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9

Molecular Mechanisms Underlying Bone Marrow Homing of Hematopoietic Stem Cells

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1. Introduction

The formation of blood cells, also called hematopoiesis, is a complex process that occurs in the bone marrow and depends on correct regulation of hematopoietic cell fate decisions. Aberrant regulation of hematopoiesis can result in the development of severe malignant and non-malignant hematological disorders, including leukemia. Hematopoietic stem cell transplantation is the most powerful treatment modality for a large number of those malignancies. Successful hematopoietic recovery after transplantation depends on homing of hematopoietic stem cells to the bone marrow and subsequent lodging of those cells in the bone marrow microenvironment.

Homing is a rapid, coordinated process in which circulating hematopoietic stem and progenitor cells actively enter the bone marrow within a few hours after transplantation (Figure 1). Rolling and firm adhesion of those cells to endothelial cells in small marrow sinusoids is followed by trans-endothelial migration across the endothelium/extracellular matrix barrier. Finally, in irradiated recipients, hematopoietic stem cells anchor to their specialized niches within the bone marrow compartment near osteoblasts and initiate long-term repopulation (Lo Celso et al., 2009). In absence of available niches in, for example, non-irradiated recipients, HSCs tend to be more randomly distributed throughout the bone marrow (Lo Celso et al., 2009). Since the first bone marrow transplantation decades ago, research has focused on understanding the mechanisms underlying homing of hematopoietic stem cells to the bone marrow. This chapter will focus on recent studies that have extended our understanding of the molecular mechanisms underlying adhesion, migration and bone marrow homing of hematopoietic stem cells.

2. Selectins and bone marrow homing

A first step in the process of bone marrow homing is initial tethering and rolling of hematopoietic stem and progenitor cells along the endothelial wall of blood vessels. It has been demonstrated that selectins play an important role in bone marrow homing of hematopoietic stem and progenitor cells by regulating these processes. Intravital microscopy in bone marrow sinoids and venules of mice deficient for individual selectins revealed that rolling of hematopoietic progenitor cells involves both P and E-selectin, but not L-selectin (Mazo et al.,

1998). Similarly, coating of a surface with immobilized P- or E-Selectin was sufficient to induce rolling of human CD34+ hematopoietic progenitor cells under flow conditions (Xia et al., 2004). A next step in bone marrow homing is transendothelial migration. This process requires firm adhesion of hematopoietic stem and progenitor cells to endothelial cells. Although CD34+ hematopoietic progenitor cells are capable of binding to fluid-phase P- and E-selectin (Xia et al., 2004), in vitro adhesion to bone marrow derived endothelial cells under static conditions has been shown not to depend on E-selectin (Naiyer et al., 1999). Transwell experiments performed to study the importance of E-selectin in migration of human hematopoietic progenitor cells through a confluent layer of bone marrow derived endothelial cells, precultured with IL-1B to induce E-selectin expression, yielded contradictory results. While Naiyer et al. have demonstrated with blocking antibodies that E-selectin is important for transendothelial migration (Naiyer et al., 1999), no significant inhibition in transendothelial migration could be observed by Voermans et al. who performed similar experiments (Voermans et al., 2000). Transplantation of lethally irradiated recipient mice deficient for both P-and E-selectin with wild type bone marrow cells resulted in reduced recruitment of hematopoietic progenitors to the bone marrow and enhanced levels of circulating hematopoietic progenitors, indicating that selectins indeed play an important role in bone marrow homing (Frenette et al., 1998).

Ligands for E-selectin include the PSGL-1 glycoform CLA, CD43 and the CD44 glycoform HCELL (Dimitroff et al., 2001; Merzaban et al., 2011). These ligands are all expressed on mouse Lin-Sca-1+c-Kit+ hematopoietic stem and progenitor cells and human CD34+ hematopoietic progenitor cells (Merzaban et al., 2011). Immune precipitation experiments revealed that although E-selectin can bind to CLA and CD43 in both mouse and human cells, the interaction between E-selectin and CD44 only occurs in human cells (Merzaban et al., 2011). These studies indicate that the molecular mechanism underlying bone marrow homing may be different for mouse and human hematopoietic stem cells. This hypothesis was confirmed by the observation that human CD34+ hematopoietic progenitor cells exhibit a stronger E-selectin binding capacity compared to mouse Lin-Sca-1+c-kit+ cells (Merzaban et al., 2011). In contrast to PSGL-1 which is also expressed in mature hematopoietic cells, CD44 appears to be predominantly expressed on primitive human CD34+ hematopoietic progenitor cells (Dimitroff et al., 2001). Rolling experiments performed under physiological flow conditions revealed that CD44 mediates E-selectin-dependent rolling interactions over a wider shear range in comparison to PSGL-1 and promotes rolling interactions on human bone marrow endothelial cells (Dimitroff et al., 2001). Silencing of CD44 expression in human cells with shRNAs was sufficient to decrease E-selectin binding under physiologic shear conditions, while enforced CD44 expression in Lin-Sca-1+c-kit+ cells conversely increased E-selectin adherence, resulting in improved bone marrow homing in vivo (Merzaban et al., 2011). In addition, treatment of mice with blocking antibodies against CD44 resulted in an increase in committed progenitors in the peripheral blood, suggesting that CD44 is important for lodging of hematopoietic progenitors in the bone marrow (Vermeulen et al., 1998). It has also been demonstrated that the selectin ligands must be alpha1-3 fucosylated to form glycan determinants such as sialyl Lewis x (sLe(x)). Inadequate alpha1-3 fucosylation of umbilical cord blood derived CD34+CD38-/low cells resulted in reduced interaction with both E-selectin and P-selectin, while increasing the level of cell-surface sLe(x) determinants augmented binding to fluid-phase P- and E-selectin, improved cell rolling on P- and E-selectin under flow and enhanced engraftment of human hematopoietic cells in bone marrows of irradiated NOD/SCID mice (Xia et al., 2004).

