

# A hostel for the hostile: the bone marrow niche in hematologic neoplasms

Daniela S. Krause<sup>1</sup> and David T. Scadden<sup>2</sup>

<sup>1</sup>Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany; and <sup>2</sup>Department of Stem Cell and Regenerative Biology and Harvard Stem Cell Institute, Harvard University, Center for Regenerative Medicine and Cancer Center, Massachusetts General Hospital, Boston, USA

## ABSTRACT

Our understanding of the biology of the normal hematopoietic stem cell niche has increased steadily due to improved murine models and sophisticated imaging tools. Less well understood, but of growing interest, is the interaction between cells in the bone marrow during the initiation, maintenance and treatment of hematologic neoplasms. This review summarizes the emerging concepts of the normal and leukemic hematopoietic bone marrow niche. Furthermore, it reviews current models of how the microenvironment of the bone marrow may contribute to or be modified by leukemogenesis. Finally, it provides the rationale for a “two-pronged” approach, directly targeting cancer cells themselves while also targeting the bone microenvironment to make it inhospitable to malignant cells and, ultimately, eradicating cancer stem-like cells.

## Introduction

Interest in the leukemic stem-like cell (LSC) niche in the bone marrow (BM) developed due to the major advances made in the understanding of the normal hematopoietic stem/progenitor cell (HSPC) niche over the last 15 years. Given that leukemia does not propagate just anywhere in the body and is difficult to grow *ex vivo*, it was thought that leukemia cells depend on the bone marrow microenvironment (BMM). Furthermore, it was also thought that LSC interact with the BMM in ways that may affect both the LSC and the BMM, perhaps modulating the molecular pathways on which normal cells depend. Examining the BMM in leukemia may then provide opportunities for altering it in such a way that it becomes less hospitable to malignant cells.

The composition of the BMM is complex and includes multiple different cell populations that have been reported to participate in normal hematopoietic stem and progenitor cell support. These include arteriolar and sinusoidal type endothelial cells, osteolineage cells, osteoclasts, osteocytes, adipocytes, sympathetic neurons, non-myelinating Schwann cells, mesenchymal stem cells, Cxcl12-abundant reticular cells, macrophages, megakaryocytes and the extracellular matrix (Figure 1).<sup>1</sup> These elements affect HSPC number, location, proliferation, self-renewal, and differentiation, and likely alter similar parameters of the LSC.<sup>2</sup> Whether the BMM participates in the emergence of leukemia is unclear, though experimental support for this has emerged in animal studies reviewed below. Finally, there is the potential for the BMM to offer opportunities therapeutically targeting BMM-LSC interactions to disadvantage malignant cells. These aspects of leukemia biology will be discussed in the context of fundamental niche concepts, as first detailed by Schofield in 1978.<sup>3</sup>

### The normal bone marrow HSPC niche

The anatomy and physiology of the normal HSPC niche is still being elucidated, but it is likely that there is heterogeneity

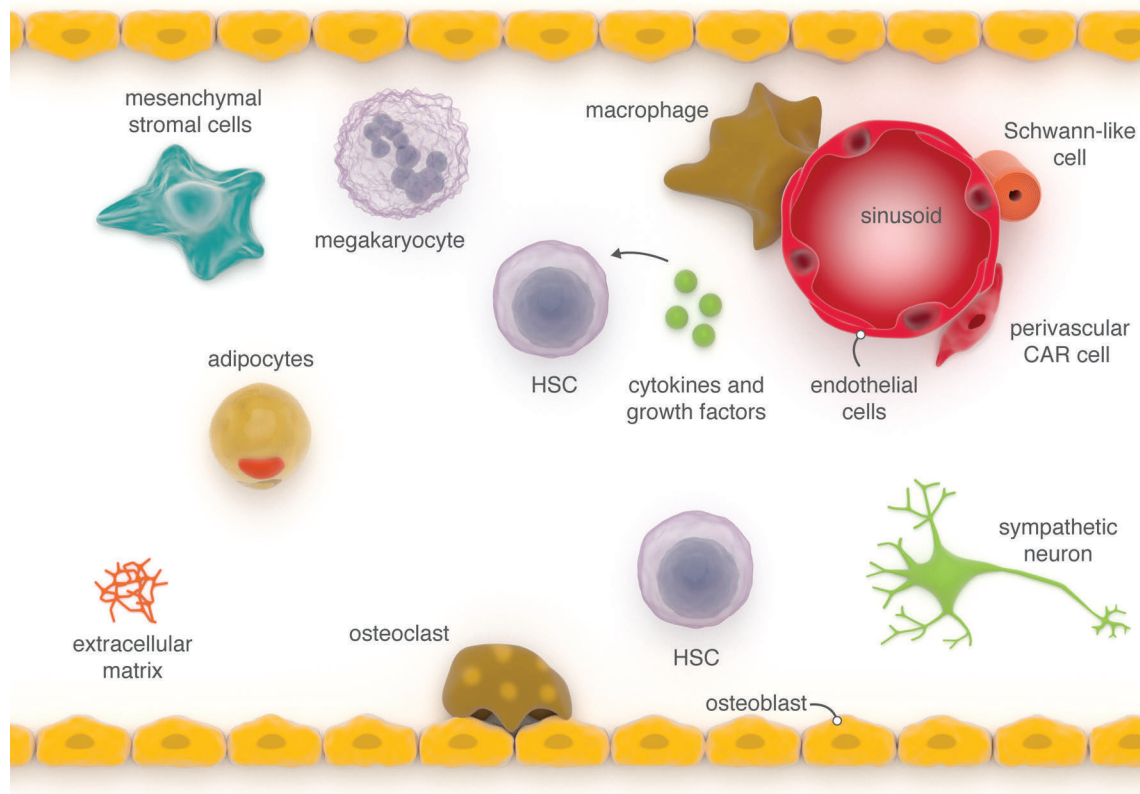
among niches as there is heterogeneity among stem cells (Figure 2). Indeed, probably there are niches that serve very different roles in different physiological contexts. For example, debate in the field still continues over the endosteal *versus* perivascular niche, yet it is likely that both exist. But different niches are important for different functions: the setting of transplantation stress (endosteal) compared with homeostasis (perivascular).<sup>1</sup> This review does not aim to reconcile these debates but rather to outline concepts and pathways that are important for the maintenance of LSC in the BMM.

The concept that vascular structures support HSPC has long been proposed and is in keeping with the growing idea that definitive hematopoiesis and establishment of a HSPC pool exists well before bone or bone marrow formation. Experimental evidence for vascular regulation of hematopoiesis was provided by the demonstration of hematopoietic regeneration occurring at sites of BM sinusoidal vascular regeneration.<sup>4</sup> Several *in vitro*<sup>5,6</sup> and *in vivo*<sup>7,8</sup> models support the notion that sinusoidal endothelial cells regulate hematopoiesis, in part by soluble factors<sup>9,10</sup> and by anatomic proximity of CD150<sup>+</sup> CD244<sup>-</sup> CD48<sup>-</sup> lineage- (SLAM marker<sup>+</sup>) HSC to BM sinusoidal vessels.<sup>11</sup> Osteoblastic cells were demonstrated to support hematopoiesis in *ex vivo* culture systems.<sup>12</sup> Evidence *in vivo* was provided by two independent studies using transgenic mice with osteoblast-specific, constitutively activated receptors for parathyroid hormone (PTH) and PTH-related peptide and mice with conditional inactivation of bone morphogenetic protein (BMP) receptor type IA (BMPRIa). In these studies, it was respectively demonstrated that a PTH-induced increase in number of osteoblastic cells<sup>13</sup> and an increase in the number of spindle-shaped N-cadherin<sup>+</sup> CD45<sup>-</sup> osteoblastic (SNO) cells<sup>14</sup> was associated with an increase in HSPC number. Conversely, the ablation of developing osteoblastic cells by conditional expression of thymidine kinase and cell killing using ganciclovir, led to a loss of progenitors of the lymphoid, erythroid and myeloid lineages.<sup>15</sup>

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Correspondence: Krause@gsh.uni-frankfurt.de or dscadden@mgh.harvard.edu



**Figure 1.** The normal bone marrow (BM) niche. The BM microenvironment is composed of multiple different cell populations that co-ordinately contribute to the regulation of hematopoiesis. The region near the endosteum is highly vascular and thought to be a site where transplanted hematopoietic stem/progenitor cells (HSPC) localize whereas sinusoids in the central portion of the marrow are thought to be the location of most HSPC under homeostasis. CAR cell: CXCL12-abundant reticular cell.

