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Review Article

Bone Marrow Homing and Engraftment Defects of Human Hematopoietic Stem and Progenitor Cells

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Abstract. Homing of hematopoietic stem cells (HSC) to their microenvironment niches in the bone marrow is a complex process with a critical role in repopulation of the bone marrow after transplantation. This active process allows for migration of HSC from peripheral blood and their successful anchoring in bone marrow before proliferation. The process of engraftment starts with the onset of proliferation and must, therefore, be functionally dissociated from the former process. In this overview, we analyze the characteristics of stem cells (SCs) with particular emphasis on their plasticity and ability to find their way home to the bone marrow. We also address the problem of graft failure which remains a significant contributor to morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). Within this context, we discuss non-malignant and malignant hematological disorders treated with reduced-intensity conditioning regimens or grafts from human leukocyte antigen (HLA)-mismatched donors.

Keywords: Stem cells; Homing; Engraftment.

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Introduction. Allogeneic hematopoietic stem cell transplantation (HSCT) currently represents one of the best standard treatment options for a variety of malignant and non-malignant hematological diseases. This approach is based on the ability of donor hematopoietic stem cells (HSC) to localize to recipient bone marrow (BM) niches. Notably, only a small percentage of infused HSCs (10%) engraft within the marrow microenvironment. This process, known as *"Homing,"* is not fully elucidated and our ability to modulate it remains incomplete. Engraftment failure is a rare but serious complication of HSCT. In order to gather the most robust evidence in this area, we performed a search of the literature available in Pubmed from January 2005 to January 2017 on "Hemopoietic stem cell homing and engraftment," "Hemopoietic stem cell homing and engraftment defects" and "Hemopoietic stem cell homing and chimerism." The present review covers the most important aspects of recent insights into the mechanisms of engraftment and defective engrafting activity of HSCs.

Biological Properties of Stem Cells. Stem cells (SCs) are ancestral precursors common to all cell types. They are responsible for the generation of the tissues that form organs during embryogenesis

and from there on maintaining the capacity of self-renewal for the entire life of the organism. The concept of stem cells dates back to the early 1960s when Till and McCulloch analyzed bone marrow to find out which components were responsible for *in vivo* blood regeneration.[1] Ten days after transplantation of syngeneic bone marrow (BM) cells in a murine model, they observed the growth of nodules in the animal spleens. These nodules, defined by the authors as "spleen colonies," appeared in proportion to the number of injected BM cells and were therefore thought to derive from a single BM cell.[2] These preliminary observations made it possible to establish two main hallmarks of HSCs, namely, their ability to renew themselves (*long-term self-renewal*) and to give rise to mature cell types with characteristic morphology and specialized functions. Before reaching a fully differentiated adult status, SCs generate intermediate cell types called *precursors* or *progenitor cells*. These cells are partially differentiated and committed to going through numerous cycles of cell division (*committed precursors)* to complete their developmental pathway in adult tissues.[3] Experiments carried out on the Drosophila fruitfly suggest two different mechanisms by which SCs can simultaneously generate identical copies of themselves as well as more differentiated progeny.[4] These two modes of cell division are referred to as *asymmetric cell division* and *symmetric cell division*. The first mode is characterized by an intrinsically asymmetric mechanism whereby only one of the two daughter cells inherit the regulating factors necessary for self-renewal and homeostatic control of the stem cell pool. Hence each single SC produces a copy of itself plus a differentiated cell (*differentiative* division).[5-7]

In the second symmetric mode, homeostatic control is maintained at the population level rather than at single cell level. Two types of symmetric division have been distinguished: a proliferative division which results in the generation of two new stem cells and a differentiation division which generates two differentiated cells.[8] Several mathematical algorithms have been developed and are currently available for the simulation of stem cell proliferation kinetics.[9]

SCs are classified as embryonic stem cells (ESCs), embryonic germ cells (EGCs) or adult stem cells (ACSs), depending on their origin and different properties. The cells that can virtually produce any kind of tissue in the body, including extra-embryonic and placental tissues, are known as *totipotent cells*. These totipotent zygote cells appear about 5-7 days after fertilization when the fertilized egg starts to divide and produces more totipotent stem cells. After about 4 days of cell division, these cells begin to specialize into *pluripotent cells* that can generate all embryonic tissues but not an entire organism. That is why totipotent stem cells are considered the most versatile among the different types of SCs.

