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Novel therapeutic strategies to target leukemic cells that hijack compartmentalized continuous hematopoietic stem cell niches

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ABSTRACT

Acute myeloid leukemia and acute lymphoblastic leukemia cells hijack hematopoietic stem cell (HSC) niches in the bone marrow and become leukemic stem cells (LSCs) at the expense of normal HSCs. LSCs are quiescent and resistant to chemotherapy and can cause relapse of the disease. HSCs in niches are needed to generate blood cell precursors that are committed to unilineage differentiation and eventually production of mature blood cells, including red blood cells, megakaryocytes, myeloid cells and lymphocytes. Thus far, three types of HSC niches are recognized: endosteal, reticular and perivascular niches. However, we argue here that there is only one type of HSC niche, which consists of a periarteriolar compartment and a perisinusoidal compartment. In the periarteriolar compartment, hypoxia and low levels of reactive oxygen species preserve the HSC pool. In the perisinusoidal compartment, hypoxia in combination with higher levels of reactive oxygen species enables proliferation of progenitor cells and their mobilization into the circulation. Because HSC niches offer protection to LSCs against chemotherapy, we review novel therapeutic strategies to inhibit homing of LSCs in niches for the prevention of dedifferentiation of leukemic cells into LSCs and to stimulate migration of leukemic cells out of niches. These strategies enhance differentiation and proliferation and thus sensitize leukemic cells to chemotherapy. Finally, we list clinical trials of therapies that tackle LSCs in HSC niches to circumvent their protection against chemotherapy.

1. Introduction

Leukemias are hematologic malignancies that are characterized by an overgrowth of white blood cells and are caused by increased monoclonal cellular proliferation in the bone marrow, resulting from (epi)genetic changes in either HSCs, lymphoid or myeloid progenitor cells [1–3]. HSCs are at the top of the hematological hierarchy as multipotent stem cells with self-renewal capacity that give rise to various types of progenitor cells and ultimately the production of mature erythrocytes, megakaryocytes, myeloid cells, and lymphocytes [4–6]. Since mature blood cells are short-lived, HSCs are required throughout life to replenish progenitor and precursor cells [5,7].

A stem cell niche is a specialized microenvironment that helps to maintain stem cell characteristics. In the bone marrow, HSCs reside in

Abbreviations: AML, acute myeloid leukemia; ANG-1, angiopoietin-1; ALL, acute lymphoblastic leukemia; AKT, protein kinase B; Ara-C, cytarabine; AXL, tyrosine kinase receptor; BCL-2, B-cell lymphoma 2; BH3, BCL-2 homology 3; BMP, bone morphogenic protein; CAR cell, CXCL12-abundant reticular cell; CBP, CREB-binding protein; c-KIT, stem cell factor receptor; CLL, chronic lymphocytic lymphoma; COX4, cytochrome c oxidase subunit 4; Ctnnb1C^{Aosb}, constitutively activated β-catenin protein; CXCR4, C-X-C receptor type 4; ECM, extracellular matrix; ERK, extracellular regulated kinases; FZ, Frizzled; GAS6, growth-arrest specific gene 6; G-CSF, colony-stimulating growth factor; GSLC, glioma stem-like cell; HA, hyaluronic acid; HHIP, human hedgehog-interacting protein; HIF-1α, hypoxia-induced factor-1α; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; IHH, Indian hedgehog; LSC, leukemic stem cell; LON, ATP-dependent protease; MAPK, mitogen-activated protein kinase; MDS, myelodysplastic syndromes; MER, tyrosine-protein kinase; MPL, thrombopoietin receptor; MPN, myeloproliferative neoplasms; MSC, mesenchymal stem cell; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa beta; NSC, neural stem cell; OB, osteoblast; OC, osteoclast; OM, osteomac; OPN, osteopontin; PI3K, phosphoinositide 3-kinase; OXPHOS, oxidative phosphorylation; RAS, retrovirus-associated DNA sequences; ROS, reactive oxygen species; SDF-1α, stromal derived factor-1α; SCF, stem cell factor; STAT, signal transducers and activators of transcription; TCF, T-cell factor; TGF-β, transforming growth factor-β; TIE2, tyrosine kinase receptor; TNF-α, tumor necrosis factor-α; TPO, thrombopoietin; VCAM-1, vascular cell-adhesion molecule-1; VEGF, vascular endothelial growth factor; VLA-4, very late antigen-4; WNT, wingless-type; XIAP, X-linked inhibitor of apoptosis protein

