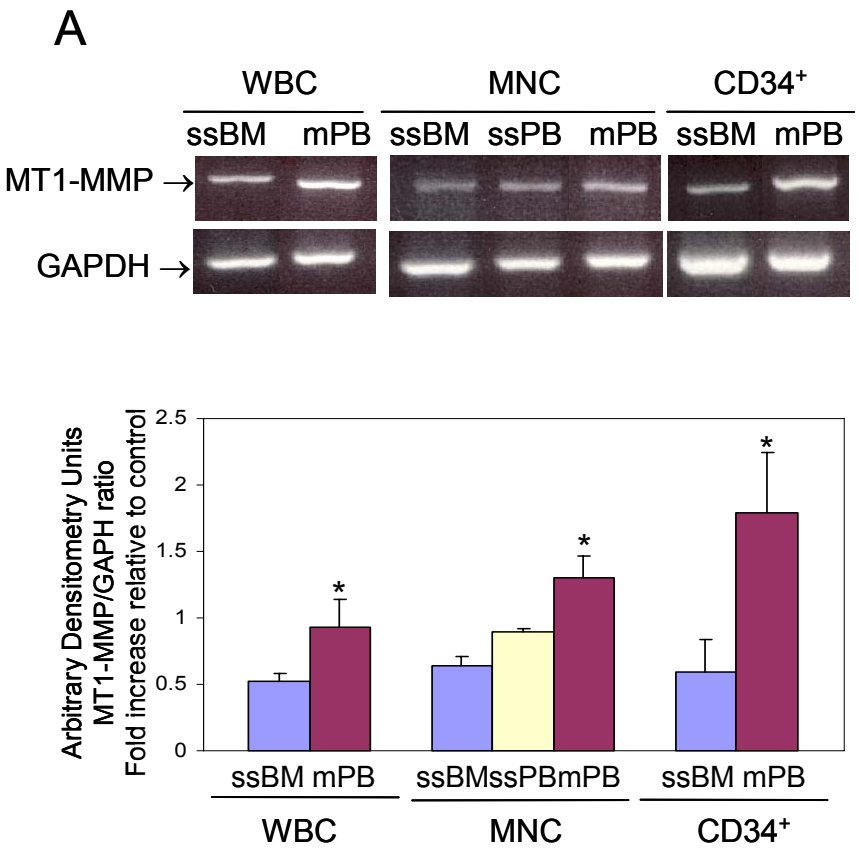

Figure 3-1. MT1-MMP expression in steady-state and mobilized hematopoietic cells. (A) RT-PCR analysis of MT1-MMP transcripts in steady-state (ss) BM and PB, and mobilized (m) PB leukocytes, MNC and $CD34^+$ cells. GAPDH was used as the mRNA control to ensure equivalence of loading. The left panel shows representative data from three independent experiments. The right panels shows mean ± standard deviations of densitometric analysis of the bands from three experiments, $*p < 0.05$.

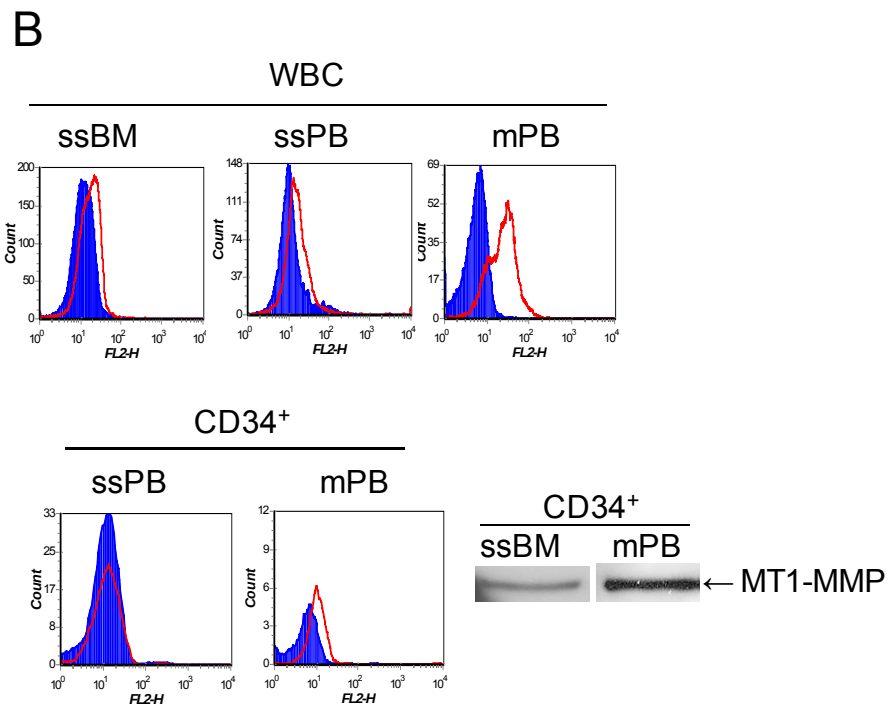

Figure 3-1. (B) FACS analysis of MT1-MMP protein expression on steady-state (ss) BM and PB and mPB leukocytes and $CD34^+$ cells. FACS analysis was carried out on three different samples and a representative histogram is shown. Filled and open histograms correspond to isotype IgG and MT1-MMP, respectively. Right panel: Western blot analysis of ssBM and mPB $CD34^+$ cells. Equal amounts of protein (30μg) as determined by the BioRad assay were loaded. Representative data from three independent experiments is shown.

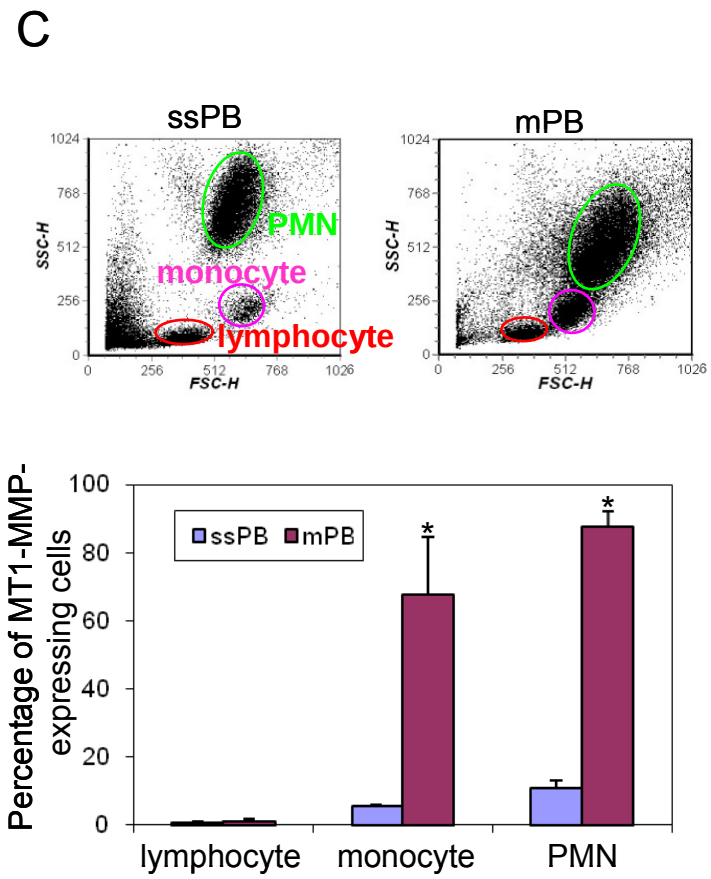

Figure 3-1. (C) Top panel: FACS analysis of steady-state nPB and mPB leukocytes showing the different populations by forward and side scatter. Bottom panel: MT1-MMP expression in each population is presented as the percentage of cells expressing MT1-MMP; the graph shows the mean ± SD of three independent experiments, *$p < 0.05$.

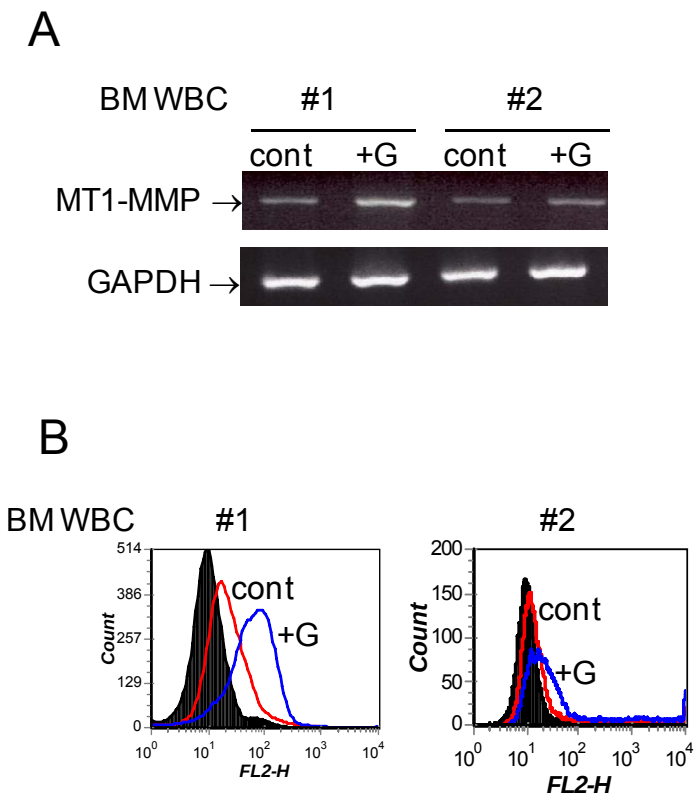

Figure 3-2. G-CSF upregulates MT1-MMP expression in HSPC. (A) RT-PCR analysis of MT1-MMP transcript from BM leukocytes obtained from two separate BM samples (BM #1 and BM #2) were stimulated for 48 h with 100 ng/ml G-CSF (+G) or not (cont). GAPDH was used as the mRNA control to ensure equivalence of loading. **(B)** FACS analysis of MT1-MMP expression on BM leukocytes (BM #1 and BM #2) stimulated for 48 h with 100 ng/ml G-CSF or not (cont). Filled and open histograms correspond to isotype IgG and MT1-MMP, respectively.

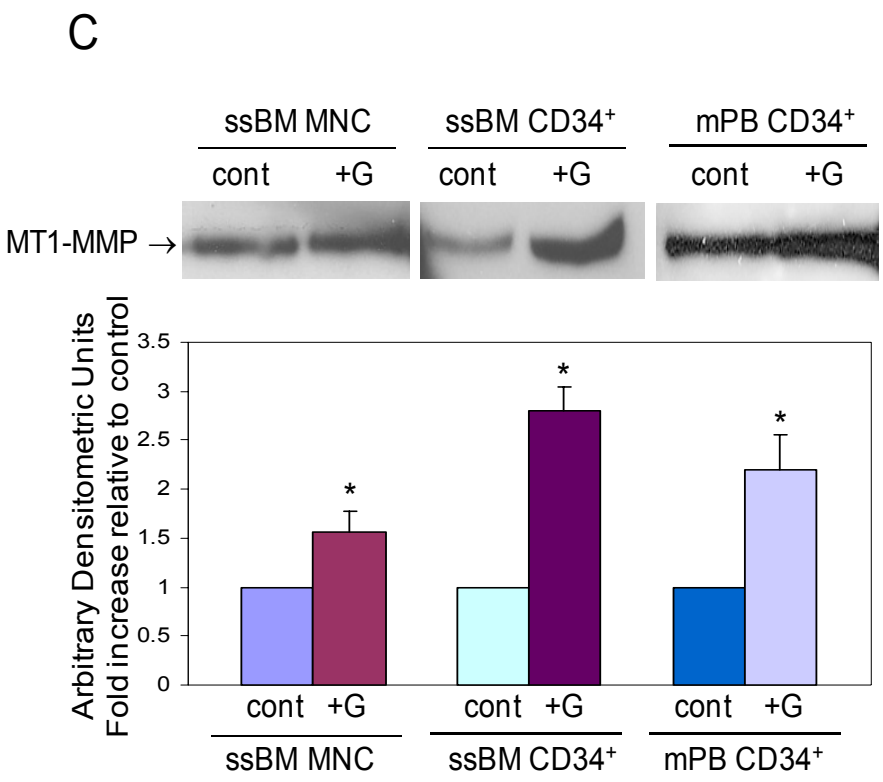

Figure 3-2. (C) Western blot analysis of MT1-MMP in BM $CD34^+$ cells, BM MNC and mPB $CD34^+$ cells stimulated or not with 100 ng/ml of G-CSF. Equal amounts of protein (30μg) as determined by the BioRad assay were loaded. The top panel shows representative data from three independent experiments. The bottom panel shows mean ± standard deviations of densitometric analysis of three experiments shown as fold increase compared to control, $*p < 0.05$.

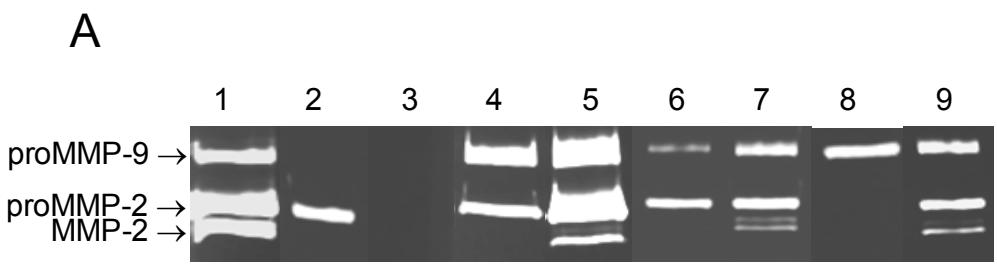

Figure 3-3. ProMMP-2 activation occurs in co-cultures of G-CSF-stimulated BM CD34$^+$ cells and inhibition of MT1-MMP-dependent trans-Matrigel migration of mPB CD34$^+$ cells and MNC. (A) Zymographic analysis of media conditioned by BM fibroblasts, steady-state BM, mPB CD34$^+$ cells or mPB MNC, co-cultures of CD34$^+$ cells with fibroblasts. Lane 1: Standard (HT1080); Lane 2: BM fibroblast; Lane 3: BM CD34$^+$ cells; Lane 4: co-culture of fibroblasts + BM CD34$^+$ cells; Lane 5: co-culture of fibroblasts + BM CD34$^+$ cells + G-CSF; Lane 6: mPB CD34$^+$ cells; Lane 7: co-culture of fibroblasts + mPB CD34$^+$ cells; Lane 8: mPBMNC cells; Lane 9: co-culture of fibroblasts + mPB MNC. In media conditioned by fibroblasts only proMMP-2 was found, while active MMP-2 is detectable in supernatants from co-cultures of fibroblasts with mPB CD34$^+$ cells, mPB MNC and BM CD34$^+$ cells stimulated with G-CSF. A representative zymogram of three separate experiments is shown.

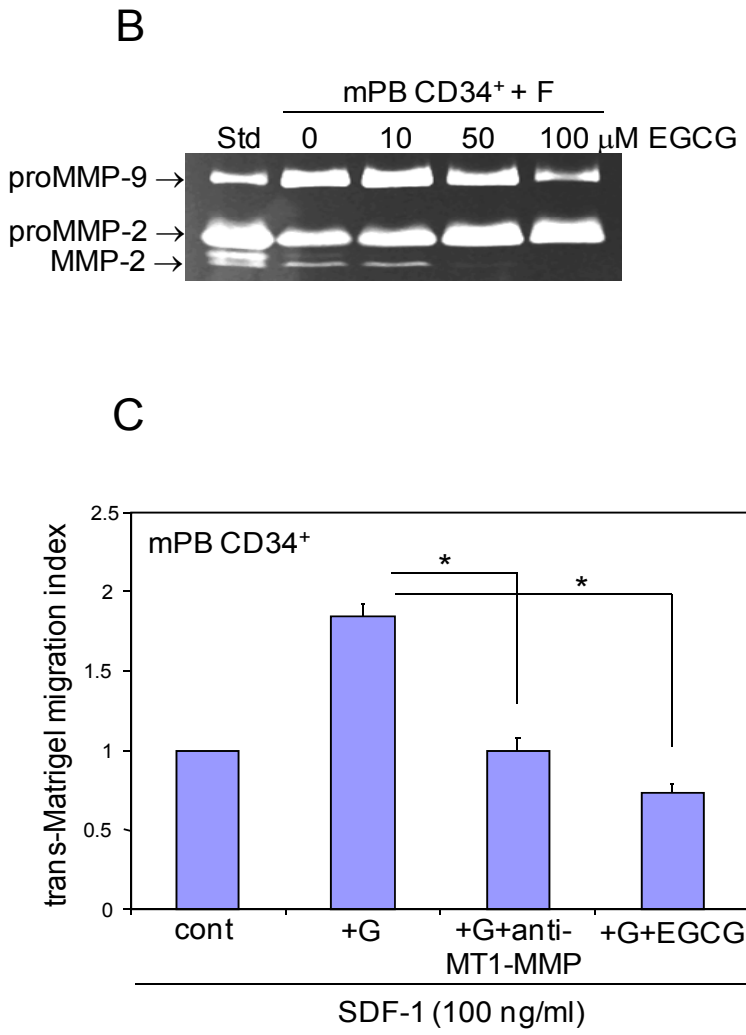

Figure 3-3. (B) Zymographic analysis of conditioned media (from co-cultures of BM fibroblasts and mPB $CD34^+$ cells) treated with different concentrations of MT1-MMP inhibitor EGCG. A representative zymogram from two separate experiments is shown. **(C)** Trans-Matrigel migration of G-CSF-stimulated mPB $CD34^+$ cells towards an SDF gradient is significantly inhibited by MT1-MMP monoclonal antibody (10 μg/ml) and inhibitor EGCG (50 μM), $*p < 0.05$.

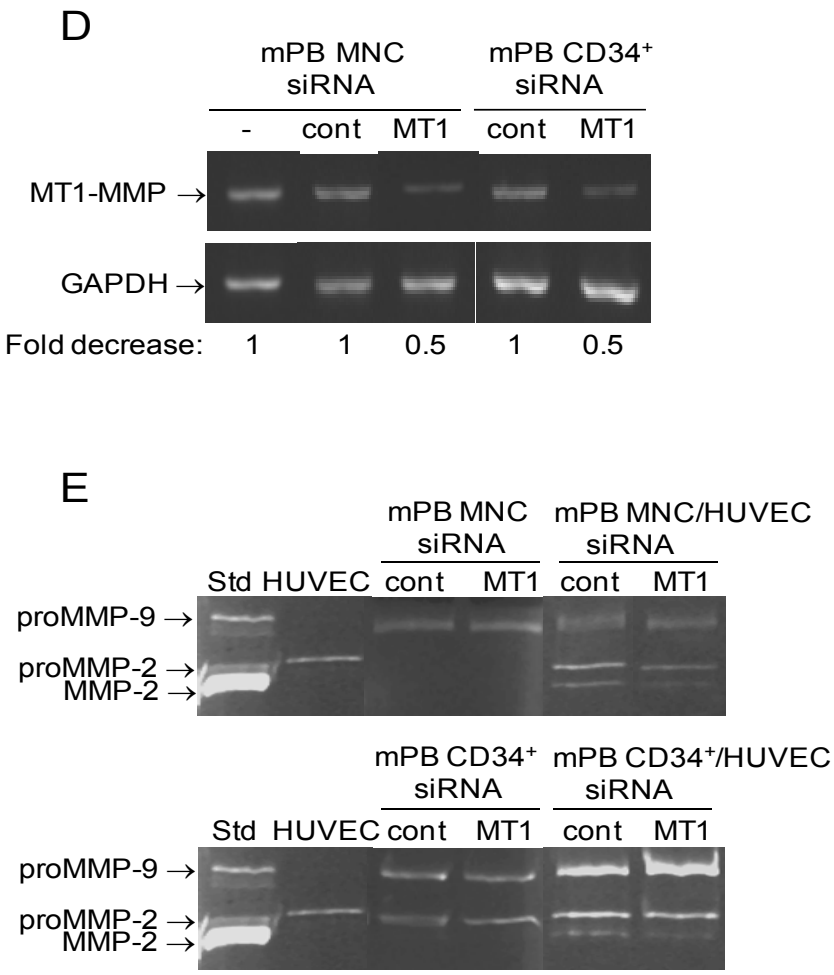

Figure 3-3. (D) RT-PCR of MT1-MMP transcripts in mPB MNC and $CD34^+$ cells transfected with either scrambled siRNA (cont) or MT1-MMP siRNA (MT1). GAPDH was used as the mRNA control to ensure equivalence of loading. **(E)** Zymographic analysis of cell-conditioned media from co-cultures of HUVEC and mPB MNC (top panel) or mPB $CD34^+$ cells (bottom panel) transfected with scrambled siRNA (cont) or transfected with MT1-MMP siRNA (MT1). A representative zymogram from two separate experiments is shown.