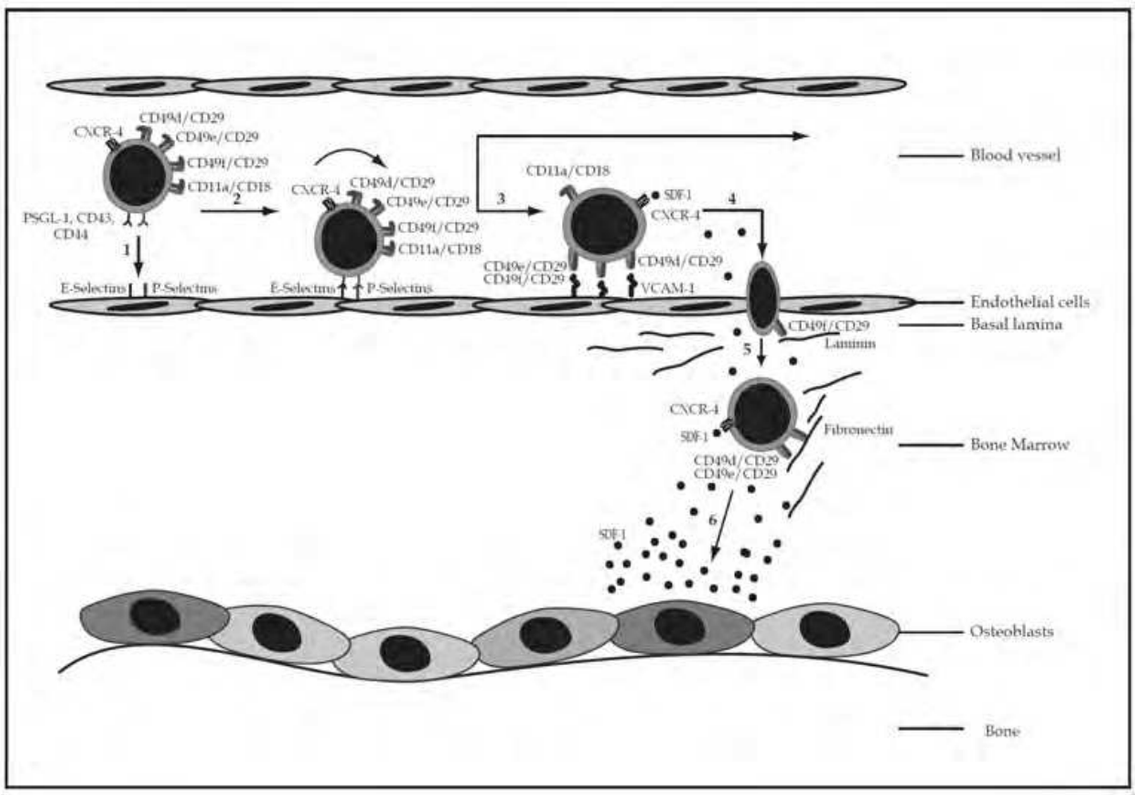

Fig. 1. Homing of hematopoietic Stem Cells to the bone marrow. 1) Initial tethering and 2) rolling are the first steps in bone marrow homing. These processes are mediated by both E- and P-selectin. 3) SDF-1 mediated integrin activation induces firm adhesion of the hematopoietic stem cells to the endothelial wall. 4) Firmly attached hematopoietic stem cells can subsequently transmigrate through the endothelial layer and 5) basal lamina, consisting of fibronectin, collagen and laminin. Integrins involved in these steps are CD49d/CD29, CD49e/CD29 and CD49f/CD29. 6) Finally, hematopoietic stem cells migrate towards the SDF-1 gradient to the osteoblasts.

3. Integrins and bone marrow homing

Integrins are, in addition to selectins, also implicated in playing an important role in regulation of bone marrow homing. Several in vitro studies with blocking antibodies have, for example, shown that both CD49d/CD29 (α4β1 or VLA-4) and CD11a/CD18 (αLβ2 or LFA-1) play an important role in adhesion of hematopoietic stem and progenitor cells to endothelial cells and subsequent transendothelial migration (Imai et al., 1999; Peled et al., 2000; Voermans et al., 2000). In addition, spontaneous migration of CD34+ hematopoietic progenitors underneath a bone marrow derived stromal cell layer, was found to be significantly inhibited by a peptide that blocks CD49d/CD29 integrin binding (Burger et al., 2003). However, adhesion of $CD34^+$ cells to fibronectin was found to be primarily dependent on CD49e/CD29 (α5β1 or VLA-5) and not CD49d/CD29 (Peled et al., 2000). In addition, chemotaxis of peripheral blood CD34+ progenitor cells on recombinant fibronectin appears to be mediated, at least in part, by CD49e/CD29 (Carstanjen et al., 2005). The importance of

both CD49d/CD29 and CD49e/CD29 in directional migration through the basal lamina, which is composed of the extracellular matrix proteins laminin, collagen, and fibronectin, has been examined utilizing a three dimensional extra cellular matrix-like gel. In contrast to the dominant role of CD49e/CD29 in facilitating static adhesion to fibronectin, SDF-1–induced directional migration of CD34+ cells was found to be dependent on both CD49d/CD29 and CD49e/CD29 integrins (Peled et al., 2000). These studies suggest that both CD49d/CD29 and CD49e/CD29 play an important role in migration of hematopoietic stem and progenitor cells in general. However, transwell migration experiments with endothelial cells from different origin showed that CD49d/CD29 is only involved in migration of hematopoietic progenitors through a confluent layer of bone marrow derived, but not human umbilical vein derived, endothelial cells (Peled et al., 2000). In addition, inhibition of CD49e/CD29 alone was not sufficient to inhibit migration through both types of endothelial cells. However, an additive effect was observed when antibodies for CD11a/CD18, CD49d/CD29 and CD49e/CD29 were mixed together (Peled et al., 2000). These results suggest that the mechanisms underlying hematopoietic stem cell migration through endothelial walls of blood vessels depends on the origin of the endothelial cells and the VCAM-1 expression level.