ESCs and induced pluripotent stem cells (iPSCs) pertain to the category of pluripotent stem cells. When pluripotent stem cells differentiate further, *multipotent cells* are formed, these cells are less plastic and more specialized and can develop into more than one cell type but never all types of cells of an organism or tissue. Examples of multipotent cells are HSCs and mesenchymal stem cells (MSCs). *Oligopotent stem cells* are further specialized and are destined to become specific types of cells. There are two kinds of hematopoietic oligolineage-restricted cells: common lymphocyte progenitors (CLPs) which are programmed to become either T or B lymphocytes or natural killer (NK) cells and common myeloid progenitors (CMPs) which are progenitors for myelo-erythroid lineages. CMPs give rise to cells that include myelomonocytic progenitors (GMPs) and megakaryocytic/erythroid progenitors (MEPs) **(Figure 1)**. More recently, an impressive study has proposed a new organization of the hematopoiesis, suggesting a readjustment in the blood hierarchy during in utero to adulthood time points.[10] Instead of a three-tiers model, the authors propose a two-tiers scheme in adult bone marrow: a top-tier which contains multipotent cells such as HSCs and multipotent progenitors, and a bottom-tier composed of committed unipotent progenitors **(Figure 2).**[10] Although often somewhat neglected by researchers in the past, *unipotent stem* cells are unique in their ability to differentiate along only one cell lineage. These cells are found in adult tissues and comparison to other stem cells have the lowest differentiation potential.[11] The potential difference between ESCs and ASCs can be summed up as follows: the former are more versatile whereas the latter are undifferentiated cells that are present in the differentiated tissue, capable of replacing cells that have died or lost function. ASCs have been identified in many different tissues including

