HHS Public Access

Author manuscript

Br J Haematol. Author manuscript; available in PMC 2015 April 01.

Published in final edited form as:

Br J Haematol. 2014 March ; 164(6): 767–778. doi:10.1111/bjh.12725.

ADVANCES IN UNDERSTANDING THE LEUKEMIA MICROENVIRONMENT

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SUMMARY

Dynamic interactions between leukemic cells and cells of the bone marrow are a feature of hematological malignancies. Two distinct microenvironmental niches in the bone marrow, the “osteoblastic (endosteal)” and “vascular” niches, provide a sanctuary for subpopulations of leukemic cells to evade chemotherapy-induced death and allow acquisition of a drug-resistance. Key components of the bone marrow microenvironment as a home for normal hematopoietic stem cells and the leukemia stem cell niches, and the molecular pathways critical for microenvironment/leukemia interactions via cytokines, chemokines, and adhesion molecules as well as hypoxic conditions, are described in this review. Finally, the genetic abnormalities of leukemia-associated stroma are discussed. Further understanding of the contribution of the bone marrow niche to the process of leukemogenesis may provide new targets that allow destruction of leukemia stem cells without adversely affecting normal stem cell self-renewal.

Keywords

Bone marrow microenvironment; leukemia; stem cell niche

INTRODUCTION

Despite significant progress achieved over the past decade in the chemotherapy-based and targeted treatments of several leukemia subsets, the relapse remains common after an initial response, indicating resistance of leukemia stem/progenitor cells to current therapies. The proof for the concept that a subpopulation of leukemic stem cells (LSCs) is solely responsible for maintenance of the leukemia was derived from the study of human acute myeloid leukemia (AML). In 1997, Bonnet and Dick established the concept of the hierarchical organization of AML. They showed that only $CD34^{++}CD38^{-}$ cells within the

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leukemic clone, regardless of the heterogeneity in maturation characteristics of the leukemic blasts, had the capacity to initiate AML growth after transplant into NOD/SCID mice (Bonnet & Dick, 1997). LSCs exhibit the unique characteristics as stem cells, including quiescence, pluripotency, and self-renewal within the bone marrow (BM) microenvironment (Warner *et al*, 2004). On the other hand, a specialized regulatory microenvironmental "niche" had been proposed more than 30 years ago (Schofield, 1978). Schofield suggested that stem cells reside in this sanctuary niche, where they receive appropriate support for maintaining self-renewal and multi-lineage differentiation capacity and are protected from environmental stress.

Subsequent studies have demonstrated that hematopoietic stem cells (HSC) reside in two distinct BM niches: the "osteoblastic (endosteal)" and "vascular" niches (Calvi *et al*, 2003; Kiel *et al*, 2005; Adams *et al*, 2006; Ding *et al*, 2012). These niches are part of a complex of BM cells that includes bone-lining cells (osteoblasts and osteoclasts), mesenchymal stem cells (MSCs), sinusoidal endothelium and perivascular stromal cells, and immune cells that play different roles in hematopoietic regulation (Nwajei & Konopleva, 2013). Cytokines and chemokines produced by BM stromal cells concentrate in particular niches secondary to varying local production and through the effects of cytokine-binding glycosaminoglycans. Chemokine (C X C motif) ligand 12 (CXCL12) positively regulates HSC homing, while transforming growth factor-β (TGF-β) and angiopoietin-1 (ANG-1) function as quiescence factors. The BM stromal cell–produced complex extracellular matrix (ECM) is involved in BM engraftment of HSCs and LSCs via vascular cell–adhesion molecule-1 (VCAM-1) or fibronectin (Miyake *et al*, 1991).

Like normal HSCs, LSCs remain dependent on signals from the hematopoiesis-regulating stromal environment for survival and proliferation (Dührsen & Hossfeld, 1996). Although the biology of LSCs shares many similarities with that of HSCs, LSCs are able to outcompete HSCs, hijacking the BM microenvironment. In the context of the "seed and soil" hypothesis, BM niches fuel the growth of leukemia cells and contribute to the therapy resistance and metastatic potential of leukemia cells by shielding LSCs (Hanahan & Coussens, 2012). There is increasing evidence that this microenvironment plays a critical role in the disease process. Not only a "microenvironment-induced oncogenesis", but also a "malignancy-induced microenvironment" has been proposed (Raaijmakers *et al*, 2010).

The key components and regulatory mechanisms (via cytokines, chemokines, adhesion molecules, and hypoxic conditions) of the two kinds of BM niche, osteoblastic and vascular, are described in this review. The genetic abnormalities of leukemia-associated stroma also are discussed. Further understanding of the contribution of the BM niches to the process of leukemogenesis may provide new targets that allow destruction of LSCs without adversely affecting normal stem cell self-renewal.

Microenvironmental niches of normal HSCs

HSCs reside and self-renew in the specialized area of the BM microenvironment called the niche. The BM niche modulates HSC quiescence, proliferation, differentiation, and migration. HSCs interact with the niche by exchanging various molecular signals, including adhesion mechanisms. The two distinct microenvironmental niches within the BM, the

osteoblastic (endosteal) and vascular niches, have been demonstrated to work in concert (Perry & Li, 2007).

The osteoblastic niche localized at the inner surface of the bone cavity with abundant bone-forming osteoblasts. In the osteoblastic niche, HSCs and osteoblasts bind to each other via adhesion molecules, and this binding contributes to the maintenance of stem cell quiescence (Iwasaki & Suda, 2009).