As described above, deletion of both P- and E-selectin in recipient mice significantly reduced bone marrow homing after transplantation of wild type HPCs. Treatment of these mice with a blocking antibody against VCAM-1, thereby prohibiting interaction with CD49d/CD29, was sufficient to further reduce bone marrow homing after transplantation (Frenette et al., 1998), suggesting that both selectins and integrins are important for optimal bone marrow homing. In addition, the capacity of cells either deficient for CD49d (Scott et al., 2003) or pretreated with CD49d antibodies (Vermeulen et al., 1998; Papayannopoulou et al., 2001; Qian et al., 2006; Carstanjen et al., 2005) to migrate to bone marrow has been shown to be impaired resulting in delayed short-term engraftment (Scott et al., 2003). Furthermore, treatment of mice with blocking antibodies against CD49d resulted in an increase in the number of committed progenitors in the peripheral blood, suggesting that CD49d is also important for lodging of hemaopoietic progenitors in the bone marrow (Vermeulen et al., 1998). Since antibodies directed against mouse CD49d can bind to both CD49d/CD29 and CD49d/ITGB7 ($\alpha 4\beta 7$), and CD49d/ITGB7 is also expressed on mouse Lin-Sca-1+c-Kit+ cells, it was hypothesized that in addition to CD49d/CD29, CD49d/ITGB7 could also be involved in bone marrow homing. Indeed, inhibition of CD49d/ITGB7or its substrate MadCam-1 significantly reduced, but not completely abrogated, bone marrow homing after transplantation (Katayama et al., 2004). In contrast, other integrins, including CD11a, appear not be involved in bone marrow homing (Vermeulen et al., 1998). Transplantation studies with hematopoietic stem cells deficient for CD18 indicated that also CD18 is not essential for bone marrow homing. However, since inhibition of CD49d/CD29 in CD18 deficient hematopoietic stem cells resulted in more dramatic reduction in bone marrow homing in comparison to inhibition of CD49d/CD29 in wild type mice, it was suggested that CD18 can contribute to bone marrow homing when the function of CD49d/CD29 is compromised (Papayannopoulou et al., 2001). In addition to CD49d, CD49e/CD29 has also been implicated in playing a role in regulation of bone marrow homing. Treatment of hematopoietic progenitors with an antibody directed against CD49e/CD29 was sufficient to partially reduce homing of those cells to the bone marrow but not to the spleen (Wierenga et al., 2006; Carstanjen et al., 2005). Another integrin implicated in regulation of bone marrow

homing is CD49f (α6). In contrast to CD49d that appears to primarily be involved in bone marrow homing of short-term repopulating hematopoietic stem cells, CD49f is thought to be important for homing of both short-term and long-term stem cells (Qian et al., 2006). In contrast, similar experiments with fetal liver cells revealed that, in contrast to CD49d which appeared to be important for homing of both hematopoietic stem and progenitor cells, CD49f is only important for homing of hematopoietic progenitors but not stem cells (Qian et al., 2007). These studies indicate that CD49d and Cd49f play differential roles during homing of cord blood and fetal liver derived hematopoietic stem and progenitor cells (Qian et al., 2007). In contrast, bone marrow homing was not affected in a more recent study in which also mouse bone marrow derived hematopoietic stem and progenitor cells pretreated with blocking antibodies directed against CD49f were transplanted in recipient mice (Bonig et al., 2009). In addition, blocking CD49f in human and primate bone marrow derived hematopoietic stem and progenitor cells, but not mobilized peripheral blood or cord blood derived cells that express little or no CD49f, resulted in enhanced bone marrow homing in a xenogeneic transplant model and significantly improved engraftment levels (Bonig et al., 2009). Finally, intravenous injection of anti-CD49f antibodies, in contrast to antibodies against CD49d integrin, did not mobilize progenitors or enhance cytokine-induced mobilization by G-CSF, suggesting that CD49f is not essential for lodging of hematopoietic stem and progenitor cells in the bone marrow (Qian et al., 2006). Additional research is required to investigate whether or not CD49f regulates bone marrow homing.

4. Chemoattractants involved in migration of hematopoietic stem cells

Chemoattractants play an important role in directing migration of hematopoietic stem and progenitor cells to the bone marrow. Several studies have demonstrated that Stromal cell Derived Factor 1 (SDF-1), also known as CXC chemokine ligand 12 (CXCL12) (Tashiro et al., 1993) acts as a chemoattractant for hematopoietic stem and progenitor cells and is important for their transendothelial migration (Aiuti et al., 1997; Naiyer et al., 1999; Mohle et al., 1998; Kim & Broxmeyer, 1998; Glass et al., 2011). Further investigation, utilizing a large panel of CC and CXC chemokines, suggested that the only chemokine capable of inducing migration of murine hematopoietic stem and progenitor cells appears to be SDF-1 (Liesveld et al., 2001; Wright et al., 2002). Although the chemokine receptors CCR3 and CCR9 were also expressed at mRNA level, their ligands could not induce migration (Wright et al., 2002). Similarly, examination of a panel of chemokines and cytokines in transendothelial migration assays revealed that SDF-1 is also important for migration of human hematopoietic progenitors through a confluent layer of endothelial cells (Liesveld et al., 2001). However, to a lesser extent, also other chemokines and cytokines, including CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), IL-8 and SCF could also induce transendothelial migration (Liesveld et al., 2001). In addition, LTD4, a ligand for CysLT(1), a G protein-coupled receptor recognizing inflammatory mediator of the cysteinyl leukotriene family, which is highly expressed in hematopoietic progenitors, has been demonstrated to up-regulate CD49d/CD29 and CD49e/CD29 dependent adhesion of hematopoietic progenitors (Boehmler et al., 2009) and to induce chemotaxis and in vitro transendothelial migration (Bautz et al., 2001). Recently, a role for the proteolysis-resistant bioactive lipids sphingosine-1-phosphate and ceramide-1-phosphate in regulation of bone marrow homing has been suggested. Conditioning of mice for transplantation resulted in enhanced levels of these lipids in the bone marrow. In addition, both lipids appear to be chemoattractants for hematopoietic stem and progenitor cells (Kim et al., 2011).

