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The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization

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The bone marrow vascular niche consists of a network of thin-walled and fenestrated sinusoidal vessels whose integrity is maintained and supported by surrounding hematopoietic cells. However, this dependence is highly reciprocal in that the bone marrow vasculature provides not only a conduit for mature hematopoietic cells to the peripheral circulation but also a site where hematopoietic progenitors, especially megakaryocytes, differentiate and set the stage for full reconstitution of hematopoiesis.

Anatomy of the Bone Marrow Vasculature

The localized presence of the hematopoietic tissue within the protected confines of the bones provides clues to the regulatory interdependence of bone and marrow beyond the obvious advantage to the marrow: a well-shielded location from which it can produce an estimated 500 billion cells per day (16, 59). The bone marrow can be subdivided into a hematopoietic cell compartment and the stroma, which is mainly composed of fibroblasts, adipocytes, nerves, and the bone marrow's vascular system (16).

Data on the bone marrow's vascular anatomy were mainly provided by careful analyses using India ink or resin injections to provide two- and three-dimensional maps (16, 35) as well as by electron microscopic studies (61) (FIGURE 1). Arterial vessels enter the marrow through foramina nutricia and then divide into several arterioles. Small arterioles and capillaries from these vessels span throughout the bone marrow and supply sinusoids, which are interconnected by intersinusoidal capillaries. The sinusoids are radially distributed around the draining central sinus, which measures ~100 μm in diameter (35). The bone marrow sinusoids are unique and are not to be compared with regular veins. The sinusoidal wall consists of a single layer of endothelial cells and is devoid of supporting cells (61). In fact, the surrounding hematopoietic marrow appears to be the major cellular moiety that supports reconstruction and remodeling of the sinusoidal microcirculation, because the rapid induction of marrow hypocellularity with cytotoxic agents or radiation is followed by a marked dilatation and collapse of the sinusoids and the central sinus (35). The lack of a regular vessel wall in sinusoids is reflected by a high level of permeability. After intravenous injection of colloidal carbon, for example, the carbon can be found deposited heav-

ily in the liver, spleen, and bone marrow (41), organs where the microvasculature has been termed "sinusoidal" to denote the fact that their endothelial cells have no connective tissue covering but are rather in direct contact with the parenchymal cells.

The Stem Cell Niche Model: Historical Background

It has long been observed that the process of healing from tissue injury is dependent on recruitment of a specialized population of cells, called somatic stem cells, to regenerate the damaged organ. The concept of the hematopoietic stem cell (HSC) niche was first proposed by Schofield in 1978 after

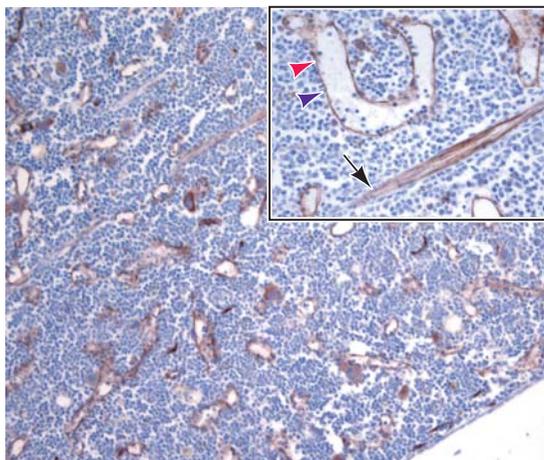


FIGURE 1. The bone marrow vascular niche comprises diverse vascular structures

Immunohistochemical staining of a paraffin bone marrow section with antiMECA-32 (panendothelial antigen) antibody demonstrates the diversity of the bone marrow's vasculature. Note the elongated arterioles (arrow) and the sinusoidal vessels (arrowheads). Megakaryocytes reproducibly stained positive for MECA-32 but were not stained by IgG control antibody. Diaminobenzidine on hematoxylin counterstain; magnification, $\times 200$ and $\times 400$ (inset).

an analysis of findings on the spleen colony-forming cell (CFU-S). He proposed that stem cells are fixed tissue cells that are prevented from differentiation and continue to proliferate as stem cells within a functionally and spatially characterized "niche" (52), where the microanatomic environment, composed of neighboring stromal cells, supports and instructs stem cells.