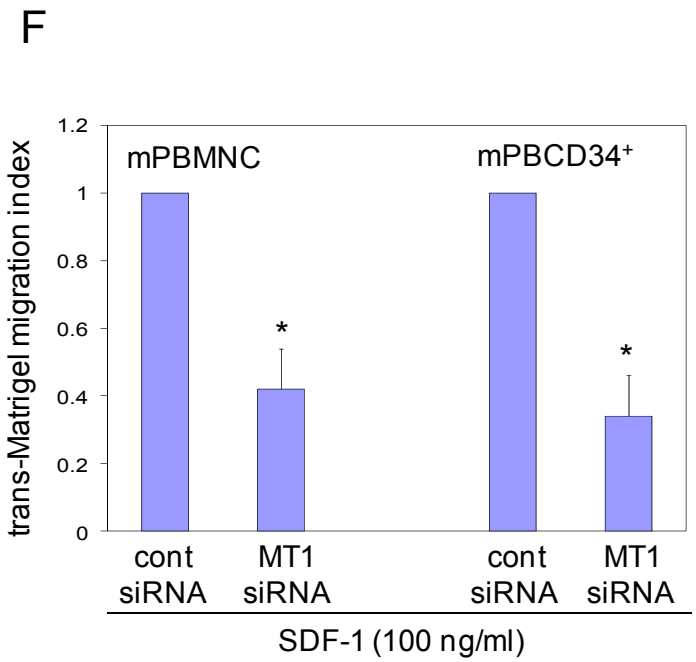

Figure 3-3. (F) Trans-Matrigel migration towards an SDF-1 gradient is inhibited in the MT1-MMP siRNA transfected cells, $*p < 0.05$. The assays were performed at least in quadruplicate for each condition. A representative of two separate experiments is shown.

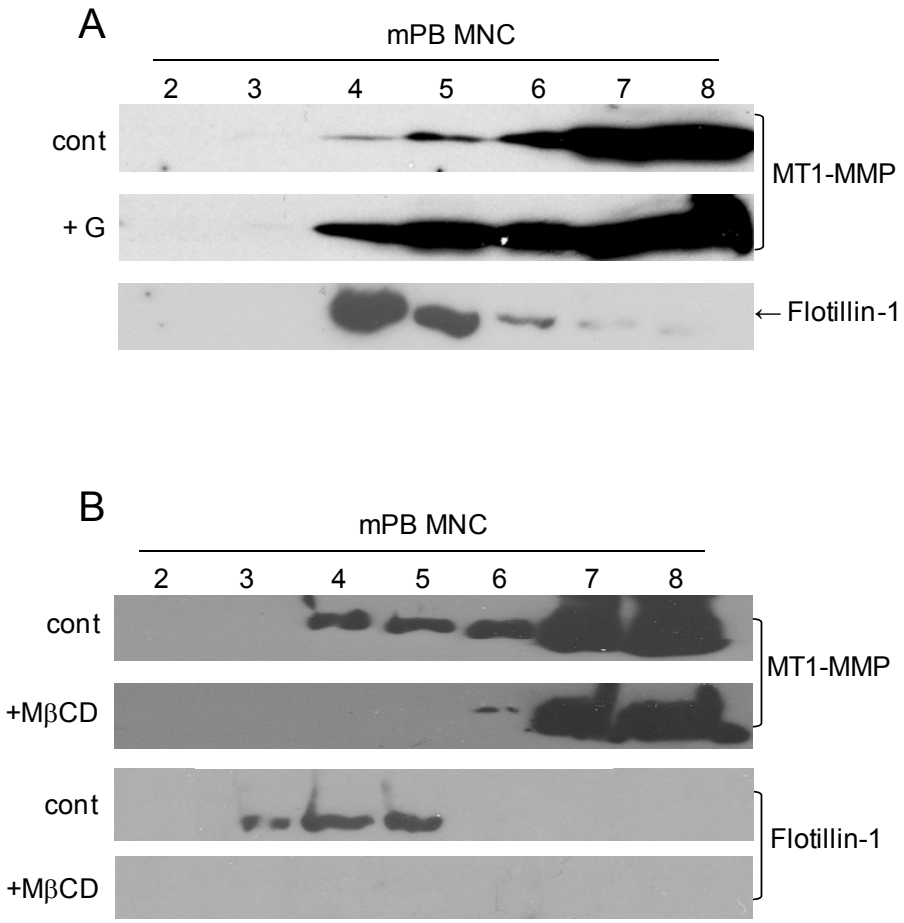

Figure 3-4. MT1-MMP is localized in the lipid rafts of hematopoietic cell membranes. (A) mPB MNC were serum-starved overnight and then stimulated or not (cont) with 100 ng/ml G-CSF for 2 h. Lysates were fractionated in a discontinuous sucrose gradient. Fractions 2-8 were analyzed by Western blotting for MT1-MMP and lipid raft marker flotillin-1. A representative of three separate experiments is shown. **(B)** MT1-MMP expression in MNC after lipid raft disruption. MNC were treated with cholesterol-depleting agent MβCD (10 mM) followed by lipid raft isolation and Western blot analysis. A representative of two separate experiments is shown.

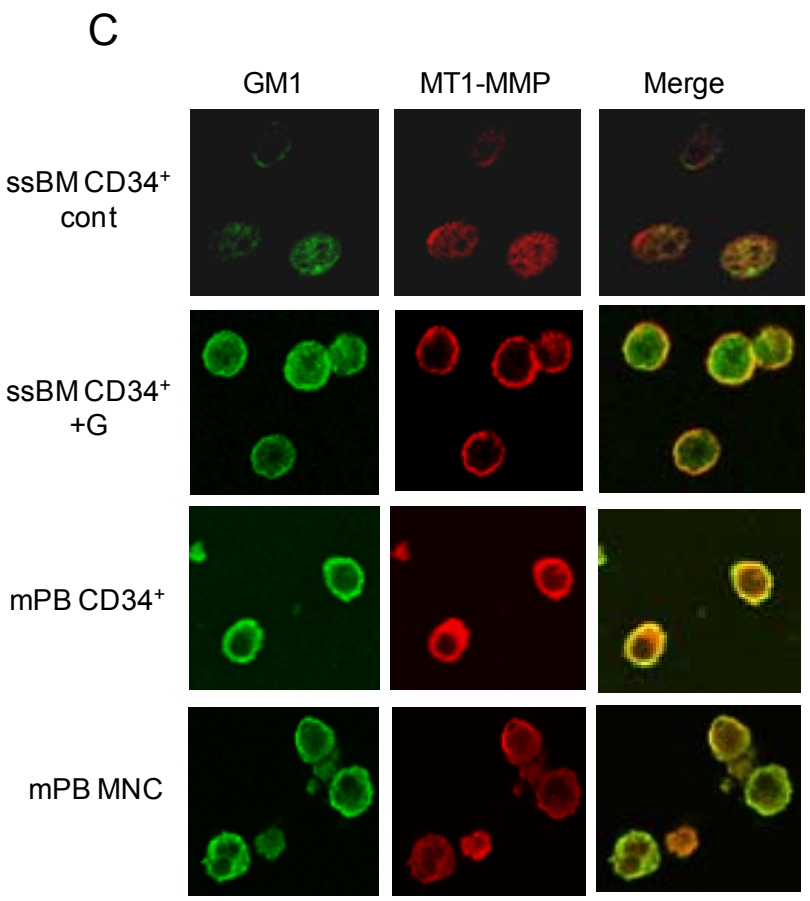

Figure 3-4. (C) MT1-MMP is localized in membrane lipid rafts of BM $CD34^+$, mPB $CD34^+$ cells and mPB MNC. BM $CD34^+$ cells were serum-starved overnight and then stimulated with 100 ng/ml G-CSF for 2 h, mPB $CD34^+$ cells and mPB MNC plated on poly-L-Lysine-coated coverslips were immunostained with MT1-MMP (red), and the lipid raft probe GM1-CTX (green). A representative of three separate experiments is shown.

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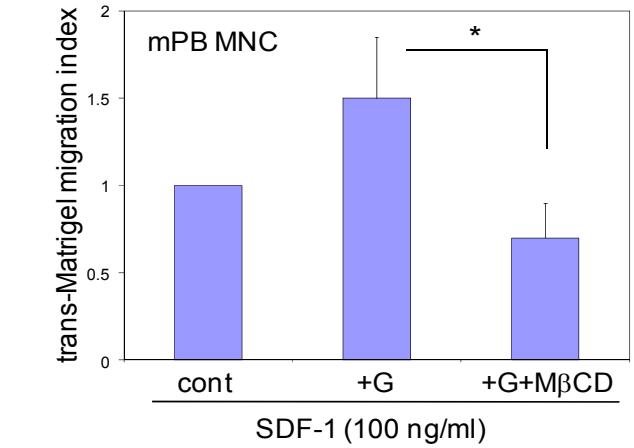

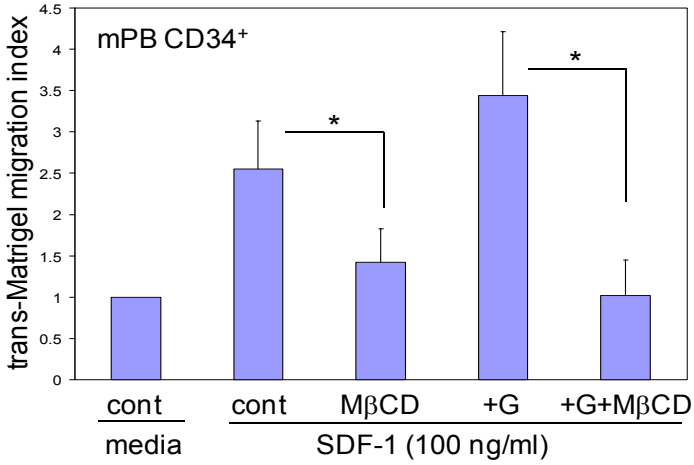

Figure 3-4. (D) Trans-Matrigel migration of mPB MNC and $CD34^+$ cells is lipid raft-dependent. MNC and $CD34^+$ cells were pretreated with MβCD (2.5 mM) for 1 h before stimulation with 100 ng/ml G-CSF for 2 h and trans-Matrigel migration towards SDF-1 (100 ng/ml) was carried out. The assays were performed at least in quadruplicate for each condition. A representative of three separate experiments is shown, $*p < 0.05$.

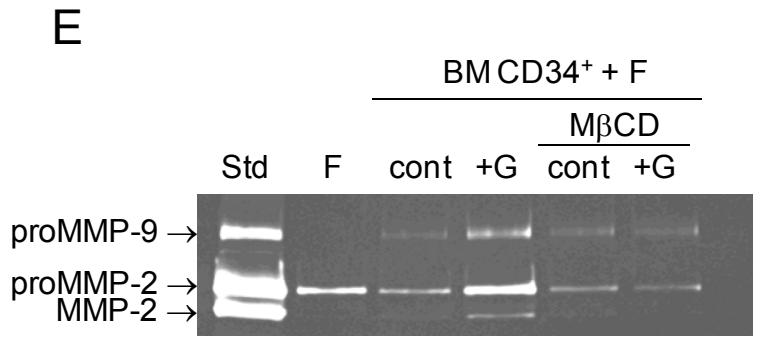

Figure 3-4. (E) ProMMP-2 activation is lipid raft-dependent. Zymographic analysis of conditioned media from co-cultures of BM fibroblasts (F) and BM $CD34^+$ cells. Co-cultures were pretreated or not (control) with MβCD for 1 h before stimulation with 100 ng/ml G-CSF (G) for 4h. The supernatants were then concentrated 5X before zymography. A representative of two separate experiments is shown.

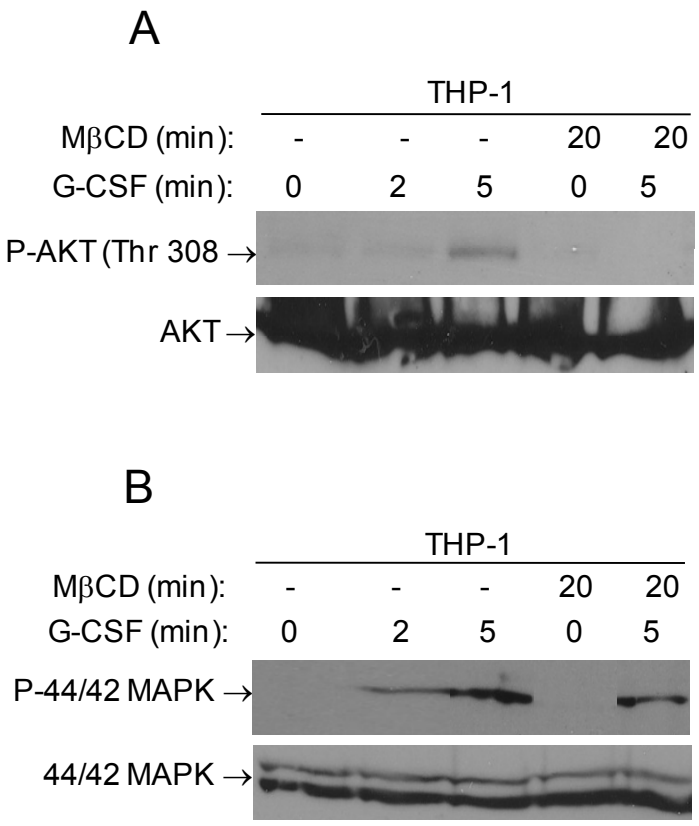

Figure 3-5. MT1-MMP upregulation by G-CSF is PI3K-dependent. (A and B) Phosphorylation of AKT and p44/42 MAPK. THP-1 cells were serum-starved overnight and then stimulated with 100 ng/ml G-CSF and/or with 10 mM MβCD at times indicated. A representative Western blot of two separate experiments is shown.

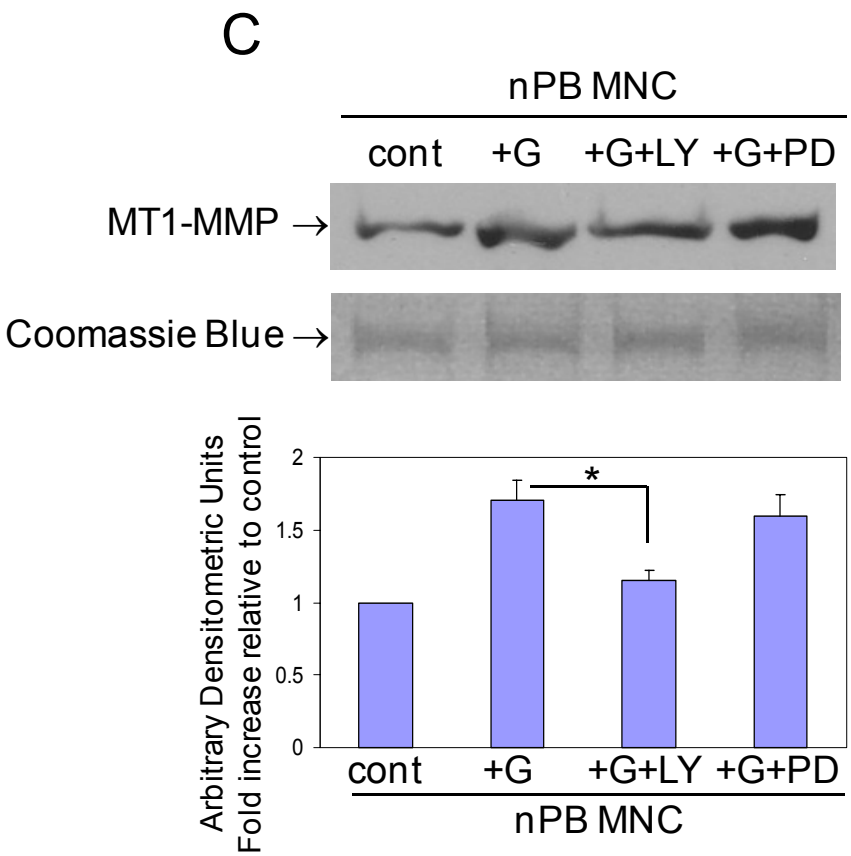

Figure 3-5. (C) MT1-MMP expression is PI3K-dependent. Western blot analysis of MT1-MMP expression in nPB MNC pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and then stimulated with 100 ng/ml G-CSF (G) for 48 h. Equal amounts of protein (30μg) as determined by the Bradford assay were loaded. The gel was stained with Coomassie blue to ensure equivalence of loading. The left panel shows representative data from three independent experiments. The right panel shows mean ± standard deviations of densitometric analysis of three experiments shown as fold increase compared to control, $*p < 0.05$.

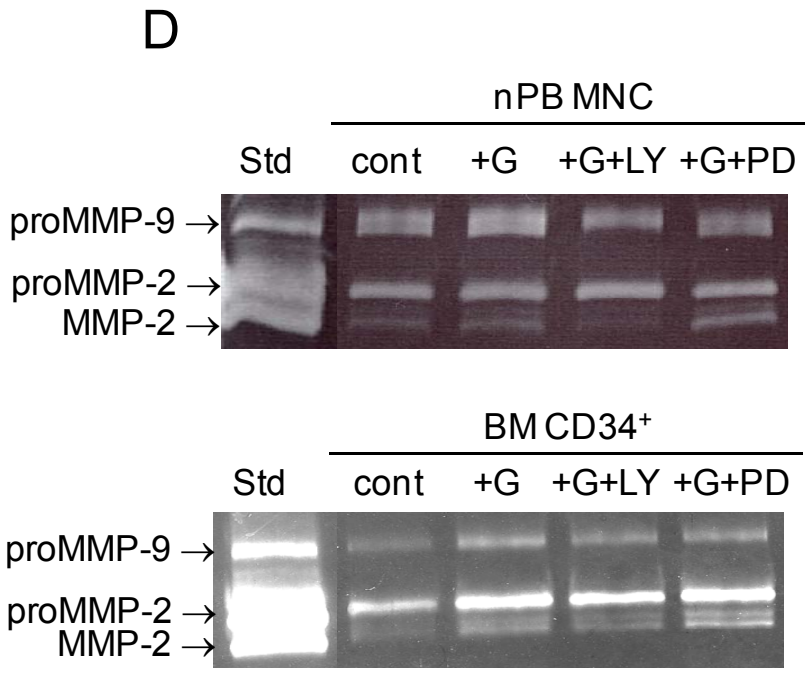

Figure 3-6. (D) ProMMP-2 activation is PI3K-dependent. Zymographic analysis of conditioned media from co-cultures of BM fibroblasts and nPB MNC (top panel) and BM $CD34^+$ cells (bottom panel). MNC and $CD34^+$ cells were plated on monolayers of BM fibroblasts and the co-cultures were pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and stimulated with 100 ng/ml G-CSF (G) for 48 h. A representative of two separate experiments is shown.

