Bone Marrow-Derived Progenitors

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Katalin Kauser
Andreas-Michael Zeiher

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Bone Marrow-Derived Progenitors

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Preface

There is excitement generated almost daily about the possible uses of stem cells to treat human disease. The ability of stem cells to acquire different desired phenotypes has opened the door for a new discipline: regenerative medicine. Much of the interest for this purpose is generated by embryonic stem cells, but their use is still controversial for moral as well as scientific reasons. Less controversial and readily available are the adult bone marrow-derived progenitors, including hematopoietic stem cells, endothelial progenitors, and mesenchymal stem cells, which are the subjects of this book. These cells can be isolated by simple procedures directly from the bone marrow or from peripheral blood after being stimulated, i.e., mobilized. By reaching sites of damage through the circulation or even after local administration, these cells can overcome the hurdles of delivery approaches that limit the success of gene therapy. Adult bone marrow-derived cells have been shown to regenerate diseased hepatocytes and contribute to neurons, blood vessels, and skeletal and cardiac muscle cells. The increasing amount of new data, sometimes with conflicting results, is making us appreciate the molecular complexity of cell differentiation and potential mechanisms of action involved in these cell-mediated processes. It is becoming increasingly important to understand the biology of these cells to potentially improve their therapeutic efficiency and to facilitate their proper therapeutic use. Examining the cell-mediated processes can ultimately lead to the discovery of pathways and molecular mechanisms of organ repair, which can be further utilized in drug development. With patients' growing attention to the most recent research developments, there is increasing medical need for a better understanding—developed through rationally designed, randomized clinical trials that will move these strategies quickly and carefully toward medical reality—to parallel the increased enthusiasm.

In this volume of the series *Handbook of Experimental Pharmacology* published by Springer, we hope to achieve the ambitious goal of providing a comprehensive overview of the currently available information related to the therapeutic utility of adult bone marrow-derived cells. Chapters in Part I focus on basic principles, including a general introduction to the different bone marrow-derived cell types, mechanisms contributing to their development and localization in the bone marrow niche, mechanisms leading to their mobilization, the current understanding about their immune plasticity, the effect

of aging, and the potential enhancement of their survival or function using cell–gene combinations. Part II is dedicated to therapeutically relevant preclinical experiences and the most recent clinical experiences with these cells for cardiac diseases, neurodegenerative disorders, liver diseases, and diabetes. The potential role of bone marrow-derived cells in tumorigenesis and their potential contribution to tumor angiogenesis are also discussed. Although their exact role in cancer pathology remains to be better understood, harnessing the ability of these cells to deliver antitumor agents provides an additional therapeutic opportunity, which is introduced within the therapeutic section.

Each chapter is written or co-authored by accomplished scientists, leading experts in their field, ensuring the delivery of up-to-date information regarding our current understanding of bone marrow-derived progenitor cell biology and its applications to specific disease indications. The editors focused their efforts on providing a balanced overview of the recent developments in the field without major interference with the content and style of the individual chapters. In some instances reiteration of basic principles in the different chapters may appear redundant when looking at the volume as a whole, but it is necessary to allow each chapter to serve as a self-standing overview of the chosen principle.

The editors thank the authors of the chapters for their excellent contributions, and Springer for its highly professional work and timely publication of the book. We would like to express our specific gratitude to Susanne Dathe from Springer for her patience and guidance throughout the development of this book. We also appreciate the interest and support of the HEP Editorial Board, specifically acknowledging Gabor M. Rubanyi among the board members for his enthusiastic support and encouragement from the very beginning of this project.

Ridgefield and Frankfurt am Main,
March 2007

Katalin Kauser
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List of Contents

List of Contributors

Addresses given at the beginning of respective chapters

Andreeff, M. , 263
Asahara, T. , 181

Beauséjour, C. , 67
Boyle, A.J. , 195

Davis, B.H. , 117
Dimarakis, I. , 243

Flores, C. , 243

Galipeau, J. , 45
Gordon, M.Y. , 243

Habib, N.A. , 243
Hall, B. , 263
Hare, J.M. , 195
Huw, L.-Y. , 37

Kan, I. , 219
Karsan, A. , 89
Kauser, K. , 37

Larrivée, B. , 89
Larsen, S.R. , 3
Lévesque, J.-P. , 3
Levičar, N. , 243
Losordo, D.W. , 167

Marini, F. , 263
Melamed, E. , 219

Offen, D. , 219

Qian, H.-S. , 37
Quraishi, A. , 167

Rasko, J.E.J. , 3
de Resende, M.M. , 37

Schuleri, K.H. , 195
Stagg, J. , 45

Taylor, D.A. , 117
Tracey, J. , 243

Winkler, I.G. , 3

Zenovich, A.G. , 117

Part I
Basic Principles

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Mobilization of Bone Marrow-Derived Progenitors

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Abstract Bone marrow (BM) is a source of various stem and progenitor cells in the adult, and it is able to regenerate a variety of tissues following transplantation. In the 1970s the first BM stem cells identified were hematopoietic stem cells (HSCs). HSCs have the potential to differentiate into all myeloid (including erythroid) and lymphoid cell lineages in vitro and reconstitute the entire hematopoietic and immune systems following transplantation in vivo. More recently, nonhematopoietic stem and progenitor cells have been identified that can differentiate into other cell types such as endothelial progenitor cells (EPCs), contributing to the neovascularization of tumors as well as ischemic tissues, and mesenchymal stem cells (MSCs), which are able to differentiate into many cells of ectodermal, endodermal, and mesodermal origins in vitro as well as in vivo. Following adequate stimulation, stem and progenitor cells can be forced out of the BM to circulate into the peripheral blood, a phenomenon called "mobilization." This chapter reviews the molecular mechanisms behind mobilization and how these have led to the various strategies employed to mobilize BM-derived stem and progenitor cells in experimental and clinical settings. Mobilization of HSCs will be reviewed first, as it has been best-explored—being used extensively in clinics to transplant large numbers of HSCs to rescue cancer patients requiring hematopoietic reconstitution—and provides a paradigm that can be generalized to the mobilization of other types of BM-derived stem and progenitor cells in order to repair other tissues.

Keywords Mobilization · Hematopoietic stem cells · Endothelial progenitor cells · Mesenchymal stem cells · Transplantation · Tissue repair

1 Introduction

In the adult, bone marrow (BM) is a source of various stem and progenitor cells that are able to regenerate a variety of tissues following transplantation. Schofield identified the first BM stem cells, which were hematopoietic stem cells (HSCs) (Schofield 1970). HSCs have the potential to differentiate into all myeloid (including erythroid) and lymphoid cell lineages in vitro and reconstitute the entire hematopoietic and immune systems following transplantation in vivo. More recently, nonhematopoietic stem and progenitor cells have been identified that can differentiate into other cell types such as endothelial progenitor cells (EPCs), contributing to the neovascularization of tumors as well as ischemic tissues (Asahara et al. 1999), and mesenchymal stem cells (MSCs), which are able to differentiate into many cells of ectodermal, endodermal, and mesodermal origins (such as adipocytes, chondrocytes, osteoblasts, hepatocytes, neurons, myocytes, and endothelial and epithelial cells) in vitro as well as in vivo (Sale and Storb 1983; Barry et al. 1999, 2001; Devine et al. 2001; Dennis and Charbord 2002).