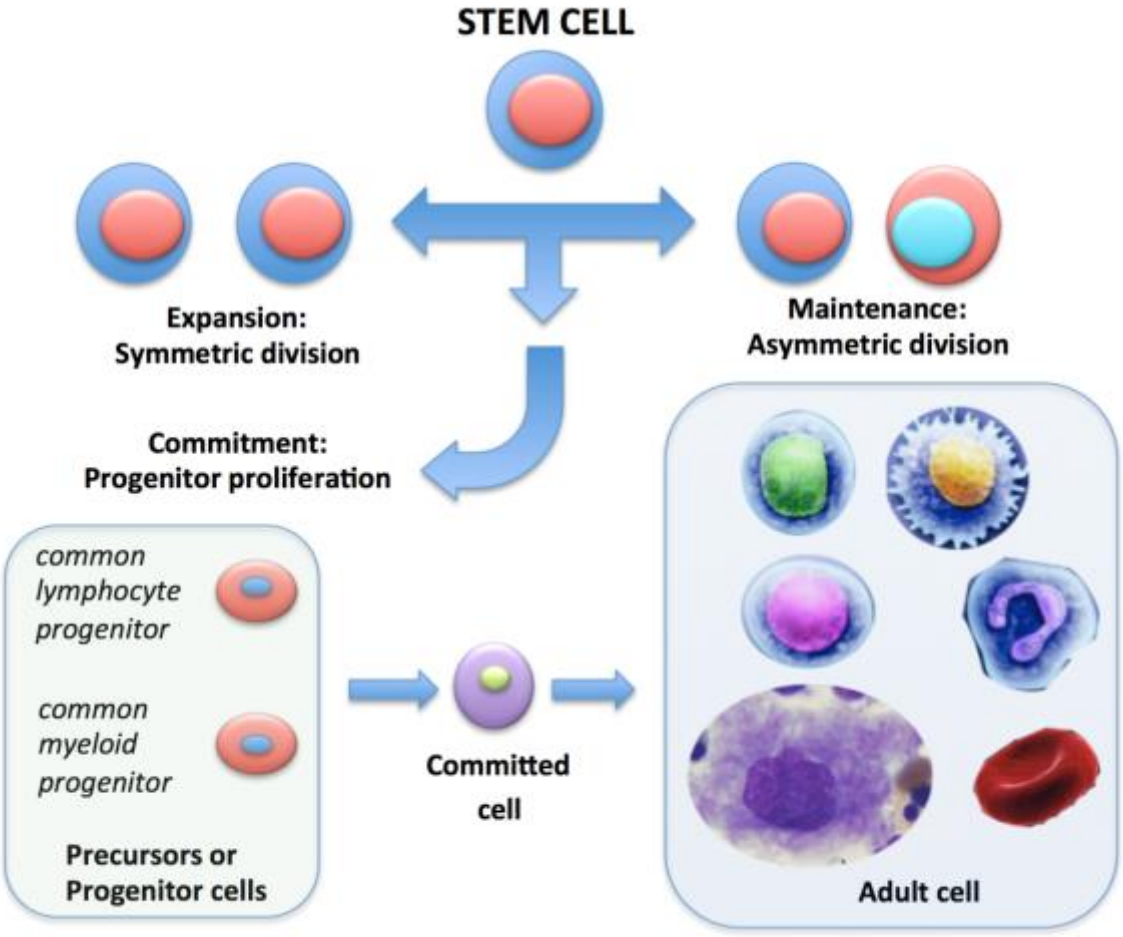

Figure 1. Hierarchical division of the stem cell in hematopoiesis

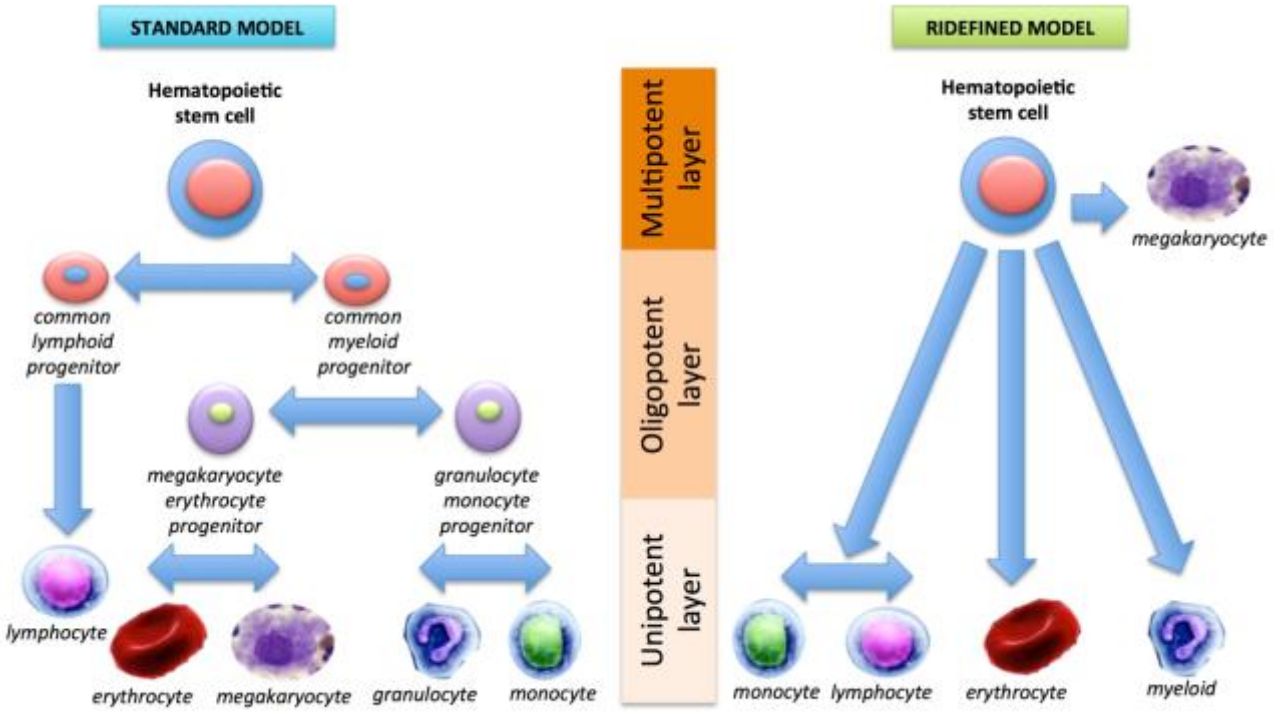

Figure 2. Redefined model of hematopoiesis. Instead of a three-tiers model, through mulitipotent, oligopotent and then unilineage progenitor, the authors proposed in adult bone marrow a two-tiers scheme: a top-tier which contains multipotent cells such as HSCs and multipotent progenitors, and a bottom-tier composed of committed unipotent progenitors.[10]

hematopoietic (blood), epidermal, muscle, neural, mesenchymal, endothelial and gastrointestinal tissues.

Most of the tissue-specific ASCs persist for prolonged periods of time in G_0 phase of cell cycle. This quiescent state of ASCs is also referred to as *homeostasis*. Differences in the expression of particular genes and transcription factors determine the transaction from the quiescent state to an active phase of the cell cycle, depending on the organism's needs.[4] Thanks to the presence of *telomeres*, the stem cell pool maintains longevity and genomic stability and is protected against damage to DNA. Telomeres are specialized repeat structures of TTAGGG and nucleoprotein complexes localized at the ends of human chromosomes. These repetitive DNA sequences at both ends of the chromosome protect cells from progressive DNA shortening and degradation during each repeated cell division.[12,13]

The fate of HSCs is also strongly influenced by the BM *microenvironment*. This microenvironment is composed of specialized microanatomical areas called *niches*. Numerous studies have shown that interactions between HSCs and their non-stem cell neighbors in the niche are critical to the maintenance of the stem cell pool in the quiescent state or promoting its self-renewal and proliferation.[14] However, this complex network of signals that occurs in the niche is far from being fully elucidated.