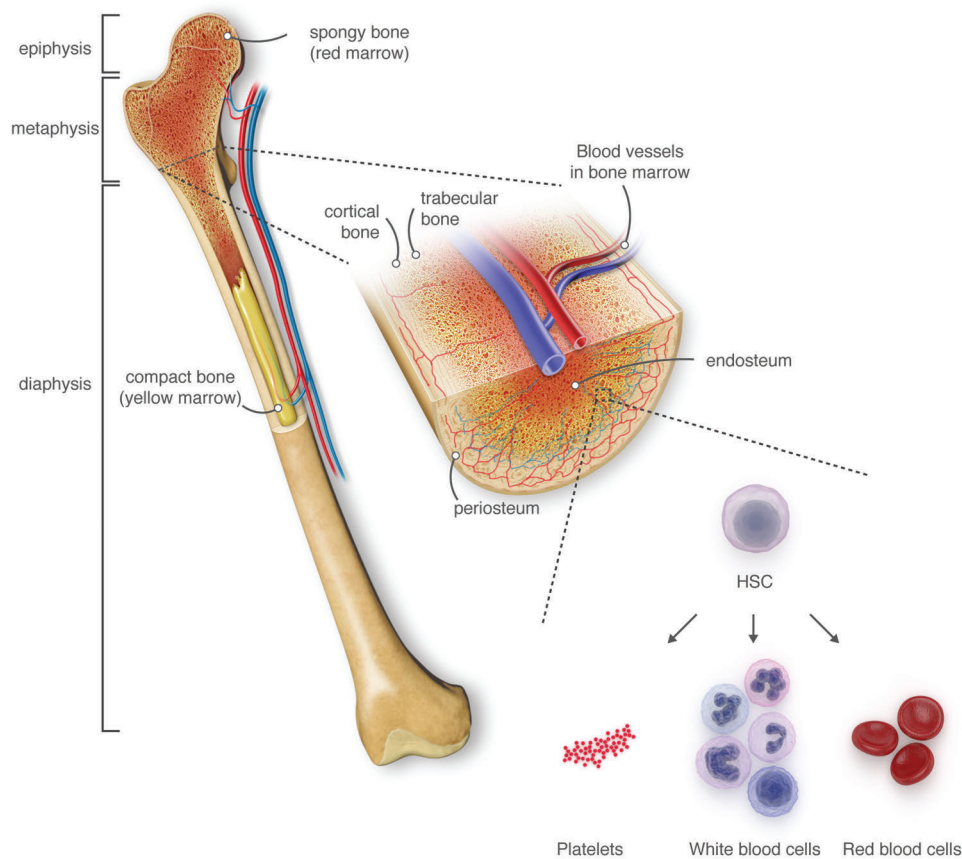
These were the first *in vivo* demonstrations of specific niche cell participants in a mammalian tissue.

These discoveries were followed by evidence that more immature perivascular mesenchymal stromal cells (MSC) maintained HSC under homeostasis. Nestin-GFP marked MSC were found in close proximity to HSC and adrenergic nerve fibers, and their depletion led to reduction of HSC.<sup>16</sup> The majority of HSC were found in the vicinity of cells expressing high amounts of CXC chemokine ligand (CXCL) 12 (CXCL12), called CXCL12-abundant reticular (CAR) cells, which are distributed throughout the BM. Deletion of CXCR4, a receptor for CXCL12, led to a reduction in HSC frequency and increased sensitivity to myelotoxic drugs.<sup>17</sup> Cell-restricted deletion of CXCL12 from endothelium or Prx1<sup>+</sup> or leptin receptor (leptinR)<sup>+</sup> cells resulted in decreased HSC. It should be noted, however, that both studies used models in which the Cre was not inducibly activated. Therefore, Cre was active throughout development and therefore all descendants of Prx1<sup>+</sup> and leptinR<sup>+</sup> cells including all bone cells could be implicated. This is balanced against the absence of an effect on HSC when osteoblastic cell-specific promoter-driven Cre activation was induced.<sup>18,19</sup> In complementary studies, it was shown that stem cell factor (SCF) is highly expressed by perivascular cells and that HSC were lost from the BMM if SCF was deleted from endothelial cells or leptin receptor (LEPR)-expressing perivascular stromal cells.<sup>20</sup> The same was not true if SCF was deleted from osteolineage or nestin<sup>+</sup> cells. However, the recombination efficiency in the different cell types was not reported.

Other work demonstrated that quiescent HSC were located close to small arterioles, frequently found in the endosteal area of the BMM and enveloped by NG2<sup>+</sup> pericytes. Activation of the cell cycle in HSC led to a redistribution from NG2<sup>+</sup> periarteriolar niches to LEPR<sup>+</sup> perisinusoidal niches, suggesting that periarteriolar niches are important for HSC quiescence.<sup>21</sup> Nestin<sup>+</sup> MSC *in vivo* are located in association with adrenergic neural fibres and HSC, which they support via the secretion of HSC-maintaining factors. The mobilization of HSPC is dependent on circadian oscillations of noradrenaline secretion and fluctuating expression of the chemokine CXCL12, suggesting that the sympathetic nervous system is heavily involved with BMM regulation.<sup>16</sup>

Blood cells themselves have been shown to be important in the BMM. Depletion of macrophages in *Macrophage Fas-induced apoptosis* (Mafia) transgenic mice or via administration of liposomes containing clodronate to normal mice led to loss of macrophages, reduction of HSC-supportive cytokines at the endosteum, and mobilization of HSC into the peripheral blood.<sup>22</sup> In addition, loss of bone marrow macrophages enhanced mobilization induced by a CXCR4 antagonist or granulocyte colony-stimulating factor.<sup>23</sup>

Another important component of the BMM is the extracellular matrix (ECM). An early work showed that heparin sulfate, a protein produced by marrow-derived stromal cells of undefined phenotype and found in the ECM of the bone marrow, is important for the adhesion of hematopoietic blast colony-forming cells to the ECM.<sup>24</sup> Consistent



**Figure 2.** Bone marrow (BM) anatomy. The normal bone marrow anatomy (here using the example of the femur) is composed of different types of bone, blood vessels and red and yellow marrow. HSPC reside in the red marrow where they differentiate into red blood cells, white blood cells and platelets via different progenitor stages (not shown). Yellow marrow represents largely adipocyte-rich regions with minimal hematopoiesis.