In steady-state conditions in adult mammals, most HSCs, EPCs, and MSCs reside in the BM, with a few HSCs (Wright et al. 2001; Abkowitz et al. 2003) and EPCs (Lin et al. 2000) circulating in the peripheral blood; circulating MSCs are usually not detectable (Lazarus et al. 1997; Wexler et al. 2003). For this reason, the stem and progenitor cells used to be isolated by BM aspiration for subsequent transplantation into patients requiring immune and hematopoi-

etic reconstitution or tissue repair. As multiple painful BM aspirations are necessary to obtain sufficient numbers of stem cells to reconstitute a patient, general anesthesia and hospitalization of the donor were required, a process posing a risk for the donor. Because of this limitation, alternative sources of transplantable stem cells have been investigated. Following stress, challenge, or stimulation of the BM compartment, a proportion of these BM stem and progenitor cells egress from the BM and circulate into the blood. This phenomenon is called "mobilization." Mobilized cells are harvested by apheresis from the peripheral blood so they can be concentrated, eventually enriched, and stored for transplantation. If sufficient numbers of stem cells can be mobilized, mobilization offers the advantage of a cost-effective, relatively safe procedure to collect transplantable stem cells. The advantage of mobilized stem cells over BM aspiration is illustrated by the speed at which mobilized hematopoietic stem and progenitors cells (HSPCs) have supplanted BM aspiration as the preferred source of HSPCs for transplantation. Since its first discovery in human patients in the 1980s, cellular support using mobilized HSPCs has now grown to the point where it is used with over 45,000 patients a year worldwide. The main reason why mobilization is preferred to BM aspiration for collection of HSPCs is increased safety and comfort for the donor, faster reconstitution, and greater disease-free survival in recipient (To et al. 1997; Korbling and Anderlini 2001).

The molecular mechanisms that drive BM stem cell mobilization and the reasons behind certain molecules eliciting that mobilization have remained a mystery for a long time. In recent years, systematic analysis of (1) the molecular mechanisms responsible for the retention of BM stem and progenitor cells at their specific niches and (2) how these mechanisms are perturbed during mobilization has shed some light on the processes behind mobilization. The identification of some of the mechanisms responsible for mobilization (although many remain to be identified) has led to the development of new molecules that will considerably improve HSPC mobilization in the clinic.

The aim of this chapter is to review the molecular mechanisms and how these have led to the various strategies employed to mobilize BM-derived stem and progenitor cells in experimental and clinical settings. Mobilization of HSPCs will be reviewed first as it is the best understood and provides a paradigm that can be generalized to the mobilization of other types of BM stem and progenitor cells.

2
Mobilization of Hematopoietic Stem and Progenitor Cells

2.1
Diversity of Mobilization Mechanisms and Resulting Kinetics

HSPC mobilization can occur in response to a variety of stimuli that can be grouped according to their molecular nature, kinetics, and efficiency to mobi-

lize HSPC. For instance, intense and prolonged physical exercise causes a limited but nevertheless significant HSPC mobilization as observed in marathon runners (Barrett et al. 1978; Bonsignore et al. 2002). Neutrophil-activating chemokines such as interleukin (IL)-8/CXCL8 or Groβ/CXCL2 induce extremely rapid and brief HSPC mobilization within minutes, whereas hematopoietic growth factors induce more sustained mobilization within days. At the other end of the spectrum, cytotoxic drugs such as cyclophosphamide (CY) or 5′-fluorouracil (5-FU) induce HSPC mobilization within weeks during the recovery that follows myeloablation of the BM. Collectively, this broad range of kinetics and these levels of mobilization suggests that the different agents induce HSPC mobilization through different mechanisms (Table 1).

Currently, the agent most commonly used to elicit HSPC mobilization in the clinical setting is granulocyte-colony stimulating factor (G-CSF) used alone or in combination with myelosuppressive chemotherapy or stem cell factor (SCF) (To et al. 1994; Korbling and Anderlini 2001). The administration of G-CSF induces a 10- to 100-fold increase in the level of circulating HSPC in humans, primates, and mice. G-CSF-induced mobilization is time- and dose-dependent, involving a rapid neutrophilia (evident within hours) and a gradual increase in HSPC numbers in the blood, peaking between 4 and 7 days of G-CSF administration in humans. Mobilization with chemotherapeutic agents such as CY occurs during the recovery phase following the chemotherapy-induced neutropenia, that is, days 6–8 in mice, and days 10–14 in humans.

In order to understand how BM HSPCs are mobilized into the blood, it is first necessary to understand why they remain in the BM in steady-state conditions.

2.2 Stem and Progenitor Cells Reside in Separate Niches Within the Bone Marrow

Hematopoietic stem cells and lineage-restricted progenitor cells (HPCs) do not distribute randomly in the BM but are localized according to their differentiation stage. The majority of true HSCs are found at the bone–BM interface (endosteum) (Lord et al. 1975; Nilsson et al. 2001) in contact with osteoblasts (Zhang et al. 2003; Arai et al. 2004), whereas more committed progenitors accumulate in the central BM (Lord et al. 1975; Nilsson et al. 2001). The first experiments to illustrate this were performed by Brian Lord in the 1970s. The BM was dissected according to its proximity to the femur shaft. Most of the short-term reconstitution cells that colonize the spleen of lethally irradiated mice (colony forming units-spleen, CFU-S) were found close to the bone, while lineage-restricted HPCs that form colonies in vitro (CFU-C), but are unable to reconstitute hematopoiesis in vivo in lethally irradiated mice, accumulated away from the bone, in the central BM (Lord et al. 1975). As CFU-S can reconstitute hematopoiesis in vivo for a few weeks (unlike true HSCs that reconstitute life-long hematopoiesis), while CFU-C cannot, it was concluded that the most

Table 1 Classes of mobilizing agents

Class of agents	Time to maximum mobilization in humans and mice	Examples	Mechanism(s)
Chemokines and their analogs	15 min to few hours	CCL3, CXCL2, CXCL8	Direct granulocyte activation
		CXCL12, CXCR4 antagonists (AMD3100)	Blockade of CXCR4 function
Stress	1–2 h	Intense exercise, ACTH	Demargination, granulocyte activation?
Polyanions	1–2 h	Fucoidans	Release of cytokines and chemokines from intracellular and extracellular stores
		Dextran sulfate, polymethacrylic acid	ND
	5 days	Defibrotide	ND
Toxins	2–3 days	Pertussis toxin	Blocked CXCR4-mediated signaling
	ND	Bacterial endotoxins	ND
Antibodies	1–2 days	Anti-CD49d, anti-VCAM-1	Direct inhibition of VLA4:VCAM-1 interaction
Growth factors	4–6 days	G-CSF, pegylated G-CSF	Granulocyte expansion/activation
		KIT ligand/SCF	Synergism with G-CSF
		IL-3, GM-CSF, Flt-3 ligand, thrombopoietin, growth hormone, erythropoietin	ND
		VEGF, angiopoietin-1	Activation of Nos3, release of MMP-9
Myelosuppressive	1–3 weeks	CY, 5-FU	Granulocyte expansion, activation following BM suppression