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HSC niches, which play an important role in regulating the behavior of HSCs with respect to homeostasis and stress responses [7–10]. The proliferation and differentiation of hematopoietic progenitor cells and their daughter cells are sufficient to maintain the homeostatic hematopoiesis under normal conditions, consisting of the production of one trillion (10^{12}) cells per day in healthy human adult red bone marrow. In such circumstances, HSCs are in a dormant quiescent state to prevent stem cell exhaustion [11–13]. Blood is a tissue with one of the highest regenerative capacities and the prevention of HSC exhaustion is extremely important considering the necessity to upregulate hematopoiesis in case of blood loss due to tissue damage or hematopoietic stress [5]. In these contexts, HSCs are forced to leave the niches to differentiate and proliferate in order to maintain hematopoiesis [13–15].

In the present review, molecular mechanisms are discussed of the crosstalk between HSCs and the three types of HSC niches that are recognized until now. Next, HSC niches are described in AML and ALL when AML/ALL cells use HSC niches to become LSCs that are quiescent and resistant to therapy [16–19]. We refer to this process as hijacking of HSC niches by leukemic cells. Hijacking of HSC niches by leukemic cells and their transformation into LSCs is considered to be the most prominent cause of tumor recurrence [18–20]. We describe the molecular interactions between LSCs and the main cell types of HSC niches, growth factors, cytokines and chemokines in HSC niches that facilitate adhesion, survival and quiescence of LSCs in HSC niches, ultimately resulting in therapy-resistance of LSCs [19]. Finally, therapeutic targeting of LSCs in HSC niches is discussed as a promising approach to treat AML and ALL more effectively.

2. HSC niches

HSCs are currently considered to reside in one of three types of HSC niches: endosteal, reticular or perivascular niches [21,22]. Some cell types, proteins and factors are shared between the three types of niches, others are considered to be unique for a specific niche type. A common factor of all types of niches is that they tightly regulate whether HSCs migrate into niches, are kept inside the niches or migrate out of the niches. This is crucial, because HSC stemness and quiescence are promoted in the niches, whereas migration out of the niches enables HSC differentiation and proliferation. The cell types, proteins and factors that are present in the two compartments are listed in Table 1.

2.1. The endosteal niche

The endosteum is the interface between bone and bone marrow which mainly consists of osteoblasts and, to a lesser extent, osteoclasts. Mature osteoblasts produce ECM and are responsible for bone formation whereas osteoclasts resorb bone and thus function in bone remodeling [4,21]. The endosteal niche is associated with the endosteum (Fig. 1A) and facilitates interactions between osteoblasts and HSCs, which keeps HSCs quiescent [19]. The main cell types of the endosteal niche that maintain stemness and quiescence of HSCs and affect their homing and mobilization are osteoblasts, osteoclasts [8] and osteomacs [4,23]. The functions of these 3 main cell types and the molecular mechanisms by which they maintain HSCs in the endosteal niche, will be discussed in the following Sections (2.1.1–2.1.3).

2.1.1. Osteoblasts

2.1.1.1. Osteoblast-promoted retention of HSCs in endosteal niches. Several interactions and intermediate molecules between osteoblasts and HSCs have been described for the endosteal niche. OPN is a matrix glycoprotein with cytokine and chemokine properties which is secreted by osteoblasts and binds to HSCs via CD44 or integrins containing a β1 subunit. This results in homing of HSCs in the endosteal niche and downregulation of HSC proliferation [7,24–26]. CD44 can also interact with the ECM component (HA), which results in homing of HSCs into HSC niches. HA is produced in the endosteum by stromal cells and hematopoietic cells under hypoxic conditions due to HIF-1α activity [27]. Furthermore, the chemoattractant SDF-1α (CXCL12) is produced by osteoblasts under hypoxic conditions and interacts with its receptor, CXCR4, which is expressed on HSCs, resulting in the retention of HSCs in the endosteal niche [7,25,26,28,29].