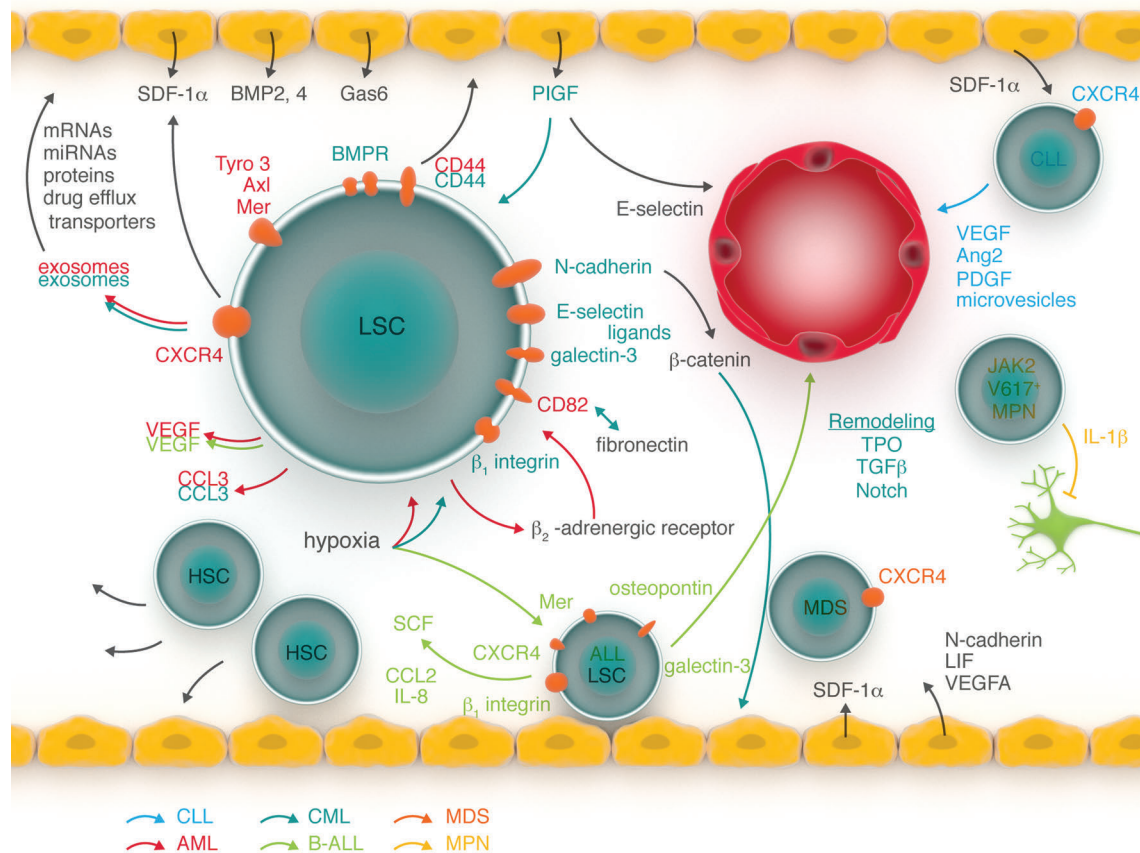
with this, inhibition of heparan sulfate led to mobilization of more and more functionally competent HSC.<sup>25,26</sup> Mice deficient for tenascin-C, another ECM protein normally expressed by stromal and endothelial cells, were unable to reconstitute hematopoiesis after myeloablation or support hematopoiesis after transplantation.<sup>26</sup> Furthermore, osteopontin, secreted by osteoblastic cells and other cell types, was found to negatively regulate HSC number and function through influencing the expression of Jagged1 and Angiopoietin-1.<sup>27,28</sup>

Oxygen tension within a given environment was thought to regulate stem cell behavior, but methods to directly study the distribution of oxygen in the BMM have only recently become available. The bioprobe pimonidazole measures reducing intermediates and in conjunction with *in vivo* microscopy suggested that the endosteal region is hypoxic.<sup>29</sup> HSPC with pimonidazole retention and increased expression of hypoxia-inducible transcription factor-1 $\alpha$  (HIF-1 $\alpha$ ) consistent with a hypoxic state preferentially localized in endosteal zones.<sup>30</sup> Using a Hoechst 33342 perfusion gradient to assess perfusion, HSC were found in areas of the BMM with the lowest Hoechst level. Furthermore, the hypoxic cytotoxic agent tirapazamine was selectively toxic to HSC.<sup>31</sup> Direct measurement of the local oxygen tension (pO<sub>2</sub>) using two-photon phosphorescence lifetime microscopy showed that the lowest (pO<sub>2</sub> ~9.9 mmHg) measurements were in perisinusoidal regions with the arteriole-rich endosteal region being less hypoxic. Cytotoxins increased pO<sub>2</sub>, suggesting that oxygen consumption accounts for the relatively low ambient oxygen level in the central bone marrow.

### Alterations of the BMM can lead to hematologic abnormalities

Based on findings in the normal bone marrow, alteration of the BMM was tested and found to lead to hematologic abnormalities. Mice with deficiency of retinoic acid receptor (RAR)  $\gamma$  are characterized by an increase in granulocytic/macrophagic progenitors and increased granulocytes in peripheral blood, bone marrow and spleen. However, when wild-type bone marrow was transplanted into these mice, the recipient mice, also developed this myeloproliferation. These data suggest that the myeloproliferation was not intrinsic to the hematopoietic cells, but arose due to alterations in the microenvironment. Increased levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) in the RAR $\gamma$ -deficient BMM contributed to the myeloproliferation.<sup>32</sup> When retinoblastoma protein (Rb) was deleted in the hematopoietic system, a similar myeloproliferation was found and HSCs were lost from the BMM due to mobilization; but these findings were dependent on Rb deletion in both hematopoietic cells and their BMM, as deletion in either one alone was not sufficient to create the phenotype.<sup>33</sup> Similarly, fatal microenvironment-dependent myeloproliferation also developed in mice with inactivation of mind bomb 1 (Mib1), a molecule necessary for the processing and presentation of Notch ligands.<sup>34</sup> In mice with osteocyte-specific deletion of the G $\alpha$  subunit of the G-protein signaling cascade, a signaling molecule downstream of the receptors for parathyroid hormone and prostaglandin, and certain  $\beta$ -adrenergic receptors myeloproliferation also developed. In the latter example, the myeloproliferation was due to increased GCSF secretion





**Figure 3. Leukemia stem cells.** Leukemia stem cells (LSC) in acute myeloid leukemia (AML; red), chronic myelogenous leukemia (CML; dark blue), B-cell acute lymphoblastic leukemia (B-ALL; green), chronic lymphocytic leukemia (CLL; pale blue), myelodysplastic syndrome (MDS; orange) and JAK2 V617F positive myeloproliferative neoplasia (MPN; yellow) interact with their bone marrow microenvironment via specific pathways. Specific details are provided in the text. SDF-1 $\alpha$ : stromal-derived factor 1 $\alpha$ ; BMP: bone morphogenetic protein; BMPR: bone morphogenetic protein receptor; Gas6: growth-arrest-specific-gene 6; VEGF (A): vascular endothelial growth factor (A); SCF: stem cell factor; IL-8: interleukin-8; PIGF: placental growth factor; TPO: thrombopoietin; LIF: leukemia-inhibitory factor; PDGF: platelet-derived growth factor  $\beta$ .

from mutated osteocytes.<sup>35</sup> The hyperproliferation in these examples was not accompanied by hematopoietic cell transformation or evidence of abnormal differentiation.