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MMP, matrix metalloproteinase; ND, not determined; SCF, stem cell factor; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VLA, very late activation antigen

primitive HPCs and HSCs reside in proximity of the bone, whereas more lineage-restricted HPCs reside in the central marrow (Lord et al. 1975). This was recently confirmed in the mouse by sorting lineage-negative (Lin^-) wheat-germ agglutinin (WGA^{dim} rhodamine 123 ($Rho123)^{dull}$ cells or Lin^- Sca-1^+ c-KIT^+ cells, which are both enriched in HSCs, and Lin^- WGA^{dim} $Rho123^{bright}$ cells, which are enriched in HPCs. HSC- and HPC-enriched populations were then labeled with the fluorescent cell tracker carboxylfluorescein diacetate succinimidyl ester (CFSE) and injected intravenously into nonirradiated syngeneic recipients. While HPCs distributed randomly in the whole BM, HSCs lodged preferentially at the bone–BM interface or endosteum (Nilsson et al. 2001, 2005). Further studies have confirmed that cells that display the antigen profile of mouse HSCs (Lin^- Sca-1^+ c-KIT^+ Tie-2^+) are in close association with bone-forming osteoblasts that line the endosteum (Calvi et al. 2003; Zhang et al. 2003; Arai et al. 2004), whereas myeloid and lymphoid HPCs are in contact with fibroblastoid elements of the central marrow that express vascular cell adhesion molecule-1 (VCAM-1) and the chemokine CXCL12 (Tokoyoda et al. 2004). This differential distribution of stem and progenitor cells within the BM is in good accord with the notion that each type of stem and progenitor cell lodges in specific niches that provide specific signals adapted to the specific requirements of these cells (Schofield 1978; Fuchs et al. 2004; Tumbar et al. 2004; Kiel et al. 2005; Nagasawa 2006; Yin and Li 2006). These niche-specific signals control survival, quiescence, proliferation, and differentiation in order to maintain a steady stock of stem and progenitor cells while producing an adequate number of mature blood cells.

2.3 Finding Your Home and Staying There: A Matter for Cell Adhesion Molecules and Chemokines

2.3.1 Bone Marrow Hematopoietic Stem Cell Niches

In addition to the anatomical evidence for an HSC niche at the endosteum, there is strong genetic evidence that osteoblasts are crucial to BM hematopoiesis. For instance, transgenic mice with increased bone formation have an increased number of HSCs in the BM. Transgenic mice expressing a constitutively active mutant of the parathyroid hormone receptor 1 (PTHR1) in osteoblasts as well as mice carrying an inducible deletion of the bone morphogenic protein (BMP) receptor 1A (BMPR1A) gene, both have an increased number of osteoblasts, increased trabeculae, and a doubling in the number of HSCs per femur despite a markedly reduced BM cavity due to increased bone formation (Calvi et al. 2003; Zhang et al. 2003). Conversely, the ablation of osteoblasts results in a drop in HSC number. In transgenic mice carrying a herpesvirus thymidine kinase (HTK) suicide gene driven by an osteoblast-specific promoter, administration

of ganciclovir specifically induces osteoblast death. Osteoblast ablation results in a rapid and strong reduction in BM cellularity and a tenfold reduction in the number of HSCs present in the BM (Visnjic et al. 2004). Importantly, HSPCs and hematopoiesis relocate to the spleen and liver of these conditionally osteoblast-ablated mice (Visnjic et al. 2004). Collectively, these data strongly suggest that osteoblasts are an essential component of the HSC niche in the BM.

To understand how bone formation and BM hematopoiesis are coupled, one has to consider the molecular interactions between osteoblasts and HSCs. Lodgment at the endosteal niche is driven by a calcium gradient (Adams et al. 2006) and an array of osteoblast-mediated adhesive interactions (Fig. 1). At the endosteum, the bone is in constant turnover with concomitant bone formation driven by osteoblasts and osteoclast-mediated bone degradation. This continuous bone degradation releases soluble Ca^{2+} in the BM fluid, thus forming a Ca^{2+} gradient. HSCs express a chemotactic Ca^{2+} receptor that senses this gradient and promotes their migration to and lodgment at the endosteal HSC niche (Adams et al. 2006). Similarly, adhesive molecules produced by osteoblasts such as osteopontin, N-cadherin, transmembrane c-KIT ligand stem cell factor (tm-SCF), and the polysaccharide hyaluronic acid keep HSCs at the endosteum (see Fig. 1). Deletion of any of these molecules or their receptors results in random distribution of HSCs in the BM following transplantation (Nilsson et al. 2003, 2005; Hosokawa et al. 2005; Stier et al. 2005; Adams et al. 2006). Interestingly, adhesive interactions mediated by osteopontin, hyaluronic acid, and N-cadherin also initiate signaling events in HSCs that together delay their proliferation (Nilsson et al. 2003, 2005; Hosokawa et al. 2005; Stier et al. 2005). Thus, these adhesive interactions not only mediate tight adhesion of HSCs to osteoblasts at the hematopoietic niche, but are also likely to regulate HSC self-renewal in vivo.

The dramatic effect of osteoblast ablation on hematopoiesis may be explained by the loss of osteoblast-mediated adhesive interactions as well as loss of the cytokines and chemokines they produce (Fig. 1). In particular, osteoblasts are the main source of (1) chemokine stromal cell-derived factor-1 (SDF-1)/CXCL12 in the BM (Semerad et al. 2005), (2) angiopoietin-1, the ligand for the tyrosine-kinase receptor Tie-2 which is expressed by HSCs (Arai et al. 2004), and (3) Jagged-1, a ligand for Notch1 also expressed at the surface of HSCs (Calvi et al. 2003). Importantly, these interactions all regulate the survival/quiescence/proliferation of HSCs (Carlesso et al. 1999; Lataillade et al. 2000; Cashman et al. 2002; Arai et al. 2004). Hence, in light of these recent findings, it is not surprising that elimination of these essential interactions by specific ablation of osteoblasts, or conversely enhancement of osteoblast-specific ligands through increased bone formation, suppresses or enhances BM hematopoiesis. Interestingly, ablation of osteoblasts results in the massive migration of HSPCs to the spleen and liver via the blood (Visnjic et al. 2004). Hence, suppression of bone formation results in HSPC mobilization.

It must be noted that an "endothelial HSC niche" has recently been suggested as some HSCs that express CD150 are found in direct contact with the many

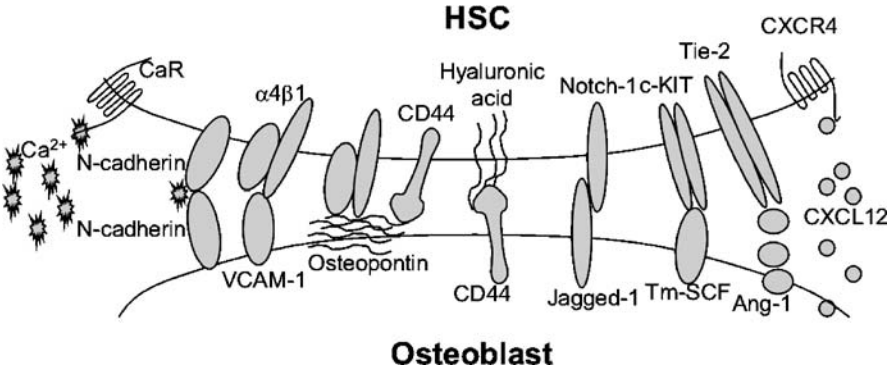

Fig. 1 Molecular interactions between HSCs and osteoblasts at the endosteal HSC niche. Attracted by CXCL12 secreted by osteoblasts and by soluble calcium released from bone degradation, HSCs expressing CXCR4 and calcium receptors lodge at the endosteum to establish direct cell–cell contact with osteoblasts through N-cadherin homotypic adhesive interactions, VCAM-1 interaction with α4β1 integrin, osteopontin with β1 integrins and CD44, hyaluronic acid with CD44. These cell–cell adhesive interactions allow Notch-1 to interact with its ligand Jagged-1, Tie-2 with angiopoietin-1 (Ang-1), and c-KIT with transmembrane SCF (tmSCF) expressed at the surface of osteoblasts