The role of SDF-1 in migration of hematopoietic stem and progenitor cells will be discussed below in more detail.

4.1 SDF-1 and bone marrow homing

SDF-1 is produced by several types of bone marrow cells (Maekawa & Ishii, 2000). In the adult human bone marrow, SDF-1 was found to be expressed by endothelial cells and along the endosteum region (Peled et al., 2000; Ponomaryov et al., 2000). SDF-1 plays an important role in many processes, including immune surveillance, proliferation, differentiation and survival of many cell types (Aiuti et al., 1997; Bleul et al., 1996; Bleul et al., 1998; Cashman & Eaves, 2000; Lataillade et al., 2000). In addition, SDF-1 is considered to be essential for migration of hematopoietic stem cells to the bone marrow (Imai et al., 1999; Peled et al., 1999a; Wright et al., 2002). To date, two receptors for SDF-1 have been identified, of which CXCR4 (LESTR/fusin), a seven-transmembrane domain G-protein coupled receptor, appears to be the most prominent (Heesen et al., 1997; Loetscher et al., 1994). CXCR4 is expressed by a variety of cell types, including hematopoietic stem and progenitor cells, T lymphocytes, endothelial, stromal and neuronal cells (Nagasawa et al., 1996; Ma et al., 1998; Mohle et al., 1998; Loetscher et al., 1994). Recently, CXCR7, another SDF-1 receptor, has been identified (Tarnowski et al., 2010). However, CXCR7 is expressed at low levels in normal human $CD34^+$ hematopoietic stem and progenitor cells and does not appear to be important for migration of those cells. In contrast, CXCR7 is highly expressed in several human myeloid leukemic cell lines and is thought to play a role in adhesion and, to a lesser extent, also in migration of those cells (Tarnowski et al., 2010).

Mouse transplantation studies have been performed to investigate the importance of SDF-1 in migration of hematopoietic stem cells to the bone marrow. Pre-treatment of human $CD34+CD38^{-/low}$ cells with a blocking antibody against CXCR4 has, for example, been demonstrated to be sufficient to impair their capacity to home to the bone marrow of immune deficient NOD/SCID mice or β2m deficient NOD/SCID mice (Peled et al., 1999b; Kollet et al., 2001; Kollet et al., 2002; Oberlin et al., 1996). In addition, up-regulation of CXCR4 expression by incubation with hematopoietic cytokines (SCF and IL-6) (Peled et al., 1999b) or over-expression of CXCR4 by viral transduction (Brenner et al., 2004; Kahn et al., 2004) resulted in enhanced bone marrow homing of human CD34+ and CD34+CD38- cells in NOD/SCID mice, which correlated with enhanced engraftment levels 6 weeks after transplantation (Peled et al., 1999b; Kollet et al., 2001; Kollet et al., 2002). Similarly, fetal liver hematopoietic stem and progenitor cells deficient for CXCR4 displayed a reduced bone marrow homing capacity compared to wild type cells (Ma et al., 1998). In addition to bone marrow homing, SDF-1 also appears to play a critical role in retention of hematopoietic stem cells in the hematopoietic stem cell niche. Enhancing the level of SDF-1 in plasma, but not bone marrow, utilizing adenoviral vectors (Hattori et al., 2001) or sulfated glycans (Sweeney et al., 2000; Frenette & Weiss, 2000; Sweeney et al., 2002) resulted in mobilization of CXCR4 expressing hematopoietic stem and progenitor cells (Hattori et al., 2001; Sweeney et al., 2002). Similarly, treatment of C3H/HeJ mice or healthy human volunteers with AMD3100, a selective CXCR4 antagonist, enhanced the number of HSCs and neutrophils in peripheral blood, again suggesting a role for CXCR4 and SDF-1 in HSC retention in BM (Broxmeyer et al., 2005).

4.2 Regulation of SDF-1 activity

Several proteolytic enzymes have been implicated in negatively regulating migration of hematopoietic stem cells by cleaving and inactivating SDF-1, including matrix metalloproteinases (MMP) 2/9 (Heissig et al., 2002; Sweeney et al., 2002; McQuibban et al., 2001), CD26 (Christopherson et al., 2002), carboxypeptidase M (Marquez-Curtis et al., 2008), carboxypeptidase N (Davis et al., 2005), neutrophil elastase (Petit et al., 2002; Levesque et al., 2002), cathepsin G (Petit et al., 2002; Levesque et al., 2002) and cathepsin K (Kollet et al., 2006). Cleavage of SDF-1 by several individual MMPs at Ser^4-Leu^5 bond of SDF-1 N-terminal domain has, for example, been demonstrated to result in reduced binding capacity of SDF-1 for CXCR-4 and reduced chemoattractant activity for hematopoietic stem and progenitor cells (McQuibban et al., 2001; Cho et al., 2010). Another protein involved in regulation of the activity of SDF-1 is the membrane-bound extracellular peptidase CD26 (DPPIV). It has been shown that a small number of umbilical cord blood derived CD34+CXCR4+ cells express CD26 and can therefore cleave the N-terminal part of SDF-1 at 2-proline (Christopherson et al., 2002). Functional studies showed that truncated SDF-1 lacks the ability to induce migration of CD34+ cells. In addition, inhibition of endogenous CD26 activity appears to be sufficient to enhance the migratory capacity of CD34+ cells towards SDF-1, indicating that CD26 abrogates SDF-1 induced migration of hematopoietic progenitors (Christopherson et al., 2002; Christopherson et al., 2003; Christopherson et al., 2006).