In contrast, osteoprogenitor cell-specific disruption of *Dicer1*, required for microRNA processing, led to a myelodysplasia-like syndrome. Furthermore, few mice developed acute myeloid leukemia (AML) with new complex genetic abnormalities that did not involve *Dicer1*.<sup>36</sup> These data are the first evidence that a specific distinct stromal cell type, in this case an osteolineage cell, can be the inciting abnormality that eventually leads to malignancy in a distinct, parenchymal cell. Subsequently, it has been reported that mutations activating  $\beta$ -catenin in osteoblasts in mice resulted in AML with high penetrance in association with increased Notch activation. These investigators also found increased  $\beta$ -catenin signaling in osteoblastic cells and increased Notch signaling in hematopoietic cells in 38% of patients with myelodysplastic syndromes (MDS) or AML.<sup>37</sup> Taken together, these findings suggest that an altered microenvironment can serve as the inciting event in hematologic neoplasia.

The phenomenon of donor-derived leukemia in patients receiving allogeneic transplantation is uncommon but may

represent a dysfunctional microenvironment sufficient to rapidly induce neoplasia.<sup>38</sup> Along these lines, studies have reported genetic abnormalities in bone marrow stromal cells, isolated via adherence to plastic, from patients with MDS or AML, although whether these can cause disease has still not been proved.<sup>39,40</sup>

### The LSC niche

#### Adhesion to the LSC niche

In 2006, the important role of the cell-surface glycoprotein CD44, which binds the extracellular matrix proteins hyaluronan, osteopontin,<sup>41</sup> and possibly E-selectin,<sup>42,43</sup> was investigated for the interaction between AML<sup>44</sup> and CML<sup>45</sup> cells with their BMM. Repopulation by human AML cells in non-obese diabetic-severe combined immune-deficient (NOD/SCID) mice treated with an activating antibody to CD44 was decreased, likely due to defective transport of LSC to supportive niches.<sup>44</sup> Others showed that high levels of CD44 were important for AML induction or relapse in mouse models of AML.<sup>46</sup>

In CML, CD44 expression was shown to be increased and to contribute functional E-selectin ligands on CML-initiating cells. Lack of CD44 led to reduced homing and engraftment of CML-initiating cells in the well described

retroviral model of CML-like myeloproliferative neoplasia.<sup>47,48</sup> It also led to decreased efficiency of induction of CML-like myeloproliferative neoplasia in wild-type recipients. CD44, in contrast, was shown to be dispensable for the induction of B-cell acute lymphoblastic leukemia.<sup>45</sup> In support of these data, lower levels of soluble CD44 in autologous hematopoietic stem cell grafts from patients with AML, plasma cell myeloma and non-Hodgkin lymphoma, shed from the surface of malignant cells, correlated with improved outcome after autologous hematopoietic stem cell transplantation.<sup>49</sup> Furthermore, treatment of humanized RAG2<sup>-/-</sup>gc<sup>-/-</sup> mice transplanted with human CD34<sup>+</sup> blast crisis CML cells with a CD44 monoclonal antibody alone or in combination with the tyrosine kinase inhibitor dasatinib led to a reduction of human progenitor cells in niches and a significant reduction of LSC self-renewal capacity upon serial transplantation.<sup>50</sup> Polymeric nanoparticle-mediated silencing of CD44 decreased surface levels of CD44 in AML cells, induced apoptosis and reduced adhesion of AML cells to mesenchymal stem cells from reamed human bone marrow.<sup>51</sup> In addition, treatment with a combination of anti-CD44 (and particularly anti-CD44v10<sup>52</sup>) and anti-CD49d antibodies dislodged CD44-expressing lymphoid cell lines, EL4 and Jurkat, in mouse bone marrow and spleen, leading to increased sensitivity to chemotherapy.<sup>53</sup> These data strongly support targeting CD44 as a means of compromising myeloid and possibly acute lymphoid malignancies, though current clinical trials are only targeting CD44 in terms of a cancer stem cell marker in solid tumors.

The importance of specialized niches for the engraftment of tumor cells in the BMM was shown by dynamic *in vivo* confocal imaging. The adhesion molecule E-selectin and stromal-derived-factor 1 (SDF-1) are expressed in certain domains in the endothelium of the BMM and represent the sites at which circulating leukemic cells, a B-ALL cell line, as well as normal HSPC and lymphocytes can engraft.<sup>54</sup> Functionally, a critical role of selectins and their ligands was shown for the engraftment of CML-initiating cells.<sup>55</sup> Also, inhibition of E-selectin with a small molecule antagonist, GMI1271, reduced the number of CML LSC.<sup>56</sup> Finally, E-selectin was reported to be over-expressed on bone marrow endothelium in AML and the absence of E-selectin markedly increased sensitivity of AML to cytarabine.<sup>57</sup>

Other adhesion molecules, such as CD82 on CD34<sup>+</sup> CD38<sup>-</sup> AML cells, were shown to inhibit adhesion to fibronectin and impaired engraftment of AML cells in immunodeficient mice.<sup>58</sup>

In the 1990s, significant *in vitro* work demonstrated a role for  $\beta$ 1-integrins in the adhesion of CML cells to fibronectin. An activating antibody to  $\alpha$ 5 $\beta$ 1, for example, led to increased adhesion of leukemia cells to fibronectin and decreased their proliferation.<sup>59</sup> Interestingly, interferon  $\alpha$  restored  $\beta$ 1 integrin-mediated adhesion of CML to stromal cells of undefined phenotype inhibiting proliferation of CML cells.<sup>60,61</sup> Similarly,  $\beta$ 1 integrins may play a role in AML, as increased binding of soluble vascular cell adhesion molecule (VCAM)-1 via the  $\beta$ 1 integrin VLA-4 was associated with longer overall survival in patients.<sup>62</sup>

Understanding of the pathways by which leukemia cells interact with their BMM (Figure 3) will continue to be of great interest, as these pathways may be targetable and thereby make the LSC more sensitive to chemotherapy and other agents.