Kiel *et al* demonstrated that HSCs, in this case a highly purified population of $CD150^{+}CD244^{-}CD48^{-}$ cells isolated via a combination of SLAM (signaling lymphocyte activation molecule) family markers, mainly reside adjacent to sinusoidal endothelium in spleen and BM (Kiel *et al*, 2005). Although a few HSCs show a preference for the BM endosteum, Kiel *et al* estimated that two thirds of HSC in the BM are adjacent to sinusoids. The vascular niche consists of sinusoidal endothelial cells lining blood vessels; it promotes proliferation and differentiation of actively cycling, short-term HSCs (Passegue *et al*, 2005).

Thus, HSCs occupy multiple niches, including the endosteum and sinusoidal endothelium, in the BM microenvironment. Coordination between the osteoblastic and vascular niches regulates HSC self-renewal, proliferation, differentiation, and mobilization in and out of the BM. Several reports demonstrated that the vascular niche may localize within close proximity to the osteoblastic niche; in such cases the endosteum forms a well-vascularized special zone which is localized near N-cadherin–positive preosteoblastic cells, and this special niche promotes expansion of HSCs in response to BM damage (Jin *et al*, 2006). A recent comprehensive study has demonstrated that quiescent HSCs associate with $NG2^{+}$ periarteriolar niches found within the endosteal bone marrow, while cycling HSC re-localize to $LEPR^{+}$ perisinusoidal niches (Kunisaki *et al*, 2013). Elucidating the anatomical and functional diversity of the bone marrow microenvironment is crucial to understanding the behavior of HSCs and to exploiting this knowledge for clinical applications.

Components of the osteoblastic (endosteal) niche

Osteoblasts—The surface of the endosteum is lined by osteoblasts and osteoclasts. Osteoblasts are progenitor bone-forming cells that work in tandem with osteoclasts in the process of osteogenesis (Schroder *et al*, 2012).

In the osteoblastic niche, signaling through Jagged-1 (Jag-1) on osteoblasts and its receptor NOTCH on HSCs is involved in the expansion of the HSC pool (Calvi *et al*, 2003). On the other hand, angiopoietin-1 (Ang-1) in osteoblasts interacts with its receptor Tie-2, a type of receptor tyrosine kinase (RTK) expressed in HSCs, which results in activation of β1-integrin and N-cadherin. This enhanced adhesion between the niche cell and the stem cell also contributes to the maintenance of stem cell quiescence (Arai *et al*, 2004).

Osteoblasts express osteopontin, a negative regulator of HSC pool size that inhibits HSC proliferation, promotes HSC apoptosis, and affects the expression of Jag-1 and Ang-1 by stromal cells (Nilsson *et al*, 2005).

CXCL12 produced by osteoblasts is the major chemoattractant for hematopoietic stem and progenitor cells (HSPCs) (Christopher *et al*, 2009). Several studies demonstrated that mice deficient in CXCL12 or its receptor CXCR4 displayed impaired BM engraftment by hematopoietic cells (Sugiyama *et al*, 2006). All of these data underline the essential role of MSCs and osteoblasts in the BM HSC niche.

Osteoclasts—Bone-resorbing osteoclasts play a major role in endochondral ossification and coordinate with osteoblasts in bone formation (Schroder *et al*, 2012).

Osteoclasts regulate osteoblastic development in establishing HSC niches and, more importantly, form the cavities that constitute the endosteal niche (Schroder *et al*, 2012). Mice lacking osteoclast activity developed severe osteopetrosis, whch is associated with extramedullary hematopoiesis (Mansour *et al*, 2012), and osteoclast inhibition by bisphosphonates in mice caused severe depression of HSC formation and delay of hematopoietic recovery (Lymperi *et al*, 2010). These observations indicate that osteoclasts participate in the initial formation as well as the maintenance of the HSC niche (Mansour *et al*, 2012). On the other hand, granulocyte colony-stimulating factor (G-CSF) increased osteoclastic activity, driving HSPCs from the BM to the periphery (Kollet *et al*, 2006). The activity of osteoclasts further elevates the local and systemic calcium ion concentration, and published studies have demonstrated that HSC engraftment at the endosteal niche is specified by the calcium-sensing receptor (CaR) expressed on HSCs (Adams *et al*, 2006). Reduced cellularity and HSC content and increased mobilization of progenitor cells have been observed in the BM of CaR-deficient mice (Adams *et al*, 2006), suggesting the relevance of osteoclasts in retaining high calcium concentration, which is critical to keep HSCs localized in close physical proximity to the endosteal surface and the regulatory niche components.

Potential role of the nervous system—Glial nonmyelinating Schwann cells, a component of the BM niche, were shown to be responsible for activation of latent TGF-β produced by a variety of BM cells (Yamazaki *et al*, 2011). Nonmyelinating Schwann cells are ensheathed autonomic nerves in contact with a substantial proportion of HSCs. The critical role of TGF-β/Smad signaling in HSC maintenance was demonstrated by the impaired long-term repopulating activity of HSCs deficient in the TGF-β type II receptor. Autonomic denervation reduced the number of active TGF-β–producing cells and led to rapid loss of HSCs from the BM, which suggests that glial cells maintain HSC hibernation by regulating activation of latent TGF-β.