endothelial sinuses that irrigate the BM (Kiel et al. 2005). It is not clear, however, whether these HSCs that locate to endothelial sinuses represent cells in transit (as HSCs permanently leak into the circulation and home back to the BM) or whether they represent a separate HSC pool that is regulated differently. What is clear, however, is that these HSCs that lodge in specific endothelial niches must utilize a separate array of adhesive interactions from the HSCs found at the endosteum. Indeed, unlike osteoblasts, BM endothelial cells that compose this endothelial niche do not express osteopontin nor N-cadherin, but express instead high levels of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), VCAM-1/CD106, P-selectin, and E-selectin (Jacobsen et al. 1996; Schweitzer et al. 1996; Sipkins et al. 2005) whose counter-receptors are all expressed at the surface of HSCs (Yong et al. 1998; Lévesque et al. 1999; Winkler et al. 2004). Similar to the adhesion molecules expressed in the endosteal niches, P- and E-selectin-mediated adhesion regulates HSPC survival, proliferation, and differentiation (Lévesque et al. 1999; Winkler et al. 2004; Eto et al. 2005). Thus, the two anatomically distinct HSC niches, endosteal and endothelial, must regulate HSC turnover differently.

2.3.2 Bone Marrow-Committed Hematopoietic Progenitor Cell Niches

Due to the number and diversity of HPCs (multipotential, myeloid, lymphoid, etc.) their niches are much less understood and defined. B lymphoid progenitor cell niches, however, have been characterized. In mice with a green fluorescent

protein (GFP) complementary DNA (cDNA) knocked into the CXCL12 gene, GFP is specifically expressed by cells expressing CXCL12, a potent chemokine and survival factor for B cells as well as HSCs and HPCs. In these mice, the most primitive B cell progenitors (pre-pro B cells) are in direct contact with BM stromal cells located in the central BM that coexpress CXCL12 and VCAM-1. In contrast, the more mature pro-B cells are in contact with a distinct population of BM stromal cells that express IL-7 but not CXCL12 (Tokoyoda et al. 2004). Interestingly, Sca-1^+ KIT^+ cells, which include HSCs, multipotential HPCs, and most pluripotent colony-forming cells, were also found in direct contact with stromal cells that coexpress CXCL12 and VCAM-1 and are scattered throughout the BM (Tokoyoda et al. 2004).

Similarly, it has been found that megakaryocytes migrate to a specific niche proximal to BM endothelial sinuses, where they directly interact with BM endothelial cells via CXCL12 and VCAM-1. Furthermore, this direct interaction between megakaryocytes and BM endothelial cells was found to be necessary for platelet production (Avecilla et al. 2004).

From these observations, it has been speculated that the endosteal niche may maintain HSCs in a quiescent state, whereas the various niches scattered throughout the BM stroma may represent proliferative niches in which HSCs and HPCs proliferate to renew the pool of mature blood leukocytes as well as erythrocytes.

2.4 What Keeps HSPCs Within the BM?

The two interactions thought to be most important in retaining HSPCs within the BM are: (1) the adhesive interaction between VCAM-1 expressed by BM stromal cells and integrin α4β1 (very late activation antigen-4, VLA-4) expressed by HSPCs, and (2) the chemotactic interaction between the chemokine CXCL12 (or SDF-1) and its sole receptor CXCR4, also expressed by HSPCs. The conditional deletion of either the integrin α4 gene or the VCAM-1 gene (its ligand) results in a permanent and robust HSPC mobilization (Scott et al. 2003; Ulyanova et al. 2005) as does the systemic administration of CXCR4 antagonists such as AMD3100 (Liles et al. 2003; Broxmeyer et al. 2005) or the systemic delivery of CXCL12 via recombinant adenoviruses (Hattori et al. 2001b). Both VCAM-1 and CXCL12 are expressed by osteoblasts, BM stromal cells, and endothelial cells, which are all cellular components of the various HSC and HPC niches in the BM. As discussed further in the following section, it is therefore not surprising that, by targeting these two critical interactions, most mobilizing agents induce mobilization of HSCs as well as most HPCs (collectively termed as HSPCs).

2.5 What Makes HSPCs Leave the BM?

2.5.1 Neutrophilia and/or Neutrophil Activation Are Essential for HSPC Mobilization

Neutrophilia always precedes the HSPC mobilization induced by physical exercise (Barrett et al. 1978), ACTH (Barrett et al. 1978), endotoxin (Cline and Golde 1977), sulfated polysaccharides and polyanions (van der Ham et al. 1977), myelosuppressive chemotherapy (To et al. 1984, 1989), chemokines (Pruijt et al. 1999; King et al. 2001), or hematopoietic growth factors (Molineux et al. 1990, 1991, 1997; Sato et al. 1994; Glaspy et al. 1997; Torii et al. 1998), and the degree of neutrophilia is correlated to the level of mobilization (Roberts et al. 1997; Krieger et al. 1999).

The critical importance of neutrophils and neutrophilia to the mobilization of HSPCs is illustrated by the fact that mice that are made neutropenic by homozygous targeted deletion of the G-CSF receptor gene (*G-CSFR*$^{-/-}$ knockout mice), or by administration of specific antineutrophil antibodies, do not mobilize in response to G-CSF, CY, or IL-8 (Liu et al. 1997; Pruijt et al. 2002; Pelus et al. 2004). Thus, neutrophils are essential for HSPC mobilization.

2.5.2 Role of Neutrophil Proteases

Mobilization with G-CSF or CY induces the accumulation of neutrophils and their precursors in the BM (Lévesque et al. 2002) with the release of large amounts of neutrophil proteases such as neutrophil elastase (NE), cathepsin G (CG), and matrix metalloproteinase-9 (MMP-9) (Lévesque et al. 2001, 2002) directly in the BM fluid. These proteases selectively cleave and inactivate the adhesion molecules and chemokines necessary for the retention of HSPC within the BM, particularly VCAM-1 (Lévesque et al. 2001), CXCL12, and the CXCL12 receptor CXCR4 (Petit et al. 2002; Lévesque et al. 2003a), as well as the tyrosine-kinase receptor c-KIT (Lévesque et al. 2003b) and transmembrane c-KIT ligand SCF (Heissig et al. 2002) whose roles in BM retention and mobilization of HSPC has also been reported (Papayannopoulou et al. 1998; Nakamura et al. 2004). Thus, active proteases released by neutrophils during mobilization disrupt the adhesive and chemotactic interactions that are essential for retaining HSPC within the BM, resulting in their release into the circulation.