2.1.1.2. Osteoblast-promoted self-renewal of HSCs. HSCs express the receptor MPL which binds TPO after secretion by osteoblasts. Interactions between TPO and MPL result in homodimerization of MPL receptors that activate both Janus kinase 2 (JAK2) signal transduction and the signal transducers and activators of transcription (STAT) pathway, which in turn activate RAS, PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways, which ultimately results in HSC self-renewal and survival [30]. Binding of TPO to MPL also upregulates HIF-1α expression and stability and may thus function in hypoxia [31]. The Notch signaling pathway also plays an important role in maintaining the HSC phenotype. Binding of osteoblastic factor Jagged-1 to its Notch-1 receptor on HSCs causes transcription of genes involved in inhibition of differentiation and an increase in self-renewal capacity of HSCs in endosteal niches. This is achieved by proteolytic cleavage of the intracellular part of Notch-1, its translocation to the nucleus and binding to cofactor recombining binding protein suppresser of hairless (RBPJ/CBF1) and co-activator Mastermind [32,33]. Osteoblasts also secrete transforming growth factor-β (TGF-β), bone morphogenic protein (BMP)-2 and BMP-7, which bind to type I and type II serine/threonine kinase receptors on HSCs. This results in SMAD translocation to the nucleus and transcription of target genes, which results in quiescence of HSCs and maintenance of the HSC phenotype [34–36]. TGF-β also activates the PI3K pathway [36] as well as the MAPK, ERK and JUN N-terminal kinase pathways via JUN and SMADs in the nucleus, resulting in transcription of target genes and subsequently HSC self-renewal and survival [37].

2.1.1.3. Osteoblasts and WNT signaling in HSCs. Osteoblasts affect both the canonical and non-canonical wingless-type (WNT) signaling pathways in HSCs that have opposite effects on HSC differentiation. The canonical WNT signaling pathway stabilizes β-catenin after binding to its receptors Frizzled (FZ) and lipoprotein receptor-related protein 5/6 (LRP5/6). Stabilized β-catenin translocates to the nucleus, where it interacts with transcription factors that promote HSC differentiation [38–41]. In the non-canonical pathways, Wnt-Ca^{2+} and Wnt-Jun N-terminal kinase pathways, β-catenin is not stabilized whereas Wnt-FZ interactions induce an increase in intracellular Ca^{2+} levels through inositol-3-phosphate or induce the Jun N-terminal kinase pathway through Rho/Rac GTPases. Non-canonical signals can then affect actin-dependent cytoskeletal reorganization and maintain the stemness of HSCs [38–42].

2.1.1.4. Effects of osteoblasts in hypoxia. Under hypoxic conditions, HSCs express the tyrosine kinase receptor TIE2 which bind to its ligand ANG-1, which are both produced by osteoblasts under the influence of HIF-1α [7,26,43]. Binding of ANG-1 to TIE2 on HSCs results in phosphorylation of TIE2 and activation of the PI3K/protein kinase B (AKT) pathway. The downstream effects are activation of p21 and nuclear factor kappa beta (NF-κB), resulting in downregulation of HSC proliferation and HSC survival, respectively [44–46]. Osteoblasts also secrete the growth factor SCF which binds to the tyrosine kinase receptor c-KIT on HSCs which downregulates HSC proliferation [21,47]. HIF-1α directly enhances the transcriptional activity of SCF [48]. SCF can also induce HIF-1α expression in HSCs which is mediated by PI3K and retrovirus-associated DNA sequences (RAS)/extracellular regulated kinases (ERK) pathways, but it is unclear whether or not SCF also plays a direct or indirect role in the stabilization of HIF-1α in hypoxic conditions [49]. The effects of hypoxia are described in more detail in Section 2.5.