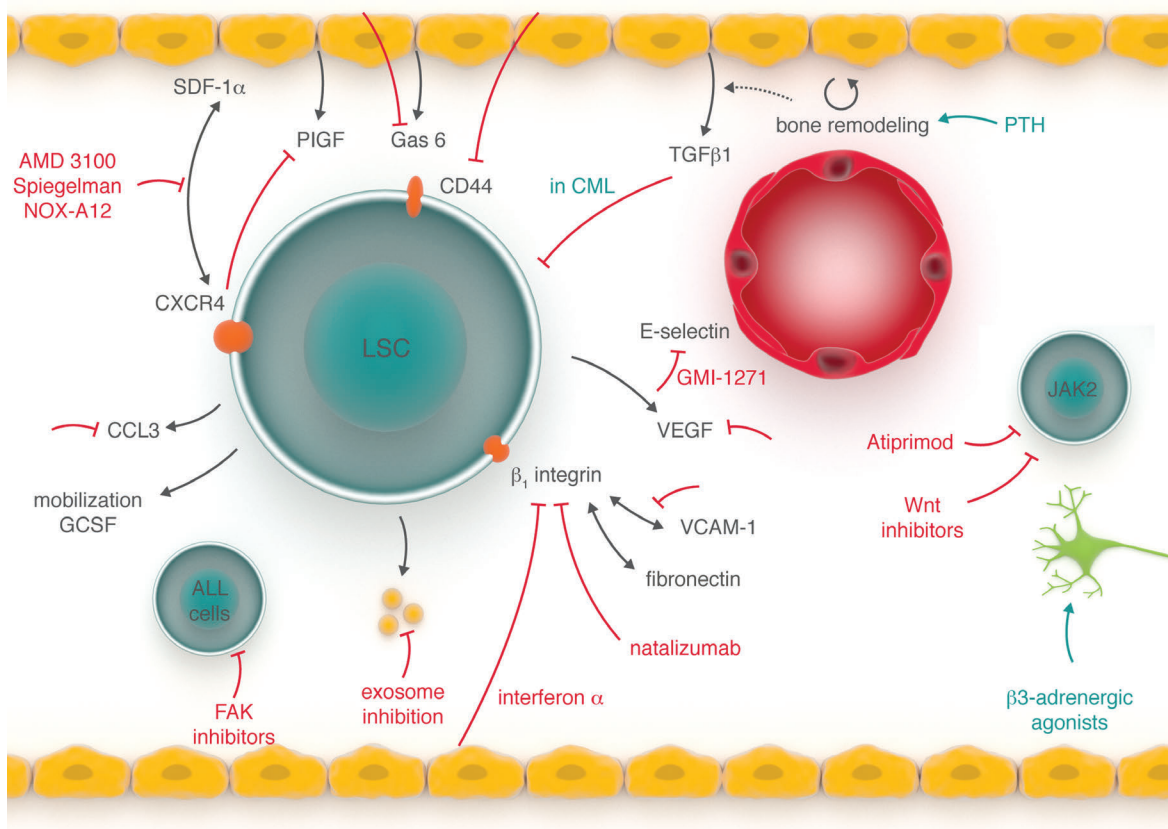
#### *Bi-directional interactions between leukemia cells and their niche*

There are several examples of the BMM influencing leukemia. For example, depletion of osteoblastic cells in the BMM of mice with AML worsened the disease with increased circulating blasts, higher tumor burden in bone marrow and spleen, and shorter survival. On the other hand, preserving osteoblastic cells by an inhibitor of serotonin production restored normal bone marrow function, reduced tumor burden and prolonged survival.<sup>63</sup> Furthermore, mixed lineage leukemias arising due to the MLL-AF9 fusion adopt differing lineage fates, depending on the cytokine milieu in which the cells were treated *in vitro* prior to injection or by altering the strain of mice.<sup>64</sup> Preliminary data generated by *in vivo* imaging suggest that BCR-ABL1<sup>+</sup> LSC may be found further away from the endosteum than normal HSC, yet this was reversed if the BCR-ABL1<sup>+</sup> LSC had previously been treated with imatinib. This may be due to upregulation of CXCR4,<sup>65</sup> or possible harboring of the T315I mutation conferring imatinib resistance.<sup>66</sup> And, finally, as described below, modulation of the BMM with parathyroid hormone led to opposite changes of the phenotype in CML versus AML.<sup>67</sup>

Just as the BMM may influence leukemia, a leukemia can shape the BMM. In a murine model of AML, osteoblastic cells were reduced and inhibited by the leukemia, as measured by decreased levels of the bone formation marker, osteocalcin. Mineralized bone was also lost. The cytokine CCL3, known to be involved in bone loss, was increased in AML cells in mice and humans suggesting that CCL3 (macrophage inflammatory protein 1 $\alpha$ ; MIP 1 $\alpha$ ) may be involved in the observed phenotype.<sup>68</sup> It is tempting to speculate that inhibition of this “molding” of the niche by leukemia cells, which likely shapes the BMM in a way most beneficial to survival of the leukemia, but most detrimental to the survival of normal HSPC, would have a negative impact on leukemia progression. Future research endeavors may be aimed at this.

In a transgenic model of BCR-ABL1<sup>+</sup> CML, the endosteal BMM was reported to be remodeled into a leukemic niche. Multipotent murine stromal cells were stimulated by leukemia cells *in vitro* to produce functionally abnormal osteoblastic cells impaired in their capacity to generate HSC-retaining and -maintaining factors. Thrombopoietin, CCL3, and direct cell-cell interactions were found to be involved in promoting the expansion of osteoblastic cells, whereas TGF- $\beta$ , Notch, and inflammatory signaling were found to play a role in the remodeling of the osteoblastic niche.<sup>69</sup> Long-term LSC in CML exhibited reduced homing and retention in the bone marrow, associated with increased granulocyte colony-stimulating factor (G-CSF) produced by leukemia cells and decreased levels of CXCL12 in the BMM,<sup>70</sup> as was observed in pediatric B-ALL.<sup>71</sup> Altered expression of cytokines in the murine CML BMM favored the growth of LSC versus normal HSC, which was partially restored by treatment with imatinib.<sup>70</sup> Ablation of osteoblastic cells in the same model of CML led to an acceleration of the leukemia phenotype and reduced survival compared to the control mice. The CML BMM is also thought to over-express the Notch ligand Jagged-1, affecting hematopoietic cell cycling.<sup>72</sup>

In the perivascular niche, autocrine and paracrine secretion of vascular endothelial growth factor (VEGF)<sup>73</sup> and other factors by leukemia cells led to proliferation of leukemia and endothelial cells in ALL<sup>74</sup> and AML.<sup>75</sup> Neo-



**Figure 4.** Leukemia stem cell targeting. Depending on the disease entity certain therapies (red) may specifically target the interaction of leukemic stem cells with their bone marrow niche. As detailed in the text, some of these novel therapies have already entered clinical trials. FAK: focal adhesion kinase. Other abbreviations as in Figure 3.

angiogenesis is a well-established phenomenon observed in AML<sup>76</sup> and ALL,<sup>77</sup> but, mechanistically, it has not received much attention, though it may provide another Achilles' heel in the treatment of leukemia.

Mesenchymal stromal cells derived from bone marrow stroma have also been shown to play a role in the LSC niche. In a xenotransplantation model of transplanted MDS-initiating Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>-</sup> stem cells, only orthotopic co-transplantation of patient-derived MSC, isolated via adherence to plastic, which are characterized by an altered differentiation pattern, gives rise to MDS-like disease in murine recipients. Factors such as N-Cadherin, insulin-like growth factor binding protein 2, VEGFA, and leukemia inhibitory factor increase the ability of MSC in MDS to promote MDS and may be involved in altering molecular expression patterns in healthy MSC when cocultured with hematopoietic MDS cells.<sup>78</sup>

In another report, levels of placental growth factor (PIGF), secreted by bone marrow stromal cells positive for the osteogenic/fibroblastoid markers Runx2, Col1a1 and alkaline phosphatase upon stimulation by the leukemia cells themselves, were elevated in CML. PIGF led to stimulation of BM angiogenesis, promotion of CML proliferation and metabolism, thereby contributing to disease aggressiveness.<sup>79</sup>