A third class of SDF-1 inhibitors includes the carboxypeptidases M and N (Marquez-Curtis et al., 2008; Davis et al., 2005). Carboxypeptidase N, which is present in human serum and plasma (Davis et al., 2005), can efficiently and specifically cleave SDF-1 at the carboxy-terminal lysine (K68) resulting in reduced SDF-1 activity and inhibition of SDF-1 mediated induction of migration of hematopoietic progenitors (Davis et al., 2005). In contrast, carboxypeptidase M is a membrane bound zinc-dependent peptidase that cleaves carboxy-terminal basic residues. This particular carboxypeptidase is expressed by stromal cells and CD34+ cells from both bone marrow and mobilized peripheral blood (Skidgel & Erdos, 1998; Marquez-Curtis et al., 2008). Carboxypeptidase M mediated cleavage of SDF-1 results in reduced chemotactic activity of hematopoietic stem and progenitor cells, which can be rescued by addition of the carboxypeptidase inhibitor DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (Marquez-Curtis et al., 2008).

Whereas high SDF-1 expression in the bone marrow is essential for normal bone marrow homing of hematopoietic stem and progenitor cells and lodging of those cells in the hematopoietic stem cell niche, during mobilization SDF-1 levels should conversely be decreased. Upon administration of G-CSF, which is used to mobilize HSPCs, an accumulation of various proteolytic enzymes including MMP-9, neutrophil elastase and cathepsin G or K (Petit et al., 2002; Levesque et al., 2002) has been observed in mouse bone marrow which correlated with a gradual decrease in SDF-1 in the bone marrow, but not circulation (Petit et al., 2002). In addition, also an enhanced SDF-1 plasma level was shown to result in up-regulation of MPP-9 in bone marrow cells and mobilization of hematopoietic stem and progenitor cells (Heissig et al., 2002). The importance of MPP-9 for mobilization of hematopoietic stem cells was demonstrated utilizing MMP-9 deficient mice. A high SDF-1 level in plasma was not sufficient to induce mobilization of hematopoietic progenitors in these mice (Heissig et al., 2002). In addition, in primary myelofibrosis, which is a chronic

myeloproliferative neoplasm characterized by constitutive mobilization of hematopoietic stem and progenitor cells into the peripheral blood (Migliaccio et al., 2008), both a high level of truncated SDF-1 and enhanced levels of proteases, including dipeptidyl peptidase-IV (CD26), neutrophil elastase, matrix metalloproteinase-2 (MMP-2), MMP-9, and cathepsin G have been observed (Cho et al., 2010). Taken together, these studies demonstrated that SDF-1 plays an important role in integrin-mediated firm arrest of human HSPCs, facilitate their transendothelial migration, and regulate bone marrow homing and retention of HSPCs in the hematopoietic stem cell niche.

4.3 Molecular mechanisms underlying SDF-1 mediated regulation of migration

To understand the molecular mechanism underlying migration of hematopoietic stem and progenitor cells, research has focused on identifying the downstream effectors of SDF-1 and CXCR4. SDF-1 has been demonstrated to induce the activity of the integrins CD11a/CD18 (Peled et al., 2000) and CD49/CD29 (Hidalgo et al., 2001; Peled et al., 2000) on CD34+ cells which allows interaction with their substrates ICAM-1 and VCAM-1, respectively.

Small guanosine triphosphatases (GTPases) that belong to the Ras superfamily of GTPases , including Rho, Rac and Cdc42, have been demonstrated to be involved in SDF-1 mediated homing and migration of hematopoietic stem and progenitor cells (Fuhler et al., 2008; del Pozo et al., 1999). The activity of Rho GTPases can be induced by tyrosine kinase receptors (Taylor & Metcalfe, 2000; Timokhina et al., 1998), integrin receptors (del Pozo et al., 2004) and chemokine receptors including SDF-1 (Cancelas et al., 2005; del Pozo et al., 1999; Fuhler et al., 2008; Shirvaikar et al., 2011). It has been demonstrated in *in vitro* assays that SDF-1 induced chemo-attraction is mediated, at least in part, by Rac (del Pozo et al., 1999; Shirvaikar et al., 2011; Wysoczynski et al., 2005). In addition, analysis of Rac2 deficient mice revealed that Rac2 is essential for lodging of HSPCs in the bone marrow. Deletion of Rac2 resulted, for example, in reduced adhesion and enhanced mobilization of hematopoietic stem cells to the circulation. Furthermore, Rac2 deficiency resulted in enhanced SDF-1 induced migration of hematopoietic stem and progenitor cells (Yang et al., 2001). An enhanced activation of Cdc42 and Rac1 was observed in these cells, suggesting a compensatory role of Cdc42 and Rac1 with regard to migration, but not adhesion (Yang et al., 2001). In addition, it was shown that SDF-1 mediated Rac activation is impaired in CD34+ cells from MDS patients. CD34+ cell from patients with myelodysplastic syndrome exhibit reduced F-actin polymerization and migration towards SDF-1 compared to normal CD34+ cells (Fuhler et al., 2008). While pharmacological inhibition of Rac1 activity in a human myeloblastic cell line (HL-60) with NSC23766 was sufficient to abrogate SDF-1 induced actin assembly and migration, over-expression of active Rac in HL-60 cells conversely restored both F-actin polymerization and migration, suggesting that Rac is essential for SDF-1–induced migration in these cells (Fuhler et al., 2008). Although over-expression of active Rac in CD34+ cells from patients with myelodysplastic syndrome resulted in increased F-actin polymerization and enhanced motility, directional migration toward SDF-1 was not improved (Fuhler et al., 2008). These studies suggest that SDF-1 mediated induction of Rac activity is important for migration of both normal and malignant hematopoietic progenitors (Fuhler et al., 2008). The role of the hematopoietic-specific guanine nucleotide exchange factor Vav1, which is an upstream regulator of Rac activity, in localization and engraftment of hematopoietic stem and progenitor cells has also been