Bone Marrow Homing. Regenerative or gene HSC-based therapy is an interesting emerging field with a huge potential for the cure of numerous congenital and acquired diseases. There has been a rapid surge in clinical trials involving HSC therapies over the last decade. These trials continue to demonstrate the importance of stem cells both in replacing damaged tissue and in providing extracellular factors capable of promoting endogenous cellular salvage and replenishment.[15-18]

A key feature of treatment with HSC is represented by their ability, once introduced into the bloodstream to reach their final destination in a distant target tissue. This intrinsic property is known as *homing*. Homing is a crucial step toward successful engraftment after HSC transplantation. It was first described several years ago as an active process that allows for migration of HSCs through the blood and vascular endothelium to different organs and BM niches. Nevertheless, the full comprehension of this mechanism with its myriad of complex molecular events remains a challenge. Homing is a process that relies on intracellular signaling and interaction between chemokines, chemokine receptors, adhesion molecules, and proteases, all of which promote HSC adhesion to microvessels. E-endothelial and P-endothelial selectin were found to be essential to cell movement (*cell rolling*) on BM microvessels **(Figure 3)**. The intimate contact with chemo-attractants promotes the expression of HSC integrins, and through interactions with several members of the Ig superfamily leads to the cell arrest on the endothelial surface. Another important role in HSC homing has been assigned to intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These two molecules have been shown to act as key factors in cell trafficking between blood and BM.[19,20] Also $\alpha_4\beta1$ integrin and lectins would seem to have a primary function in HSC attachment to marrow stromal cells.[19] Several studies have reported that $\alpha_4\beta1$/ligand interaction contributes to cellular tethering and rolling. Additionally, it has been shown that the homing ability of normal donor cells decreases after treatment with anti-$\alpha_4\beta1$.[21-23] Further evidence suggesting the involvement of $\alpha_4\beta1$-integrin in the homing process is given in the points below.

i) $\alpha_4\beta1$ is widely expressed in both stem and progenitor cells, exceeding expression of both L-selectin and β2-integrin taken together;
ii) $\alpha_4\beta1$ is constitutively active in HSC and progenitor cells;
iii) $\alpha_4\beta1$ is usually inactive in committed cells.[24-26]

The main ligand of $\alpha_4\beta1$ in committed cells is VCAM-1. It can, therefore, be reasonably assumed that all functions are likely to be accomplished through their interaction. However, homing mediated by VCAM-1 may rely on other pathways.

Another important role in homing has been assigned to concentration of stromal-cell-derived factor-1 (SDF-1) ligand which increases in the BM microenvironment after conditioning regimens for HSC transplantation **(Figure 4)**.[27] SDF-1 is a chemokine isolated from stromal fibroblasts, and it is abundantly expressed by osteoblasts, endothelial cells and a subset of reticular cells in the osteoblast and vascular niches of the bone marrow.[28] SDF-1 is highly conserved among