A large body of evidence suggests that HSCs and hematopoietic progenitor cells (HPCs) are not randomly distributed in the bone marrow but rather are localized close to the endosteum of the bone (11, 16, 28) and more recently around blood vessels (23, 42, 56). In addition, the embryonic marrow shows the first hematopoietic colonies next to the endosteum, and hematopoietic foci of regeneration in irradiated dogs are also located close to the endosteum (16). In 1975, Shackney described a gradient of cell development in the bone marrow, with undifferentiated cells being located along the endosteum and differentiation and maturation being associated with centripetal movement toward the highly vascularized bone marrow cavity (54). A few years later, it had been suggested that the stromal environment itself might determine the quality of hematopoiesis (43).

Two landmark reports introduced the concept that localization of stem cells to specific niches, for example the osteoblastic niche in the bone marrow, was a dynamic process where stem cells can be shuttled from a quiescence-favoring microenvironment to the vascular zone to undergo differentiation (19, 52). Subsequently, three recent papers confirmed these results, showing that the osteoblastic niche provides signals for the maintenance of repopulating cells in an undifferentiated state (5, 11, 67). Importantly, it has recently been demonstrated that these spatial differences in hematopoietic tissues do not reflect or translate into the properties of the harbored stem cells themselves (22).

With the increased characterization of the molecules and cellular components that comprise the endosteal niche came the notion that stromal structures like the bone marrow sinusoidal vessels could serve as alternative cellular scaffolds upon which hematopoietic cells could reside and mature. To delineate the bone marrow sinusoidal network as a separate anatomic and functional entity from the endosteal zone, the name "vascular niche" was employed. Whereas the endosteal zone is thought to favor quiescence, the centrally located vascular niche serves as a location that allows differentiation and ultimately mobilization to the peripheral circulation (1). Ultrastructural studies have demonstrated that differentiated rather than immature hematopoietic cells have a close association with the bone marrow microvasculature (58,

60). Strikingly, nearly all mature megakaryocytes were located adjacent to the thin-walled sinusoids, and whole megakaryocytes were shown to be able to transmigrate through intact endothelial cells (61, 62). This observation is not limited to thrombopoiesis but can be applied to erythroid and B-lymphoid progenitors, as these lineages have also been reported to reside in defined niches within the marrow (9, 51). These findings thus point to progenitor-stromal cell interactions as being critical determinants in the maturation process, further reinforcing the idea of stem cell niches (46) as microanatomic structures that are both permissive and instructive for stem cell differentiation.

Upon initial isolation by selective intrinsic adhesive interactions of endothelial cells, the bone marrow endothelium could be cultured and studied *in vitro* (21, 48, 49). With the advent of bone marrow endothelial cell (BMEC) cell lines, in addition to primary cultures, came the molecular characterization of BMECs with regard to their adhesive properties, their response to angiogenic and chemokinetic factors, and their contribution to supporting HSC differentiation (49). Finally, the rapidly evolving field of molecular biology opened up the door for the study of knockout animals like thrombopoietin (TPO)-deficient or TPO receptor (Mpl)-deficient mice or transgenic animals expressing marker genes, like Tie2-LacZ mice, thereby adding functional *in vivo* evidence for the interdependence of the bone marrow parenchyma and the sinusoidal vasculature (6, 24).