investigated. Deletion of Vav1 in hematopoietic stem cells has been demonstrated to result in impaired responses to SDF1α, dysregulated Rac/Cdc42 activation and a reduction of in vitro migration. In addition, intravital microscopy assays revealed that transplantation of Vav1 deficient hematopoietic stem and progenitor cells results in impaired early localization near nestin(+) perivascular mesenchymal stem cells after transplantation (Sanchez-Aguilera et al., 2011). Recently, another upstream regulator of Rac activity has been identified. In contrast to Rac, the activity of R-Ras, a member of the Ras family, is inhibited upon SDF-1 stimulation. Deletion of R-Ras resulted in enhanced levels of Rac1/2 activity, while expression of a constitutively active R-Ras mutant resulted in down-regulation of Rac1-activity. Deletion of R-Ras in hematopoietic stem and progenitor cells resulted in increased directional migration. This phenotype could be reversed by inhibition of Rac. Furthermore, R-Ras deficient mice showed enhanced responsiveness to G-CSF for progenitor cell mobilization and exhibited decreased bone marrow homing (Sanchez-Aguilera et al., 2011).

Another important mediator of hematopoietic progenitor cell migration is the GTPase Rho (Bug et al., 2002; Ghiaur et al., 2006; Gottig et al., 2006). It has been demonstrated that SDF-1 mediated release of intracellular Ca^{2+} stores requires activation of Rho GTPases, but not Rac or Cdc42 (Henschler et al., 2003). Depletion of intracellular Ca^{2+} resulted in reduced SDF-1 induced migration and bone marrow homing of hematopoietic progenitors (Henschler et al., 2003). In addition, over-expresssion of dominant negative RhoA by retroviral transduction in mouse cells (C57BL/6J mice) resulted in decreased migration of hematopoietic progenitor cells towards SDF-1 and reduced integrin-mediated adhesion (Henschler et al., 2003). Furthermore, over-expression of RhoH, a GTPase deficient type of Rho (Sahai & Marshall, 2002), in hematopoietic stem and progenitor cells resulted in impaired activation of Rac GTPases, defective actin polymerization and impaired chemotaxis. In contrast, inhibition of RhoH expression in these cells conversely stimulated SDF-1–induced migration in vitro (Gu et al., 2005). In addition, it has been demonstrated that Epac1, a nucleotide exchange protein for the GTPase Rap1, which is directly activated by cAMP, can also improve the adhesive and migratory capacity CD34+ hematopoietic progenitor cells (Carmona et al., 2008), suggesting that Rap1 may also play a role in bone marrow homing.

Endolyn (CD164), a type I integral transmembrane silomucin (Chan et al., 2001; Zannettino et al., 1998), which is recruited to CXCR4 upon SDF-1 stimulation (Forde et al., 2007) was shown to play an important role in SDF-1 mediated migration of human CD133+ hematopoietic stem and progenitor cells (Forde et al., 2007). Inhibition of CD164 in CD133+ cells with 103B2, a specific mAb, resulted in a reduction of migration towards SDF-1, but not CCL1, CCL5, CCL17, CCL19, CCL20, CCL21, CCL22 and CXCL3. A similar inhibition in SDF-1 mediated migration of CD133+ cells was observed after siRNA mediated knock-down of CD164 (Forde et al., 2007). Knock-down of CD164 resulted in a significant reduction in SDF-1 mediated activation of PI3K and PKCζ (Forde et al., 2007). Both PI3K and PKCζ have been implicated in playing an important role in SDF-1 mediated migration of $CD34^+$ cells. Inhibition of PKCζ, for example, reduced SDF-1 induced migration of CD34+ cells and reduced engraftment levels after transplantation (Petit et al., 2005). Furthermore, injection of inhibitory PKCζ pseudosubstrate peptides resulted in mobilization of murine progenitors to the circulation, suggesting an important role for PKCζin SDF-1-dependent regulation of hematopoietic stem and progenitor cell motility and localization (Petit et al., 2005) The role of PI3K in regulation of bone marrow homing will be discussed in the next section.

In addition to regulating the activity of downstream effectors, SDF-1 has also been demonstrated to regulate the expression of specific target genes. Stimulation of peripheral blood mononuclear, Jurkat or HeLa cells has, for example, been demonstrated to result in a rapid increase in expression of the ubiquitin-specific protease 17 (USP17) (de la Vega et al., 2011). A role for this protease in regulation of migration of hematopoietic progenitor cells has been examined in vitro. Inhibition of USP17 in these cells showed decreased chemotaxis towards SDF-1, whereas over-expression of USP17 conversely resulted in increased chemotaxis. Interestingly, CXCR4 levels were not affected by inhibition or over-expression of USP17, suggesting that USP17 modulates the down-stream signaling of the CXCR4 receptor. shRNA mediated inhibition of USP17 expression resulted in decreased polymerization of actin and tubulin and reduced membrane ruffling. In addition, upon SDF-1 stimulation, the GTPases, RAC1, Cdc42 and RhoA were not transported to the plasma membrane, thereby prohibiting their activiation (de la Vega et al., 2011). In addition, CD9, a member of the tetraspanin superfamily (Boucheix et al., 1991) that is widely expressed in hematopoietic and non-hematopoietic cells, has been shown to be a SDF-1 responsive gene. Microarray analysis with human umbilical cord blood derived CD34+ cells revealed that short-term exposure to SDF-1 resulted in up-regulation of CD9 mRNA expression both in $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^{-/low}$ cells (Leung et al., 2011). A role for CD9 in migration and adhesion of human cord blood derived hematopoietic stem and progenitor cells was investigated utilizing a neutralizing CD9 antibody (Leung et al., 2011). Although actin polymerization was not affected, the calcium influx and transendothelial migration towards a SDF-1 gradient was reduced by this antibody (Leung et al., 2011). In contrast, adhesion of progenitor cells to fibronectin and human umbilical vein endothelial cells was enhanced (Leung et al., 2011). Transplantation experiments revealed that in NOD/SCID mice, pre-treatment of human CD34+ cells with a neutralizing CD9 antibody resulted in inhibition of homing to bone marrow and spleen. However, enhanced CD9 expression in CD34+ cells with ingenol 3,20-dibenzoate (IDB), a protein kinase C agonist which was shown to induce CD9 expression in CD34+ cells, did not result in enhanced bone marrow homing (Desmond et al., 2011).