In support of this model is the fact that specific NE inhibitors reduce mobilization by 60%–70% in the mouse (Petit et al. 2002; Pelus et al. 2004), and the administration of the serine-protease inhibitor serpina1/α_1-antitrypsin (a physiological inhibitor of both NE and CG) completely blocks mobilization (van Pel et al. 2006). Similarly, anti-MMP-9 monoclonal antibodies that block MMP-9 proteolytic function inhibit mobilization induced by IL-8 (by 90%)

and G-CSF (by 40%) in the mouse (Pruijt et al. 1999; Pelus et al. 2004). In humans, raised plasma NE and MMP-9 concentrations correlate significantly with the level of HPC mobilization (Lévesque et al. 2001). A similar increase in blood NE, CG, MMP-9, and cleaved soluble VCAM-1 has been observed in idiopathic myelofibrosis and polycythemia vera patients, correlating with the level of constitutive HSPC mobilization that occurs with these conditions (Xu et al. 2005; Passamonti et al. 2006). It thus appears that mobilizing agents that induce the accumulation, activation, or accumulation and activation of neutrophils in the BM (e.g., G-CSF, cytotoxic, IL-8, Groβ) disrupt the proteolytic balance within the BM, maintenance of which is essential to the regulation of the BM microenvironment, homeostasis, and HSPC trafficking.

It must be noted, however, that body fluids are normally loaded with naturally occurring protease inhibitors to protect tissues from proteolytic damage. For instance, blood contains approximately 2 mg/ml α_2-macroglobulin, an inhibitor of a wide range of proteases, and approximately 1 mg/ml serpina1/α1-antitrypsin and serpina3/α1-antichymotrypsin, both of which are specific inhibitors of NE and CG. BM extracellular fluids are devoid of α_2-macroglobulin (which is produced by the liver and cannot diffuse through the endothelial cell barrier because of its large size) but contain large amounts of serpina1 and serpina3 (Winkler et al. 2005). Unlike blood serpins, which are produced by the liver, BM serpins are transcribed and produced within the BM to protect the BM stroma from neutrophil serine proteases (Winkler et al. 2005). We have recently demonstrated that during mobilization induced by either G-CSF or CY, the levels of these serpins drop dramatically within the BM (from a few mg/ml to below detection) boosting the levels of active neutrophil serine proteases with concomitant cleavage and inactivation of molecules essential for the retention of HSPCs (Winkler et al. 2005). Interestingly, serpina1 expression by the liver and hence in the plasma remains unchanged during mobilization (Winkler et al. 2005). That the downregulation of serpina1 and serpina3 is critical to mobilization is also supported by the recent finding that prior administration of human serpina1 into mice prevents HSPC mobilization in response to IL-8 (van Pel et al. 2006). Therefore, the downregulation of serpin expression is likely to be a permissive step enabling the accumulation of active neutrophil proteases in the BM extracellular fluid.

Despite this array of convergent observations, there remains a discrepancy between the results of studies using short-term, systemic administration of protease inhibitors or conditional gene deletions (many of which alter mobilization) and those using mice where the targeted gene has been deleted throughout development (where mobilization is generally not altered). For example, systemic administration of specific NE or CG inhibitors decreases mobilization (Petit et al. 2002; Pelus et al. 2004) while mice knocked out for NE and CG mobilize normally even in the presence of a soluble MMP inhibitor (Lévesque et al. 2004). Functional redundancy between proteases may be partly responsible for these conflicting results, as neutrophils express many

other proteases that were not targeted or might have been overexpressed to compensate for the lack of NE and CG.

2.5.3 The Role of Bone-Forming Cells

Recently we have found that in addition to cleavage by neutrophil proteases, CXCL12 messenger RNA (mRNA) levels also drop in the BM of mobilized mice, suggesting that an alternative mechanism may also be involved (Semerad et al. 2005). By sorting various mouse BM cell populations, we found the $Lin^{-}CD45^{-}CD31^{-}CD51^{+}$ population that is enriched in osteoblasts most actively transcribed CXCL12 (Semerad et al. 2005). This is of particular interest as osteoblasts have been recently identified as an essential component of the HSC endosteal niche (Visnjic et al. 2001; Calvi et al. 2003; Zhang et al. 2003; Zhu and Emerson 2004).

A common side effect of G-CSF administration is bone pain, which affects 80% of mobilized donors (Vial and Descotes 1995). This may be due to the dramatic reduction in bone turnover that occurs with G-CSF administration (Takamatsu et al. 1998). Systemic administration of G-CSF rapidly inhibits osteoblast-mediated bone formation as well as increasing bone degradation by osteoclasts in both human and mouse (Takamatsu et al. 1998). Osteocalcin, a bone matrix protein specifically produced by osteoblasts, is a good indicator of bone formation and osteoblast activity. In humans, osteocalcin concentration in the plasma drops during HSPC mobilization, and this drop is significantly correlated with the number of CFU-GM mobilized in the peripheral blood (Takamatsu et al. 1998). Similarly in mice, the concentration of osteocalcin mRNA in the bone marrow drops approximately 50-fold during G-CSF administration (Semerad et al. 2005), while the number of osteoblasts lining the endosteum is reduced. The mechanisms by which osteoblasts are inhibited during HSPC mobilization are still poorly understood as none of the cytokines used to induce mobilization directly binds to osteoblasts or alters their function (Lévesque et al. 2005; Semerad et al. 2005; Katayama et al. 2006). It is not a direct effect of G-CSF as osteoblasts do not express the G-CSF receptor and do not respond to G-CSF in vitro (Semerad et al. 2005). It has recently emerged that sympathetic nerves that extend through the BM and bones may play an important role in the maintenance of osteoblast function and HSC mobilization (Katayama et al. 2006). Using mice knocked out for the uridine diphosphate-galactose ceramide galactosyltransferase (an enzyme necessary for myelin synthesis) gene or for the dopamine β hydroxylase (the enzyme converting dopamine to norepinephrine) gene, it has been shown that functional sympathetic nerves are necessary for HSPC mobilization and osteoblast inhibition in neonatal mice, but to a much lesser extent in adults (Katayama et al. 2006). As adrenergic nerves express G-CSF receptors and osteoblasts express β_2 adrenergic receptors, it is possible that G-CSF may directly act on

these nerves eliciting osteoblast inhibition and HSPC mobilization. However, lethally irradiated *G-CSFR*$^{-/-}$ adult recipients reconstituted with wild-type BM cells (thus containing *G-CSFR*$^{-/-}$ nerves with *G-CSFR*$^{+/+}$ hematopoietic cells) mobilize normally in response to G-CSF or CY (Liu et al. 2000). Similarly, HSPC mobilization can be completely abrogated by neutrophil depletion (Liu et al. 1997, 2000; Pruijt et al. 2002; Pelus et al. 2004) despite apparently normal neuronal function. Taken together, it is therefore likely that the predominant mechanisms behind mobilization in adults are independent of neurons.

Collectively, these data indicate that mobilization follows the inhibition of bone-forming osteoblasts, which in turn would lead to a decrease in both CXCL12 production and number of HSPC endosteal niches. Therefore, HSPC mobilization may involve at least two underlying mechanisms. The first mechanism involves the release of active proteases by BM neutrophils that cleave and inactivate chemotactic and adhesive molecules that retain HSPC within the BM, particularly CXCL12, c-KIT, tmSCF, and VCAM-1. Since most HSPCs are in direct contact with BM stromal and endothelial cells expressing these molecules and express receptors for these molecules, the protease-mediated mechanism could be responsible for the mobilization of multipotential, myeloid and lymphoid progenitors located in the central BM (Fig. 2).