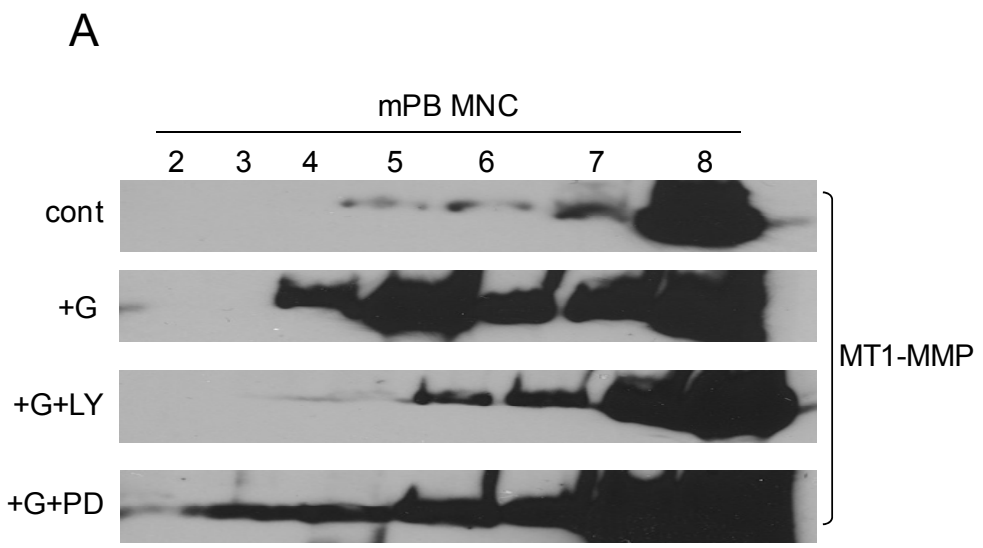

Figure 3-6. MT1-MMP incorporation into lipid rafts is PI3K−dependent. (A) mPB MNC were serum-starved overnight, pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and then stimulated with 100 ng/ml G-CSF (G) for 2 h. Lysates were fractionated in a discontinuous sucrose gradient. Fractions 2-8 were analyzed by Western blotting for MT1-MMP. A representative of two independent experiments is shown.

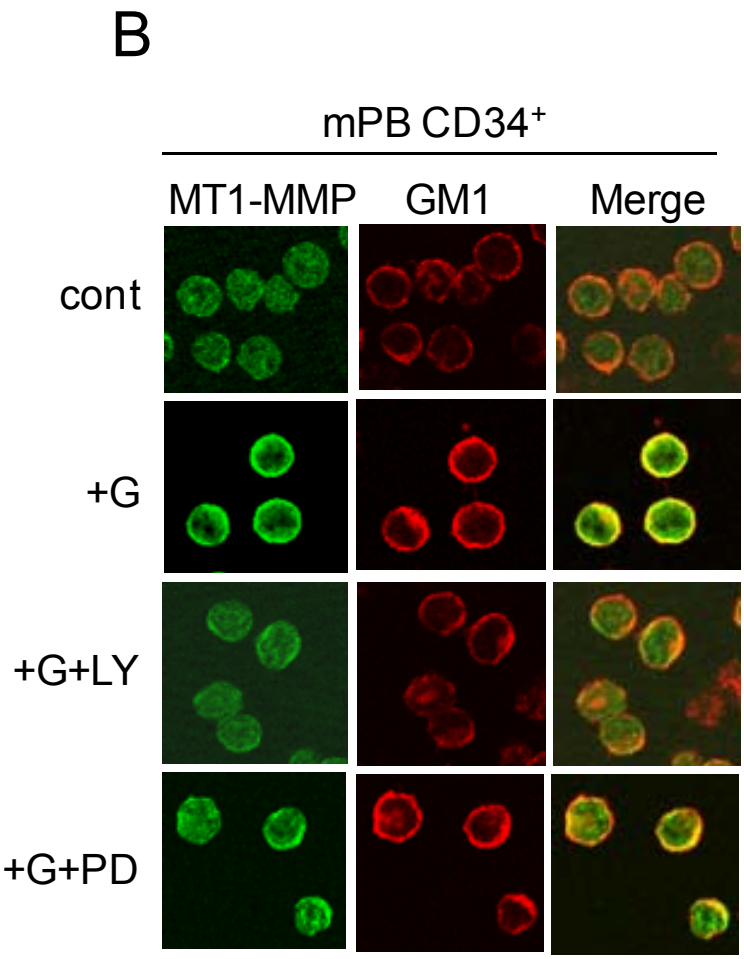

Figure 3-6. (B) mPB $CD34^+$ cells were serum-starved overnight, pretreated with 30 μM LY290042 or 50 μM PD98059 for 1 h and then stimulated with 100 ng/ml G-CSF for 2 h, plated on poly-L-Lysine-coated coverslips and immunostained with MT1-MMP (green), and GM1-CTX (red). A representative of two separate experiments is shown.

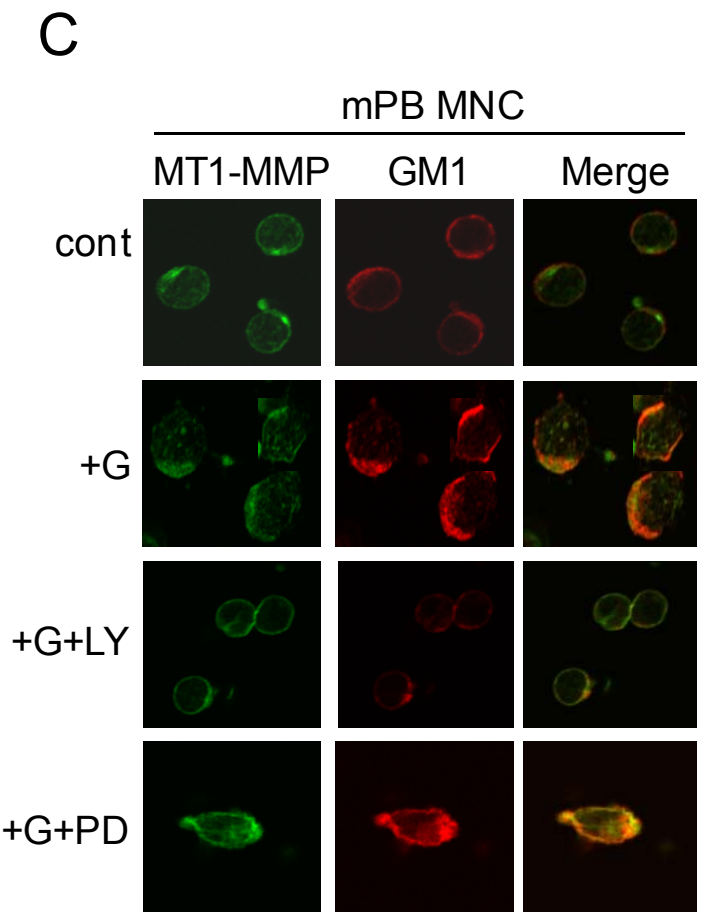

Figure 3-6. (C) mPB MNC were serum-starved overnight, pretreated with 30 μM LY290042 or 50 μM PD98059 for 1 h and then stimulated with 100 ng/ml G-CSF for 2 h, plated on fibronectin-coated coverslips and immunostained with MT1-MMP (green) and F-actin (red). A representative of two separate experiments is shown.

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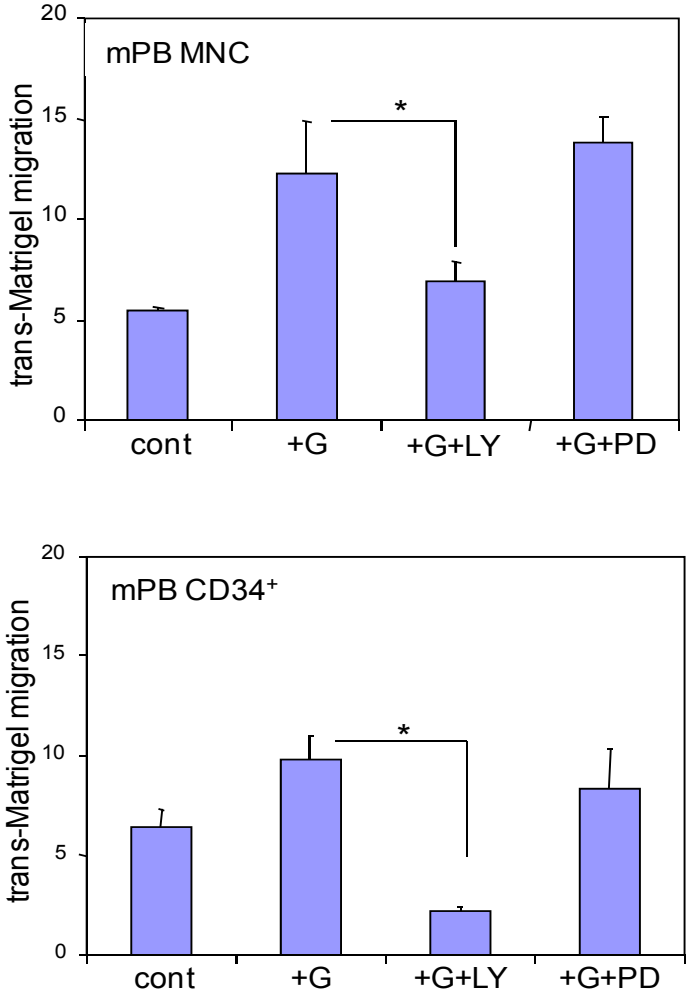
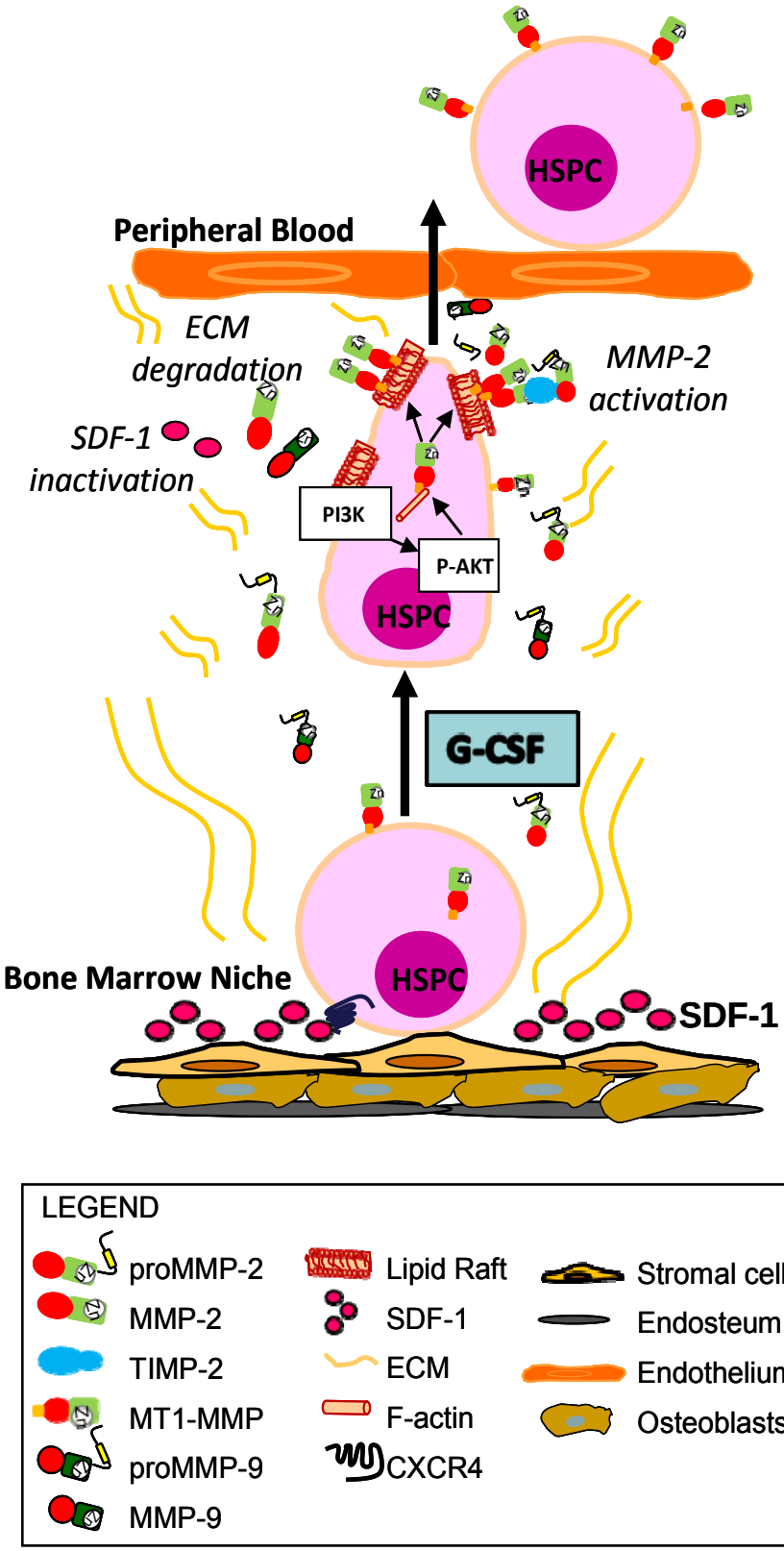


Figure 3-6. (D) Trans-Matrigel migration of mPB MNC and $CD34^+$ cells is PI3K-dependent. MNC (top panel) and $CD34^+$ cells (bottom panel) were pretreated with 30 μM LY290042 or 50 μM PD98059 for 1 h; and stimulated with 100 ng/ml G-CSF for 2 h before trans-Matrigel migration. The assay was performed at least in quadruplicate for each condition. A representative of three separate experiments is shown, $*p < 0.05$.


HSPC
Peripheral Blood
ECM
degradation
MMP-2
activation
SDF-1
inactivation
PI3K
P-AKT
HSPC
G-CSF
HSPC
Bone Marrow Niche
SDF-1
LEGEND
proMMP-2
MMP-2
TIMP-2
MT1-MMP
proMMP-9
MMP-9
Lipid Raft
SDF-1
ECM
F-actin
CXCR4
Stromal cells
Endosteum
Endothelium
Osteoblasts

F**igure 3-7. Schema of MT1-MMP localization and regulation in HSPC during G-CSF induced mobilization.** In steady-state expression of MT1-MMP on BM HSPC cell surfaces and its proteolytic activity is low. Stromal cells secrete SDF-1 whose interaction with CXCR4 retains HSPC in the BM niches. During G-CSF mobilization, G-CSF activates the PI3K-AKT pathway in a lipid raft-dependent manner. Phosphorylation of AKT induces cytoskeletal re-organization and recruits MT1-MMP into the lipid rafts where it can elicit several functions. MT1-MMP exhibits pericellular proteolysis by degrading the ECM, inactivating SDF-1 and activating proMMP-2 secreted by stromal cells. Activation of proMMP-2 leads to a cascade of activation of other MMPs including MMP-9, and matrix remodeling and also inactivation of SDF-1. Activation of these proteases thus facilitates HSPC egress from the BM niches into circulation.

Chapter 4 Hyaluronic acid and thrombin upregulate MT1-MMP through PI3K and Rac-1 signaling and prime the homing-related responses of cord blood hematopoietic stem/progenitor cells

4.1 Abstract

One of the hurdles of cord blood (CB) transplantation is delayed hematopoietic engraftment. Previously, we demonstrated that supernatants isolated from leukapheresis products (SLP) of G-CSF-mobilized patients primed the homing of hematopoietic stem/progenitor cells (HSPC) by enhancing their chemotactic responses to stromal cell-derived factor (SDF)-1 and stimulating matrix metalloproteinases (MMPs) MMP-2 and MMP-9. Since membrane type (MT)1-MMP activates proMMP-2 and localizes proteolytic activity at the leading edge of migrating cells, in this study we investigated whether MT1-MMP contributes to the priming of the homing-related responses of CB HSPC. We found that components of SLP such as hyaluronic acid (HA) and thrombin: (i) increase the secretion of proMMP-9 and transcription and protein synthesis of MT1-MMP in CB $CD34^+$ cells; (ii) increase the levels of active MMP-2 in co-cultures of $CD34^+$ cells with endothelial cells; (iii) increase the chemoinvasion across reconstituted basement membrane Matrigel of $CD34^+$ cells towards a low SDF-1 gradient (20 ng/mL); and (iv) activate mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Rac-1 signaling pathways. Inhibition of PI3K and Rac-1 by their respective inhibitors LY290042 and NSC23766, attenuated MT1-MMP expression in CB cells leading to reduced proMMP-2

[1]A version of this chapter has been submitted to Stem Cells and Development 2010

activation and HSPC trans-Matrigel chemoinvasion towards SDF-1. Thus, our data suggest that MT1-MMP plays an important role in the homing-related responses of HSPC, and we propose that pretreatment of CB HSPC with HA or thrombin before transplantation could improve their homing and engraftment.

4.2 Introduction

Umbilical cord blood (CB) is increasingly used as an alternative source of hematopoietic stem/progenitor cells (HSPC) for allogeneic transplantation in pediatric patients; however, in adult patients, its application is significantly restricted by the limited number of HSPC available from a single CB unit and as a consequence engraftment is delayed [1,2]. HSPC must home to the bone marrow (BM) after their i.v. infusion in order to engraft and enable hematopoietic recovery. The mechanism of homing is still not fully understood despite extensive studies. It is believed that this is a multi-step process requiring (i) extravasation of HSPC, (ii) migration across the extracellular matrix (ECM) in a matrix metalloproteinase (MMP)-dependent manner, and (iii) lodgement in BM niches [3,4]. In the BM microenvironment stromal cells secrete stromal-cell derived factor (SDF)-1, a chemokine which strongly chemoattracts HSPC that express its cognate receptor CXCR4. The SDF-1–CXCR4 axis activates cell surface adhesion molecules such as very late antigen (VLA)-4, VLA-5, CD44 and lymphocyte function-associated antigen 1, and mediates firm arrest of HSPC on BM endothelium [5-7]. It also facilitates trans-endothelial migration of HSPC by upregulating the basement membrane-degrading enzymes MMP-2 and MMP-9

[8,9], and plays a central role in their retention, survival and proliferation in the BM niches [3,4].