Developmental Evidence for a Vascular Niche in Hematopoiesis

The existence of vascular niches for HSCs and HPCs is logical from a developmental point of view: the development of the closed circulatory system in vertebrates (which are more efficient in terms of hemodynamics compared with the "open systems" of invertebrates, where hematopoietic cells can freely diffuse between the blood and interstitial spaces) posed the problem that hematopoietic cells had to be incorporated into the bloodstream. The solution to this problem rested with the establishment of a common precursor for hematopoietic cells and endothelial cells: the hemangioblast (13, 39). The colonization of hematopoietic organs in development encompasses a multistep process. From the production site in the aorta-gonad-mesonephros region, HSCs travel to the fetal liver, where they expand and finally reach the bone marrow and spleen, thereby settling in their respective stem cell niches (65). Mice deficient in the chemokine stromal cell-derived factor-1 (SDF-1) display a defect in this hematopoietic colonization of the bone marrow by HSCs from the

peripheral circulation during embryogenesis (34). Interestingly, enforced expression of SDF-1 in vascular endothelial cells could rescue this bone marrow colonization defect, suggesting that endothelial cells in the bone marrow are essential for the colonization of the fetal bone marrow by HSCs in the presence of SDF-1 (4).

There is a large body of evidence for a developmental interconnection of blood and endothelial cells during almost every stage of ontogenesis. Blood islands in the yolk sac can only develop in association with flk-1-positive vascular precursor cells (55). Endothelial cells from the yolk sac are able to promote HPC proliferation *in vitro* (29), and HSCs are found in close contact with endothelial cells at any time point during development. In the human embryo, CD34+ cells can be detected within the vessel wall of the aorta at embryonic day 35 (63) and later in perivascular locations of the fetal liver (40) as well as in the adult bone marrow (6, 67). HPCs were histologically observed to originate from endothelial cells in the dorsal aorta (15). Moreover, hematopoiesis in the human bone marrow has been shown to develop exclusively in specific structures organized by vascular cells (12).

Together, these developmental findings support a strong interdependence of HSCs/HPCs and endothelial cells embryologically, which extends to the adult.

In Vitro Characteristics of BMECs

Until the isolation of BMECs in 1993, the bulk of published studies on the bone marrow stromal cells' influence on hematopoiesis relied on bone marrow fibroblasts' contribution. Although the functional contribution of the endothelial stromal component was unknown, it was obvious that the function of the BMEC was key to a mechanistic understanding of the blood cell-producing capability of the bone marrow (49). Studies on other endothelial cell types like human umbilical vein endothelial cells (HUVECs) had previously elucidated that transendothelial trafficking was dependent on the expression of surface receptors or adhesion molecules, which were inducible by inflammatory cytokines (57, 68). Therefore, the release of mature blood cells as well as HSC/HPC mobilization and homing were likely to be regulated by similar mechanisms (49). Indeed, BMECs were consecutively found to support the proliferation and differentiation of hematopoietic progenitors *in vitro* via production of various cytokines and also possibly via physical contact (47–49). Reciprocally, coculturing megakaryocytes and BMECs resulted in survival prolongation of BMECs, probably because megakaryocytes secrete the endothelial cell survival factor VEGF-A (33), another

excellent example of the interdependent interactions of BMECs and hematopoietic cells (6).

Compared with HUVECs or lung-derived endothelial cells, BMECs were more potent inducers of HPC adhesion and migration (20, 66). Compared with other organ-specific endothelial cells, BMECs express lower levels of von Willebrand factor (69) and constitutively express cytokines (47–49) and adhesion molecules like VCAM-1 and E-selectin (2, 53). Whereas the heparan sulfate sulfation patterns of BMECs and HUVECs are different (36), adhesion molecule expression and regulation of this expression pattern by cytokines were found to be comparable in BMECs and HUVECs (49).