Sympathetic nervous system regulation of HSCs residing in BM niches via norepinephrine signaling has been reported (Katayama *et al*, 2006). Katayama *et al* showed that G-CSF–induced adrenergic activity resulted in suppression of osteoblasts, decreasing CXCL12 synthesis by osteoblasts and thus increasing HSPC mobilization in the BM microenvironment. Lucas *et al* further demonstrated that chemotherapy-induced nerve injury impaired hematopoietic regeneration and that neuroprotection induced by deletion of *TP53* in sympathetic neurons or neuroregeneration induced by administration of 4-methylcatechol or glial-derived neurotrophic factor promoted hematopoietic recovery in a murine model (Lucas *et al*, 2013). These reports indicate that sympathetic nerves in the BM promote the

survival of constituents of the BM niche. In humans, however, Bonig and Papayannopoulou (2013) reported observing no difference in mobilization of stem cells by G-CSF in stem cell donors taking a noradrenalin-reuptake inhibitor or beta-receptor blocker.

Regulatory T cells—In immune-suppressive stem cell niches called immune-privileged sites, multiple mechanisms cooperate to prevent immune attack and enable prolonged survival of foreign allografts without immunosuppression. Fujisaki *et al* (2011) demonstrated co-localized accumulation of HSPCs with regulatory T (T(reg)) cells on the endosteal surface in the calvarial and trabecular BM, which was lost after the depletion of T(reg) cells in their non-immunosuppressed mouse model. These results suggest that T(reg) cells participate in creating the BM niche, which provides a relative sanctuary from immune attack and supports stem-cell function.

Components of the vascular niche

CXCL12-abundant reticular cells—CXCL12 (SDF-1α), a chemokine elaborated by stromal cells, functions through its receptor CXCR4, a seven-transmembrane G-coupled receptor protein. CXCL12 attracts CXCR4-expressing HSCs to stromal surfaces. CXCL12–CXCR4 signaling is involved in homing of HSC into BM, activates several integrins, and supports survival of colony-forming progenitor cells (Sugiyama *et al*, 2006). CXCL12-secreting cells include osteoblasts (Christopher *et al*, 2009), CXCL12-abundant reticular (CAR) cells (Sugiyama *et al*, 2006), and Nestin-positive stromal cells (Mendez-Ferrer *et al*, 2010), all components of the BM niches.

Most CAR cell populations express PPARγ, Runx2, and Osterix in the BM, and short-term ablation of CAR cells *in vivo* severely impaired the adipogenic and osteogenic differentiation potential of BM cells, indicating that CAR cells are adipo-osteogenic bipotential progenitors (Omatsu *et al*, 2010). In the sinusoidal areas of the BM in which HSCs predominantly reside, the HSCs have direct contact with CAR cells, which secrete higher levels of CXCL12 than osteoblasts. Moreover, depletion of CXCR4 leads to reduction of the HSC population, suggesting that CXCL12–CXCR4 chemokine signaling plays an essential role in maintaining the HSC pool (Sugiyama *et al*, 2006).

Similarly, the mobilization of HSCs into the peripheral blood induced by CXCL12 downregulation or CXCR4-selective antagonism further indicates a role for CXCL12 in retaining HSC in BM niches (Sugiyama *et al*, 2006).

Nestin-positive mesenchymal stem cell and leptin receptor–positive cells—Whereas the nature of the true “mesenchymal stem cells” remains enigmatic, specific MSCs named Nestin-positive MSCs have been reported to participate in the regulation of BM niches (Raaijmakers *et al*, 2010). Nestin-positive MSCs constitute an essential HSC niche component, co-localizing with HSCs and adrenergic nerve fibers (Mendez-Ferrer *et al*, 2010). Frenette and colleagues reported that depletion of Nestin-positive MSCs in an *in vivo* model significantly reduced BM homing of hematopoietic progenitors and HSC content in the BM (Mendez-Ferrer *et al*, 2010). They also reported that the selective downregulation of HSC retention genes in Nestin-positive MSCs was induced by the reduction of CD169-positive BM macrophages, which led to reduced BM CXCL12 levels and egress of HSPCs

to the bloodstream (Chow *et al*, 2011). It has been further demonstrated that the conditional deletion of stem cell factor (SCF) from endothelial cells or leptin receptor–expressing perivascular stromal cells, including Nestin-positive stromal cells and CAR cells, significantly reduced HSC number, whereas SCF deletion from hematopoietic cells, osteoblasts, and Nestin-cre– or Nestin-creER–expressing cells did not affect HSC number (Ding *et al*, 2012).

Thus, heterogeneous stromal cells contribute to HSC maintenance through various mechanisms.

LEUKEMIC MICROENVIRONMENT

Bone marrow niche as a “foster home” for LSCs

The leukemic clone is organized as a hierarchy, and LSC behavior is modulated by interactions and signals received within their BM microenvironment. For LSC survival, proliferation, and differentiation, both the osteoblastic and vascular niches are critical (Calvi *et al*, 2003; Arai *et al*, 2004; Nilsson *et al*, 2005; Kiel *et al*, 2005). LSCs share certain features of self-renewal and differentiation with HSCs, and the molecules that mediate the interaction between LSCs and the BM niche, including adhesion molecules (Messinger *et al*, 1996), and CXCL12-mediated CXCR4 signaling for homing and mobilization within the BM (Messinger *et al*, 1996) are similar to those of HSCs.