Table 1
Cell types and proteins in periarteriolar and perisinusoidal compartments of HSC niches that are involved in binding of HSCs and LSCs in HSC niches and release from the niches. Abbreviations: ANG-1a, angiopoitin-1; BMP, bone morphogenic protein; CAR cell, CXCL12-abundant reticular cell; c-KIT, Mast/stem cell growth factor receptor; CXCR4, C-X-C receptor type 4; ECM, extracellular matrix; G-CSF, granulocyte colony-stimulating growth factor; HSC, hematopoietic stem cell; MPL, thrombopoietin receptor; MSC, mesenchymal stem cell; OPN, osteopontin; SCF, stem cell factor; SDF-1α, stromal derived factor-1; TGF-β, transforming growth factor-β; TIE2, receptor tyrosine kinase; TPO, thrombopoietin; VCAM-1, vascular endothelial cell-adhesion molecule; VLA-4, very late antigen-4; WNT, wingless type.

Cell types and proteins	Function
Periarteriolar compartment of HSC niches	
Osteoblasts	Synthesis of ECM including bone [4,21]
Osteoclasts	Bone resorption [4,7,21,51]
Osteomacs	Macrophages localized near osteoblasts and osteoclasts, mediate optimal mineralization and secrete SDF-1α, ANG-1 and c-KIT [4,23]
CAR cells	Secretion of high levels of SDF-1α and SCF and maintenance of HSC phenotype [21,25,26,59,60]
MSCs	HSC mobilization and maintain HSC phenotype by expressing SDF-1α, SCF, VCAM-1, OPN c-KIT, ANG-1 and interleukin 7 [21,26,70]
N-cadherin	Adhesion of HSCs to osteoblasts and maintenance of HSC phenotype [8,21]
CXCR4 and SDF-1α	Retention and maintenance of $CXCR4^+$ HSCs in the niche [7,24–26,28]
VCAM-1 and VLA-4	Maintenance of HSC phenotype via interaction with integrin VLA-4 [66]
SCF and c-KIT	Maintenance of HSC phenotype by binding to receptor c-KIT on the HSC membrane [21,47]
OPN and CD44 or β1 integrins	Anchorage of HSCs to the niche by binding CD44 or β1 integrins and maintenance of HSC phenotype [7,24–26]
TIE2 and ANG-1	HSC retention and maintenance of HSC phenotype [44–46]
MPL and TPO	HSC retention and maintenance of HSC phenotype [4,30]
CatK	Degradation of SDF-1α, migration of HSCs out of the niche [13,15,28]
G-CSF	Induction of migration of HSCs out of the niche by reducing SDF-1α levels and downregulating CXCR4 on HSCs [54,58]
TGF-β	Maintenance of HSC phenotype [34–37]
BMP2 and BMP7	Maintenance of HSC phenotype [34–36]
Notch pathway	Maintenance of HSC phenotype and inhibition of differentiation [32,33]
WNT pathway	Maintenance of HSC phenotype and inhibition of differentiation [38,39,42]
Perisinusoidal compartment of HSC niches	
Endothelial cells	Production of SDF-1α and SCF for homing and maintenance of HSC phenotype [64]
MSCs	HSC mobilization and maintenance of HSC phenotype by expressing SDF-1α, SCF, VCAM-1, OPN, c-KIT, ANG-1 and interleukin 7 [21,26,70]
CAR cells	Secretion of high levels of SDF-1α and SCF and maintenance of HSC phenotype [21,25,26,59,60]
SDF-1α and CXCR4	Retention and maintenance of $CXCR4^+$ HSCs in the niche [7,24–26,28]
VCAM-1 and VLA-4	Retention of HSCs in the niche via interaction with integrin VLA-4 [66]
SCF and c-KIT	Maintenance of HSC phenotype by binding to receptor c-KIT on the membrane of HSCs [21,47]
OPN and CD44 or β1 integrins	Anchorage of HSCs to the niche by binding CD44 or β1 integrins and maintenance of HSC phenotype [7,24–26]
TIE2 and ANG-1	Maintenance of HSC phenotype via interaction with receptor TIE2 [44–46]
Interleukin-7	Maintenance of HSCs [21,26,70]