Megakaryocyte progenitors mature and randomly disintegrate *in vitro*, producing proplatelet-like fragments. Although this is an artificial process and not a physiological phenomenon, it can be used to measure chemokinetic and potentially thrombopoiesis-stimulating activity (18). For example, *in vitro* fragmentation of megakaryocytes is increased when CXCR4-positive megakaryocytes migrate through a layer of BMECs in response to the chemokine CXCL-12 (SDF-1). The presence of BMECs is obligatory for SDF-1 to induce *in vitro* proplatelet formation, suggesting that cellular contact of megakaryocytes with BMECs is necessary for thrombopoiesis (6, 18). Among the known megakaryocyte-active chemokines, SDF-1 has been shown to increase the affinity and migratory capacity of megakaryocytes across BMECs, and FGF-4 was found to support the adhesion of megakaryocytes to BMECs, thereby enhancing their survival and maturation (6).

Despite the proceedings in the functional characterization of microvascular BMECs as described above, the data concerning the cellular origin of BMECs are still incomplete, and a full phenotypic characterization of this cell type has not yet been achieved (6).

In Vivo Data on the Function of the Bone Marrow Vascular Niche

Our group has previously shown that the translocation of megakaryocyte progenitors to the vicinity of bone marrow vascular sinusoids was sufficient to induce megakaryocyte maturation as well as platelet production, even in the absence of TPO signaling (6). This process was demonstrated to be dependent on chemokines like SDF-1 and FGF-4, which restored both platelet counts in the peripheral blood and megakaryocyte concentration in the bone marrow in TPO^{-/-} and Mpl^{-/-} mice to wild-type levels. SDF-1 and FGF-4 are known to induce the expression of adhesion molecules, including very late antigen (VLA)-4 on megakary-

ocytes and VCAM-1 on BMECs (7, 8). Trans-endothelial migration of megakaryocytes results in proplatelet formation and platelet release, a complex but highly orchestrated process that is dependent on the direct cellular interaction of megakaryocytes with BMECs via these adhesion molecules. In fact, disruption of BMEC VE-cadherin-mediated homotypic intercellular adhesion interactions results in a profound inability of the vascular niche to support megakaryocyte differentiation and to act as a conduit to the periphery (6). The molecular mechanism by which the proper structural integrity of endothelial cells leads to a cellular platform conducive to HSC support is under study. However, a recent report (24) suggests that angiogenic remodeling concomitantly involves the activation and expression of molecules on the BMECs, which results in TPO-independent thrombopoiesis. Evidence from *in vitro* studies indicates that compartmentalized megakaryocyte apoptosis is necessary to form proplatelets (14). However, the notion that platelet formation *in vivo* resembles the *in vitro* process, including proplatelet formation, is controversial, and the role of megakaryocyte apoptosis *in vivo* has not been established (25).

Recovery from Myelosuppression as a Model for Bone Marrow Angiogenesis

Whereas it has long been known that hematopoietic regeneration and revascularization of the bone marrow cavity after radiation exposure are temporally related and that there is no hematopoietic regeneration without vascular reconstitution of the bone marrow (16, 58), the functional significance of this finding has only recently been recognized. Taking advantage of a hematopoietic regeneration model after myelosuppression with cytotoxic agents or whole-body irradiation, the interdependence of the bone marrow sinusoidal network and hematopoietic cells as well as the dependence of megakaryocyte maturation on intact microvasculature has been demonstrated in our laboratory (6, 24).

Myelosuppression, such as radiation exposure of the blood-forming bone marrow, leads not only to apoptosis of cycling hematopoietic cells, but also to the destruction of the bone marrow vasculature. Because the intricate network of sinusoids lack a regular vessel wall, they are especially affected by ionizing radiation and they display ultrastructural signs of necrosis (58), marked dilation (35), and overt breakdown with plasma and blood cell leakage (16, 24). Indeed, the bone marrow sinusoids seem to be supported by their neighboring hematopoietic cells themselves. Losing this sup-

port means losing stability, leading to hemorrhage within the bone marrow cavity after radiation or myelosuppressive chemotherapy. In the process of hematopoietic regeneration, the sinusoids are reconstructed. These processes—hematopoiesis and angiogenesis—occur hand in hand. Although myelosuppression with 5-fluorouracil (5-FU) destroys hematopoietic cells and sinusoidal endothelial cells, it only minimally affects HSCs or vascular progenitor cells in G_0 of the cell cycle. The model of recovery from myelosuppression (typically with 5-FU) is therefore a valuable tool to study the factors that promote hemangiogenic recovery of the bone marrow.