A second mechanism targets the osteoblasts that form the HSC niche by decreasing their number and function (Fig. 2). Ultimately, this inhibition not only results in decreased CXCL12 expression and release by osteoblasts, but also in a net decrease in the number of functional HSC niches at the endosteum. Thus, this mechanism could be involved in the mobilization of most primitive HSCs residing at the endosteum, by depleting the endosteal niches, and forcing their migration to more a central location within the BM where protease-dependant mechanisms could take the relay to force their egress into the peripheral blood (Fig. 2).

2.6 Strategies to Increase HSPC Mobilization

2.6.1 Why the Need to Enhance Mobilization

The dose of mobilized CD34$^+$ HSPCs infused correlates directly with the speed of recovery to acceptable levels of neutrophils and platelets and the overall survival of transplanted patients (To et al. 1986; Sheridan et al. 1994; Siena et al. 2000). A minimal threshold of 2–5×10^6 CD34$^+$ cells/kg is necessary for successful hematopoietic reconstitution (Demirer and Bensinger 1995; To et al. 1997; Siena et al. 2000). Furthermore, it now appears that the higher the dose of CD34$^+$ cells infused the shorter is the leukopenic period and the faster is the recovery.

A significant and well-documented problem with G-CSF is patient-to-patient variability in HSPC mobilization which extends over 2.5 orders of

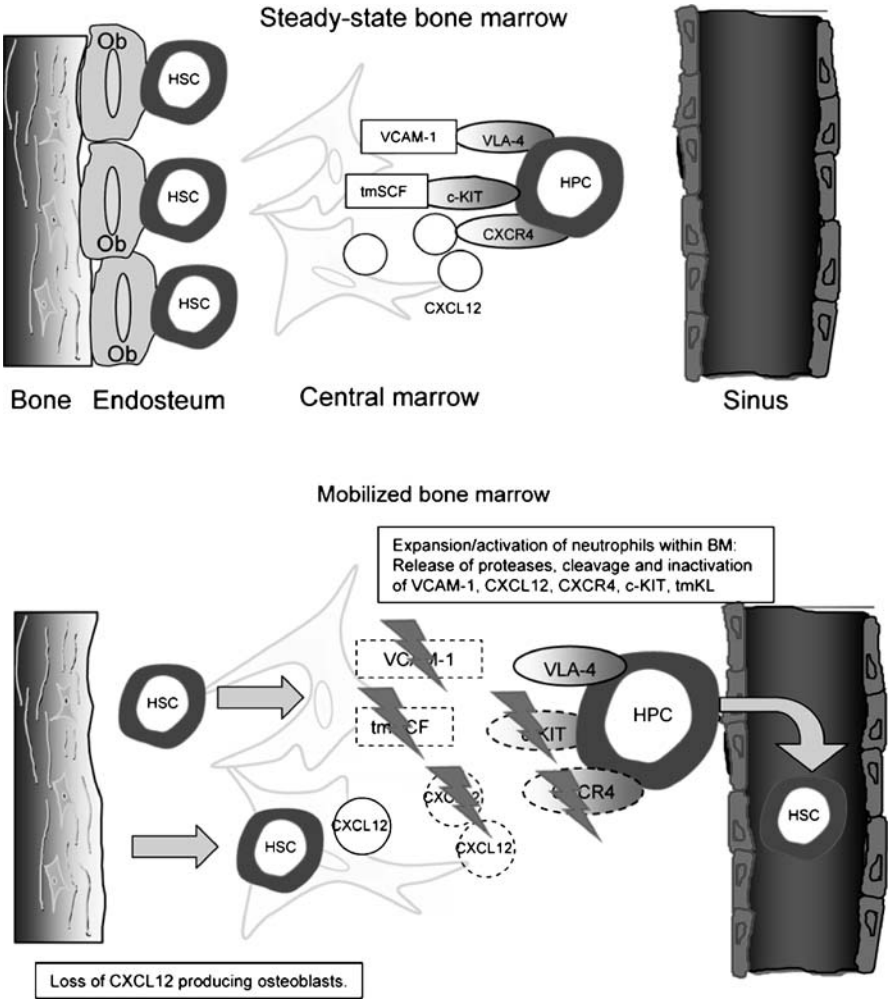

Fig. 2 A two-step model of HSPC mobilization. The inhibition of osteoblasts results in a loss of the HSC endosteal niche and decrease production of the chemokine CXCL12 causing the migration of HSCs away from the endosteum into the central marrow. In parallel, neutrophils expand and accumulate within the BM, releasing proteases. Because the expression of protease inhibitors is downregulated, neutrophil proteases accumulate in an active state, cleaving and inactivating molecules essential to HSPC retention in the BM such as VCAM-1, tmSCF, c-KIT, CXCL12, and CXCR4

magnitude (Roberts et al. 1995; Villalon et al. 2000). A consequence of this variability is the failure to harvest sufficient numbers of peripheral blood HSPC to reach minimal thresholds in 5%–10% of allogeneic healthy donors and up to 50%–60% of patients who have already undergone several courses of high-dose chemotherapy (mobilized for autologous transplantation) (Bensinger

et al. 1995; Demirer and Bensinger 1995; Brown et al. 1997; Villalon et al. 2000). Thus, the discovery of more efficient mobilizing agents is paramount to improve transplantation outcome.

2.6.2 Chemical Stabilization of G-CSF

The commercially available form of human G-CSF used to mobilize HSPCs (Filgrastim or Neupogen, Amgen, Thousand Oaks, CA) in clinics is produced in recombinant form in *Escherichia coli* and is thus unglycosylated. Unglycosylated G-CSF is more sensitive to proteolytic degradation by both NE and CG (El Ouriaghli et al. 2003; Carter et al. 2004), the very same proteases that become active during mobilization. More recently, the recombinant human G-CSF produced in *E. coli* has been stabilized by attaching long chains of polyethylene glycol ("pegylation"). Because of its increased resistance to proteases and lower renal filtration, due its large size, this pegylated G-CSF (Pegfilgrastim or Neulasta, Amgen, Thousand Oaks, CA) persists at pharmacologically active concentrations in the plasma over 4 days following a single injection. As a consequence, a single injection of pegylated G-CSF is sufficient to induce robust mobilization in rodents and humans instead of a 4–5 day course of daily injections of nonpegylated G-CSF (de Haan et al. 2000; van Der Auwera et al. 2001). In addition, transplantations using cells mobilized with pegylated G-CSF or progenipoietin-1 (a fusion protein made of G-CSF and Flt-3 ligand) display (1) reduced graft-vs-host disease (GvHD) in fully mismatched murine models and (2) increased graft-vs-leukemia reaction (GvL) (Morris et al. 2004, 2005; Kiel et al. 2005). Because of its improved stability and its strong immunomodulatory functions, the use of pegylated G-CSF for HSPC mobilization is likely to increase rapidly and replace nonpegylated G-CSF.

2.6.3 Stem Cell Factor

In the last 15 years, a number of hematopoietic growth factors have been tried for their potential to mobilize HSPCs, with varying degrees of success. For instance, GM-CSF, IL-3, IL-1, and IL-6 showed little potential, while ligands of tyrosine kinase receptors such as SCF synergized when used with G-CSF (To et al. 1994; Roberts et al. 1997; Stiff et al. 2000; To et al. 2003). Of those, the KIT ligand/SCF is the only one that has made it to the bedside. Because of the strong synergistic effect of SCF with other hematopoietic growth factors, and particularly G-CSF, this combination of SCF together with G-CSF increases by a factor 5 to 20 the number of HSPCs mobilized by G-CSF alone. Despite its very potent effect on HSPC mobilization, SCF is no longer used the USA due to a high incidence of mast cell-mediated reactions despite antihistamine

prophylaxis (Stiff et al. 2000). SCF, however, is still used in Canada, Europe, and Australia to boost mobilization in patients who have failed to respond adequately in response to G-CSF alone.