HSPC collected from mobilized peripheral blood (mPB) by leukapheresis engraft significantly faster after transplantation as compared to those from CB or BM [10]. We previously reported that several molecules [platelet derived microparticles (PMV), complement C3a, thrombin, hyaluronic acid (HA) and fibrinogen] accumulate in the blood during G-CSF mobilization and the leukapheresis procedure [11]. These molecules are present in the supernatants of leukapheresis products (SLP) and prime the chemotactic responses of HSPC towards SDF-1 by incorporating CXCR4 into membrane lipid rafts and upregulating MMP-2 and MMP-9. HA is an important component of the BM ECM and through interactions with its receptor CD44 and the SDF-1–CXCR4 axis, promotes HSPC homing to BM and their retention in the BM niches [12]. On the other hand, thrombin through proteolytic activation of its receptor PAR-1, elicits numerous cellular responses in platelets and endothelial cells such as induction of adhesion molecules, production of chemokines, activation of proMMP-2, cytoskeletal reorganization and migration [13].

MMP-2 and MMP-9 belong to a family of Zn^{2+}-binding, Ca^{2+}-dependent endopeptidases whose substrates include ECM proteins, growth factors, chemokines and cytokines [14-16]. They are secreted as proenzymes, and are activated by membrane type (MT)-MMPs that are anchored on the cell surface. MT1-MMP forms a ternary complex with tissue inhibitor of matrix metalloproteinases (TIMP)-2 and proMMP-2; then another MT1-MMP molecule,

free of TIMP-2, activates proMMP-2 [17]. MT1-MMP cleaves ECM substrates (collagens, laminin, fibronectin, proteoglycan), non-ECM substrates such as SDF-1, IL-8 and MCP-3, and cell surface molecules (CD44, integrin $\alpha_v\beta_3$, syndecan-1) [18-20]. MT1-MMP also mediates pericellular proteolysis associated with tumor cell migration, metastasis and angiogenesis [17-19] and migration of endothelial cells and monocytes [21,22]. Previously we demonstrated that MT1-MMP is involved in the trans-Matrigel migration of human mesenchymal stem cells, CB $CD34^+$ cells and megakaryocytic progenitors towards an SDF-1 gradient [23,24] and other investigators have shown in a murine model that MT1-MMP mediates homing of $CD34^+$ cells [25]. However, the molecular mechanisms of MT1-MMP regulation during human HSPC homing have not been characterized.

Cell migration is a tightly coordinated series of events in which reorganization of the actin cytoskeleton and cell polarization towards a chemoattractant are regulated by several signaling pathways [26]. An important regulator of actin cytoskeletal dynamics and F-actin polymerization is the phosphatidylinositol 3-kinase (PI3K)-AKT pathway. Activation of PI3K leads to accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the cell's leading edge and ultimately to actin polymerization, lamellipodia formation, and directional cell movement [27]. Inhibition of PI3K has been shown to abrogate HSPC migration towards SDF-1 [28]. Another group of important regulators of actin cytoskeleton and cell migration are the Rho-family GTPases including Rac, Rho and Cdc42 [29]. We previously demonstrated that SDF-1–CXCR4 signaling during HSPC homing occurs in the lipid rafts where CXCR4 and Rac-1 assemble

together, thus allowing an optimal chemotactic response to SDF-1 [11]. HA and thrombin, through their respective receptors CD44 and PAR-1, promote cytoskeletal rearrangements and cell migration by activating Rac GTPase [30,31]. Rac GTPases also play an important role in the control of gene expression, and activation of proliferation and survival pathways [29]. Rac-1 regulates intracellular signaling during HSPC homing and engraftment as murine Rac-$1^{-/-}$ HSPC failed to engraft in the BM of irradiated recipient mice [32,33].

In this study we investigated the priming effect of HA and thrombin in the homing-related responses of HSPC and characterized the function and regulation of MT1-MMP in this process by demonstrating the involvement of PI3K and Rac-1 signaling pathways.

4.3 Materials and methods

4.3.1 Cells and cultures

CB samples were collected with mothers' informed consent and in accordance with the guidelines by the University of Alberta Health Research Ethics Board. Light density mononuclear cells (MNC) from CB were separated using a 60% Percoll gradient (1.077 g/ml, GE Healthcare, Baie D'Urfe, PQ) and enriched for $CD34^{+}$ cells using the Miltenyi MACS Technology (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions and as previously described [8].

Human umbilical vein endothelial cells (HUVEC, a gift from Dr. Allan Murray, Dept. Of Medicine) were isolated from several umbilical cords, pooled, and cultured on gelatin-coated flasks in medium M199 containing 10 mM L-glutamine, 250 IU/ml penicillin-streptomycin (all from Invitrogen, Burlington,

ON), 20% bovine growth serum (BGS, Hyclone, ThermoFisher Scientific, Nepean, ON) and endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), grown to sub-confluence, trypsinized and washed in serum-free IMDM. Cells (1 x 10^5/ml) were plated in gelatin-coated 24-well cell culture plates. For co-culture experiments, CB $CD34^+$ cells (suspended in serum-free IMDM at 2 x 10^6 cells/ml) were seeded onto the HUVEC monolayers, and HA (50 μg/ml) or thrombin (10 U/ml) (both from Sigma, St Louis, MO) were added or not (control) to the co-cultures. In some experiments, the co-cultures were preincubated for 1 h with PI3K inhibitor LY290042 (30 μM) or p44/p42 MAPK inhibitor PD98059 (50 μM), or Rac1 inhibitor NSC23766 (50 μM) (all from Calbiochem, La Jolla, CA) before adding HA or thrombin. Supernatants were from these co-cultures were collected after 48 h incubation at 37ºC in 5% CO_2. The myelomonocytic U937 cell line was obtained from the American Type Culture Collection (Rockville, MD), grown in RPMI media supplemented with 10% BGS and used for phosphorylation experiments (see below).

4.3.2 RT-PCR analysis

Expression of MMP-9, MMP-2 and MT1-MMP mRNA was evaluated using RT-PCR. Briefly, total RNA was isolated from cell pellets using TRIZOL (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Concentrations were determined by measuring the absorbance at 260 nm in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). PCR reactions were carried out in an Eppendorf Mastercycler (Westbury, NY) as described [23]. Sequences of MMP-9, MMP-2, MT1-MMP and GAPDH were

obtained from GenBank (Los Alamos, NM) and used to design primer pairs. PCR products were run on 2% agarose gels containing 100 μg/ml ethidium bromide (Sigma), visualized under UV light and photographed using the FluorChem Imaging System (Alpha Innotech, San Leandro, CA). Semi-quantitative evaluation by densitometric analysis of the bands in each sample was carried out using AlphaEaseFC Software (Alpha Innotech). The relative level of target mRNA was calculated from the ratio between the intensities of the target primer and the GAPDH bands.

4.3.3 Zymography

In order to evaluate MMP-2 and MMP-9 activities, zymography assays were performed on media conditioned by CB $CD34^+$ cells alone or by co-cultures of $CD34^+$ cells with HUVEC as described previously [8]. Briefly, cells in serum-free IMDM at a concentration of 2 x 10^6 cells/ml were incubated or not (control) with HA (50 μg/ml) or thrombin (10 U/ml) for 48 h at 37°C, 5% CO_2. After centrifugation the cell-conditioned media were collected and applied onto a 12% SDS-PAGE gel containing 1.5 mg/ml gelatin (Sigma). Clear bands at 92 kDa and 72 kDa against a Coomassie blue background indicated the presence of latent forms of MMP-9 and MMP-2, respectively. The intensity of the bands was analyzed by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

4.3.4 Western blotting

For MT1-MMP total protein analysis, the cells were lysed for 30 min in lysing buffer (1% Triton, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing protease inhibitors (1 mM PMSF and 2 mM protease inhibitor cocktail) (all from Sigma), followed by sonication and centrifugation at 14000 rpm for 10 min at 4^{o}C. The protein concentrations in lysates were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The cell lysate was separated under denaturing conditions in a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad), followed by blocking with 5% fat-free dried milk in Tris-buffered saline and 0.05% Tween 20. MT1-MMP was detected with a rabbit anti-MT1-MMP (Abcam, Cambridge, MA) and a secondary antibody (goat anti-rabbit, HRP-conjugated IgG, Pierce Biotechnology, Rockford, IL). Chemiluminescence detection was performed using the Super Signal West Pico system (Pierce Biotechnology) and exposed on X-Ray film. The intensities of the bands were analyzed (using Commassie blue as loading control as described [34]) by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

4.3.5 SiRNA electroporation

SiRNA was targeted against 21-nucleotide sequences of MT1-MMP (Dharmacon, Lafayette, CO) as described [35]. A control siRNA sequence was generated from a scrambled MT1-MMP sequence. CB CD34^{+} cells (5 x 10^{6}) were electroporated with siRNA oligonucleotides (5 μg) using a nucleofector kit (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions, and incubated for 24 h in RPMI + 10 % BGS at 37^{o}C and 5% CO_2. Viability of the transfected

cells was determined by the trypan blue exclusion test and was >80% and efficiency of MT1-MMP knockdown was determined by RT-PCR.

4.3.6 Trans-Matrigel migration assay

Cell migration was determined using the trans-Matrigel migration assay as we described in detail previously [8]. CB $CD34^+$ cells that had been preincubated with or without HA (50 μg/ml) or thrombin (10 U/ml) were loaded in the upper compartments of Boyden chambers (2 x 10^5/chamber) and incubated for 3 h at 37°C and 5% CO_2. The lower compartments of the chambers were filled with serum-free media with (or without) SDF-1α (20 ng/ml or 100 ng/ml), and polycarbonate filters (Costar/Nucleopore, Toronto, ON) coated with 25 μg of Matrigel (Collaborative Biomedical Products) were placed between the upper and lower chambers. Percentage migration was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded. In some experiments, before the migration assay was conducted, CB $CD34^+$ cells were pre-incubated for 1 h with (-)-epigallocatechin 3-gallate (50 μM) (EGCG, Sigma), LY290042 (30 μM), PD98059 (50 μM), or NSC23766 (50 μM) before stimulation with HA or thrombin for 2 h.

4.3.7 Confocal analysis

CB $CD34^+$ cells stimulated with HA were plated on cover slips coated with fibronectin or poly-L-lysine (Sigma) for 2 h, fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked with 1% BSA in PBS and incubated with the monoclonal mouse anti-MT1-MMP antibody (R&D Systems,

Minneapolis, MN). Goat anti-mouse AlexaFluor-488 (Invitrogen) was used as a secondary antibody. F-actin was detected using AlexaFluor 564-phalloidin (Invitrogen). Finally, they were mounted in ProLong anti-fade reagent (Invitrogen) and examined using an LSM510 laser scanning confocal microscope (Carl Zeiss, Germany).

4.3.8 Phosphorylation of intracellular pathway proteins and Rac activation assay

Phosphorylation of intracellular kinases was evaluated by Western blot analysis as described previously [11]. U937 cells were kept in RPMI + 0.5% BSA overnight at 37^{o}C and 5% CO_2 to render the cells quiescent. Cells were then stimulated with HA (50 μg/ml) or thrombin (10 U/ml) for 0 and 5 min at 37°C and lysed as described above. Phosphorylation of 44/42 MAPK and AKT proteins was detected by protein immunoblotting using rabbit anti-Phospho-p44/42 MAPK and rabbit anti-Phospho-AKT antibodies (Cell Signalling Technology, Danvers, MA) followed by goat anti-rabbit, HRP-conjugated IgG secondary antibody. The blots were then stripped and reprobed with total rabbit anti-p44/42 MAPK and total rabbit anti-AKT (Cell Signaling Technology) to ensure equal loading. The intensities of the bands were analyzed by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

Activation of Rac-1 GTPase was carried out using the Rac activation assay kit (Millipore, Billerica, MA) as described previously [11]. U937 cells were rendered quiescent by incubating them in RPMI + 0.5% BSA overnight at 37^{o}C and 5% CO_2. Cells were preincubated with LY290042 (30 μM), or PD98059 (50

μM) for 20 min before stimulation with HA (50 μg/ml) or thrombin (10 U/ml) for 5 min at 37°C and lysed with MLB lysis buffer (50 mM Tris, pH 7.4, 10% glycerol, 200 mM NaCl, 1mM Na_2VO_3, 25mM NaF, 1 mM PMSF and 2 mM protease inhibitor cocktail). Cell lysates were precleared by centrifugation and incubated with PAK binding domain-tagged agarose (10 μg) at 4 ^oC for 1 h. The agarose beads were then washed 3 times with MLB buffer and boiled in Laemmeli sample buffer. Activated Rac was detected by Western blotting using monoclonal Rac antibody (Millipore). The intensities of the bands were analyzed by densitometry using the Alpha Innotech imaging system (San Leandro, CA).

4.3.9 Statistical analysis

Arithmetic means and standard deviations were calculated and statistical significance was defined as $p < 0.05$ using Student's *t* test.

4.4 Results

4.4.1 HA and thrombin upregulate MT1-MMP in BM and CB CD34$^+$ cells

Previously we showed that SLP increased secretion of proMMP-2 and -9 in CB CD34$^+$ cells [11]. In this study, we determined whether individual components of SLP, mainly HA and thrombin, have similar effects. Using RT-PCR we found that HA and thrombin significantly upregulated mRNA transcripts for MMP-9 (p = 0.001 and 0.008, respectively) and MT1-MMP (p = 0.003 and 0.002, respectively) up to 1.5-fold (Figure 4-1A). Zymographic analysis of media conditioned by CB CD34$^+$ cells stimulated with HA or thrombin showed that proMMP-9 secretion was significantly increased (p = 0.007 and 0.004,

respectively) up to 2-fold compared to unstimulated cells (Figure 4-1B). Furthermore, using Western blot we found that HA and thrombin also significantly increased MT1-MMP protein levels in CB $CD34^+$ cells (p = 0.006 and 0.001, respectively) up to 2-fold (Figure 4-1C).

4.4.2 MT1-MMP activates proMMP-2 in co-cultures of CB $CD34^+$ cells with HUVEC stimulated with HA and thrombin and mediates trans-Matrigel migration of $CD34^+$ cells towards an SDF-1 gradient

As a part of the homing process, $CD34^+$ cells extravasate through the endothelium, and basement membrane-degrading MMPs are thought to be involved in their trans-migration [4,7]. As shown in Figure 4-1B and Figure 4-2A, CB $CD34^+$ cells secreted only proMMP-9, and HUVEC only proMMP-2. However, in co-cultures of these cells we detected active MMP-2 (Figure 4-2A). Moreover, the levels of active MMP-2 increased when these co-cultures were stimulated with either HA or thrombin (Figure 4-2A) suggesting that an upregulation of MT1-MMP expression in $CD34^+$ cells resulted in enhanced activation of proMMP-2 secreted by HUVEC. To further confirm the involvement of MT1-MMP in proMMP-2 activation in this co-culture system, we silenced MT1-MMP in CB $CD34^+$ cells by siRNA transfection. This led to a 50% reduction of MT1-MMP gene expression compared to cells transfected with control (scrambled) siRNA or untransfected cells (Figure 4-2B). The transfected CB $CD34^+$ cells were then co-cultured with HUVEC and the co-cultures stimulated with HA or thrombin. We found using zymography that, media conditioned by co-cultures of CB $CD34^+$ cells transfected with MT1-MMP

siRNA did not show distinct active MMP-2 bands (Figure 4-2C), indicating that expression of MT1-MMP on $CD34^+$ cells is required for proMMP-2 activation.

To examine whether the upregulated MT1-MMP expression in CB $CD34^+$ cells translates to their enhanced migration, we performed trans-Matrigel migration assays. We found that, HA and thrombin significantly increased (p = 0.007 and 0.008, respectively) trans-Matrigel chemoinvasion of CB $CD34^+$ cells towards a low SDF-1 gradient (20 ng/ml) which was comparable to chemoinvasion of unstimulated cells migrating towards a high SDF-1 gradient (100 ng/ml) (Figure 4-2D). Moreover, the priming effects of HA and thrombin in the chemoinvasion of CB $CD34^+$ cells towards an SDF-1 gradient were significantly abrogated (p = 0.001 and 0.002, respectively) by the potent MT1-MMP inhibitor EGCG (Figure 4-2D). Furthermore, chemoinvasion of CB $CD34^+$ cells transfected with MT1-MMP siRNA was significantly lower (HA: p = 0.002, thrombin: p = 0.003) than of those cells transfected with scrambled (control) siRNA (Figure 4-2E).

4.4.3 HA and thrombin-induced MT1-MMP upregulation is PI3K-dependent

Next we investigated the intracellular signaling pathways activated by HA and thrombin. We found that HA and thrombin induced phosphorylation of AKT (Ser476) and p44/42 MAPK within 5 min (Figures 4-3A and 4-3B) in U937 cells. To determine whether MT1-MMP is regulated by these signaling pathways, we preincubated CB MNC with the PI3K inhibitor LY290042 and the p44/p42 MAPK inhibitor PD98059 prior to HA or thrombin treatment. We found that upregulation of MT1-MMP by HA or thrombin was abrogated by LY290042

whereas PD98059 had no effect (Figure 4-3C), indicating that HA or thombin-induced upregulation of MT1-MMP is PI3K-dependent. Moreover, zymographic analysis of media conditioned by co-cultures of CB $CD34^+$ cells with HUVEC pretreated with LY290042 before HA or thrombin stimulation, showed reduced level of active MMP-2 (Figure 4-3D), indicating that activation of proMMP-2 is also PI3K-dependent.

MT1-MMP has been shown to associate with F-actin, which is necessary for localization of MT1-MMP to the migration front [19]. To determine the subcellular localization of MT1-MMP after HA stimulation, CB $CD34^+$ cells stimulated with HA were plated on fibronectin-coated coverslips, stained with anti-MT1-MMP antibody and F-actin, and evaluated using confocal microscopy. We found that upon stimulation with HA, MT1-MMP co-localized with F-actin in the lamellipodia; moreover, this co-localization was attenuated by LY290042 but unaffected by PD98059 (Figure 4-3E). PI3K inhibition by LY290042 also resulted in significantly reduced (HA: $p = 0.002$, thrombin: $p = 0.007$) trans-Matrigel chemoinvasion of CB $CD34^+$ cells towards SDF-1 (Figure 4-3F).

4.4.4 Intracellular crosstalk between signaling pathways

We previously showed that components of SLP (fibrinogen and fibronectin) activate Rac-1 resulting in enhanced responsiveness of hematopoietic cells towards an SDF-1 gradient [11]. Here we found that HA and thrombin also activate Rac-1 in U937 cells and furthermore, this activation is PI3K-dependent (Figure 4-4A). U937 cells preincubated with LY290042 prior to HA or thrombin stimulation showed reduced Rac-1 activation, whereas preincubation with

PD98059 had no effect (Figure 4-4A). It has been reported that Rac can in turn activate PI3K [27], and indeed we observed that U937 cells preincubated with the Rac-1 inhibitor NSC23766 attenuated P-AKT phosphorylation (Figure 4-4B) while having no effect on phosphorylation of MAPK (Figure 4-4C), indicating a crosstalk between PI3K and Rac-1 signaling pathways.