Using this model, our group established that matrix metalloproteinase-9 (MMP-9) activity, by releasing soluble Kit ligand from its membrane-bound state, is important for the translocation of progenitors to the vascular zone, thereby allowing them to differentiate, and MMP-9-deficient animals display severely impaired hemangiogenic recovery after 5-FU myelosuppression (19). Targeted disruption of vascular endothelial cadherin (VE-cadherin, CD144) homotypic interactions after 5-FU with neutralizing monoclonal antibodies interfered with the reconstruction of sinusoidal BMECs and blocked VCAM-1 expression on BMECs. Therefore, administration of this antibody (clone E4G10) impaired not only vascular reconstruction but also megakaryocyte recovery, which is apparently dependent on physical interaction with the vascular niche (6). Similarly, antibody treatment directed against CXCR4 after 5-FU in *c-Mpl*^{-/-} mice abrogates rebound thrombocytosis and results in a reduction of megakaryocytes as well as a depleted vascular niche. These data suggest that inhibition of CXCR4 not only blocks the translocation of megakaryocytes to the vascular niche but might also impair the recruitment of HSCs (6).

In the same way, Tie2 (the endothelial receptor for angiopoietin-1 and -2) expression is significantly downregulated in the bone marrow vasculature during steady-state hematopoiesis. After myelosuppression with cytotoxic agents or radiation as well as after stimulation with VEGF-A and angiopoietin-1, we found an upregulation of Tie2 in the bone marrow vascular endothelial cells (24). As in other organs, Tie2 expression was higher in arterioles than on the venous side of the bone marrow's vascular bed (3) (FIGURE 2A). Inhibition of Tie2 signaling resulted in impaired reconstruction of the vascular niche as well as in delayed hematopoietic recovery. The bone marrow displayed a seemingly paradoxical finding, with an accumulation of mature megakaryocytes in the bone marrow in the face of peripheral thrombocytopenia (FIGURE 2B).