2.1.1.5. Direct contacts between osteoblasts and HSCs. Osteoblasts that are spindle-shaped and N-cadherin-positive have contacts with N-cadherin-positive HSCs. These calcium-dependent intercellular adhesions between osteoblasts and HSCs induce quiescence and dedifferentiation of HSCs but the underlying mechanisms are not fully understood [8,21]. However, recent imaging studies that applied fluorescence microscopy failed to demonstrate any direct contacts between osteoblasts and HSCs. Therefore, it is doubtful whether these N-cadherin-mediated interactions exist [45,50].

2.1.2. Osteoclasts

Osteoclasts play a complex role in endosteal niches. On the one hand, release of calcium during bone resorption by osteoclasts attracts and retains HSCs in endosteal niches, because HSCs possess calcium-sensing receptors [7,51]. Signaling through calcium-sensing receptors on HSCs involves G-protein coupled receptor subunits $G_{i\alpha}$ and $G_{q\alpha}$ with subsequent inhibition of cyclic AMP generation and activation of the MAPK pathway and phospholipase C, respectively. It ultimately results in retention of HSCs in the endosteal niche [52]. Besides, osteoclasts release growth factors such as TGF-β in a similar fashion as osteoblasts and contribute to the regulation of HSC quiescence and the maintenance of the stem cell pool [34–36].

On the other hand, osteoclasts release proteases, such as cathepsin K and matrix metalloproteinase 9, in stress situations such as bleeding and surgery [28]. Proteases released by osteoclasts during stress can degrade SDF-1α and OPN and downregulate the retention signal of HSCs to the niche and thus cause HSC migration out of HSC niches [13,15,28]. Furthermore, SDF-1α levels in niches can be reduced by G-CSF (see below), which also downregulates expression of vascular cell-adhesion molecule-1 (VCAM-1), SCF, ANG-1, and SDF-1α by nestin-positive mesenchymal stem cells (MSCs), which also induces migration of HSCs out of niches [26,50,53]. Nestin-positive MSCs are present in low abundancy in the endosteal niche but play a significant role in HSC mobilization [26,54,55].

2.1.3. Osteomacs

Osteomacs are macrophages that are intercalated throughout the endosteum and are localized in close proximity of endosteal osteoblasts and osteoclasts. Osteomacs are crucial for osteoblast function, since loss of osteomacs results in depletion of osteoblasts and abrogation of bone mineralization by osteoblasts [4,23]. The interaction mechanisms between osteomacs and osteoblasts are unknown [23], but the interaction between osteomacs and osteoclasts is understood better. Granulocyte colony-stimulating growth factor (G-CSF) is secreted by osteoclasts and binds to its receptor on osteomacs [23]. This depletes osteomacs and ultimately results in depletion of osteoblasts and reduced levels of osteoblast-secreted cytokines that are required for HSC retention in endosteal niches, such as SDF-1α, ANG-1 and c-KIT [23,56]. This results in migration of HSCs out of endosteal niches. G-CSF synthesis is regulated by adrenergic neurons [54] by the transcription factor growth factor independence-1 [57] and through control of mRNA stability [58]. G-CSF mRNA in osteomacs is stabilized in response to interleukin 1, tumor necrosis factor-α (TNF-α) or lipopolysaccharides. Conversely, stabilization of G-CSF mRNA is reversed by interleukin 10 [58].