In a murine model of JAK2 V617F<sup>+</sup> myeloproliferative neoplasia abrogation of the regulatory innervation of the BMM by sympathetic nerve fibres was shown to be an essential component of the pathogenesis of MPN. In

patients and mice with MPN sympathetic nerve fibres, supporting Schwann cells ensheathing the neural fibres and nestin<sup>+</sup> MSC were reduced in the BMM. The loss of MSC and neural damage were due to the production of interleukin-1 $\beta$  by malignant HSC, and depletion of nestin<sup>+</sup> cells led to acceleration of MPN. However, treatment of mice with  $\beta$ 3-adrenergic agonists as sympathomimetic agents restored the loss of nestin<sup>+</sup> MSCs and blocked MPN progression via an indirect reduction of MPN cells.<sup>80</sup> These findings have led to initiation of a clinical trial, as described below. In AML, leukemia-induced impairment of the sympathetic nervous system in the BMM led to an increased infiltration of the BMM with leukemic cells in a murine model of MLL-AF9<sup>+</sup> AML. AML development altered the quiescence of Nestin<sup>+</sup> niche cells, favoring the differentiation of mesenchymal stem and progenitor cells towards osteoblastic cells, while not favoring the periarteriolar niche cells known to support HSC. Leukemogenesis was promoted by the  $\beta$ 2 adrenergic receptor expressed on stromal cells in the BMM, suggesting that the sympathetic nerve system may be "hijacked" by the malignant cells in order to promote and advance the neoplasm.<sup>81</sup>

Just as in the normal HSC niche, hypoxia may play a role in the leukemic BMM. Expression of HIF-1  $\alpha$ , up-regulated by hypoxia and other factors, though this correlation may be controversial, was associated with a negative prognostic impact on survival of patients with pre-B-cell ALL. Stroma-mediated AKT/mTOR signaling further induced HIF-1  $\alpha$ , while blockade of mTOR restored the



chemosensitivity of B-ALL cells.<sup>82</sup> In a murine model of CML, HIF-1  $\alpha$  is involved in regulating cell cycle propagation and apoptosis and, therefore, promotes LSC survival via increased expression of *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* in LSCs.<sup>83</sup> Hypoxia also led to decreased apoptotic rates and increased clonogenicity and repopulating efficiency of BCR-ABL1<sup>+</sup> cells, while knockdown of HIF1- $\alpha$  reversed the enhanced clonogenicity during hypoxic conditions.<sup>84</sup> High expression of HIF-1 $\alpha$  is found in AML1-ETO<sup>+</sup> AML cells and is associated with inferior outcomes while co-expression of AML1-ETO and HIF1 $\alpha$  leads to an increased cellular proliferation rate *in vitro* and more aggressive leukemia in mice.<sup>85</sup> Knockdown of HIF-1  $\alpha$  and the use of the HIF inhibitor echinomycin abrogated the colony-forming unit activity and eradicated LSC in human AML, as tested by serial transplantation assays in xenotransplants.<sup>86</sup> Deregulation of HIF-2  $\alpha$ , which impaired the long-term repopulating ability of human CD34<sup>+</sup> cells, impaired the engraftment of human AML, suggesting that HIF-2  $\alpha$  also affects AML cell interactions with the BMM.<sup>87</sup> These studies suggest that hypoxia with deregulation of HIF1- $\alpha$ , and possibly HIF2- $\alpha$ , alters LSC function, including chemoresistance. However, the hypoxia field in the LSC (and HSC) niche, the role of HIF-1 in hypoxia, as well as the location of LSC (and HSC) in relation to hypoxic areas in the BMM all remain a subject of scientific debate and warrant further research.

### Exosomes

Exosomes are lipid vesicles or microparticles (<1  $\mu$ m) secreted by cancer cells, MSC, macrophages, dendritic cells, B and T cells, mast cells and endothelial cells.<sup>88</sup> They are associated with the bidirectional transfer of mRNAs, microRNAs, drug efflux transporters and other proteins between cancer and stroma cells leading to alteration of gene expression in neighboring cells.<sup>89</sup> They may be a means by which a cancer modulates its environment. It was demonstrated that primary leukemia cells and leukemia cell lines release microvesicles containing coding and non-coding RNAs that enter cells of the microenvironment, altering their secretion of growth factors and reprogramming the LSC niche.<sup>90,91</sup> Co-culture of a CML cell line with endothelial cells led to exosomes containing the microRNA miR-126 being shuttled into the endothelial cells, led to reduced expression of VCAM-1 and CXCL12 in endothelial cells, and decreased motility and adhesion of LAMA84 cells. Transfection of a miR-126 inhibitor into the endothelial cells reversed these effects.<sup>92</sup> Treatment of LAMA84 cells or mice xenografted with human CML cells promoted the colony-forming ability of LAMA84 cells in methylcellulose and tumor size, respectively, due to an increase in the anti-apoptotic molecules BCL-w, BCL-xl, and survivin, and a reduction of the pro-apoptotic molecules BAD, BAX and PUMA in the leukemia cells.<sup>93</sup> Exosomes appear to be a means by which cancer cells communicate with each other and their environment, though their role has not yet been fully elucidated. Exactly how exosomes are formed, how they incorporate their "message", and how this may be functionally blocked represent important issues for further research.

### The LSC niche in B-cell acute lymphoblastic leukemia

The niche in B-ALL appears to be distinct from the LSC niches in AML and CML, possibly reflecting differences in myeloid and lymphoid niches of normal cells.<sup>82,85,94</sup>

The metabolomic<sup>95</sup> and functional abnormalities of niche cells from patients with B-ALL have long been known.<sup>96,97</sup> For example, some Philadelphia chromosome-positive (Ph<sup>+</sup>) ALL cells coexpress markers of endothelial cells (VE-cadherin and others) and B-lineage progenitors (Ph<sup>+</sup> VE-cadherin<sup>+</sup>).<sup>98</sup> E2A/PBX1-positive B-cell precursor ALL interacts with the BMM through Gas6/Mer with Gas6 promoting survival of ALL cells and preventing their apoptosis after chemotherapy.<sup>99</sup>

Acute lymphoblastic leukemia blasts adhere to osteopontin secreted by osteoblastic and ALL cells. Inhibition of osteopontin led to better eradication of B-ALL cells in synergism with Ara-C.<sup>100</sup> The plasma levels of the chemokines CCL2 and IL-8 were increased in children with B-ALL due to stimulation of bone marrow stromal cells by leukemia cells. CCL2 and IL-8 promoted the adhesion of CD105<sup>+</sup> CD29<sup>+</sup> CD44<sup>+</sup> CD14<sup>+</sup> CD34<sup>+</sup> and CD45<sup>+</sup> MSC generated via adherence to plastic, to B-ALL cells, and enhanced the survival and proliferation of MSC.<sup>101</sup> Similar to the role of the SDF-1 $\alpha$ /CXCR4 axis in AML, CXCR4 mediates homing of B-ALL cells to the BMM, as evidenced by inhibition with a small molecule antagonist of CXCR4. Furthermore, the combination of nilotinib, a second-line tyrosine kinase inhibitor, or vincristine with a CXCR4 antagonist prolonged survival or reduced leukemia burden in murine models.<sup>102</sup>

An interesting pathway employed by B-ALL cells is the  $\beta$ 1-integrin/focal adhesion kinase (FAK) pathway. FAK is a non-receptor tyrosine kinase constitutively active in BCR-ABL1<sup>+</sup> B-ALL cells. Integrin  $\alpha$ 5 $\beta$ 1 (VLA-5) is more highly expressed on B-ALL cells after serum starvation, and its inhibition leads to decreased adhesion of Ph<sup>+</sup> leukemia cells to fibronectin and synergistically induces apoptosis in conjunction with imatinib. Various strategies to block the activity of integrin led to impaired engraftment of leukemia cells in xenotransplantation experiments.<sup>103</sup> BCR-ABL1<sup>+</sup> B-ALL, positive for the Ikaros (IKZF) mutation, are associated with a worse prognosis in patients, led to a more aggressive phenotype in mice, and are characterized by chemoresistance to tyrosine kinase inhibitors in association with a stromal adhesion phenotype. Treatment with the FAK inhibitors VS-4718 and VS-6063 abolished stromal adhesion of *Ikzf1*-mutant B-ALL cells and induced apoptosis, but had virtually no effect on *Ikzf1* WT B-ALL cells. In combination with dasatinib, VS-4718 and VS-6063 decreased the viability of *Ikzf1*-mutant BCR-ABL1<sup>+</sup> B-ALL cells from mice and human patients cultured on stroma.<sup>104</sup> Inhibiting FAK may be a means of impairing B-ALL interaction with the BMM and has been introduced into clinical trials in solid tumors and B-ALL.