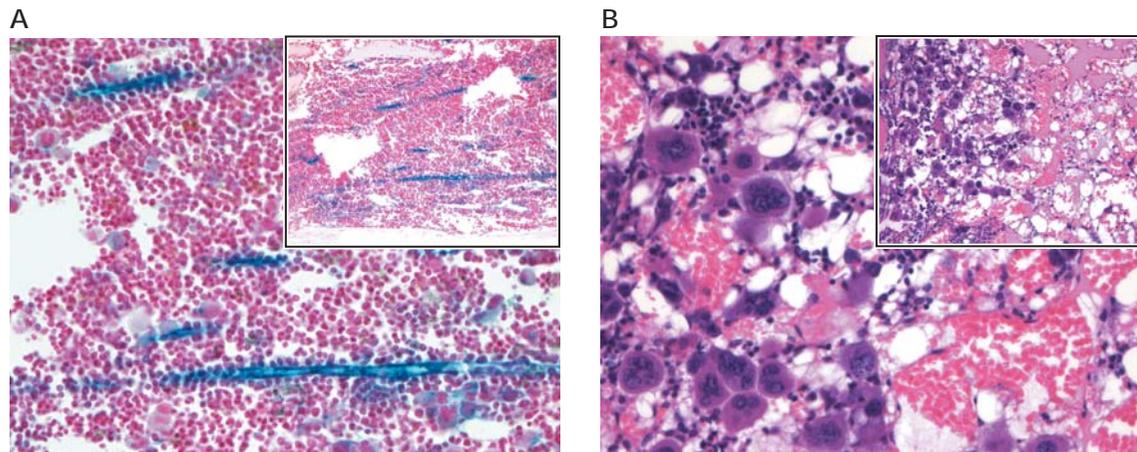


FIGURE 2. Vascular Tie2 contributes to regeneration after myelosuppression

A: Tie2^{LacZ} mice were injected with a myelosuppressive dose of 5-fluorouracil (5-FU; 250 mg/kg) and were killed 14 days after chemotherapy. Staining for LacZ activity using X-Gal shows high-level Tie2 expression in the bone marrow vascular niche. The blue-stained vessels predominantly resemble the elongated bone marrow arterioles. Paraffin section counterstained with nuclear fast red; magnification $\times 200$ (inset) and $\times 400$. B: concomitant application of 5-FU and the decoy angiopoietin receptor Tie2Fc results in accumulation of mature megakaryocytes in the face of peripheral thrombocytopenia 10 days after myelosuppression. Note the disrupted microvascular microarchitecture and the abundance of multinucleated megakaryocytes. Paraffin section counterstained with hematoxylin and eosin; magnification $\times 200$ (inset) and $\times 400$.

Stem Cell Mobilization and Homing: A Function of the Vascular Niche

The BMEC's function as the barrier between the peripheral circulation and the bone marrow parenchyma not only implies a role in the permanent process of blood cell production but also indicates that they are key to understanding both stem cell mobilization and homing. Whereas these phenomena are the basis for the clinical success of both HSC harvesting and transplantation, the functional meaning of stem cells occurring in the peripheral circulation of adult organisms is still a matter of debate (1, 65).

It has been almost 30 years since the first clinical documentation that chemotherapy can result in the appearance of HPCs in the peripheral blood (38). Since that time, administration of chemotherapy or granulocyte-colony-stimulating factor (G-CSF) to patients has become the de facto standard to induce HSC mobilization for harvesting. Furthermore, mobilized peripheral blood progenitor cells have become the preferred source for clinical transplantation (64). Moreover, the success of bone marrow transplantation by intravenous infusion relies on the ability of HSC/HPC to "home" or localize and engraft in the recipient's bone marrow. This process requires a cascade of events, which includes specific molecular recognition, cell-cell adhesion/disengagement, transendothelial migration, and functional repopulation of the depleted bone marrow stem cell niche (31). SDF-1, CXCR4, and adhesion molecules [VLA-4, leukocyte function antigen (LFA)-1, etc.] are required at high levels for efficient homing of circulating HSC/HPCs

into the bone marrow niche, as has been demonstrated by a variety of experiments that block or enhance the aforementioned factors. Newly infused circulating cells interact with the varied vascular beds via adhesion molecule binding. Adhesion molecules on the HPC involved in the process of rolling are VLA-4 (CD49d), LFA-1 (CD11a), and hyaluronan binding-cellular adhesion molecule (HCAM/CD44). Whereas the complementary binding partners on the BMECs are VCAM-1 (CD106), ICAM-1 (CD54), and E- and P-selectin (CD62E and CD62P) (30). After initial recognition in the rolling step, mainly mediated by E-selectin, P-selectin, and VCAM-1, firm adhesion proceeds by the binding of VLA-4, VLA-5 (CD49e), and LFA-1.

It has been recently determined that chemokine stimulation of HSCs and BMECs by SDF-1 (CXCL12) leads to an enhancement in transendothelial and stromal migration via activation of adhesion molecules, in addition to its well-known ability to stimulate motility (44, 45). Of interest, a parallel finding is that proteoglycans can bind and present SDF-1 to CXCR4 on HSCs. More importantly, it has been shown that proteoglycan-presented SDF-1 on endothelium under shear flow conditions is highly efficient at increasing CD34+ adhesion and at inducing transendothelial migration. Copresentation of chemokines via an adhesive matrix is capable of inducing directed cell migration, independent of a soluble chemokine spatial gradient. SDF-1 induction of transendothelial migration thus might not depend on the absolute concentration of SDF-1 in solution but