4.4.5 HA- and thrombin-induced MT1-MMP upregulation is Rac-1-dependent

Next we determined whether MT1-MMP expression and function are modulated by Rac-1. We found that in CB MNC upregulation of MT1-MMP by HA or thrombin is abrogated by the Rac-1 inhibitor NSC23766 (Figure 4-5A), indicating that HA- or thrombin-induced upregulation of MT1-MMP is Rac-1-dependent. Moreover, zymographic analysis of conditioned media from co-cultures of CB $CD34^+$ cells with HUVEC pretreated with NSC23766 before HA or thrombin stimulation showed reduced levels of active MMP-2 (Figure 4-5B), suggesting that activation of proMMP-2 is also Rac-1-dependent. Moreover, treatment of CB $CD34^+$ cells with NSC23766 attenuated co-localization of MT1-MMP with F-actin (Figure 4-5C) and significantly reduced (HA: $p = 0.002$, thrombin: $p = 0.006$) their trans-Matrigel chemoinvasion towards SDF-1 (Figure 4-5D).

4.5 Discussion

A major concern in CB transplantation is the limited number of HSPC available in a single CB unit, and as a consequence, HSPC engraftment and

hematopoietic recovery are significantly delayed [1]. Several strategies such as ex vivo expansion of CB HSPC before transplantation, transplantation with more than one CB unit, and intramedullary injection of CB HSPC have so far been tested, all with limited success [2]. We previously demonstrated that SLP or their components such as PMV or C3a anaphylatoxin primed chemotactic responses of HSPC to SDF-1 which significantly improved HSPC homing in a murine transplant model [11]. Here we provide new evidence that two other components of SLP, namely HA and thrombin, prime homing-related responses of HSPC towards SDF-1 by upregulating MT1-MMP expression. This in turn leads to proMMP-2 activation and increased chemoinvasion towards SDF-1, processes which are both PI3K- and Rac-1-dependent.

First we demonstrated that HA and thrombin upregulate MT1-MMP in CB HSPC at both the mRNA and protein levels. Then we showed that MT1-MMP is involved in homing-related responses of CB HSPC. Earlier we proposed that SDF-1 could facilitate HSPC homing to the BM by stimulating the secretion of MMP-2 and MMP-9 [9]. In this work we demonstrated that although CB $CD34^+$ cells themselves do not secrete proMMP-2, when they are co-cultured with endothelial cells, active MMP-2 can be detected. Furthermore, we found that this activation is strongly upregulated by HA and thrombin, suggesting that high MT1-MMP expression on CB $CD34^+$ cells could be responsible for activation of proMMP-2 secreted by HUVEC. This was confirmed when we found that in co-cultures of HUVEC with CB $CD34^+$ cells that had been transfected with MT1-MMP siRNA, active MMP-2 was not detectable. Activation of proMMP-2 is

important not only because active MMP-2 plays a crucial role in matrix remodeling but also because it initiates a cascade of activation of other MMPs including MMP-9 [16].

Moreover, MT1-MMP itself plays an active role in cell migration by exhibiting pericellular proteolysis [18]. Previously we showed that MT1-MMP mediates trans-Matrigel chemoinvasion of CB $CD34^+$ cells and megakaryocytic progenitors [24] and here we provide further evidence of MT1-MMP's role in CB HSPC migration by demonstrating that specific inhibition of MT1-MMP by siRNA significantly abrogated their migration across reconstituted basement membrane. This is consistent with previous studies where MT1-MMP was reported to be required for endothelial transmigration of monocytes [22]. MT1-MMP inhibition by monoclonal antibody has been shown to attenuate human HSPC homing in a NOD/SCID mouse model and homing of MT1-$MMP^{-/-}$ c-Kit^+ cells has been demonstrated to be lower than that of wild type cells in a chimeric mouse model [25].

Cell migration towards a chemotactic gradient requires reorganization of the cytoskeleton and F-actin polymerization leading to cell polarization and lamellipodia formation in the direction of the signal [26]. MT1-MMP has been shown to be localized in specialized F-actin-rich domains such as invadipodia in migrating tumor cells, and lamellipodia in endothelial cells and monocytes [18,22]. Consistent with this, upon HA stimulation we observed co-localization of MT1-MMP and F-actin in membrane protrusions of CB $CD34^+$ cells. It has been suggested that in tumor cells MT1-MMP associates with CD44, which in turn is

associated with F-actin and the cytoskeleton [20]. Moreover, in $CD34^+$ cells SDF-1 was shown to promote CD44 binding to immobilized HA leading to the formation of membrane protrusions where CD44 co-localized with F-actin and facilitated trans-endothelial migration and homing of HSPC [12]. Although the molecular mechanisms of this phenomenon are not clear, the investigators suggested that CD44 binds to MMPs and subsequent shedding of CD44 enables cell detachment from the substrate and facilitates HSPC migration. Interestingly, it was recently reported that shedding of CD44 by MT1-MMP in HSPC is required for their detachment from BM niches and G-CSF induced mobilization [25].

One of the important F-actin regulators is the PI3K-AKT axis which has been demonstrated to regulate mRNA as well as protein expression of MT1-MMP and proMMP-2 activation in highly invasive Lewis lung carcinoma H-59 cells and endothelial cells [36,37]. Moreover, MT1-MMP induction and invasion of H-59 cells were abrogated by the PI3K-AKT axis inhibitors wortmannin and rapamycin but not by MAPK inhibitor PD98059 [36]. Consistent with this, we demonstrated that although HA and thrombin activated both PI3K and MAPK, MT1-MMP expression and proMMP-2 activation were only PI3K-dependent, same as the chemoinvasion of $CD34^+$ cells. Another important regulator of F-actin polymerization is Rac-1 GTPase which has also been shown to modulate MT1-MMP expression and proMMP-2 activation in tumor cells [38,39]. It has been demonstrated that Rac-1 promotes the homophilic complex formation of MT1-MMP (which is critical for proMMP-2 activation) at the lamellipodia and

promotes cell migration [39]. In agreement with this we showed that HA and thrombin activated Rac-1 GTPase which modulated MT1-MMP expression and proMMP-2 activation in hematopoietic cells and increased their chemoinvasion.

It has also been suggested that intracellular crosstalk and positive feedback loops between PI3K and Rac-1 GTPase signaling pathways during chemotaxis lead to localized signal amplification which in turn results in enhanced F-actin polymerization and cell migration towards the chemotaxis gradient [27,28,40,41]. During chemotaxis, chemokine receptors such as CXCR4 sense the spatial distribution of their ligands, in this case SDF-1, and the information is transduced in the cell to initiate signaling events for polarized actin assembly. PI3K and Rac play important roles in signal transduction from chemokine receptors to induce actin polymerization. Activation of PI3K leads to recruitment of its substrate PIP3 at the cell surface. PIP3 has been shown to regulate actin polymerization through Rac [27,41], which induces lamellipodia formation. Rac in turn enhances accumulation of PIP3 at the leading edge thus forming a positive feedback loop. Accordingly, here we demonstrated that in hematopoietic cells, both PI3K and Rac-1 GTPase pathways are activated by HA and thrombin. Moreover, we showed that intracellular crosstalk occurs between these signaling pathways since inhibition of PI3K by LY290042 attenuated Rac-1 activation, and inhibition of Rac-1 GTPase by NSC23766 attenuated phosphorylation of AKT. Based on our results we suggest that HA and thrombin cooperate with the SDF-1−CXCR4 axis by activating PI3K and Rac-1, thus allowing signal amplification of a weak SDF-1 gradient. This signal amplification increases MT1-MMP cell surface expression

and proMMP-2 activation and ultimately enhances chemoinvasion of HSPC towards a low SDF-1 gradient.

In summary, we present evidence that HA and thrombin activate PI3K and Rac-1 signaling pathways, and intracellular crosstalk between these pathways leads to signal amplification, reorganization of the cytoskeleton, and recruitment of MT1-MMP to the cell surface where several functions are elicited including proMMP-2 activation and increased chemoinvasion towards SDF-1 (Figure 4-6). This could lead to enhanced homing-related responses of HSPC and could speed up their engraftment. However, further in vivo studies in murine models are warranted before clinical trials using ex vivo-primed CB grafts with HA or thrombin are initiated.

4.6 References

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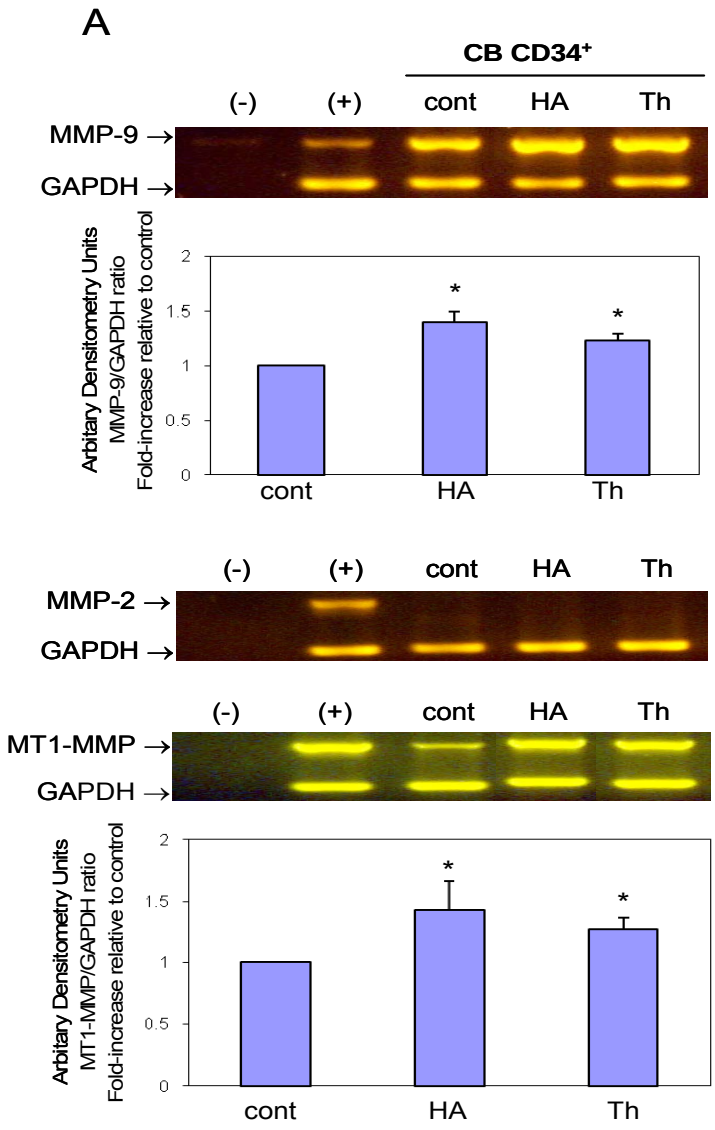

Figure 4-1. HA and thrombin upregulate MMP-9 and MT1-MMP expression in CB HSPC. (A) RT-PCR analysis of MMP-9, MMP-2 and MT1-MMP transcript from CB $CD34^+$ cells stimulated or not (cont) for 48 h with HA or thrombin (Th). GAPDH was used as the mRNA control to ensure equivalence of loading. The HT1080 cell line was used a positive control (+) and no cDNA as negative control (-) for HGF mRNA expression.The top panel show representative data from three independent experiments. The bottom panel shows mean ± standard deviations of densitometric analysis of three experiments shown as fold-increase compared to control, $*p < 0.05$.

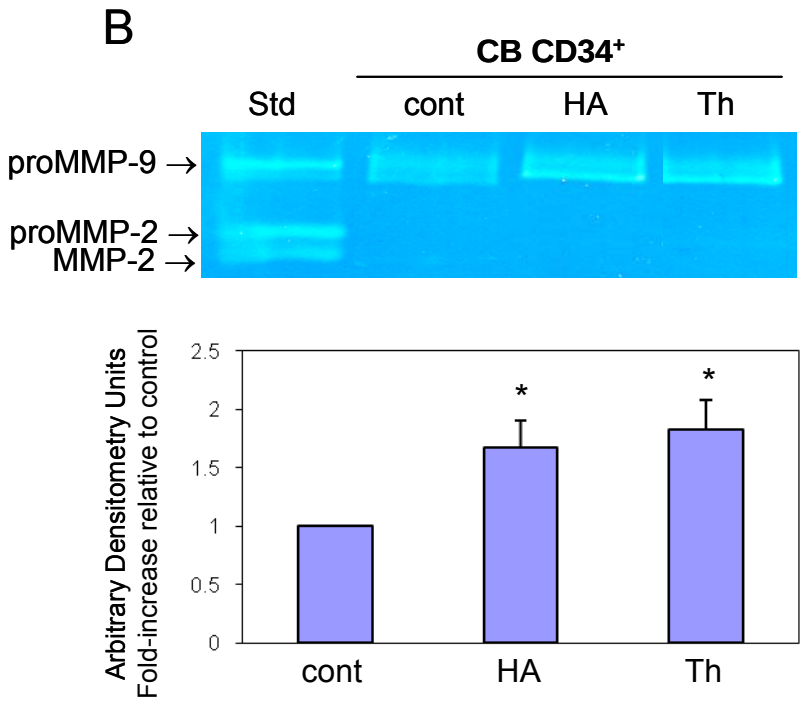

Figure 4-1. (B) Zymographic analysis of proMMP-9 expression on CB $CD34^+$ cells stimulated or not (cont) for 48 h with HA or Th. The top panel shows representative data from three independent experiments. The bottom panel shows mean ± standard deviations of densitometric analysis of three experiments shown as fold increase compared to control, $*p < 0.05$.

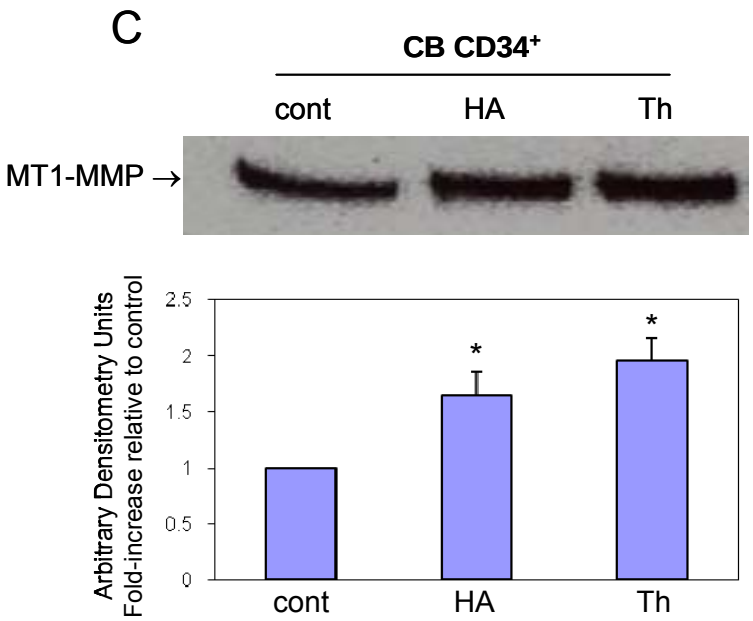

Figure 4-1. (C) Western blot analysis of MT1-MMP in CB $CD34^+$ cells stimulated or not (control) with HA or Th. Equal amounts of protein (30μg) as determined by the Bradford assay were loaded. The gel was stained with Coomassie blue to ensure equivalence of loading. The top panel shows representative data from three independent experiments. The bottom panel shows mean ± standard deviations of densitometric analysis of three experiments shown as fold-increase compared to control, $*p < 0.05$.

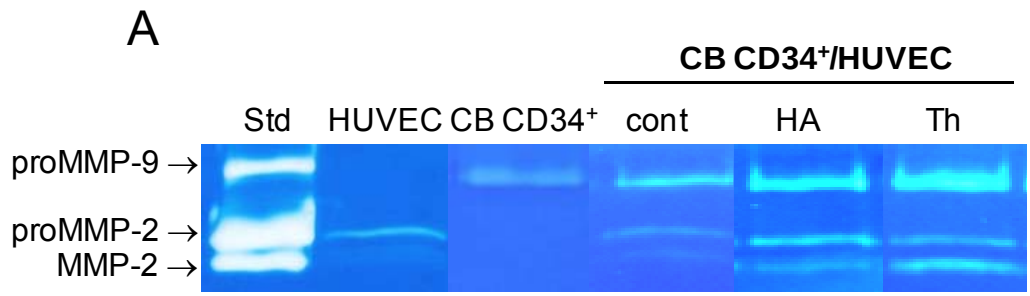

Figure 4-2. HA and thrombin upregulate proMMP-2 activation in co-cultures of CB CD34$^+$ cells with HUVEC and prime chemoinvasion of CB CD34$^+$ cells towards a low SDF-1 gradient. (A) Zymographic analysis of media conditioned by endothelial cells (HUVEC), CB CD34$^+$ cells, co-cultures of CD34$^+$ cells with HUVEC (cont), co-cultures of CD34$^+$ cells with HUVEC stimulated with HA or thrombin (Th). In media conditioned by HUVEC only proMMP-2 was found, while active MMP-2 is detectable in supernatants from co-cultures of CB CD34$^+$ cells with HUVEC stimulated with HA or Th. A representative of three independent experiments is shown.

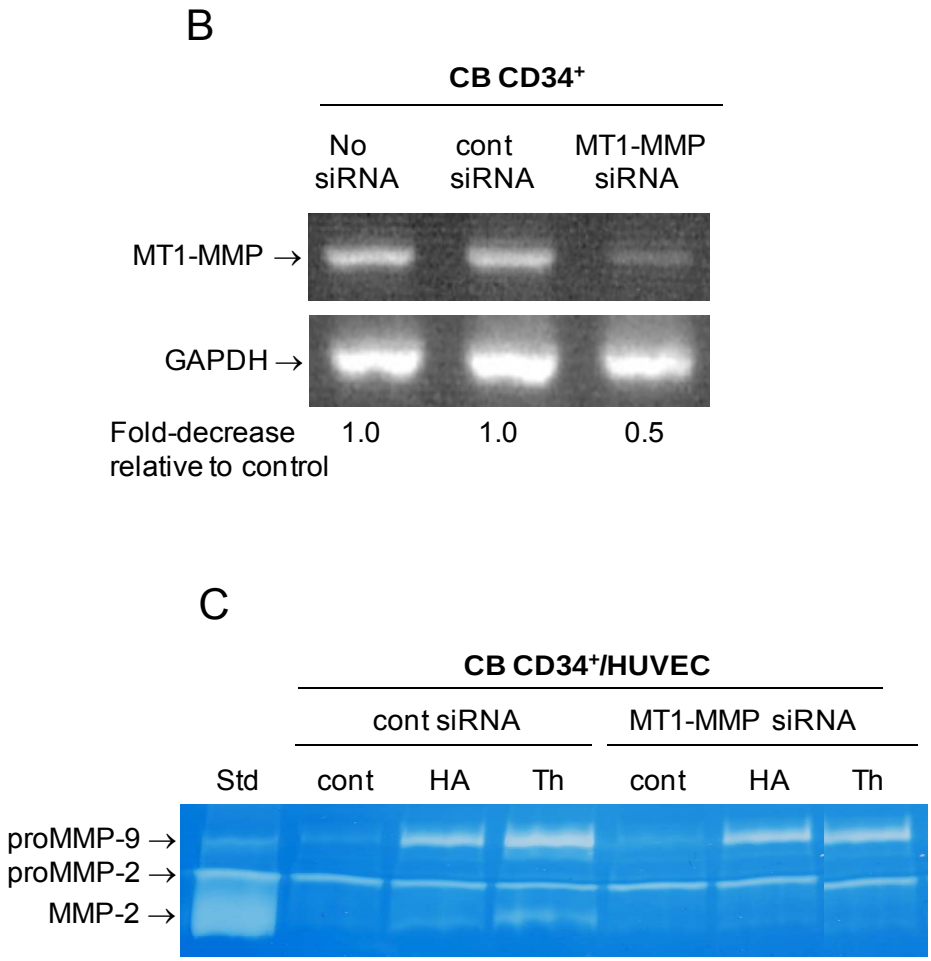

Figure 4-2. (B) RT-PCR of MT1-MMP transcripts in CB CD34$^+$ cells transfected with either scrambled siRNA (cont) or MT1-MMP siRNA. GAPDH was used as the mRNA control to ensure equivalence of loading. **(C)** Zymographic analysis of cell-conditioned media from co-cultures of HUVEC and CB CD34$^+$ cells transfected with scrambled (cont) siRNA or transfected with MT1-MMP siRNA. A representative zymogram from two independent experiments is shown.