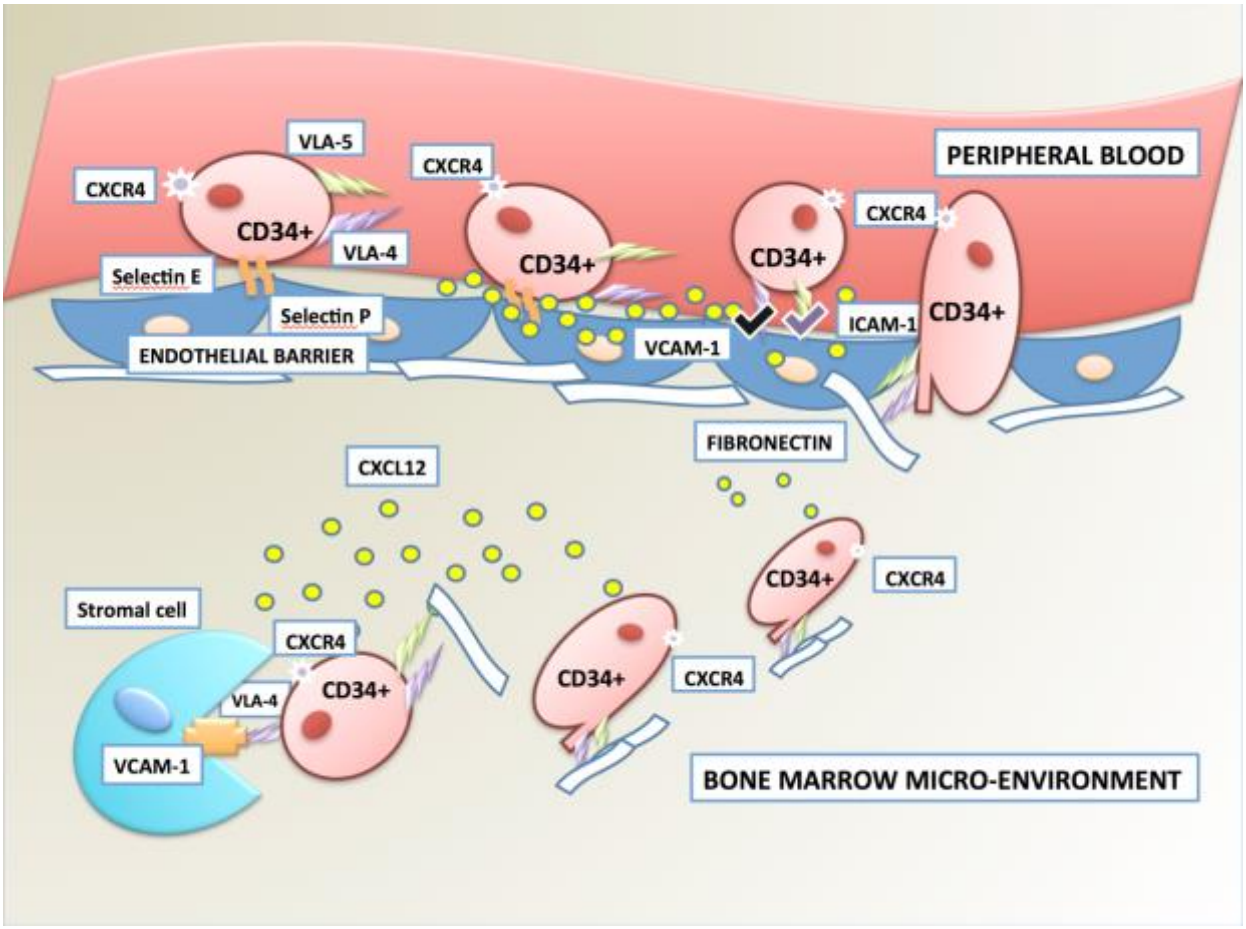

Figure 3. Migration and homing of HSCs into the bone marrow microenvironment. E- endothelial and P- endothelial selectin were found to be important to cell movement (*cell rolling*) and promote weak HSC adhesion to bone marrow microvessels. The expression of the chemokine receptor CXCR4 on the HSC surface promotes cell activation via CXCL12 factor. Following stronger interaction between LFA-1/ICAM-1 and VLA-4/VCAM-1, HSCs arrest on the endothelial surface and migrate through basal lamina. The migration is also promoted by VLA-4 and VLA-5 interaction with fibronectin, present in the extracellular matrix.