5. The PI3K/PKB signalling module and bone marrow homing

Correct regulation of the Phosphatidylinositol-3-Kinase (PI3K) / Protein Kinase B (PKB/c-Akt) signaling module is essential for multiple processes during hematopoiesis. Phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$, the most important substrate for PI3K, can be phosphorylated upon extracellular stimulation, resulting in the formation of phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$) (Hawkins et al., 2006). $PI(3,4,5)P_3$ subsequently serves as an anchor for pleckstrin homology (PH) domain-containing proteins, such as Protein Kinase B (PKB/ c-akt) (Burgering & Coffer, 1995). Activation of PI3K and its downstream effector Protein Kinase B (PKB/c-Akt) has been observed in leukemic cell lines stimulated with SDF-1 (Ganju et al., 1998). A positive role for PI3K/PKB in regulation of SDF-1 induced migration of hematopoietic stem cells was therefore suggested. However, it has been shown that Protein Phosphatase 2A plays an important role in positively regulating SDF-1 mediated migration of human hematopoietic progenitors by inhibition of PKB activity (Basu et al., 2007). Similarly, inhibition of PKB activity in $CD34^+$ cells for over 24 hours appears to be sufficient to reduce their adhesion to bone marrow derived stromal cells and to induce their basal migratory capacity (Buitenhuis et al., 2010). Transwell

migration experiments through a confluent layer of human umbilical vein endothelial cells revealed that the observed reduction in firm adhesion does not ameliorate the induced migratory capacity of $CD34^+$ cells pre-treated with a PKB inhibitor (Buitenhuis et al., 2010). In addition, ectopic expression of constitutively active PKB in CD34+ cells conversely induced firm adhesion and reduced the basal level of migration. Although it cannot be excluded that transient activation of PI3K/PKB activity by SDF-1 is important for induction of migration, these studies suggest that prolonged activation of PKB activity is detrimental for migration of $CD34^+$ cells. The role of PI3K in regulation of bone marrow homing was initially examined utilizing mice deficient for SHIP (SH2-containing inositol-5'-phosphatase), a negative regulator of PI3K (Damen et al., 1996). Transplantation of lethally irradiated recipients with HSCs from SHIP deficient mice resulted in diminished repopulation, suggesting that constitutive activation of PI3K impairs the ability of HSCs to home to and to be retained in the hematopoietic stem cell niche in the bone marrow. Assessment of bone marrow homing revealed that $SHIP^{-/-}$ hematopoietic stem and progenitor cells indeed traffic to the bone marrow and spleen with significantly reduced efficiency compared to wild type cells. Although it is evident that constitutive activation of PI3K plays a critical role in regulation of hematopoiesis per se (Buitenhuis et al., 2008), these results indicate that the inability of SHIP deficient hematopoietic stem cells to engraft and sustain long-term hematopoiesis can be, at least partially, explained by their impaired ability to home to the bone marrow (Desponts et al., 2006). Deletion of Phosphate and tensin homologue (PTEN), another critical negative regulator of PI3K signaling that dephosphorylates $PI(3,4,5)P_3$ resulting in the formation of $PI(4,5)P_2$ (Maehama & Dixon, 1998) only decreased bone marrow homing when PTEN deficient HSCs were transplanted into non-irradiated recipients. These results suggest that, although PTEN deficient hematopoietic stem cells are capable of migrating to the bone marrow, their performence is reduced compared to competeting wild-type hematopoietic stem cells when vacant niches are limited (Zhang et al., 2006). Although both PTEN and SHIP act on the main product of PI3K activity, $PI(3,4,5)P_3$, the products generated are distinct, which could explain the differences between SHIP and PTEN deficient hematopoietic stem cells in terms of bone marrow homing (Dowler et al., 2000; Golub & Caroni, 2005). Recent findings demonstrated that, similar to deletion of SHIP, constitutive activation of PKB in human hematopoietic progenitors cells is sufficient to significantly inhibit homing of these cells to the bone marrow and spleen of β2 microglobulin -/- NOD/SCID mice (Buitenhuis et al., 2010). In contrast, although transplantation of C57 BL/6 mice with bone marrow cells from *5-fluorouracil* treated mice that ectopically expressed constitutively active PKB resulted in reduced engraftment levels, bone marrow homing was only modestly impaired 18 hours after transplantation (Kharas et al., 2010). To investigate whether inhibition of PKB activity would be sufficient to conversely improve bone marrow homing, human hematopoietic progenitor cells, pre-treated with a PKB inhibitor for 24 or 48 hours, were injected into recipient mice. Flow cytometric analysis, 22 hours after transplantation, revealed that transient inhibition of PKB activity prior to transplantation is sufficient to improve bone marrow homing (Buitenhuis et al., 2010). In addition, while constitutive activation of PKB appears to be detrimental for bone marrow homing, engraftment levels and hematopoietic recovery, inhibition of PKB activity prior to transplantation, resulting in an induction of bone marrow homing, conversely enhanced engraftment levels in recipient mice. Together, these studies demonstrated that correct regulation of PI3K/PKB is essential for migration of hematopoietic stem and progenitor cells to the bone marrow after transplantation, which is essential for optimal engraftment and hematopoietic recovery (Buitenhuis et al., 2010; Desponts et al., 2006; Kharas et al., 2010).