2.2. The reticular niche

The name of reticular niches is derived from reticular cells, which are stromal cells that produce large amounts of SDF-1α (CXCL12) and are therefore called CXCL12-abundant reticular (CAR) cells [21,25,26,59,60]. The regulatory mechanisms with respect to CAR cell function and their SDF-1α production are currently unclear. CAR cells are scattered throughout the bone marrow and have long processes that create a network [21,61] (Fig. 1B). In fact, immunohistochemical

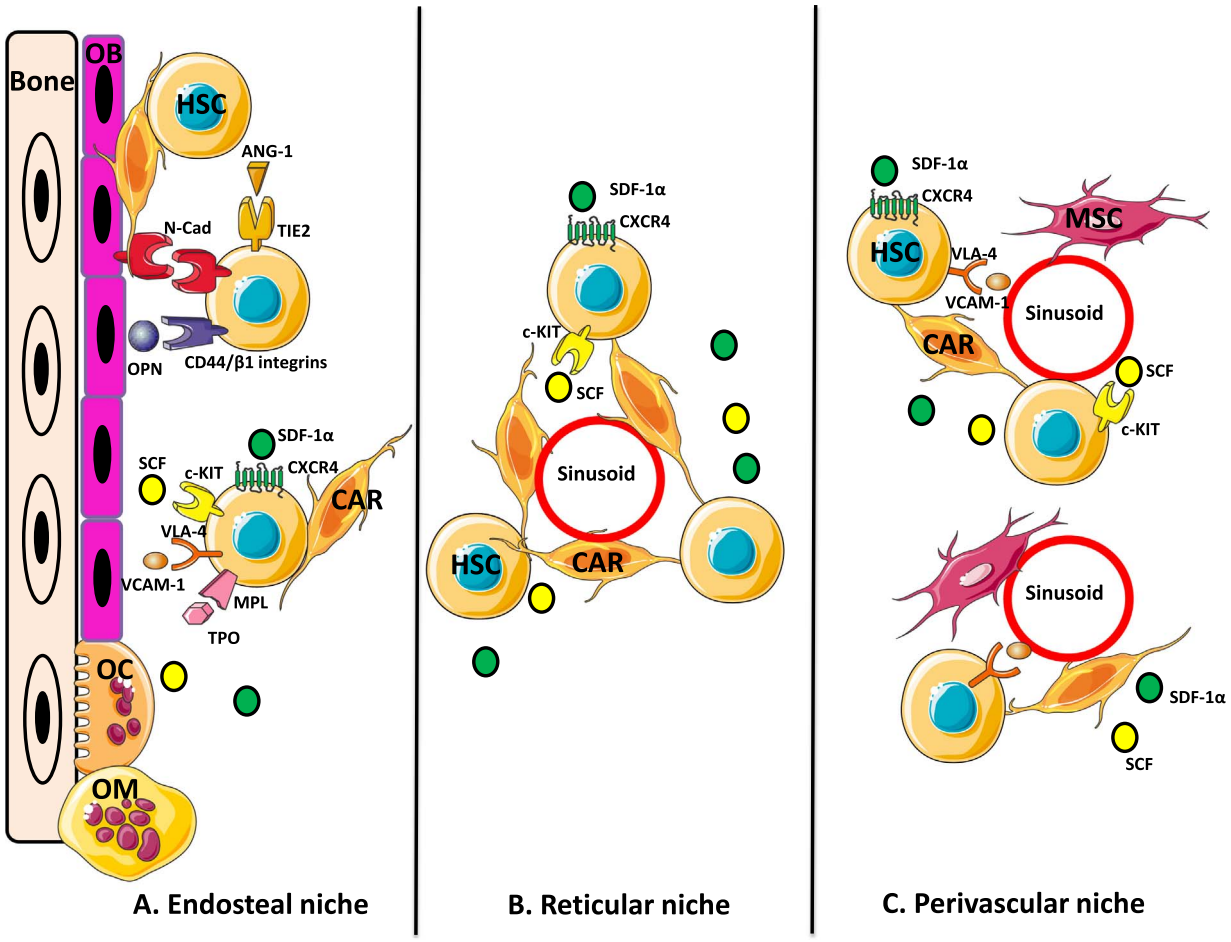

Fig. 1. Three types of HSC niches. A. The most essential cell types in the endosteal niche are OBs, OCs, OMs and CAR cells. These cell types facilitate adhesion of HSCs to the endosteal niche. The following interactions have been reported to be involved: N-CAD, SDF-1α/CXCR4, OPN/CD44/β1 integrins, ANG-1/TIE2, SCF/c-KIT, VCAM-1/VLA-4 and TPO/MPL. B. CAR cells around sinusoids in the reticular niche produce large amounts of SDF-1α and SCF, which maintain the HSC phenotype. C. HSCs are maintained in the perivascular niche by the SDF-1α/CXCR4, SCF/c-KIT and VCAM-1/VLA-4 axes. HSC maintenance factors are produced by nestin-positive MSCs. Abbreviations: ANG-1, angiopoietin-1; c-KIT, stem cell factor receptor; CAR cell, CXCL12-abundant reticular cell; CXCR4, C-X-C receptor type 4; HSC, hematopoietic stem cell; N-CAD, N-cadherin; MPL, TPO receptor; OB, osteoblast; OC, osteoclast; OM, osteomac; OPN, osteopontin; MSC, mesenchymal stem cell; SCF, stem cell factor; SDF-1α, stromal derived factor-1α; TIE2, tyrosine kinase receptor; TPO, thrombopoietin; VCAM-1, vascular cell-adhesion molecule-1; VLA-4, very late antigen-4.

analysis has shown that 97% of HSCs in perivascular niches (see Section 2.3) and 100% of HSCs in endosteal niches are in contact with processes of CAR cells [21,61]. CAR cells are located around endothelium of sinusoids and do not express the pan-endothelial marker platelet/endothelial cell-adhesion molecule (PECAM)/CD31 or α-smooth muscle actin, indicating that these cells are different from endothelial cells, pericytes and smooth muscle cells [21,60]. CAR cells also secrete large amounts of SCF and are crucial to keep HSCs in an undifferentiated state [62]. When SDF-1α and SCF production by CAR cells is down-regulated, which occurs during hematopoietic stress, HSCs migrate out of the niche [21,61,63].