2.6.4
Chemokines and Their Analogs: The AMD3100 Success Story

As reviewed earlier, CXCR4 is central to the retention of HSCs and HPCs within the BM, and perturbations of the CXCR4:CXCL12 chemotactic interaction within the BM results in mobilization. Since exogenous CXCL12 is rapidly cleared from the circulation, injection of CXCL12 has a limited effect on mobilization. Therefore more stable CXCR4 agonists and antagonists were designed to induce mobilization. The first series of compounds comprises a shorter cyclic version of CXCL12 (Perez et al. 2004; Pelus et al. 2005). A second category of compounds comprises small synthetic nonpeptide molecules such as the bicyclam AMD3100 (Mozobil, AnorMED-Genzyme, Cambridge, MA). AMD3100 was designed as a CXCR4 antagonist to treat human immunodeficiency virus (HIV) infection, as CXCR4 is a coreceptor of HIV. Although AMD3100 was efficacious in blocking HIV entry into T cells in vitro, clinical trials were stopped because of its long-term toxicity and low effect on HIV viral load (Hendrix et al. 2000; Hendrix et al. 2004). However, when administered for a few days, AMD3100 induces potent HSPC mobilization and strongly synergizes with G-CSF, increasing mobilization by one to two logs over G-CSF alone (Liles et al. 2003; Devine et al. 2004; Broxmeyer et al. 2005; Flomenberg et al. 2005). There are currently eight clinical trials from phase I to phase III ongoing in the USA to further evaluate the safety and efficacy of AMD3100 to increase G-CSF-induced HSPC mobilization in autologous transplantations (multiple myeloma, non-Hodgkin's lymphoma) as well as in healthy donors for transplantation into patients affected with a variety of hematopoietic diseases. These diseases include myelodysplastic syndrome, multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's disease, and acute myelogenous, acute lymphoblastic, chronic myelogenous, and chronic lymphocytic leukemia (http://www.clinicaltrials.gov).

3
Mobilization of Bone Marrow Endothelial Progenitor Cells

3.1
Mobilization of Pro-angiogenic Progenitor Cells

Following limb ischemia or myocardial infarct, rapid reperfusion and reestablishment of circulation is critical in order to limit the extent of tissue damage and necrosis. Since terminally differentiated endothelial cells isolated from

the vascular lumen (i.e., from umbilical cord or from aorta) have an enormous capacity to proliferate and migrate in vitro, postnatal neovascularization was thought to involve the migration and proliferation of differentiated endothelial cells from neighboring preexisting vessels. In 1997, however, Asahara et al. reported the existence of a subpopulation of $CD34^+$ cells isolated from human peripheral blood that adhere to fibronectin, proliferate, and differentiate in culture into cells that display the hallmarks of endothelial cells: spindle-shaped morphology, formation of tube-like structures, incorporation of fluorescent acetylated low-density lipoproteins (AcLDL), expression of endothelial nitric oxide synthase (eNOS)/Nos3 and vascular endothelial growth factor receptor 2 (VEGFR2)/kinase insert-domain containing receptor (KDR). Furthermore, when injected intravenously into immunodeficient mice with a prior hind-limb ischemia, these cells colonized the neovascularized ischemic limb, suggesting that they directly contributed to the neovascularization process and as such were labeled "endothelial progenitor cells" or EPCs (Asahara et al. 1997). From this pioneering observation, a flurry of studies followed in order to determine whether these putative EPCs that circulate in steady-state blood could be mobilized to accelerate postischemic revascularization. Two years later, the same group showed that administration of human vascular endothelial growth factor 165 ($VEGF_{165}$) into mice mobilized cells displaying these EPC characteristics. Furthermore, VEGF administration resulted in a 50% increase of the neovascularization taking place in the cornea following corneal injury. When these experiments were repeated in chimera mice transplanted with BM cells from transgenic mice expressing LacZ under the Tie-2 promoter, administration of VEGF resulted in the contribution of $LacZ^+$ cells in the neovascularized cornea. $LacZ^+$ cells were very rare in the neovascularized cornea of control mice injected with bovine serum albumin, proving that these endothelial-like cells in neoformed vasculature were (1) coming from the BM and (2) mobilized into the circulation in response to VEGF (Asahara et al. 1999). Subsequently, it was shown that EPCs are mobilized by many of the cytokines that mobilize HSPCs such as VEGF-A, angiopoietin-1 (Hattori et al. 2001a), G-CSF (Orlic et al. 2001), GM-CSF (Takahashi et al. 1999), or erythropoietin (Heeschen et al. 2003). In these experiments, evidence of EPC mobilization was demonstrated using *Tie-2*LacZ BM transplantation chimera with enhanced neovascularization of damaged tissues and enhanced contribution of $LacZ^+$ bone marrow-derived cells in neoformed vessels following corneal injury, hind-limb ischemia, or acute myocardial ischemia.

3.2 What Are Endothelial Progenitor Cells? A Simple Question, a Complex Answer

That the phenotypic profile of HSCs and HPCs is now well-established in both humans and mice has greatly helped our understanding of HSPC mobilization. Transplantation of single-sorted cells and clonal analyses have resolved the

identity of HSCs and most types of HPCs (Morrison and Weissman 1994; Kondo et al. 1997, 2003; Akashi et al. 2000). This knowledge has enabled the precise localization of HSCs and HPCs in a variety of tissues in adults and during development, follow their trafficking in vivo, or purify them to analyze their fundamental biological properties as well as their potential to reconstitute the various lineages of the hematopoietic and immune systems. In respect to EPCs, their proper identification and characterization has been a lot more challenging, and to date their precise nature remains still open to debate.

EPCs are still currently defined as cells that, upon culture, form colonies of adherent spindle-shaped cells displaying characteristics of endothelial cells such as the incorporation of AcLDL and binding of von Willebrand factor (vWF) and BS-1 lectin. While this functional definition is sufficient to enable the identification of molecules that accelerate neovascularization and/or enhance EPC mobilization in vivo, it gives little insight on the nature, ontology, and biology of the cells responsible for this effect, whether it is due to the mobilization of so-called EPCs and/or to comobilized hematopoietic cells secreting pro-angiogenic cytokines.

The task of precisely identifying and defining EPCs has been hampered not only by the lack of specific markers but also by the very nature of endothelial cells, since they share many markers with hematopoietic cells. This promiscuity of endothelial vs hematopoietic markers is likely to be due to the fact that during development they derive from a common cell, the hemangioblast. The task is also complicated by the fact that many hematopoietic cells are mobilized together with EPCs, and these hematopoietic cells secrete a wide range of proangiogenic cytokines and directly contribute to the repair of the ischemic tissue, particularly macrophages and granulocytes (Balsam et al. 2004; Minatoguchi et al. 2004; De Palma et al. 2005).