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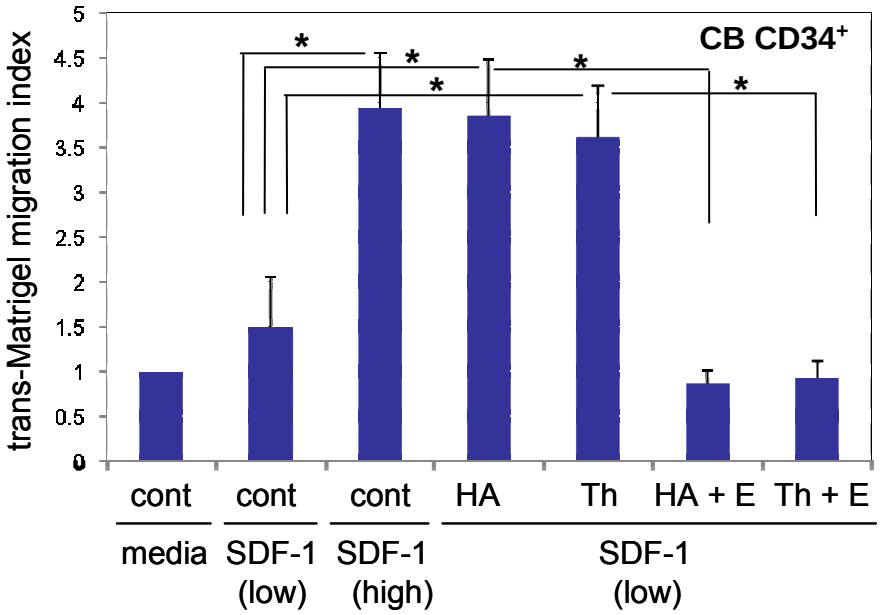


Figure 4-2. (D) Trans-Matrigel migration of CB $CD34^+$ cells towards media alone (cont), low SDF-1 (20 ng/ml), high SDF-1 (100 ng/ml), $CD34^+$ cells stimulated with HA (50 μg/ml) or thrombin (10 U/ml) towards low SDF-1 (20 ng/ml), and cells pre-incubated with MT1-MMP inhibitor EGCG (E, 50 μM) before stimulation with HA, thrombin towards low SDF-1 (20 ng/ml). Data are pooled from triplicate samples from three independent experiments, $*p < 0.05$.

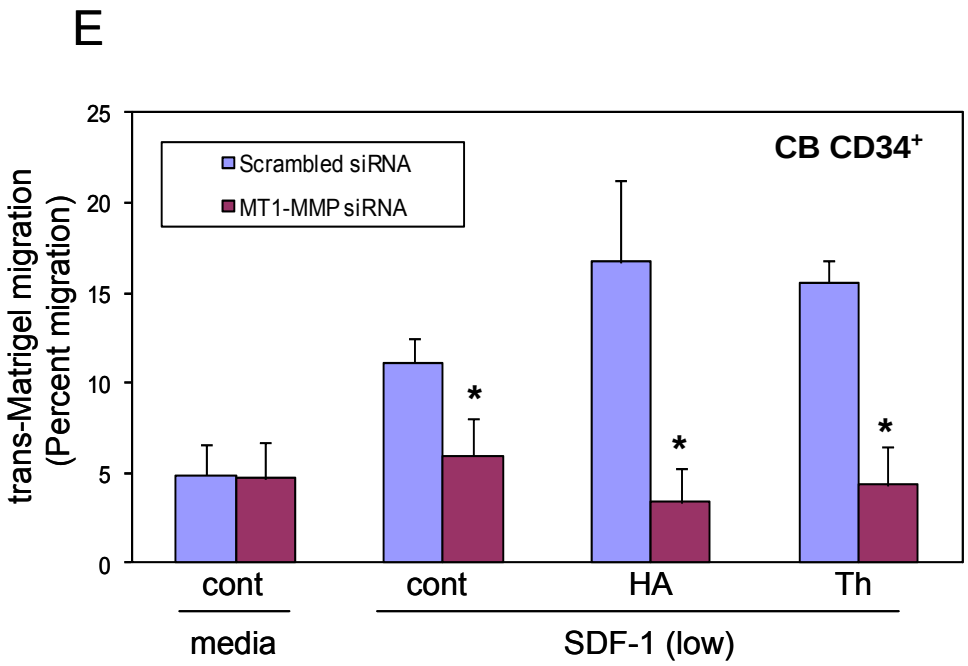


Figure 4-2 (E) CB $CD34^+$ cells were electroporated with either a scrambled siRNA or MT1-MMP siRNA. Trans-Matrigel migration towards an SDF-1 gradient was significantly inhibited in the MT1-MMP siRNA transfected cells ($p < 0.05$). The assays were performed at least in quadruplicate for each condition. A representative of two separate experiments is shown.

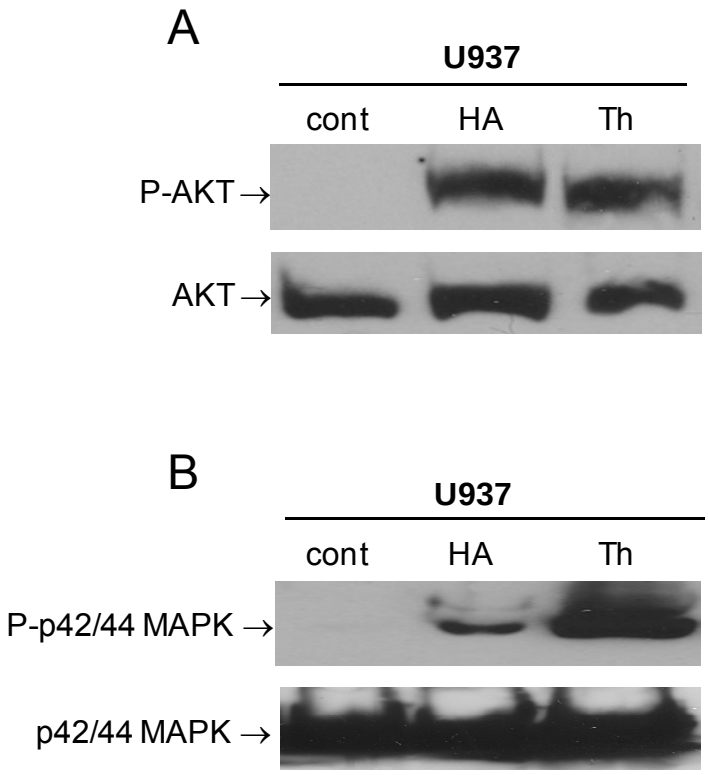


Figure 4-3. MT1-MMP upregulation by HA and thrombin is PI3K-dependent. (A and B) Phosphorylation of AKT and p44/42 MAPK. U937 cells were serum-starved overnight and then stimulated with 50 μg/ml HA or 10 U/ml thrombin (Th) for 5 min. A representative Western blot from two independent experiments is shown.

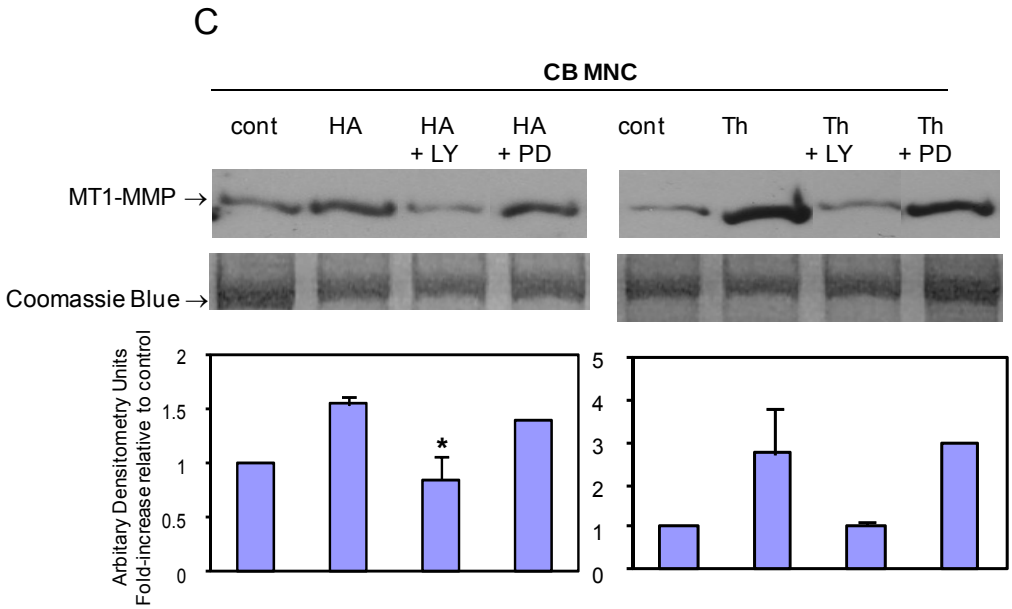


Figure 4-3. (C) MT1-MMP expression is PI3K-dependent. Top panel: Western blot analysis of MT1-MMP expression in CB MNC pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and then stimulated with 50μg/ml HA (left panel) or 10 U/ml thrombin (right panel) for 48 h. Equal amounts of protein (30μg) as determined by the Bradford assay were loaded. The gel was stained with Coomassie blue to ensure equivalence of loading. Top panel: A representative Western blot of two separate experiments is shown. Bottom panel: mean ± standard deviations of densitometric analysis of two experiments shown as fold-increase relative to control.

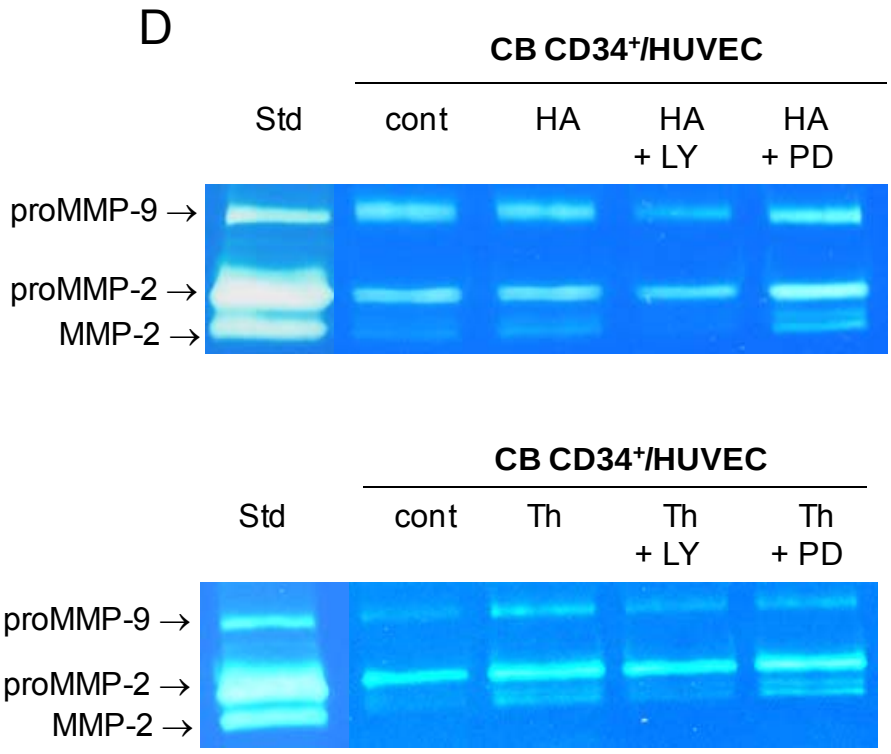


Figure 4-3. (D) ProMMP-2 activation is PI3K-dependent. Zymographic analysis of conditioned media from co-cultures of HUVEC and CB $CD34^+$ cells pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and then stimulated with 50 mg/ml HA (top panel) or 10 U/ml thrombin (bottom panel) for 48 h. A representative zymogram of two independent experiments is shown.

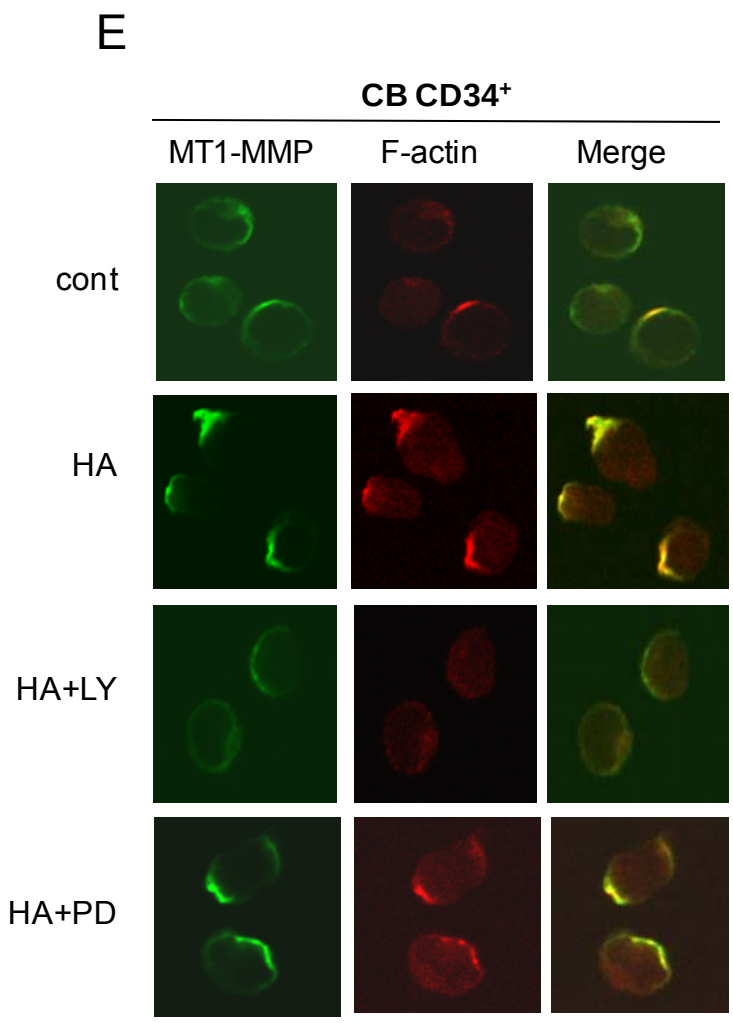


Figure 4-3. (E) CB $CD34^+$ cells were pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 1 h and then stimulated with 50 mg/ml HA for 2 h, plated on fibronectin-coated cover slips and immunoassayed with MT1-MMP (green) and F-actin (red).

F

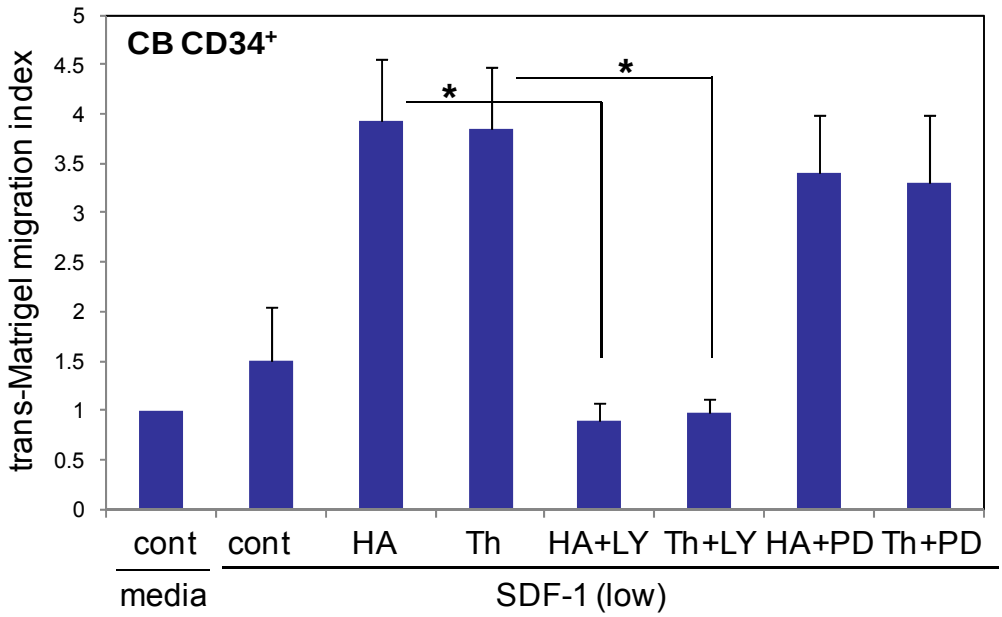


Figure 4-3. (F) Trans-Matrigel chemoinvasion of CB CD34$^+$ cells is PI3K-dependent. CB CD34$^+$ cells were pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) and stimulated with 50 μg/ml HA or 10 U/ml thrombin for 2 h before trans-Matrigel chemoinvasion towards SDF-1 low (20 ng/ml). Data are pooled from triplicate samples from three independent experiments, *$p < 0.05$.

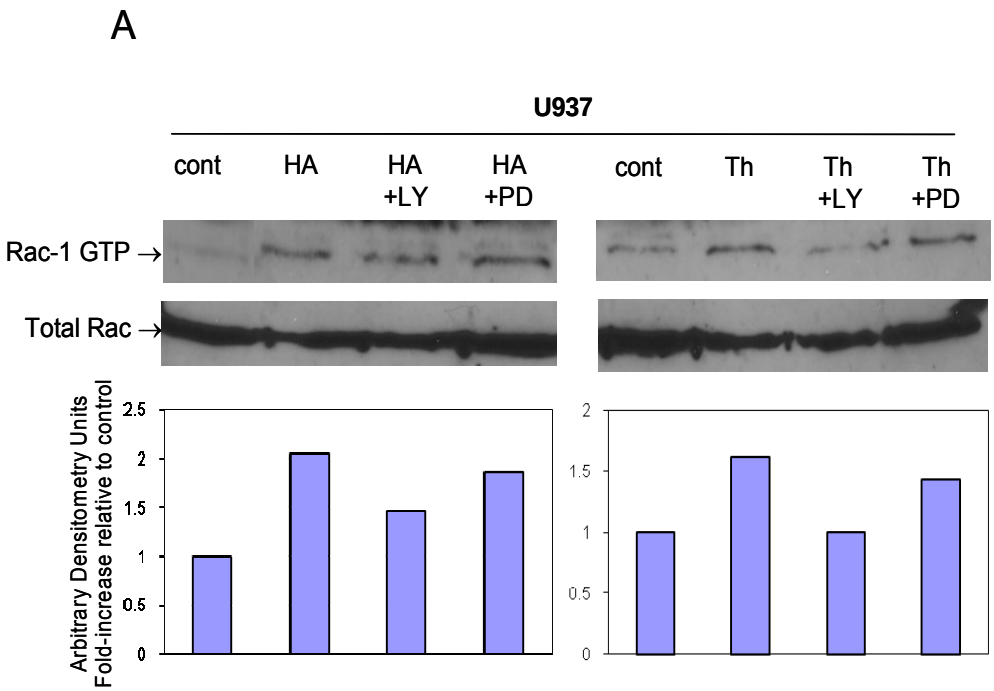


Figure 4-4. Intracellular crosstalk between signaling pathways. (A) Detection of GTP-bound (active) form of Rac-1 in U937 cells. Cells were serum-starved overnight, pretreated with 30 μM LY290042 (LY) or 50 μM PD98059 (PD) for 20 min and then stimulated with 50 mg/ml HA (left panel) or 10 U/ml thrombin (right panel) for 5 min. Top panels: activated Rac was detected by Western blot. Bottom panels: densitometric analysis shown as fold-increase relative to control. A representative Western blot of two independent experiments is shown.