However, LSCs differ from HSCs in their dysregulated activation of key pathways regulating proliferation, survival, and abilities to invade and spread (Lane, 2012). Recent studies indicate that BM niche components contribute to LSC engraftment into the niches; to leukemia development, survival, and drug resistance; and to determination of leukemia phenotype by providing the necessary cytokines and cell contact–mediated signals to LSCs (Raaijmakers *et al*, 2010). These findings suggest the roles of the normal HSC niche in leukemia pathogenesis (Lane, 2012). On the other hand, LSCs themselves create their “foster home,” inducing reversible changes in BM stromal cell function or composition that result in survival of the leukemic cells (Dührsen *et al*, 1996). Suppression of normal hematopoiesis in leukemia patients with relatively low tumor burden may reflect disruption of normal hematopoietic progenitor cell (HPC) BM niches and creation of leukemia niches by leukemic cells (Colmone *et al*, 2008).

In a murine model, transplanted leukemic cells initially migrated toward the CXCL12-positive vascular niches, which overlap with normal HPC niches; after leukemia growth *in vivo*, CXCL12 production in the leukemia vascular niche was markedly downregulated, and newly transplanted normal $CD34^+$ cells migrated to tumor niches by virtue of SCF abundantly secreted by leukemic cells (Colmone *et al*, 2008). These findings indicate that the signaling mechanisms of BM niches are altered such that the niches are “hijacked” by LSCs (Li & Neaves, 2006) and remodeled as their “foster home.”

CXCR4–CXCL12 interactions and leukemic cell migration to bone marrow niches

The poor prognosis of acute leukemia afforded by current treatments is mainly due to the relapse of the disease following chemotherapy. Interaction of LSCs and BM niches is

recognized as the major cause of this acute leukemia relapse. Chemokine CXCL12 elaborated by osteoblasts, CAR cells, or Nestin-positive MSCs is one of the key factors mediating the crosstalk between leukemic cells and the BM niches and regulates the homing and engraftment of LSCs into the BM niche.

Levels of the CXCR4 receptor for CXCL12 are significantly elevated and highly responsive to CXCL12 in primary leukemic cells, including B-cell chronic lymphocytic leukemia (B-CLL) (Mohle *et al*, 2000), B-cell acute lymphocytic leukemia (ALL) (Shen *et al*, 2001), and to a lesser degree AML (Raaijmakers *et al*, 2010). Associations between CXCR4 expression and poor outcome in patients with B-CLL (Ishibe *et al*, 2002), pre-B-ALL (Crazzolara *et al*, 2001), or AML (Rombouts *et al*, 2004; Konoplev *et al*, 2007) have been reported.

Inhibition of CXCL12–CXCR4 interactions resulted in abolishment of CXCL12-induced chemotaxis; inactivation of prosurvival signaling pathways, including phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) and signal tranducer and activator of transcription 3 (STAT3); and decreases in stromal protective effects on chemotherapy-induced apoptosis in CLL and AML cells (Zeng *et al*, 2006). The small-molecule reversible CXCL12–CXCR4 inhibitor plerixafor completely blocked CXCL12-induced chemotaxis, attenuated the migration of pre-B-ALL cells into BM stromal cell layers, and enhanced the cytotoxic and antiproliferative effects of vincristine and dexamethasone (Juarez *et al*, 2003). In a murine model of acute promyelocytic leukemia (APL), administration of plerixafor in combination with chemotherapy triggered an increase of circulating APL cells with decreased tumor burden and improved overall survival compared to chemotherapy alone (Nervi *et al*, 2009). Novel fully human antibody to CXCR4 BMS-936564/MDX-1338 induced apoptosis in AML cell lines and exhibited single agent antitumor activity in the in vivo AML models (Kuhne *et al*, 2013).

CXCR4 expression has been reported to be higher in Flt3/internal tandem duplication AML than in FLT3/wild-type AML (Rombouts *et al*, 2004). Additional preclinical data indicate that the Flt3 axis participates in the CXCR4-mediated trafficking of transformed hematopoietic cells and that CXCR4 inhibition increased sensitivity of FLT3-mutated leukemic cells to the FLT3 inhibitor sorafenib under stromal co-culture conditions. Furthermore, CXCR4 inhibitor AMD3465, alone or in combination with G-CSF, induced mobilization of AML progenitor cells into circulation and reduced AML burden in mice, which resulted in prolonged survival in response to sequenced sorafenib treatment, presumably through recruitment of leukemic cells out of their protective microenvironmental niches (Zeng *et al*, 2009). Similarly, treatment with plerixafor combined with TGFβ-neutralizing antibody 1D11 and cytarabine decreased leukemia burden and prolonged survival in a leukemia mouse model, proving that TGFβ and CXCL12, produced abundantly in the BM niche, play a role in AML chemoresistance (Tabe *et al*, 2013).

Primary chronic myelogenous leukemia (CML) blasts show attenuated migration to CXCL12 and decreased CXCR4 expression (Peled *et al*, 2002). BCR-ABL tyrosine kinase inhibitor imatinib restored CXCR4 expression under MSC co-culture conditions, which in turn induced migration of CML cells to the BM microenvironment niches, where quiescent

CML progenitor cells acquired stroma-mediated chemoresistance (Jin *et al*, 2008). Pathological crosstalk between BCR/ABL and the CXCR4 pathway is modulated by Src family tyrosine kinase Lyn in CML cells, which disrupts chemokine signaling and chemotaxis and increases the ability of immature cells to escape from the BM (Tabe *et al*, 2012).