Mobilized EPCs express CD34, CD133, VEGFR2, and VE-cadherin in humans and are Lin^- c-KIT^+ Sca-1^+ $VEGFR2^+$ VE-$cadherin^+$ in mice (Rafii and Lyden 2003). However, this phenotype is shared with that of HSCs. Other frequently used markers are CD31 and CD146 (P1H12). While CD31 is expressed by many nucleated hematopoietic cells in the BM, CD146 is expressed by a subset including HSCs. Amazingly, CD45, a transmembrane phosphatase exclusively expressed by nucleated hematopoietic cells, is very rarely included in these studies. It is therefore difficult to assess whether mobilized EPCs are of hematopoietic or endothelial origin, and whether accelerated revascularization following ischemia is due to mobilized EPCs, mobilized hematopoietic cells, or both. To illustrate the importance of this still unresolved question, a subpopulation of $CD45^+$ $CD11b^+$ monocytes expressing Tie2 (as HSCs and EPCs do) play a critical role in tumor neovascularization and ischemia revascularization (De Palma et al. 2005). These Tie2-expressing monocytes express proangiogenic factors, and their ablation suppresses tumor neovascularization. Reciprocally, incorporation of Tie2-expressing monocytes into Matrigel plugs implanted under the skin promotes robust angiogenesis, suggesting that

recruitment of these Tie2-expressing monocytes to the site of ischemic injury is sufficient to support revascularization (De Palma et al. 2005).

The hematopoietic nature of mobilized EPCs is suggested by a recent study showing that injection of monoclonal antibodies blocking α4-integrin function mobilize EPCs (Qin et al. 2006) in parallel to HSPCs (Papayannopoulou and Nakamoto 1993; Papayannopoulou et al. 1995). The ability of blood cells to form endothelial colonies (defined as mobilized EPCs) was contained within the $CD45^+$ $\alpha4\text{-integrin}^+$ population, which comprises only hematopoietic cells (as CD45 is exclusively expressed by hematopoietic cells). Unfortunately, the potential of $CD45^-$ cells was not tested, and it is therefore impossible to conclude from this study whether the entire EPC activity is contained within the $CD45^+$ population.

The possibility that mobilized hematopoietic cells are essential to the revascularization of ischemic tissues is further suggested by the finding that ischemia-induced revascularization could be due to "hemangiocytes," a subpopulation of HPCs expressing VEGFR1 together with CXCR4 (Jin et al. 2006). Unlike endothelial cells, these hemangiocytes do express CD45 and CD11b (and are therefore of hematopoietic origin), but do not express VE-cadherin, vWF, E-selectin, or smooth muscle α-actin (Grunewald et al. 2006). The hemangiocytes are mobilized following VEGF-A administration and home to the site of ischemia due to the release of CXCL12 by fibroblasts surrounding the damage vessels. Once homed to ischemic vessels, the hemangiocytes may release proangiogenic paracrine factors that stimulate the proliferation of adjacent endothelial cells from the damaged vessel, or of mobilized EPCs that have been recruited the site of ischemia by a similar mechanism (Grunewald et al. 2006).

3.3 Is Re-vascularization Due to EPC Mobilization?

It is intriguing that many of the agents that mobilize HSPCs also induce revascularization post ischemia and are therefore presumed to mobilize EPCs (e.g., VEGF-A, placental growth factor, G-CSF, GM-CSF, erythropoietin, CXCL12, function-blocking anti-α4-integrin). This raises the question about whether revascularization is due to mobilized EPCs, and if so, is this function due to intrinsic properties of EPCs or rather BM leukocytes that comobilize with them, such as Tie2-expressing monocytes, granulocytes, and hemangiocytes (Balsam et al. 2004; Minatoguchi et al. 2004; Ingram et al. 2005; Jin et al. 2006; Kopp et al. 2006).

An answer to this difficult question has been provided by Shahin Rafii's group. The blood concentration of cytokines such as thrombopoietin/megakaryocyte growth and differentiation factor (TPO), soluble SCF, erythropoietin, and GM-CSF and of CXCL12 chemokine rises dramatically between 24 and 72 h following hind-limb ischemia. Furthermore, mice deficient for TPO, its receptor c-Mpl, G-CSF, or GM-CSF exhibit reduced ischemia-induced revascular-

ization, demonstrating that in vivo revascularization is critically dependent on endogenous cytokines that also induce mobilization of HSPCs (Jin et al. 2006). Interestingly, *MMP-9*$^{-/-}$ mice, in which the shedding of tmSCF into soluble SCF is compromised, also exhibit impaired postischemia revascularization (Jin et al. 2006). TPO and soluble SCF induce the release of CXCL12 stored in platelet granules both in vitro and in vivo, as there is no rise in CXCL12 blood concentration following ischemia in thrombocytopenic *TPO*$^{-/-}$ or c-*mpl*$^{-/-}$ mice (Jin et al. 2006). Revascularization is critically dependant on CXCL12 as systemic delivery of CXCL12 following infection with recombinant adenoviruses expressing CXCL12 restores postischemic revascularization in *TPO*$^{-/-}$ or c-*mpl*$^{-/-}$ mice (Jin et al. 2006). Finally, these authors show that the rise in plasma CXCL12 mobilizes BM VEGFR1^{+} CXCR4^{+} hemangiocytes, which then home to damaged vessels in response to the release of CXCL12 and VEGF-A by the surrounding hypoxic tissue (Jin et al. 2006). In favor of this model, injection of purified hemangiocytes into *MMP-9*$^{-/-}$ ischemic mice (which have impaired hemangiocyte mobilization and revascularization) restores revascularization. Thus, accelerated revascularization following mobilization may not involve the mobilization of EPCs but rather that of hemangiocytes, which are nonendothelial.

The intimate link between acceleration of revascularization and mobilization of BM hematopoietic cells is further illustrated in mice deficient for eNOS/Nos3. *Nos3*$^{-/-}$ mice have impaired neovascularization following ischemia. This intracellular enzyme is expressed by endothelial cells and myocytes but not by BM hematopoietic cells. It produces nitric oxide (NO) by converting L-arginine into citrulline. Once released by endothelial cells, NO is a vasodilator that relaxes smooth muscle cells and increases vessel permeability, as well as promotes endothelial cell survival and proliferation. Increased Nos3 activity and NO release are characteristic of the ischemic response. Although Nos3 is not expressed by BM hematopoietic cells, the deletion of the Nos3 gene reduces BM hematopoietic cell survival and recovery in response to cytotoxic injury (particularly in response to 5-FU), reduces EPC and HSPC mobilization in response to VEGF, and impairs revascularization following hind-limb ischemia (Aicher et al. 2003). This effect is due to nonhematopoietic cells, as lethally irradiated *Nos3*$^{-/-}$ recipients reconstituted with wild-type BM cells have impaired mobilization and revascularization whereas wild-type recipients reconstituted with *Nos3*$^{-/-}$ BM cells mobilize normally in response to VEGF-A. The effect of Nos3 on mobilization and revascularization seems to involve MMP-9 because (1) MMP-9 release is reduced in the BM of *Nos3*$^{-/-}$ mice (Aicher et al. 2003), and (2) MMP-9 cleaves tmSCF into soluble SCF, a critical step of VEGF-induced and 5-FU-induced mobilization of EPCs and HSPCs (Heissig et al. 2002; see Fig. 3). Therefore, although not expressed in hematopoietic cells, Nos3 regulates the homeostasis of the BM stroma that regulates the fate of both EPCs and HPCs.

Therefore, from these studies, it is clear that a population of still not well defined EPCs is mobilized from the BM into the blood, and that this mobi-