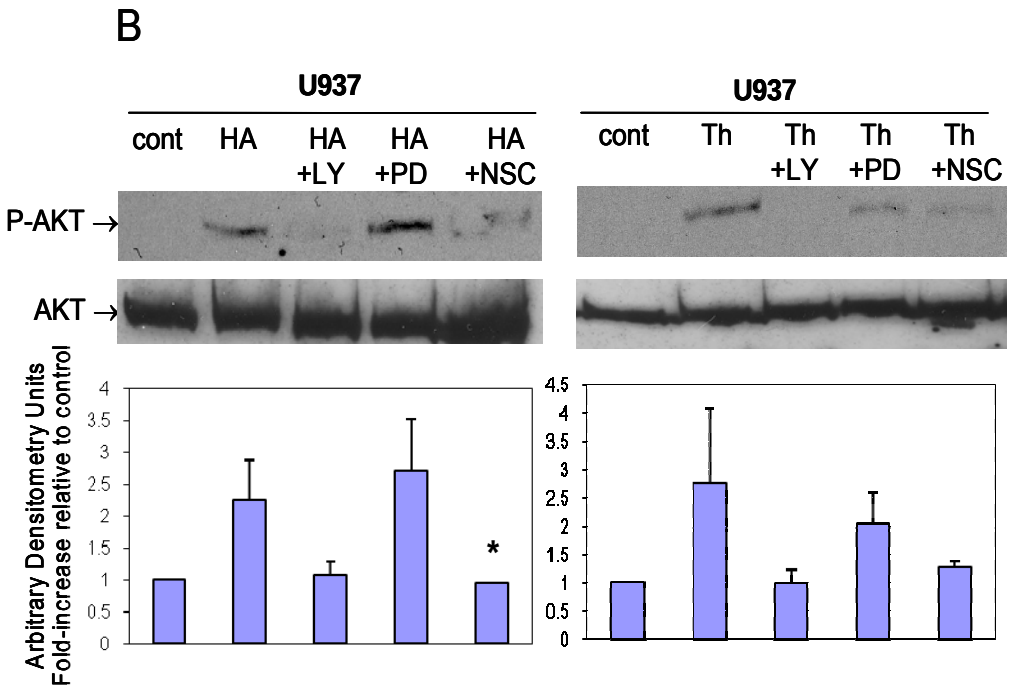


Figure 4-4. (B) Phosphorylation of AKT. U937 cells were serum-starved overnight, pretreated with 30 μM LY290042, 50 μM PD98059 or 50 μM NSC23766 (NSC) for 20 min and then stimulated with 50 mg/ml HA (left panel) or 10 U/ml thrombin (right panel) for 5 min. Top panel: a representative Western blot of two independent experiments is shown. Bottom panel: mean ± standard deviations of densitometric analysis of two experiments shown as fold-increase relative to control, $*p < 0.05$.

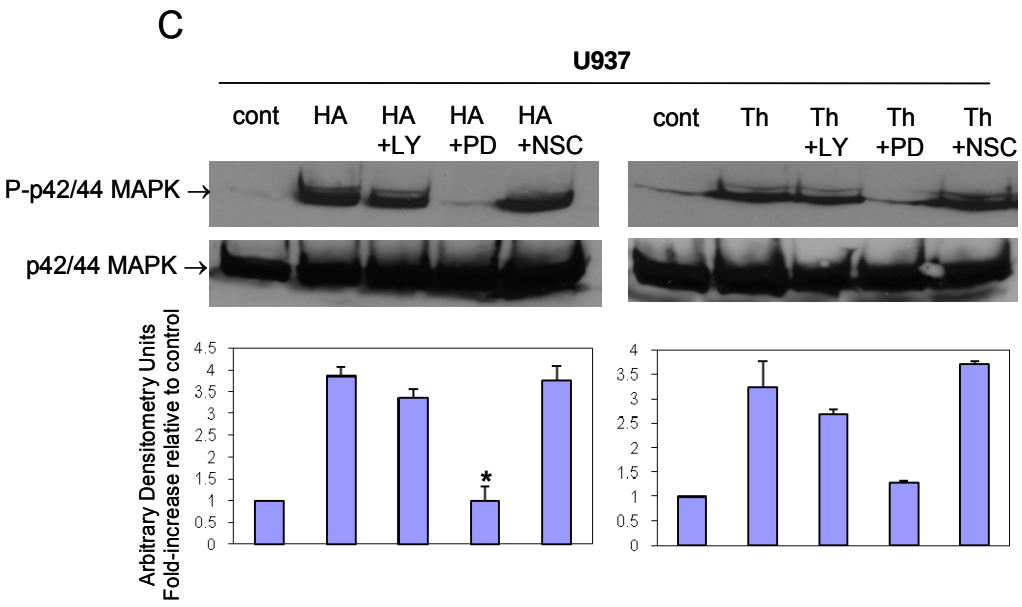


Figure 4-4. (C) Phosphorylation of p44/42MAPK. U937 cells were serum-starved overnight, pretreated with 30 μM LY290042, 50 μM PD98059 or 50 μM NSC23766 for 20 min and then stimulated with 50 mg/ml HA (left panel) or 10 U/ml thrombin (right panel) for 5 min. Top panel: a representative Western blot of two independent experiments is shown. Bottom panel: mean ± standard deviations of densitometric analysis of two experiments shown as fold-increase relative to control, $*p < 0.05$.

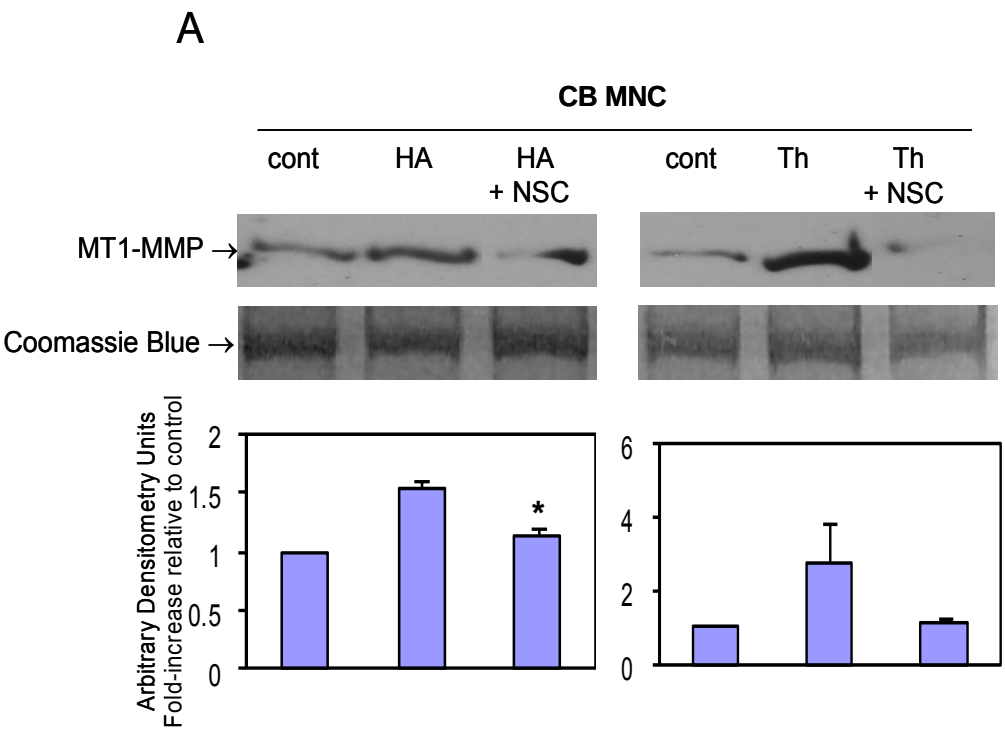


Figure 4-5. MT1-MMP upregulation by HA or thrombin is Rac-1-dependent. **(A)** MT1-MMP expression is Rac-1–dependent. Western blot analysis of MT1-MMP expression in CB MNC pretreated with 50 μM NSC23766 (NSC) for 1 h and then stimulated with 50μg/ml HA (left panel) or 10 U/ml thrombin (Th) (right panel) for 48 h. An equal amount of protein (30μg) as determined by the Bradford assay, was loaded. The gel was stained with Coomassie blue to ensure equivalence of loading. Top panel: a representative Western blot of two independent experiments is shown. Bottom panel: mean ± standard deviations of densitometric analysis of two experiments shown as fold-increase relative to control, $*p < 0.05$.

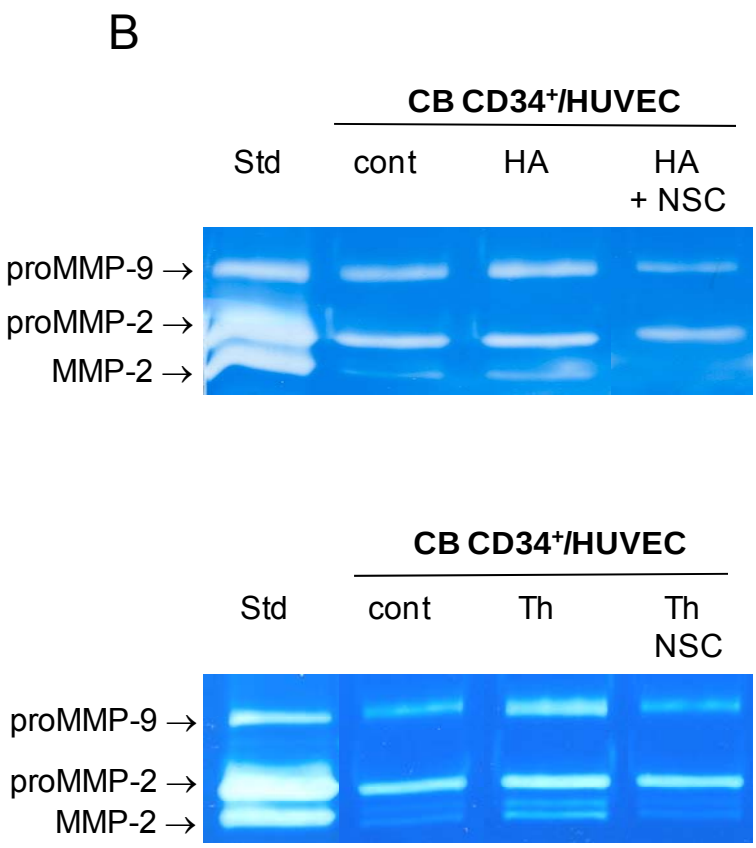


Figure 4-5. (B) ProMMP-2 activation is Rac-1-dependent. Zymographic analysis of conditioned media from co-cultures of HUVEC and CB $CD34^+$ cells pretreated with 50 μM NSC23766 for 1 h and then stimulated with 50 mg/ml HA (left panel) or 10 U/ml thrombin (right panel) for 48 h. A representative zymogram from two independent experiments is shown.

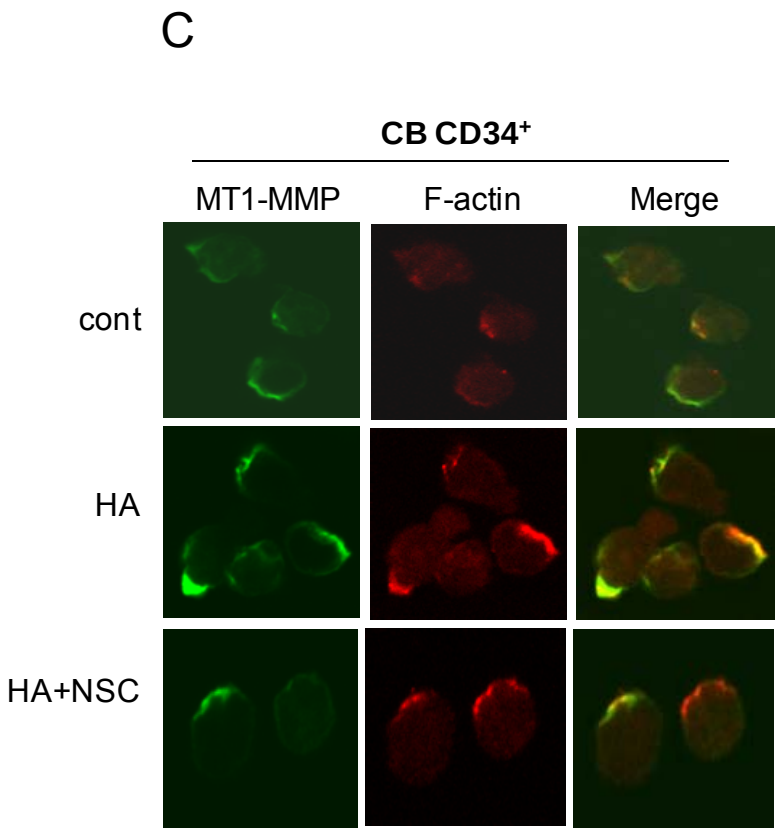


Figure 4-5. (C) CB $CD34^+$ cells were pretreated with 50 μM NSC23766 for 1 h and then stimulated with 50 mg/ml HA for 2 h, plated on fibronectin-coated cover slips and immunostained with MT1-MMP (green) and F-actin (red).

D

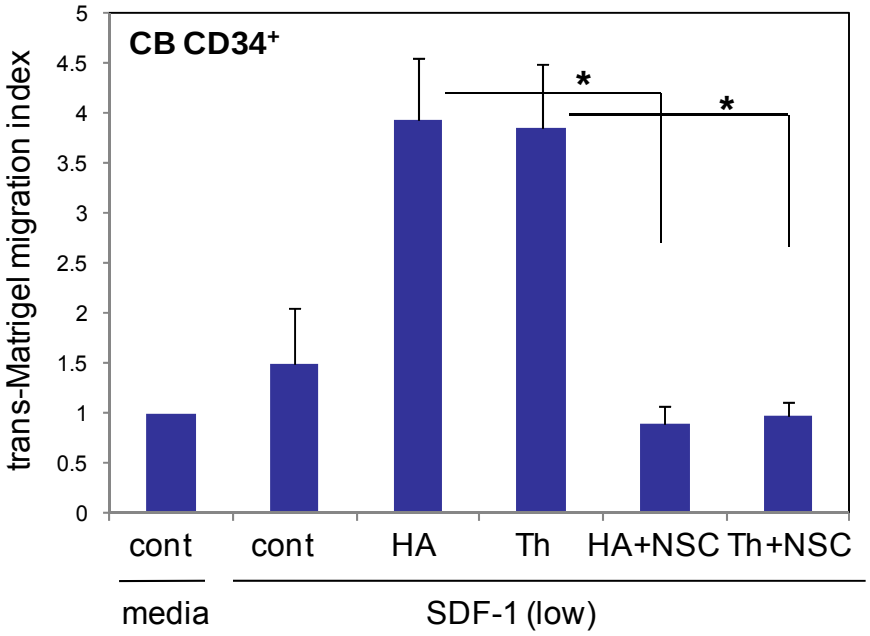
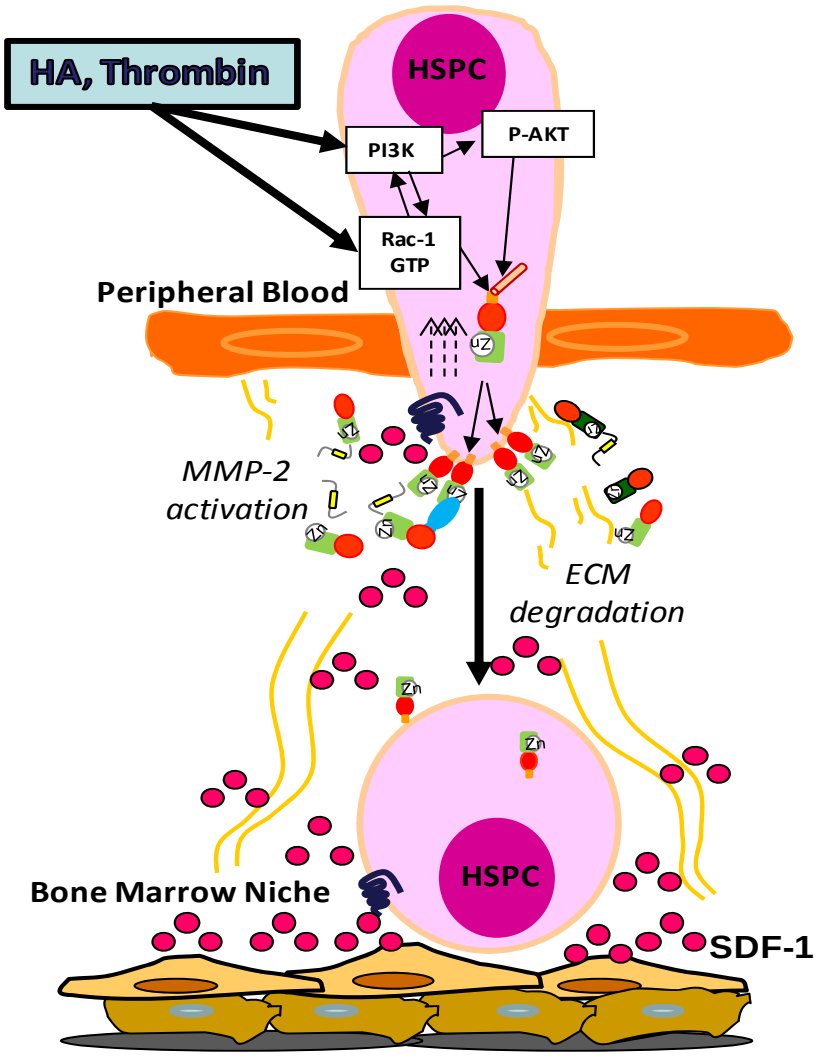


Figure 4-5. (D) Trans-Matrigel chemoinvasion of CB $CD34^+$ cells is Rac-1-dependent. CB $CD34^+$ cells were pretreated with 50 μM NSC23766 for 1 h and then stimulated with 50 μg/ml HA or 10 U/ml thrombin for 2 h before trans-Matrigel chemoinvasion towards low SDF-1 (20 ng/ml). Data are pooled from triplicate samples from three independent experiments ($p < 0.05$).