These results suggest that CXCL12–CXCR4 interactions in the BM microenvironment contribute to the chemoresistance of leukemic cells and that disruption of these interactions by CXCR4 inhibitors represents a rational strategy for blocking LSC homing to a BM niche and/or sensitizing leukemic cells to chemotherapy or kinase inhibitors.

LSC niche and adhesion molecules

Adhesion to the stromal niche is crucial for LSCs because it directly supports self-renewal, proliferation, and arrest of differentiation and protects from damage by chemotherapy or kinase inhibitors. The transmembrane glycoprotein CD44, existing as a standard isoform (CD44s) and a range of variant isoforms (CD44v), is a key regulator of LSC homing to BM niches and maintenance of their primitive state (Jin *et al*, 2006). CD44 modulates interactions of LSCs with hyaluronan; ECM components, including heparin sulfate; and a range of growth factor ligands to promote CD44/ligand/RTK complex formation and signal transduction (Nervi *et al*, 2009). The glycosaminoglycan hyaluronan is highly concentrated in the endosteal region (Avigdor *et al*, 2004). CD44–hyaluronan interactions contribute to self-renewal, proliferation, differentiation, homing to BM, and preservation of the integrity of the stem cell genome by decreasing DNA damage and enahancing DNA repair (Williams *et al*, 2013).

CD44/ligand/RTK signaling has been shown to modulate microRNA expression to regulate promoter methylation status and gene expression (Williams *et al*, 2013). Through this mechanism, CD44 participates in reprogramming of leukemia cells to exhibit a more stem cell-like LSC phenotype, which could be an elemental mechanism in promoting leukemic progression and chemoresistance (Williams *et al*, 2013). On the other hand, cancer stem cells (CSCs) have been shown to further synthesize hyaluronan to recruit tumor-associated macrophages into the CSC niche (Jinushi *et al*, 2011), which then recruits adjacent stromal cells into the CSC niche.

Niche stromal cells secrete numerous growth factors, many of which are known to moderate stem cell functions such as self-renewal and stem cell fate. CD44s and CD44v bind to growth factors, activating an impressive range of RTKs. Although activities of CD44s or CD44v have been reported to be similar in hyaluronan-mediated regulation of HSC differentiation (Herrlich *et al*, 2000) and MSC homing to BM (Avigdor *et al*, 2004), the mechanisms of preferential CD44v expression have been shown to be potentially useful in enforced maturation of self-renewing LSCs. For example, variant isoforms of CD44v4–10 have been demonstrated as the primary CD44 isoform expressed during maturation of CML progenitor cells into myeloid cells (Herrlich *et al*, 2000).

Cells in the BM niche also express integrins, which are cell adhesion receptors that link extracellular adhesion molecules with the intracellular actin cytoskeleton (Redondo-Munoz

et al, 2008). Integrins are known to be required for lodging of LSCs in the BM niche (Redondo-Munoz *et al*, 2008). Integrin heterodimers, made up of one of 18α subunits and one of 8β subunits, regulate cell–cell adhesion, growth factor receptor signaling, cell lineage specification, differentiation, survival, proliferation, and migration (Prowse *et al*, 2011). Many of these functions parallel with CD44 expression, suggesting integrin–CD44 interactions (Williams *et al*, 2013). Indeed, integrins are known to link to CD44 through their interactions with selectins. Homing of HSPC to BM requires a coordinated sequence of four steps, including E-selectin receptor/ligand interaction; engagement of CXCL12–CXCR4 signaling, resulting in activation of very late antigen–4 (VLA-4; integrin α4β1); VLA-4 adherence to VCAM-1; and transmigration on endothelium (Sackstein *et al*, 2011). VLA-4 binds to CD44v to form a docking complex for pro–matrix metalloproteinase-9, which is associated with transendothelial migration and invasion through Matrigel of B-CLL cells (Redondo-Munoz *et al*, 2008). Mudry *et al* (2000) showed that the maximum viability of ALL cells during exposure to cytarabine and etoposide required interaction with the MSC adhesion molecule VCAM-1. Conditional deletion of alpha4 sensitized BCR-ABL(+) leukemias to nilotinib, and pharmacological VLA4 blockade with antibody Natalizumab prolonged survival of NOD/SCID recipients of primary ALL when combined with chemotherapy, indicating the role of this integrin in chemoresistance of lymphoid malignancies (Hsieh *et al*, 2013). Very recently, Miller *et al* demonstrated that *ITGB3* knockdown impaired homing, downregulated LSC transcriptional programs, and induced differentiation via the intracellular kinase Syk without affecting normal HSPCs (Miller *et al*, 2013).

Modulation of the LSC stem cell niche via hypoxia/HIF-1α signaling

The endosteum of the bone–BM interface has been shown to be hypoxic: the average pO_2 in BM is approximately 55 mmHg, and the mean O_2 saturation 87.5% (Harrison *et al*, 2002). Leukemic cells are able to proliferate even under hypoxic conditions, indicating that the cells are able to adapt to these conditions. Abnormalities including elevated CXCL12, vascular endothelial growth factor (VEGF), and SCF levels, as well as increased acidity and hypoxia, were reported in leukemia BM (Mohle *et al*, 2000; Benito *et al*, 2011). It has been reported that the oxygen-regulated component hypoxia-inducible transcription factor alpha (HIF-1α) was overexpressed in clusters of leukemic cells in BM specimens from ALL patients (Wellmann *et al*, 2004).