### Chemoresistance mediated by the BMM in leukemia

Several mechanisms leading to chemoresistance in leukemia cells mediated by the BMM have been described. LSC in AML home to and engraft within the endosteal, osteoblast-rich area of the bone marrow in newborn NSG mice. AML cells appeared to be protected from apoptosis induced by chemotherapy at that site.<sup>105</sup> In a murine model of ALL, and in human patients, small foci of leukemia-propagating cells remained after chemotherapy. These persisting leukemia cells secreted cytokines leading to the recruitment of Nestin<sup>+</sup>, mostly Vimentin<sup>+</sup> and partially  $\alpha$ -smooth muscle actin<sup>+</sup> stromal cells and the formation of a protective niche. The niche itself provided the protease Furin, in order to process the growth differ-

entiation factor 15 (GDF15), a member of the transforming growth factor  $\beta$  superfamily, which conferred chemoresistance on the leukemia-propagating cells.<sup>106</sup> The tumor microenvironment also inhibits the attack of tumor cells, which have been targeted by antibodies, by macrophages and macrophage-mediated killing. Cyclophosphamide, however, a frequently used chemotherapeutic agent, induced a secretory phenotype in ALL cells leading to the release of CCL4, IL8, VEGF, and TNF $\alpha$  from treated tumor cells, inducing infiltration and phagocytosis by macrophages in the BMM.<sup>107</sup> These data suggest that the choice of the chemotherapeutic regimen may influence the immunological homeostasis in the BMM. The interaction between vascular cell adhesion molecule 1 (VCAM-1) expressed in the bone marrow microenvironment and the  $\beta$ 1 integrin very late antigen-4 (VLA-4) expressed on leukemia cells was shown to play an important role in the activation of NF- $\kappa$ B and chemoresistance in leukemia cells in the BMM.<sup>108</sup> Another protein known to mediate chemoresistance in CML<sup>109</sup> and ALL<sup>110</sup> is Galectin-3 (Gal-3), a lectin with the ability to bind  $\beta$ -galactosides. Co-culture of 5 CML cell lines with HS-5 stromal cells induced the expression of Gal-3 in the leukemic cell lines. The signaling pathways activated by enforced expression of Gal-3 induced resistance to BCR-ABL1-specific tyrosine kinase inhibitors, as well as chemotherapeutic agents due to increased leukemia cell proliferation and decreased induction of apoptosis. *In vivo* overexpression of Gal-3 led to enhanced lodgement of leukemia cells in the BMM.<sup>109</sup> Treatment of CML cells with a tyrosine kinase inhibitor (TKI) promotes migration towards stromal cells, characterized by their adhesion to plastic, *in vitro*<sup>111</sup> and towards osteoblastic cells *in vivo*<sup>66</sup> where it promotes resistance to imatinib via redistribution of CXCR4 into the lipid raft fraction in the cell membrane.<sup>111</sup>

Altered expression of c-Myc<sup>112</sup> or activation of Stat3<sup>113</sup> by the BMM have been reported to affect chemosensitivity of AML and CML, respectively. Co-culture experiments with human mesenchymal stromal cells and CML led to the identification of N-cadherin receptor as a mediator of stroma-induced resistance to TKIs. N-cadherin-mediated adhesion to MSCs, previously adhered to plastic, increased the nuclear translocation and transcriptional activity of  $\beta$ -catenin, thereby linking Wnt-mediated  $\beta$ -catenin signaling with protection of leukemia cells from TKI therapy by the BMM.<sup>114</sup> In myeloproliferative neoplasia positive for the JAK2(V617F) mutation, treatment with the potent JAK2 inhibitor atiprimod only inhibited the growth of the malignant cells in *in vitro* experiments in the absence of stroma, which was shown to be due to the secretion of various cytokines by the stromal cells in co-culture experiments.<sup>115</sup>

### Modeling the LSC niche in 3D

Much effort has been made to model the LSC niche in 3 dimensions. In 2009 a "biomimetic osteoblast niche" was constructed using bio-derived bone as a scaffold. On this scaffold, MSC from CML patients were seeded and differentiation into osteoblastic cells was induced. Compared to cultures in a 2-dimensional system, the 2-dimensional system yielded a higher number of CD34<sup>+</sup> and CD34<sup>+</sup> CD38<sup>-</sup> cells, higher numbers of colony-forming units and a higher number of long-term culture-initiating cells.<sup>116</sup> Another strategy was the creation of ectopic niches using

polyurethane scaffolds that had been coated with human MSC. Upon subcutaneous implantation of these scaffolds, which, eventually, became vascularized and showed the presence of osteoclasts and adipocytes, into NOD/SCID mice, engraftment of primary human AML cells was supported.<sup>117</sup> Furthermore, the leukemia cell lines K562, HL60 and Kasumi-6 were shown to grow best on polyurethane scaffolds that had been precoated with collagen type I compared to scaffolds made of other materials.<sup>118</sup> Decellularized matrices have been employed for the expansion of hematopoietic progenitor cells from umbilical cord blood<sup>119</sup> and a mixture of human MSC, generated via prior adhesion to plastic, endothelial colony-forming cells and matrigel has been implanted subcutaneously into immunodeficient mice, leading to engraftment of normal hematopoietic cells and leukemic cells in one study,<sup>120</sup> and in hypoxic conditions in another study.<sup>121</sup> 3D models used to test drug resistance have demonstrated their superiority over 2D models, as leukemic cells cultured in 3D exhibited greater resistance to drug-induced apoptosis compared to cells cultured 2-dimensionally or in suspension, and differences were observed in the response of leukemia cell lines to chemotherapy when cultured in a 3D system.<sup>122</sup> Improved modeling of the LSC (and HSC) niche may help improve pre-clinical assessment of drug response. However, controversy still remains as to the most suitable composition of the scaffold. Furthermore, most 3D scaffolds are inefficient at modeling the physico-chemical properties of the niche, such as pH, shear forces, oxygen and cytokine gradients.

### Targeting the LSC niche

Most of the current strategies to target the BMM in leukemia involve impairment or inhibition of the CXCR4/SDF1 $\alpha$  axis. The CXCR4 inhibitor AMD3100 arrested the proliferation of AML cell lines and initiated changes suggesting differentiation<sup>123</sup> and in a murine model of acute promyelocytic leukemia, co-treatment with the CXCR4 inhibitor AMD3100 and chemotherapy led to a decreased tumor burden and prolonged survival.<sup>124</sup> Similarly, the effects of the FLT3 inhibitor lestaurtinib, the BCR-ABL1-targeting tyrosine kinase inhibitor nilotinib or vincristine were enhanced in various models of ALL when combined with an antagonist of CXCR4.<sup>102,125</sup> These results may be due to altered localization in the bone marrow. A clinical trial employing an antibody to CXCR4 in AML ([clinicaltrials.gov](http://clinicaltrials.gov) identifier:01120457) or a peptidic inhibitor to CXCR4 in CML ([clinicaltrials.gov](http://clinicaltrials.gov) identifier:02115672) are in progress or recruiting, respectively.