HA, Thrombin
HSPC
PI3K
P-AKT
Rac-1
GTP
Peripheral Blood
MMP-2
activation
ECM
degradation
HSPC
Bone Marrow Niche
SDF-1

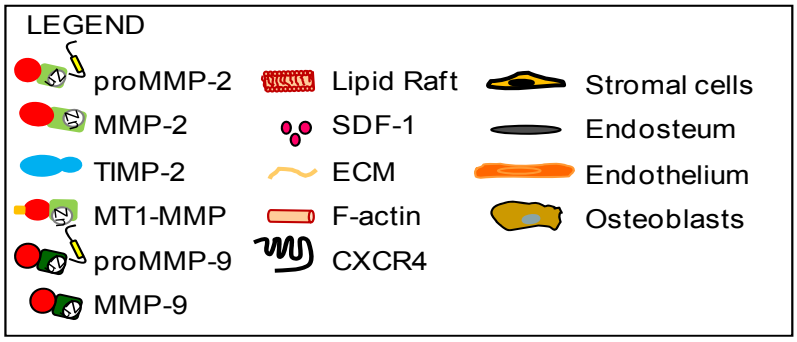

LEGEND
proMMP-2
MMP-2
TIMP-2
MT1-MMP
proMMP-9
MMP-9
Lipid Raft
SDF-1
ECM
F-actin
CXCR4
Stromal cells
Endosteum
Endothelium
Osteoblasts

Figure 4-6. Schema of MT-MMP localization and regulation in HSPC during HA- and thrombin-primed HSPC homing. Priming molecules HA and thrombin (Th) activate PI3K-AKT and Rac-1 signaling pathways in HSPC which leads to incorporation of CXCR4 and Rac-1 GTPase into membrane lipid rafts. Intracellular crosstalk between PI3K and Rac-1 signaling pathways leads to accumulation of F-actin and MT1-MMP to the cell surface. MT1-MMP at the migration front exhibits pericellular proteolysis by degrading the ECM, shedding cell surface adhesion molecules such as CD44, and activating proMMP-2 secreted by endothelial cells. Activation of pro-MMP-2 leads to an activation cascade of other MMPs including MMP-9 which leads to enhanced matrix remodeling and chemoinvasion of $CD34^+$ cells towards SDF-1 and conceivably their homing to BM niches.

Chapter 5 General discussion and future directions

HSPC transplantation has been used in the treatment of various malignant and non-malignant hematological and non-hematological disorders (Table 1-1); however, challenges still remain. Although G-CSF-mobilized HSPC are used for majority (> 90%) of transplantations, significant numbers of patients/donors (5-40%) either mobilize poorly or fail to mobilize [1-3]. On the other hand, the use of CB as a source of HSPC for transplantation is limited due to delayed engraftment [4,5]. While considerable progress has been made in elucidating the mechanisms of HSPC mobilization and homing, the basic biology of these processes needs further clarification.

Trafficking of HSPC from the BM to circulation and homing back to the BM requires their extravasation through endothelial and basement membrane barriers, and migration through extracellular matrix (ECM), a process which is facilitated by several molecules including MMP-2 and MMP-9 [6-8]. These MMPs are secreted in their latent form and require activation for them to become functional. This thesis focuses on the role of MT1-MMP, a protease involved in activation of proMMP-2 during mobilization and homing of HSPC.

In Part I (Chapter 3), the evidence for MT1-MMP's involvement in mobilization is presented. First, I observed that both mature leukocytes as well as immature $CD34^+$ cells from in vivo G-CSF-mobilized (m) PB highly expressed MT1-MMP compared to their steady-state (ss) BM and ssPB counterparts (Figure 3-1A-C). Furthermore, cell surface expression of MT1-MMP in in vivo-mobilized polymorphonuclear cells (PMN) and monocytes was 8 and 12 times higher than in

their respective ssPB counterparts (Figure 3-1C). Upregulated MT1-MMP in PMN is important since PMN are known to be essential for mobilization, as neutropenic mice do not mobilize [9]. Furthermore, release of granulocytes from the BM always precedes mobilization of HSPC as shown in murine models [10,11]. Aside from stimulating MT1-MMP, G-CSF exerts its effects on other signaling axes that also result in upregulation of MT1-MMP. For example, we observed that hepatocyte growth factor (HGF) increases in the plasma of mobilized patients, and that G-CSF, through induction of HGF, upregulates MT1-MMP expression in BM PMN [12]. In addition, activation of the complement cascade (CC) by G-CSF triggers a series of reactions generating various bioactive peptides including the C5 cleavage fragments (C5a and $_{desArg}$C5a) which have been shown to play an important role in mobilization [11,13]. In particular, we recently demonstrated that C5a increases MT1-MMP expression in PMN and mononuclear cells (MNC), contributing to a highly proteolytic microenvironment that is conducive to the egress of HSPC from the BM [14]. MT1-MMP causes pericellular degradation by processing several ECM components (such as gelatin, fibronectin, laminin, vitronectin and fibrillar collagens) [15,16]. This is further substantiated by my observation that G-CSF-induced migration of MNC and HSPC through the reconstituted basement membrane Matrigel (containing collagen type IV, laminin, entactin) is MT1-MMP-dependent (Figure 3-3C and 3-3F).

The next evidence of the role of MT1-MMP in the BM microenvironment derives from observation of co-cultures of HSPC and stromal cells stimulated by

G-CSF. MT1-MMP expressed by HSPC activated proMMP-2 secreted by BM stromal cells (Figures 3-3A and 3-3D). Active MMP-2 could subsequently initiate a cascade of activation of other MMPs including MMP-9 and MMP-13 [17], which not only degrade the extracellular matrix (ECM) but also disrupt adhesive interactions between HSPC and their niches [6]. One of the most potent of these interactions is that of SDF-1, produced by stromal cells and osteoblasts, and its receptor CXCR4, expressed by HSPC. Recently, we demonstrated that C5a, apart from upregulating MT1-MMP, decreases CXCR4 expression in PMN, which disrupts their chemotaxis towards SDF-1. Moreover, this impaired chemoattraction is partially restored by the potent MT1-MMP inhibitor EGCG (which also inhibits proMMP-2 activation, Figure 3-3B), suggesting that MT1-MMP contributes towards reduced retention of HSPC in the BM microenvironment [14].

Additionally, reduced retention of HSPC in the BM during G-CSF-induced mobilization is also due to a decrease in active SDF-1 in the BM which coincides with peak proteolytic activity [6]. SDF-1 can be cleaved and inactivated by several proteases which are activated during mobilization such as neutrophil elastase (NE), cathepsin G (CG), MMP-2 and MMP-9, CD26, carboxypeptidase M (CPM) and MT1-MMP [18-22]. Nevertheless, SDF-1 levels are decreased in mice deficient in MMP-9, NE and CG, suggesting that either there is redundancy in their proteolytic activity or they are not necessary for mobilization [23]. However, a recent report showed that in a chimeric mouse model, mobilization of MT1-MMP$^{-/-}$ c-kit^{+} cells was very low compared to wild-type cells [24].

Furthermore, administration of anti-MT1-MMP antibody in NOD/SCID mice abrogated mobilization of human HSPC [24], underscoring the important role of MT1-MMP in HSPC mobilization and the fact that other proteases are involved in this process.

A low SDF-1 level in the BM during cytokine-induced mobilization has been suggested to result from suppression of SDF-1-producing osteoblasts and although mechanism of this phenomenon is not completely understood [25,26], it has been suggested to occur in light of an observed increase in osteoclast activity [27]. Active bone remodeling takes place during G-CSF mobilization, and increased cathepsin K and MMP-9 secretion by osteoclasts has been implicated in high bone turnover [27,28]. Similarly, MT1-MMP also plays an important role in bone remodeling as shown by the fact that MT1-MMP$^{-/-}$ mice exhibit severe skeletal defects [29]. MT1-MMP is also known to be expressed by osteoclasts [30] and recently it was demonstrated that MT1-MMP was necessary for macrophage fusion during multinucleated osteoclast formation and differentiation [31]. Moreover, MT1-MMP, independently of its catalytic activity, associated with adapter protein p130Cas through its cytoplasmic tail, and regulated Rac-1 signaling at the cell surface, thereby facilitating macrophage motility and fusion during osteoclastogenesis [31]. Accordingly, 8-day-old MT1-MMP$^{-/-}$ mice exhibited impaired osteoclast function, which did not however,result in increased bone mass since osteoblast function is also compromised in these mice [29,31]. These results suggest that MT1-MMP could have a dual role in bone development

[31]. Nevertheless, the role of MT1-MMP in bone remodeling during G-CSF-induced mobilization requires further investigation.

Furthermore, I present evidence of the subcellular localization of MT1-MMP and the regulation of its activities during mobilization. Several studies have shown that in migrating cells MT1-MMP associates with F-actin and is relocalized to lamellipodia and invadipodia [32,33]. Accordingly, I demonstrated that in HSPC MT1-MMP associates with F-actin at the cell surface upon stimulation with G-CSF (Figure 3-4C). Accumulating evidence also suggests that relocalization of MT1-MMP to the sites where ECM degradation takes place involves a complex interplay between endocytic and exocytic mechanisms [34]. In resting cells, MT1-MMP expression on the cell surface is low, as I showed for steady-state BM cells, because it is quickly endocytosed [34]. Endocytosis of MT1-MMP is mediated through either clathrin-coated pits or caveolae [33-35].

Caveolar expression of MT1-MMP has been demonstrated in tumor and endothelial cells and, moreover, transient and regulated association of MT1-MMP in these specialized membrane domains has been shown to be important for its function and activities during invasion and cell migration [36,37]. Accordingly, in this study, I demonstrated that MT1-MMP associates with lipid rafts in hematopoietic cells, and furthermore this association is regulated by the PI3K-AKT axis that leads to proMMP-2 activation and enhanced HSPC migration (Figures 3-4 and 3-6). Association of MT1-MMP with caveolae and lipid rafts has been demonstrated to increase the invasiveness of PC-3 prostate tumor cells, and it has been suggested that this could be a mechanism to localize MT1-MMP in

contact with other pericellular proteins and the ECM [38]. MT1-MMP and caveolin-1 are required for invadipodia formation, with caveolin-1 regulating the transport of lipid raft-associated MT1-MMP at the invadipodia [39]. MT1-MMP recruitment to the invadipodia has also been demonstrated to be regulated through the lipid raft-associated vesicular SNARE Ti-VAMP [40]. The molecular mechanisms of MT1-MMP association with lipid rafts in hematopoietic cells thus warrant further investigation, especially since these mechanisms could also be mediating the trafficking of other lipid raft-resident proteases such as the GPI-anchored CD26 and CPM that are important in mobilization, as we and others have shown [21,22].

To fully understand the significance of MT1-MMP in HSPC mobilization, in vivo studies in MT1-MMP$^{-/-}$ mice need to be carried out. MT1-MMP deficiency in mice, although not embryonically lethal, causes multiple developmental defects including impaired growth, angiogenesis and bone turnover, and results in death at a very early age [29]. Hence, in vivo mobilization experiments in these mice are not possible; however, future experiments with conditional knock-out mice could provide further insights into MT1-MMP function and its regulation during G-CSF-induced HSPC mobilization.

In Part II (Chapter 4), I present evidence of the role of MT1-MMP in the homing of CB HSPC. Previously, we demonstrated that HSPC primed by supernatants of leukapheresis products (SLP) or their components [fibrinogen, fibronectin, complement C3a, platelet-derived microvesicles (PMV), hyaluronic acid (HA), thrombin, C1q] respond better to an SDF-1 gradient [41-44].

Furthermore, murine HSPC that have been primed with C3a, PMV or SLP before transplantation engrafted faster in lethally irradiated mice [41-43]. This priming effect was due to increased incorporation of CXCR4 and Rac-1 GTPase into membrane lipid rafts and to increased MMP-2 and MMP-9 secretion, indicating that the SDF-1−CXCR4 axis cooperates with MMPs during HSPC homing [41]. In this thesis, the effects of two priming molecules, HA and thrombin, on the modulation of MT1-MMP expression and its activity were investigated.

First, I present evidence that HA and thrombin upregulated MT1-MMP in CB HSPC (Figure 4-1A and 4-1C). Secondly, I showed that HA- and thrombin-primed chemoinvasion of HSPC towards a low SDF-1 gradient was MT1-MMP-dependent (Figures 4-2D and 4-2E). This is consistent with our previous findings in which we demonstrated that SDF-1 upregulates MT1-MMP in CB HSPC and megakaryocytic progenitors, and promotes their homing-related responses [45], and with a recent study where inhibition of MT1-MMP abrogated homing of human HSPC in a NOD/SCID mouse model [24]. It has been established that the SDF-1−CXCR4 axis promotes the chemotaxis not only of normal but also of tumor cells [46,47]. Recently, it was reported that the coordinated interaction of CXCR4 and MT1-MMP is required for melanoma cell metastasis to lungs [48]. CXCR4 was required for the initial phases of melanoma cell chemotaxis and their arrival in the lungs, whereas MT1-MMP was necessary for subsequent invasion and dissemination of the tumor [48]. We can therefore speculate that a coordinated interaction between CXCR4 and MT1-MMP is also required by HSPC for their homing to the BM.

Casting more light on the function of MT1-MMP in the BM microenvironment during HSPC homing, I present evidence from co-cultures showing that MT1-MMP expressed in CB HSPC (upregulated by HA and thrombin), activated proMMP-2 secreted by endothelial cells (Figures 4-2A and 4-2C). Active MMP-2 could thereby participate in the extravasation process and facilitate homing by activating other MMPs and degrading ECM barriers. In this respect, MT1-MMP has been shown to facilitate trans-endothelial migration of monocytes through clustering of MT1-MMP at the lamellipodia upon contact with activated endothelial cells or the immobilized endothelial ligands VCAM-1 and ICAM-1 [49].

Furthermore, I investigated the molecular mechanisms of MT1-MMP regulation and priming of homing-related responses in CB HSPC and showed that HA and thrombin activated the PI3K-AKT signaling axis and Rac-1 GTPase (Figures 4-3A and 4-4A). MT1-MMP expression and proMMP-2 activation were also dependent on both these signaling pathways (Figures 4-3C and 4-5A). Recently it was demonstrated that in melanoma cells MT1-MMP accumulates intracellularly after SDF-1 stimulation through activation of the Rac-Erk1/2 pathway, and is redistributed to the cell surface in a PI3K-dependent manner after the cells have been in contact with components of Matrigel [48]. Here, I demonstrated that intracellular crosstalk between these pathways leads to signal amplification of a low SDF-1 gradient, leading to enhanced MT1-MMP expression on CB HSPC cell surfaces and increased HSPC chemoinvasion towards SDF-1. Thus, agents that positively regulate the SDF-1−CXCR4 axis

may prime the homing-related responses of CB HSPC by upregulating MT1-MMP. However, further investigations in a murine model to determine the priming effect on MT1-MMP modulation during in vivo HSPC homing and engraftment are warranted.

Aside from the involvement of MT1-MMP in cell trafficking which is presented here, new evidence suggests it may also play a role in stem cell differentiation. HSPC reside within specialized niches in the BM where their maintenance and the regulation of their self-renewal and differentiation are believed to depend on the extrinsic signals provided by the niche microenvironment [50,51]. Interestingly, impairment in myeloid and erythroid cell differentiation was observed in 12-day-old MT1-MMP$^{-/-}$ mice [52], but this study is preliminary and requires further confirmation. BM is a reservoir not only for HSPC but also for mesenchymal stem cells (MSC). MT1-MMP expression in MSC has been shown to be modulated by several cytokines secreted in the BM microenvironment [53]. Recently, it was demonstrated that MT1-MMP not only mediates MSC trafficking through interstitial ECM both in vitro and in vivo but also controls their osteogenic differentiation in 3-D collagen I matrix [54]. Thus, MT1-MMP could modulate the BM microenvironment in several ways: e.g., by remodeling ECM and bone; cleaving and releasing ECM-tethered growth factors and chemokines; and modulating differentiation of HSPC and MSC. Therefore, determining the role of MT1-MMP in the BM niche would provide more insights into mechanisms of regulation of HSPC and MSC.

Significance

The findings in this thesis imply an important role for MT1-MMP in HSPC mobilization and homing and suggest that modulation of MT1-MMP could become a potential target for development of therapeutic strategies that could improve transplantation outcomes. A better understanding of the molecular mechanisms that regulate HSPC mobilization has already led to the development of new mobilizing agents that improve transplantation outcomes. For example, recent use of Plerixafor (AMD3100) which reversibly binds CXCR4 and disrupts SDF-1−CXCR4 interactions has demonstrated that a combination of AMD3100 and G-CSF resultes in greater mobilization efficacy compared to G-CSF alone [55,56].

The main problem with G-CSF-induced mobilization is the variable kinetics of mobilization as shown by the significant number of patients/donors who either mobilize poorly or fail to mobilize. Findings from this thesis indicate that positive regulation of MT1-MMP increases migration/mobilization of HSPC, and hence the development of mobilizing agents that increase MT1-MMP expression could enhance mobilization efficiency. For example, cytokines such as HGF that upregulate MT1-MMP expression in HSPC [12] could also synergize with G-CSF and increase mobilization efficiencies in patients who mobilize poorly with G-CSF alone. This could be tested in a clinical setting following confirmatory results from murine models.

On the other hand, potential inhibitors such as green tea polyphenol EGCG which inhibits MT1-MMP expression and proMMP-2 activation, and

statins which like methyl-β-cyclodextrin (MβCD) disrupt lipid raft formation could inhibit MT1-MMP incorporation into lipid rafts and thereby negatively affect HSPC mobilization. Recently it was reported that donor treatment with atorvastatin (which induces T-cell hyporesponsiveness) prior to G-CSF-mobilization, reduced the incidence of acute GvHD in recipients without compromising immunological control over malignancy [57]. However, implications of work done in this thesis suggest that patients/donors who are on statins could mobilize poorly. Therefore, caution should be exercised in the use of these drugs or excessive consumption of green tea at the time of mobilization.

Understanding the basic biology of HSPC self-renewal, differentiation and migration/homing has led to the design of strategies to increase the limited number of HSPC available in a single CB unit (by ex vivo expansion of $CD34^+$ cells using various cytokine cocktails before transplantation) [4,5]. Another strategy includes ex vivo exposure of CB HSPC to agents such as C3a and PMV that prime their chemotactic responses by positively regulating the SDF-1−CXCR4 axis, as we previously showed in murine models [40-42]. Interestingly, this strategy of ex vivo exposure of CB HSPC to C3a is already being evaluated in clinical trial [58]. Therefore I suggest that agents such as HA and thrombin described in this thesis that upregulate MT1-MMP expression and increase the responsiveness of CB HSPC towards SDF-1, could enhance their homing efficiency and engraftment, leading to improvement in transplantation outcomes. Therefore, cord blood units could be pretreated with HA or thrombin prior to transplantation. However, the time of exposure and dose for ex vivo pretreatment

especially with respect to thrombin needs to be be evaluated in murine models in order to avoid complications with coagulation and to ensure optimal homing and engraftment.

Moreover, a better understanding of the molecular mechanisms of HSPC migration is not only beneficial for designing novel therapeutic strategies but also could be applied to the promotion of homing of other stem cells such as MSC which share common migration mechanisms.

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