Notably, HIF-1α was demonstrated to regulate *CXCL12* gene expression in endothelial cells, resulting in selective *in vivo* expression of CXCL12 in ischemic tissue, which increased migration and homing of circulating CXCR4-positive progenitor cells into the ischemic tissue (Ceradini *et al*, 2004). In AML, total and surface CXCR4 expression were upregulated under hypoxic conditions in leukemic cell lines and patient samples (Fiegl *et al*, 2009). Consistent with the findings that HIF-1α regulates CXCR4 (Staller *et al*, 2003), these data suggest that a hypoxic BM microenvironment represents a conditional stem and progenitor cell niche in which HIF-1α–induced stabilization and activation of CXCL12–CXCR4 signaling facilitates recruitment and retention of leukemic progenitor cells.

One of the most advertised functions of hypoxia and HIF-1α is upregulation of growth factor VEGF and stimulation of angiogenesis. The microvasculature is an active component of the BM microenvironment and is responsible for supplying appropriate oxygen and nutrients. VEGF secreted by leukemic cells activates receptors on both leukemic and endothelial cells and plays a vital role in the growth of leukemia cells (Ferrara *et al*, 2003). Increased angiogenesis is observed in myelodysplastic syndrome (Korkolopoulou *et al*, 2001), AML (Hussong *et al*, 2000), ALL (Koomagi *et al*, 2001) and multiple myeloma (Rajkumar *et al*, 2000). VEGF was found to inhibit apoptosis in leukemic cells after exposure to etoposide and doxorubicin by inducing Mcl-1, a member of the prosurvival Bcl-2 family, and to promote the survival of multiple myeloma cells by inducing Bcl-2 via VEGF receptor 2 (Dias *et al*, 2002). The direct HIF-1α inhibitor PX-478 decreased expression of hypoxia-mediated, but not normoxic, VEGF expression with antitumor activity against tumor xenografts (Koh *et al*, 2008).

CML-associated oncogene *BCR-AB1L1* has been shown to induce *VEGFA* and *HIF1A* gene expression via a phophoinositide-3 kinase (PI3K)/mTOR–dependent pathway (Mayerhofer *et al*, 2002). Tyrosine kinase inhibitor imatinib inhibited c-KIT–induced HIF-1α activity and VEGF expression in small cell lung cancer cells (Litz *et al*, 2006). These findings indicate that the activation of c-KIT by SCF could be followed by HIF-1α –mediated VEGF expression.

In a very recent review, Bonig and Papayannopoulou (2013) discussed the significance of the observation that, although the lower oxygen tension in the BM than in arterial blood is an inevitable consequence of physics, it is difficult to define the BM stem cell niche as severely hypoxic. They argued that the high abundance of capillaries in cancellous bone is incompatible with the hypoxia in BM niches, and that histological images of HSCs indicate that HSCs reside in proximity to the proliferative progenitor cells throughout the BM (Kiel & Morrison, 2008) but not in anatomically separate compartments. This notion is supported by a recent quantitative imaging study of HSCs which demonstrated that despite their preferential endosteal localization, HSCs closely interact with bone marrow microvessels, and yet exhibit a hypoxic profile, indicating regulation of the hypoxic HSC state through cell-intrinsic mechanisms rather than lack of blood supply (Nombela-Arrieta *et al*, 2013). It remains to be determined whether abundant marrow hypoxia reported in leukemic bone marrows (Benito *et al*, 2011) regulated through similar or distinct mechanisms. Whereas both leukemia progenitor cells and LSCs reside in the “hypoxic” BM microenvironment, they have distinct metabolic states; progenitor cells are engaged in active cycling and contain many mitochondria, whereas LSCs are quiescent and exhibit few mitochondria (Suda *et al*, 2011). The transition from stem to progenitor cell corresponds to a critical metabolic change, namely from glycolysis to oxidative phosphorylation (Suda *et al*, 2011). For the stress resistance of LSCs, long-term quiescence and self-renewal may be crucial, and leukemic cells’ survival and proliferation is critically regulated by the transition mechanisms from stem to progenitor cell, corresponding to metabolic alteration, oxygen concentration, cytokine stimulation, or cell contact regulation (Suda *et al*, 2011).

Leukemogenesis: role of the bone marrow microenvironment

Dysfunction of a BM niche may contribute to leukemogenesis via interaction through adhesion, supplying abundant growth factors and immunosuppression that promote proliferation and/or inhibit apoptosis (Jones & Wagers, 2008). Overproduction of apoptosis-inducing cytokines by T cells triggers BM failure and then leads to transformation through clonal selection and adaption of modified BM microenvironment-resistant HSCs, which develop into clonal neoplasms such as AML, myelodysplastic syndromes, and paroxysmal nocturnal hemoglobinuria (Tavor & Petit, 2010). In a recent report, Schepers et al. dscribe how malignant myeloid cells profoundly reprogram the endosteal BM osteoblasts into the pro-inflammatory bone marrow niche that supports LSC while creating an inhospitable environment for normal HSCs (Schepers *et al*, 2013). Leukemogenic transformation of normal cells triggered by alterations of the BM microenvironment has been observed in patients with BM failure syndromes such as aplastic anemia (Sands *et al*, 2013). Conditional knockout of *DICER1*, a gene that regulates microRNA processing, in osteoblastic precursors has been shown to result in BM failure and leukemia predisposition. *DICER1* deletion caused reduced expression of *SBDS*, the gene mutated in Schwachman-Bodian-Diamond syndrome. Deletion of *SBDS* in mouse osteoprogenitors induced myelodysplasia and the development of AML (Raaijmakers *et al*, 2010). These findings highlight the suggestion that primary stromal dysfunction can result in secondary neoplastic disease, supporting the concept of niche-induced oncogenesis.