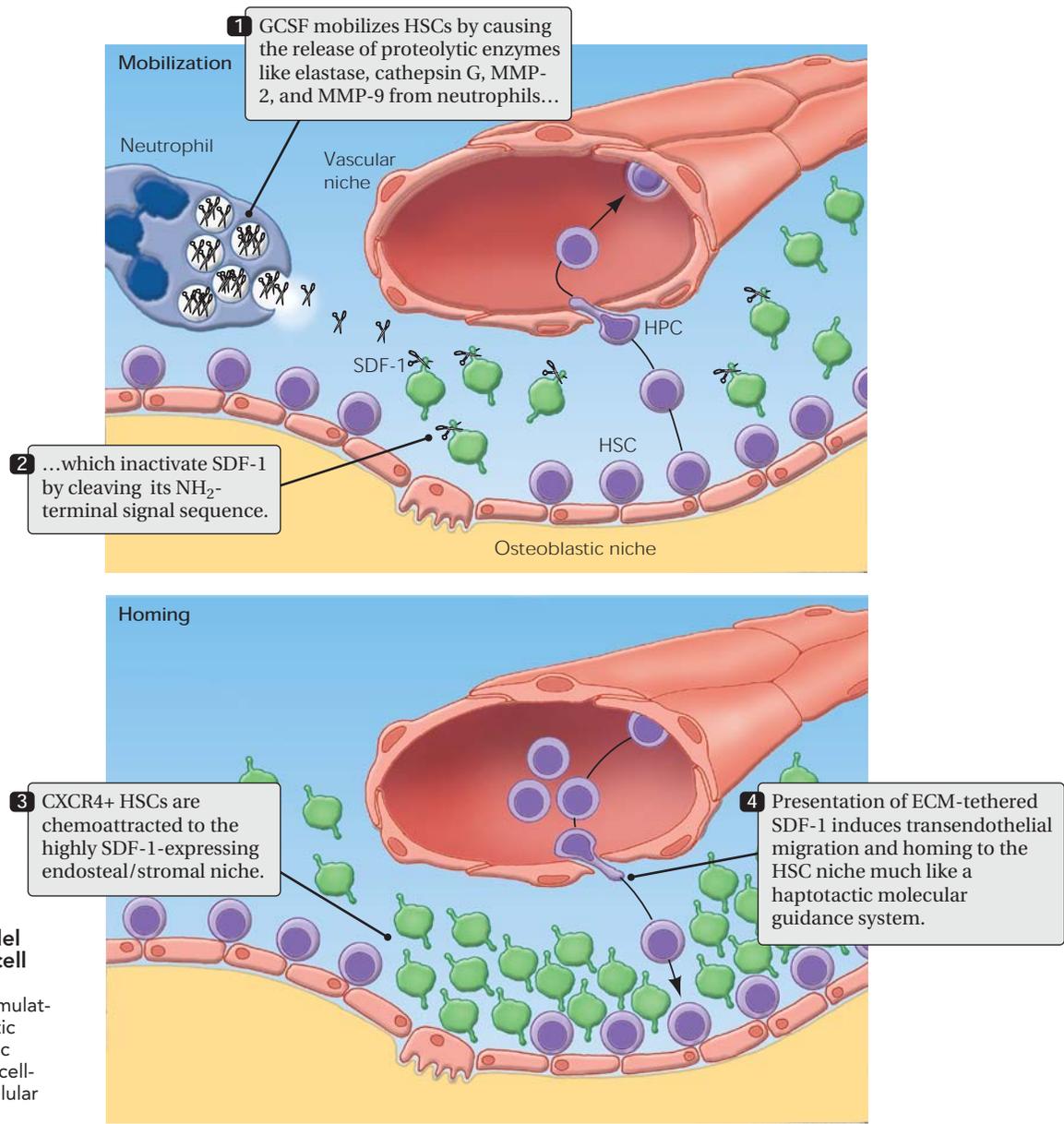


FIGURE 3. Simplistic model for hematopoietic stem cell mobilization and homing

GCSF, granulocyte colony-stimulating factor; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; SDF, stromal cell-derived factor; ECM, extracellular matrix.