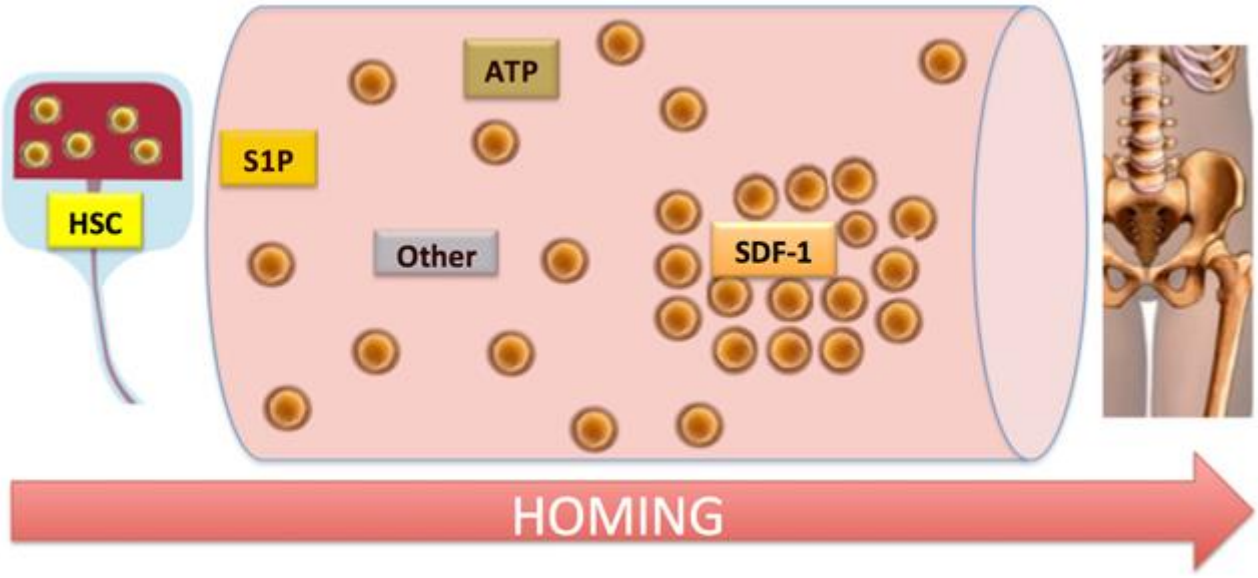

Figure 4. Schematic representation of HSC homing. HSCs infused into blood are more responsive to stromal cell-derived factor (SDF)-1 gradient between bone marrow and blood compared to other factors that are upregulated after transplantation conditioning regimen (S1P, ATP).

species and constitutively produced in many tissues. At the basal homeostatic concentration, SDF-1 interacts as a ligand with the G-protein coupled receptor CXCR4, promoting HSC quiescence and survival. The expression of the chemokine receptor CXCR4 on the HSC surface

promotes migration and homing into or from the BM.[29] Mouse embryos knocked out for SDF-1 or CXCR4 show multiple lethal defects, as well as the absence of BM homing by HSCs. Activation of the CXCR4 receptor by SDF-1 is one of the transductional axes most studied in recent years because of its fundamental importance in regulating trafficking of HSCs to and from the BM. It has also been reported that CXCR4-depleted human cells are insensitive to mobilization with agonists or antagonists of the CXCR4 receptor.[30] Secretion of SDF-1 in the bone marrow oscillates in a circadian manner. This process, although not fully understood, also involves the activity of the beta3-adrenergic (AdR) receptor.[31]

SDF-1-CXCR4 interaction triggers chemotaxis via intracellular GTPase proteins (heterotrimeric G-proteins, typically Gαi subunits).[32] After binding to SDF-1, CXCR4 undergoes down-modulation and ubiquitination of the C-terminus (C-ter) by E3 ubiquitin ligase, in this way promoting receptor degradation or its recycling via the endosomal pathway.[33,34]

Other potential factors involved in the homing process are the extracellular nucleotides (eNTPs), such as adenosine triphosphate (ATP) and uridine triphosphate (UTP), recently described as having a fundamental role in the modulation of HSC migration in the presence of SDF-1. Since extracellular UTP improves HSC migration toward SDF-1 gradients, pretreatment with eUTP, it is likely to increase homing of HSCs to the BM significantly as has been demonstrated in immunodeficient mice.[35] The aforesaid eNTPs act through P2 nucleotide receptors (P2Rs); particularly P2YRs. These seven transmembrane-spanning receptors, also referred to as G-protein coupled receptors, activate their signal transduction pathway via activation of phospholipase C or activation/inhibition of adenylate cyclase.[36]

Although the influence of SDF-1 on HSC chemotactic responses has been well established, [37,38] its role in the different molecular pathways underlying the early stages of homing remains a highly discussed and contentious issue.[39,40] Indeed, evidence has been produced of HSC homing to the BM independent of the SDF-1–CXCR4 axis. Several observations support this evidence. In 1999, Qing Ma and colleagues showed that CXCR4-deficient HSCs could successfully seed BM and give rise to all blood lineages in an SDF-1- independent manner.[41] A study of HSC homing in a murine model made refractory to SDF-1 by incubation and co-injection with AMD3100 (a CXCR4 receptor antagonist) showed normal or only slightly reduced BM cellularity. In yet another study, HSCs in which CXCR4 had been knocked down using an SDF-1 intrakine strategy were competent to engraft. Myeloablative conditioning for transplantation most likely induces a highly proteolytic BM microenvironment that leads to SDF-1 proteolytic degradation, thereby harshly sharpening its chemotactic homing gradient.[42-44]

Adamiak and colleagues recently confirmed the involvement of the bioactive phosphosphingolipid sphingosine-1-phosphate (S1P) as a potent chemotactic factor for HSCs. They performed hematopoietic transplantation in mice deficient in BM-expressed sphingosine kinase 1 (Sphk1−/−), using HCs from normal control mice as well as mice in which floxed CXCR4 (CXCR4fl/fl) had been conditionally deleted. They found that homing and engraftment in the Sphk1−/− mice was defective after transplantation of CXCR4−/− BM cells, indicating that SIP expressed in the BM microenvironment was involved in the homing process.