LSCs that receive the support of a BM niche for their survival may in turn associate with deregulation of the BM niche by their dominant proliferation-promoting signals. It has been shown that beta-catenin signaling has a central role in the self-renewal of CML and AML stem cells. Zhao *et al* demonstrated that beta-catenin deletion caused a profound reduction in the ability of mice to develop BCR-ABL–induced CML (Zhao *et al*, 2007). Wang *et al* showed that, in murine LSCs derived from MLL-AF9-induced leukemias, the Wnt/beta-catenin signaling pathway was required for self-renewal (Wang *et al*, 2010). Thus, activation of the self-renewal pathways through Wnt/beta-catenin signaling can be caused by microenvironmental stimuli (Konopleva & Jordan, 2011). N-cadherin and Wnt-β-catenin axis was recently shown to play an important role in microenvironment- mediated protection of CML LSCs from tyrosine kinase inhibitor treatment, suggesting this axis a potential; new target for eradication of residual leukemia in CML patients (Zhang *et al*, 2013). On the other hand, myeloma cells inhibited Wnt activation in the microenvironment through release of soluble dickkopf homolog 1 (DKK1), which caused an increase in the concentration of RANKL and a decrease in osteoprotegerin production, resulting in increased activation of osteoclasts and bone destruction (Qiang *et al*, 2008). It has been reported that activation of NF-κB or the absence of its inhibitor IκBα in myelopoietic cells changed the nonhematopoietic compartment, resulting in increased numbers of dysplastic hematopoietic cells with progression into secondary AML via upregulated perinatal expression of Jag-1 in $I\kappa B\alpha^{\Delta/\Delta}$ hepatocytes and activation of NOTCH1 in neutrophils (Rupec *et a*l, 2005). These findings indicate that a premalignant hematopoietic disorder can be initiated by non-hematopoietic cells with inactive IκBα, conceivably via activation of the Notch pathway. Jagged/Notch activation has been shown to result in increased numbers of HSCs and niche expansion (Calvi *et al*, 2003). Additional studies demonstrated that the tumor suppressor

FBXW7, which negatively regulates cyclin E, NOTCH, and c-MYC protein levels, plays a role in maintaining HSC quiescence and repressing potential oncogenic activity of HSCs (Matsuoka *et al*, 2008).

Several lines of experimental evidence have suggested that genetic changes in the BM microenvironment contribute to or are required for leukemogenesis. Walkley *et al* reported that dysfunction of the retinoblastoma protein (RB), a central regulator of the cell cycle and a tumor suppressor, or of retinoic acid receptor γ (RARγ) in the BM microenvironment contributes to development of preleukemic myeloproliferative disease. They demonstrated that the widespread inactivation of RB but not myeloid-specific loss of RB resulted in extramedullary hematopoiesis and myeloproliferative disease in the murine hematopoietic system (Walkley *et al*, 2007a). The microenvironment-induced myeloproliferative-like disorder was also observed in $RAR\gamma^{-/-}$ mice because of the RARγ-deficient microenvironment (Walkley *et al*, 2007b)

Similarly, deficiency of phosphatase and tensin homolog (PTEN), a tumor suppressor and an antagonist of the PI3K pathway, in both hematopoietic cells and the microenvironment resulted in myeloproliferation that progressed to overt leukemia/lymphoma (Yilmaz *et al*, 2006). However, inducible PTEN deletion in hematopoietic cells in the presence of a wild-type BM microenvironment promoted HSC depletion without evidence of myeloproliferation or leukemic development (Yilmaz *et al*, 2006). These findings indicate the importance of interactions between hematopoietic cells and the BM niche/microenvironment and suggest that additional genetic mutations within the BM microenvironment may be necessary for leukemic transformation.

Intriguingly, several studies have implicated a previously unrecognized link between microenvironment and cancer metabolism. A high level of asparagine secretion by MSCs has been shown to cause asparaginase resistance of ALL cells that reside in MSC niches, and this protective effect correlated with levels of asparagine synthetase expression in MSCs (Iwamoto *et al*, 2007). Further, recent study by Zhang et al. has shown that bone marrow stroma conversion of cystine to cysteine is crucial for the survival of CLL cells *ex vivo* and their protection against oxidative damage (Zhang *et al*, 2012). In turn, AML blasts alter the immune microenvironment via release of high concentrations of arginase II, which suppresses T cell proliferation, polarizes surrounding monocytes into a suppressive M2-like phenotype, and finally inhibits proliferation and differentiation of murine granulocyte-monocyte progenitors and human $CD34^{+}$ progenitors (Mussai *et al*, 2013). These findings directly implicate metabolic features of the perturbed bone marrow microenvironment as a prerequisite for leukemia-stroma interplay. Further characterization of the key mechanisms governing this metabolic exploitation of the supporting bone marrow niche may yield novel therapeutic targets to render the microenvironment less promiscuous for the genetically altered leukemia cell.