rather on the immobilized chemokine fraction bound to components of the extracellular matrix (ECM) and bone marrow stromal cells, termed “haptotactic gradient” (37, 38). Chemokine immobilization and presentation on the vascular niche and ECM can thus be envisioned as a molecular road map for the migrating HSC.

There is a large body of evidence suggesting that homing and mobilization are diametric processes when it comes to expression patterns of adhesion molecules and chemokine receptors on hematopoietic progenitor cells (17). On the other hand, even with the introduction of presupposed CXCR4 antagonists, such as AMD3100, into clinical practice, it is unclear just how exactly the SDF-1/CXCR4 signaling pathway contributes to stem cell homing/engraftment and mobilization (10).

In support of the hypothesis that a gradient shift

can cause HPC mobilization are the data on the mechanism of action of GCSF. GCSF induces proteolytic enzymes like elastase, cathepsin G, MMP-2, and MMP-9, which inactivate SDF-1 by cleaving its NH₂-terminal signal sequence (32). In addition, MMP-9 is known to cleave membrane-bound Kit ligand from bone marrow stromal cells, thereby increasing soluble Kit ligand levels and mobilizing HPCs (19). In fact, the gradual proteolytic changes of human and murine bone marrow induced by GCSF is correlated with a gradual decrease in SDF-1, accompanied by a gradual increase in CXCR4 expression (26). Thus a simplistic model can be envisioned in which CXCR4+ HSCs are chemoattracted to the highly expressing SDF-1 endosteal/stromal niche until there is a physiological requirement for mobilization. Homing would be the reverse process in this simplifying model

(FIGURE 3). Conflicting data from other groups about the role of proteases in stem cell trafficking point to the possibility of redundant protease activity as well as to differences among different mouse strains in mobilization ability (27, 50).

Conclusion

The bone marrow vasculature provides the barrier between the hematopoietic compartment and the peripheral circulation. Therefore, it is the decisive anatomic structure that allows blood cell production as well as stem cell mobilization and homing. Recently, the bone marrow vascular niche's role in these processes has been further elucidated, and previous paradigms of cytokine functions in the process of stress hematopoiesis have been challenged by showing that TPO is dispensable for platelet production in c-Mpl^{-/-} and TPO^{-/-} mice as long as megakaryocyte progenitor interaction with the bone marrow vascular niche is available.

The interdependence of hematopoietic and endothelial cells during development is evident, but even in adult animals, the bone marrow parenchyma and vascular network do not seem to be able to live without each other. This reciprocal cellular addiction can be studied well in a model where hemangiogenic reconstitution after myelosuppression is observed in the bone marrow. Using this method, our group has established a role for the vascular niche in hematopoietic progenitor differentiation in addition to its apparent function in blood cell release to the periphery.

The precise molecular and cellular determinants that endow the bone marrow sinusoidal endothelium with the unique capacity to support hematopoiesis have yet to be discovered. There is no doubt that, compared with other organ-specific vasculature, expression of hemangiogenic factors specifically by the BMEC confers these cells with the capacity to selectively support hematopoiesis. One major technical obstacle for researchers to study hemangiogenesis is the localization of the marrow within bone. Decalcification procedures required for histological sectioning are potentially harmful to tissue antigens, thereby making immunohistological methods rather difficult and presenting a major hurdle for proper characterization of HSC:BMEC interactions. However, selective deactivation of genes that modulate hemangiogenesis will provide an instructive platform for identifying genes that support hemangiogenesis. ■

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