SIP levels in the BM are regulated by a balance in activity between type 1 SP-1 kinase (Sphk1) and S1P lyase, which has the role of degrading S1P.[45] Since 2010, it has been observed that S1P is a potent chemoattractant for HSCs, much stronger than SDF-1.[46]

It has also been suggested that HSC homing could be improved by inhibiting CD26 protein (DPPIV/dipeptidyl peptidase IV). Peptidase CD26 removes dipeptides from the amino terminus of proteins, and it is has been demonstrated that endogenous CD26 expression on donor cells downregulates homing and engraftment. Therefore, it can be reasonably assumed that by deleting or inhibiting CD26, it would be possible to increase HSC transplantation efficiency.[42]

Besides the BM microenvironment, other individual genetic factors can have an impact on successful engraftment of HSCs. For example, HSC homing is influenced by several molecules involved in inflammatory and other signaling pathways of innate immune response.[47,48]

Ratajczak and colleagues describe how innate immunity derived factors are external modulators

of the SDF-1–CXCR4 axis. Because SDF-1 is extremely susceptible to degradation by proteolytic enzymes, its availability in biological fluids may be somewhat limited. However, the authors observed that at a minimum near threshold doses, SDF-1 was still able to exert a robust chemotactic influence on engraftment. They showed that chemotactic responsiveness of HSCs to several different types of homing gradients could be modulated by ex vivo manipulations, using a strategy that takes advantage of a hematopoietic stem and progenitor cell (HSPC) - priming approach. Homing of HSPCs can be enhanced by ex vivo cell exposure to C3a (cleavage fragments of the third protein component of the complement cascade). A trial evaluating this procedure is currently ongoing at the Masonic Cancer Center, University of Minnesota.[49]

Another molecule that should be tested in the clinical setting as a potential priming factor is cathelicidin LL-37, a physiological factor secreted by BM stromal cells with a more powerful priming potential than C3a.[50]

Despite the many questions that still need to be answered, all these molecules could support a rationale for the development of innovative strategies aimed at improving HSC engraftment.

Hemopoietic Stem Cell Homing and Engraftment Defects. Graft failure remains an important complication of allogeneic HSCT because of the high morbidity and mortality associated with this event. Two different clinical forms of defective engraftment have been distinguished: graft failure (GF) and poor graft function (PGF), both characterized by a primary or secondary form.[51]

Graft failure is defined as absolute neutrophil count of 0.5 x 10^9/L and/or platelet count of < 20 x 10^9/L. Primary graft failure is defined as failure to achieve absolute neutrophil count (ANC) ≥ 0.5 x 10^9/L for at least 3 consecutive days or ANC above 0.5 × 10^9/L, without donor engraftment (autologous recovery). In secondary graft failure, patients fail to sustain an absolute neutrophil count of ≥ 0.5 x 10^9/L after attainment of primary donor engraftment or fail to sustain a platelet count of ≥ 20 x 10^9/L, despite neutrophil engraftment. Consequently, initial donor engraftment with neutrophil recovery is followed by loss of the functioning graft.

Both in primary and secondary graft failure, chimerism may vary from a full recipient status to a mixed condition in which donor and recipient cells coexist. Primary graft failure following myeloablative conditioning regimens generally determines deep and irreversible aplasia, often requiring re-transplantation. In secondary graft failure, autologous recovery is common, particularly after HSCT with reduced intensity conditioning (RIC); however, residual pancytopenia and bone marrow hypocellularity may persist.[52]

From a pathogenetic viewpoint, graft failure is determined by the alloreactive immune responses of residual host immune effector cells that survive the conditioning regimen.[51] Although the underlying mechanisms are not entirely known,[53] it has been shown that residual host T cells with specific anti-donor or suppressive activity play a fundamental role, both in HLA matched and mismatched settings. Also, recipient natural killer (NK) cells are involved in the pathogenetic pathways leading to graft failure. Their cytotoxic activity against donor HSCs has been attributed to the inability of inhibitory killer immunoglobulin-like receptors (KIRs) on the NK cell surface to recognize HLA class I molecules expressed on donor cells.[54] On the contrary, donor regulatory T cells (Tregs and Tr1) and mesenchymal stem cells (MSC) seem to facilitate engraftment and cco-transplantation of these cells with HSCs appears to have the potential to reduce the risk of graft failure.[55-56] Donor-specific HLA antibodies have also been found associated with an increased risk of graft failure, mainly in HLA-mismatched and haploidentical transplantation.[57-58]

Overall, the incidence of graft failure has been reported to be between 3 and 15%, in relation to the different sources of HSCs and transplant regimens.[51,52,-59-62] Several variables have been investigated as potential risk factors associated with primary or secondary graft failure. In a large retrospective study of 967 patients suffering from hematological malignant and non-malignant disorders, the parameters increasing the risk of graft failure were T-cell depletion, HLA-mismatched grafts, non-malignant disorders and reduced-intensity conditioning. Conversely, a total nucleated cell dose of ≥ 2.5 x 10^8/kg conferred a reduced risk. Furthermore, primary or secondary graft failure was associated with lower survival rates in malignant than in non-malignant

disorders.[61] Recent data, retrospectively collected from 4684 consecutive patients who underwent unrelated donor HSCT from 2006 to 2012, showed in univariate analysis that only the type and status of disease at the time of transplantation (complete remission versus no complete remission) were significant risk factors for graft failure.[62]