CONCLUSION

By elucidating the role of the BM microenvironment in the pathogenesis of hematologic tumors, recent studies have provided insight into the molecular mechanisms involved in

stem cell activation and homing to the BM niche (Table I, Figure 1). This better understanding of the nature of HSCs and their niches is expected to provide an alternative approach to the treatment of various serious diseases, including leukemia, in clinical practice. Further understanding of the contribution of the BM niche to the process of leukemogenesis may provide new targets that allow destruction of LSCs without adversely affecting normal stem cell self-renewal.

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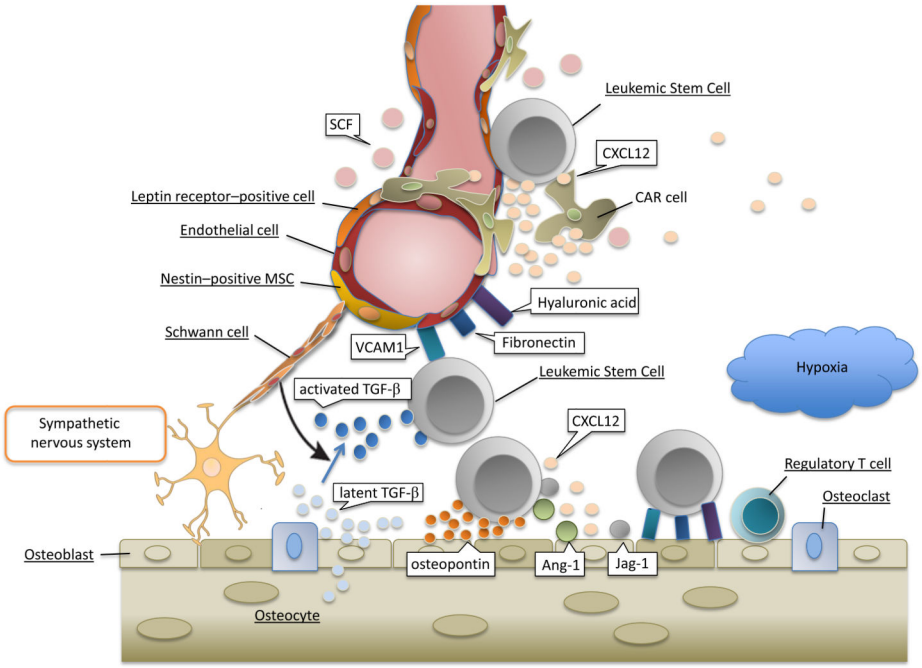

Figure 1. Key components of the leukemic bone marrow microenvironment

Components of normal HSC niches consist of multiple cell types including osteoblasts, Cxcl12-abundant reticular (CAR) cells, nestin-positive mesenchymal stem cells (MSCs), *Lepr*-expressing perivascular cells, endothelial cells and Schwann cells wrapping sympathetic nerve fibers. LSCs hijack HSC marrow spaces including perivascular and endosteal niches. The BM stromal cells and osteoblasts produce complex extracellular matrix (ECM) such as vascular cell–adhesion molecule-1 (VCAM-1), fibronectin and hyaluronic acid, which facilitate engraftment and adhesion of LSCs. Osteoblasts within endosteal niches generate transforming growth factor-β (TGF-β), angiopoietin-1 (Ang-1) and Jagged-1 (Jag-1) that in turn promote leukemia cells dormancy and decrease their chemosensitivity. CAR cells, nestin-positive MSCs, Leptin receptor-positive perivascular cells, and endothelial cells may play role for leukemia cells migration to perivascular microenvironment via cytokines, chemokines, and adhesion molecules. Inhibition of leukemia / stroma interactions causes increased leukemia cells cycling and homing to perivascular niches which can potentially be used for chemosensitization to target domant LSCs.

Table I

Cellular components of hematopoietic bone marrow niches.

Components of Niche	Molecule *(receptor molecule in HSC and LSC)	Reference
Osteoblastic (endosteal) niche		
Osteoblasts	Jagged1 *(Notch)	Calvi *et a* l, 2003
	Angiopoietin-1 (Tie-2)	Arai *et al* , 2004
	Osteopontin (β1-integrin)	Nilsson *et al* , 2005
Osteoclasts		Kong *et al* , 1999; Schroder *et al* , 2012
Regulatory T cells		Fujisaki *et al* , 2011
Vascular niche		
CXCL12-abundant reticular (CAR) cells	CXCL12 (CXCR4)	Nagasawa *et al* , 1996; Sugiyama *et al* , 2006; Nagasawa *et al* , 2011
Nestin-positive mesenchymal stem cells		Mendez-Ferrer *et al* , 2010
Leptin receptor–expressing stromal cells**		Ding *et al* , 2012
CD169-positive macrophages		Chow *et al* , 2011
Glial cells		Yamazaki *et al* , 2011
Extracellular matrix	Hyaluronan (CD44)	Jin *et a* l, 2006; Krause *et al* , 2006
	Fibronectin, VCAM-1 (VLA-4)	Miyake *et al* , 1991; Garcia-Gila *et al* , 2002
Hypoxic environment	(HIF-1α)	Mortensen *et al* , 1998; Jensen *et al* , 2000
High-calcium environment	(Calcium-sensing receptor)	Adams *et al* , 2006

** leptin receptor–expressing stromal cells include Nestin-positive MSCs and CAR cells