The molecular mechanisms underlying PKB mediated regulation of migration and bone marrow homing are, thus far, incompletely understood. Although PKB mediated inhibition of migration has been demonstrated to involve RAC1 (Farooqui et al., 2006), NFAT (Yiu & Toker, 2006; Yoeli-Lerner et al., 2005) and $p27^{Kip1}$ (Baldassarre et al., 2005; Viglietto et al., 2002; Wu et al., 2006) in non-hematopoietic cell lines, their importance for migration of hematopoietic stem and progenitor cells remains to be investigated. As described above, adhesion and migration of HSCs depend on correct integrin and selectin expression and regulation of integrin activity. PKB and its downstream effector GSK-3 have initially been shown to play an important role in recycling of the CD49e/CD29 and CD51/CD61 ($\alpha v\beta 3$) integrins to the membrane in NIH 3T3 fibroblasts, resulting in enhanced cell spreading and adhesion (Roberts et al., 2004). Ectopic expression of PKB in human hematopoietic stem and progenitor cells has been demonstrated to enhance the level of CD49d, while inhibition of PKB activity conversely reduces expression of both CD49d and CD18 (Buitenhuis et al., 2010), providing a potential mechanism by which PKB induces adhesion and inhibits migration. Although it is evident that integrins play an important role in adhesion and migration of cells, the importance of these molecules in PKB mediated inhibition of migration remains to be investigated. In addition, CXCR4 expression has been demonstrated to be reduced in SHIP deficient hematopoietic stem cells, suggesting that activation of PI3K also impairs their response to SDF-1(Zhang et al., 2006).

6. Conclusion

Allogeneic HSC transplantation is the preferred treatment modality for a number of hematological malignancies. To allow normal long-term hematopoiesis to occur after transplantation, correct regulation of homing of hematopoietic stem and progenitor cells to the bone marrow and subsequent lodging of those cells into the hematopoietic stem cell niche is essential. As described above, this is a coordinated multistep process that is regulated by chemokines, integrins and selectins. Initial tethering and rolling of hematopoietic stem and progenitor cells along the endothelial wall of blood vessels are the first steps in this process. It has been demonstrated that both P and E-selectin play an important role in rolling of HSCs. In addition to selectins, integrins are also implicated in playing an important role in regulation of bone marrow homing. Both studies with blocking antibodies and knockout mice have revealed that CD49d/CD29, CD49e/CD29, CD49f, and CD49d/ITGB7 play an important role in adhesion of hematopoietic stem and progenitor cells to endothelial cells and subsequent transendothelial migration. In addition, both CD49d/CD29 and CD49e/CD29 integrins appear to be involved in mediation of SDF-1–induced directional migration of CD34+ cells through the basal lamina. In addition, although, under normal circumstances, CD18 appears not to be essential for bone marrow homing of hematopoietic stem cells, CD18 can contribute to bone marrow homing when the function of CD49d/CD29 is compromised. Although multiple chemokines are capable of inducing transendothelial migration of hematopoietic stem cells, the chemokine SDF-1 appears to be the most prominent chemokine involved in bone marrow homing. In addition, SDF-1 also appears to play a critical role in retention of hematopoietic stem cells in the hematopoietic stem cell niche. Regulation of SDF-1 activity by a variety of proteolytic enzymes has been demonstrated to play an important role in migration of hematopoietic stem cells to and from the bone marrow. The molecular mechanism underlying SDF-1

mediated regulation of HSC migration has been investigated extensively. Thus far, multiple downstream effectors have been identified, including CD164, the GTPases Rac, Rho, and Cdc42, and the signalling molecules PI3K and PKCζ. In addition, the SDF-1 responsive genes CD9, USP17, both implicated in regulation of hematopoietic stem cell migration, have been indentified. Finally, SDF-1 has been demonstrated to induce the activity of integrins which allows interaction with their substrates. Although activation of PI3K and its downstream effector Protein Kinase B (PKB/c-Akt) has been observed in leukemic cell lines stimulated with SDF-1, suggesting a positive role for PI3K/PKB in regulation of SDF-1 induced migration of hematopoietic stem cells, the above described studies clearly implicate the PI3K/PKB signalling module in playing a critical role in negatively regulating migration of HSCs and bone marrow homing.

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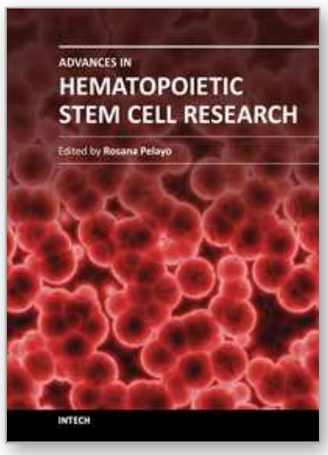

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This book provides a comprehensive overview in our understanding of the biology and therapeutic potential of hematopoietic stem cells, and is aimed at those engaged in stem cell research: undergraduate and postgraduate science students, investigators and clinicians. Starting from fundamental principles in hematopoiesis, Advances in Hematopoietic Stem Cell Research assemble a wealth of information relevant to central mechanisms that may regulate differentiation, and expansion of hematopoietic stem cells in normal conditions and during disease.

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