Over the past years, umbilical cord blood (UCB) has increasingly been used as a source of HSCs for allogeneic transplantation. Compared to marrow or mobilized peripheral blood stem cell grafts from adult donors, significant delays in neutrophil and platelet engraftment have been observed. Equally important limitations of this stem cell source are poor immune reconstitution and an increased risk of graft failure, at least partly due to defects in the homing capacity of these cells. Poor homing of UCB cells has been associated with low levels of fucosylation of cell surface molecules that are responsible for binding to P- and E-selectins expressed in the BM microenvironment.[60] Other factors linked to graft failure are low stem cell dose, major AB0 incompatibility, female donor grafts for male recipients and myeloproliferative disease.[51]

Poor graft function (PGF) is characterized by the presence of an initial full donor engraftment. In the primary form, bone marrow cellularity remains low, and patients present persistent cytopenias.[51] In the secondary form, a prompt recovery is followed by a progressive decrease in blood counts. This defect has an incidence after HSC transplantation ranging between 5 to 25%.[63] Several factors have been reported to be associated with PGF, but the most relevant condition is represented by graft versus host disease (GVHD).[64] A chronic inflammatory status, with overexpression of cytokines such as tumor necrosis factor alfa (TNF-α) and interferon gamma (IFN-γ), may lead to a decrease in HSC renewal and proliferation and thus determine peripheral cytopenias.[65,66]

Mixed chimerism (MC) after HSCT is an immunological condition characterized by the simultaneous presence of different proportions of both donor- and host-derived cells. This condition can be transient and evolve in the direction of graft failure or complete chimerism (CC), or persist for an extended period. Polymerase chain reaction (PCR) based on the amplification of variable number tandem repeats (VNTRs) or short tandem repeats (STRs) is currently the most common technique used to monitor this condition.[67] In malignant hematological disorders, MC anticipates secondary graft failure and relapse. Therefore, early detection of this condition is essential to ensure therapeutic interventions capable of reinforcing the graft, such as donor lymphocyte infusion (DLI).[68]

Achievement of persistent MC in patients transplanted for a chronic non-malignant disease like thalassemia or sickle cell disease may lead to tolerance of donor cells toward host tissues with no further need for immunosuppressive therapy. Moreover, residual donor hematopoiesis may be sufficient to eliminate transfusion dependency.[69-71] After transplantation for thalassemia, MC occurs within the first 100 days with an overall incidence ranging from 30% to 45%. This condition may be stable or evolve to CC or rejection (secondary graft failure). Three levels of MC have been established in thalassemia with different risk categories for progression to rejection: 1) grade 1, residual host cells <10%, rejection rates of 3-12%; 2) grade 2, residual host cells ranging between 10 - 25%, rejection rates of 10-50%; 3) grade 3, > 25% residual host cells, rejection rates of 50-90%.[69] Variables reported to be associated with MC in thalassemia are conditioning regimens, the dose of infused HSCs and the severity of patient clinical conditions before transplantation.[70] In recent years, it has been observed that induction of MC is an effective way of inducing tolerance and sustained graft function. Reprogramming of the immune system of the recipient to deliberately establish MC has been investigated in the solid organ transplant setting with the aim of improving the outcome and overall survival rates.[71]

Conclusions. Homing is a fascinating mechanism that allows HSCs to reach the BM microenvironment, engraft and proliferate. This property has been exploited both in auto and allo HSC transplant settings and is currently attracting considerable attention in the field of gene and regenerative therapy. Increasing advances in gene delivery techniques have led to a surge of clinical trials over the past decade. The possibility of using HSCs as possible carriers of modified genes using viral vector delivery approaches is rapidly evolving. Gene therapy with HSCs has an enormous potential, and different clinical trials have resulted in functional cures for several inherited diseases.[72] New insights on how

transplanted HSCs can reach the BM and which factors influence the homing process are thus critical.

Graft failure continues to be a major contributor to morbidity and mortality after allogeneic HSCT in patients with malignant and non-malignant diseases, particularly when treated with reduced-intensity conditioning regimens or grafts from HLA-mismatched donors. Such cases require close surveillance and regular monitoring of chimerism. On the other hand, deliberate induction of mixed chimerism by modulating the host immune system could represent an attractive way to improve graft survival in the future.

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