An RNA oligonucleotide in L-configuration, the Spiegelmer NOX-A12, neutralized CXCL12, leading to decreased chemotaxis of CLL cells and increased sensitivity to chemotherapy.<sup>126</sup> But in the retroviral model of BCR-ABL1-induced CML, combination of AMD3100 with dasatinib led to improved control of disease; however, this was at the price of an increased rate of symptoms due to involvement of the central nervous system by leukemia cells.<sup>127</sup> This suggests that the combination of CXCR4 antagonism with tyrosine kinase inhibitors or chemotherapy may be beneficial in the setting of minimal residual disease.

Other strategies to inhibit the LSC niche are encouraging (Figure 4). Using a murine transduction/transplantation model of CML and recipient mice with osteoblastic-cell specific activation of the receptor for PTH- and PTH-relat-



ed peptide, it was demonstrated that modulation of the BMM can lead to an alteration of the leukemia phenotype, as these transgenic recipient mice died significantly later than the wild-type controls, and the incidence of CML was reduced. Even treatment of wild-type mice with PTH led to a 15-fold reduction of LSC, as measured by limiting dilution analysis in secondary recipients of saline- versus PTH-treated BCR-ABL1<sup>+</sup> donor bone marrow. Upon extension of these studies to the xenotransplant setting, it was shown that treatment of NOD SCID IL2R $\gamma^{-/-}$  (NSG) mice with PTH led to significantly decreased engraftment of human BCR-ABL1<sup>+</sup> cells. It was shown that PTH-induced bone remodeling led to a release of supra-physiological levels of TGF $\beta$ 1 from the bone matrix suppressing the growth of CML cells, which express the receptor for TGF $\beta$ 1. However, the same was not applicable to AML, suggesting that the niches in these two myeloid leukemias differ significantly.<sup>67</sup> A clinical trial is being initiated in eight centers in Germany in which patients who have been receiving a tyrosine kinase inhibitor for at least six months will also receive PTH, in order to test if any remaining transcript levels of BCR-ABL1 can be rendered undetectable.

In the vascular niche in AML<sup>57</sup> and CML<sup>56</sup> preliminary data show that treatment with a small molecule inhibitor of E-selectin, which leads to an increase of HSC quiescence and self-renewal in the normal HSC niche,<sup>128</sup> may lead to reduction of LSC. In AML, a clinical trial using the pan-E-selectin inhibitor GMI-1271 has been initiated (*clinicaltrials.gov identifier 02306291*). Based on findings described above, the sympathomimetic agent mirabegron is being used in a clinical trial testing its efficacy in Jak2<sup>+</sup> myeloproliferative neoplasia (*clinicaltrials.gov identifier 02311569*) and patients with B-ALL are currently being recruited for a clinical trial testing the efficacy of the FAK inhibitor VS-4718 (*clinicaltrials.gov identifier 02215629*). The inhibition of Axl and its interaction with Gas6, secreted by stromal cells with fibroblastic/mesenchymal morphology in the BMM, and abolishment of the chemoprotective niche established by the Gas6-Axl paracrine axis in AML<sup>129</sup> or inhibition of CCL3, as mentioned above, in conjunction with chemotherapy may prove a feasible strategy for targeting the niche. The Bruton tyrosine kinase (BTK) inhibitor PCI-32765 inhibits CXCL12-mediated migration and  $\alpha$ 4 $\beta$ 1 integrin-mediated adhesion to fibronectin and VCAM-1 of lymphoma cell lines and primary CLL cells.<sup>130</sup> Other modalities of treatment resulting from mechanisms involved in the interaction of leukemia cells with their niche stated above, are inhibition of receptors for stromal-derived cytokines, such as inhibition of placental growth factor (PIGF)/VEGFR1 signaling or inhibitors of adhesive interactions of CML stem cells with stroma, including inhibition of N-cadherin-mediated activation of Wnt- $\beta$ -catenin<sup>114</sup> or  $\beta$ 1 integrin-mediated adhesion.<sup>104,108</sup> An exciting and novel idea would be to inhibit leukemia-mediated “molding” of the niche, for example in the form of inhibition of exosome-mediated changes such as neovascularization in the BMM. In this respect, carboxyamidotriazole-*orotate* may have properties targeting both the cancer cells directly, as well as the tumor microenvironment, though this needs to be tested in further studies.<sup>131</sup> Furthermore, it was recently shown that cytokines such as IL-6, secreted by CML cells, modulate normal HSPC

leading to their increased proliferation, altered differentiation ability, and reduced potential for repopulation and self-renewal. This effect, which also led to a similar gene expression pattern in normal and malignant cells, could be efficiently blocked by anti-IL6 therapy and, thereby, treated the disease.<sup>132</sup> In MPN, similarly, it has recently been established that malignant and non-malignant cells equally produce inflammatory cytokines, which signal *via* the Jak-Stat3 pathway and lead to splenomegaly and systemic symptoms; effects which are efficiently inhibited by inhibitors of the Jak kinase.<sup>133</sup> This suggests that targeting of the cytokines secreted by hematologic malignancies, which influence their microenvironment to promote the survival of malignant cells, may be another feasible strategy to improve current therapies.

Finally, progress has been made in modeling the interaction of the BMM with LSC *in vitro* to test for new inhibitory compounds. Using an *in vitro* stromal co-culture with either LSC or their normal counterparts, a sufficiently robust screening platform was generated with which over 14,000 compounds could be tested; some that would not have been found using leukemia cell lines alone.<sup>134</sup> By defining compounds capable of inhibiting LSC but not normal cells or stroma, compounds that were validated in human samples, and some that were active strictly in the presence of stroma, were defined. A clinical trial to test for at least one result is being planned. Therefore, the interplay between LSC and the BMM might be used for direct targeting or it may be used for compound discovery, with the view that cells will respond differently in the context of supportive stromal cells.

### An outlook on LSC niche biology

Targeting the LSC niche with an effort to augment existing therapies in the form of chemotherapy or therapy with tyrosine kinase inhibitors, other small molecule inhibitors or antibodies in order to, ultimately, eradicate LSC is a powerful strategy, which is still not being fully exploited. However, even our knowledge of the normal HSC niche is only recent and is in no way complete, so efforts must first be directed at understanding the pathophysiology of the LSC niche. Only then can treatment modalities modulating the LSC niche be rationally designed. While exciting progress is being made with regards to 3D and mathematical modeling, *in vivo* imaging of the LSC (and HSC) niche and *in vivo* models for hypo-thesis testing, understanding the niches is a complex process. Multiple cell types, extracellular matrices, pH, oxygenation status, cytokine networks, cell-cell interactions and mechanical forces all come into play. Understanding niche biology with human leukemia samples is difficult, as diagnoses are primarily made on bone marrow aspirates that disrupt bone marrow architecture. Therefore, xenogeneic models may ultimately prove to be extremely useful.

Importantly, however, the microenvironment is increasingly recognized to be an important component of cancer and its response to therapy. Clinicians and researchers recognize that, even with targeted leukemia therapy, we are frequently not eradicating LSC, a prerequisite for cure of our patients, and that adjuvant therapies are needed. Targeting the tumor microenvironment in leukemia may prove a useful approach to complement existing therapies, increasing our armamentarium against hematologic cancers.

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