2.3. The perivascular niche

HSCs and endothelial cells are derived from the same embryonic progenitor cells, called hemangioblasts. Vasculogenesis and hematopoiesis occur concurrently during embryonal development. HSCs are attached to the endothelium of sinusoids in the central part of perivascular HSC niches (Fig. 1C) [64,65]. Endothelial cells express VCAM-1, which interacts with the integrin very late antigen-4 (VLA-4) on the membrane of HSCs, and this ligand-receptor interaction facilitates retention of HSCs in perivascular niches. The interaction between VCAM-1 and VLA-4 results in phosphorylation of focal adhesion kinase pp12SFAK in HSCs, facilitating retention of HSCs in perivascular niches [66]. VCAM-1 expression is regulated by TNF-α produced by immune cells in the bone marrow. TNF-α triggers intracellular activation of MAPK and NF-κB in HSCs, which downregulates the transcription of various genes, such as that of VCAM-1, due to the combined effects of the transcription factors activating protein-1, GATA and NF-κB [67]. VCAM-1 downregulation results in mobilization of HSCs out of the niche via VCAM-1-VLA-4 signaling (see Section 2.1.1) [68]. Endothelial cells and nestin-positive MSCs produce SDF-1α and SCF [25,69,70] that maintain HSCs in their undifferentiated state [45,71]. Nestin-positive MSCs express HSC maintenance factors, such as not only SDF-1α and SCF, but also c-KIT, ANG-1, interleukin-7, VCAM-1 and OPN [21,26,70]. On the other hand, nestin-positive MSCs in perivascular niches are associated with adrenergic neurons of the sympathetic nervous system. Noradrenalin signaling downregulates MSC activity, thus leading to HSC mobilization by downregulation of SDF-1α levels in the niche and CXCR4 expression on HSCs [26,54,72].

2.4. The vasculature of red bone marrow in relation to hypoxia

The vasculature of red bone marrow is complex and relatively poorly investigated, particularly in humans. It differs from the microvasculature of yellow bone marrow which is undoubtedly related with its function. The microvasculature is the bridge between the arterial system that supplies blood and the venous system that drains the blood

from the bone marrow cavity [73]. Yellow bone marrow contains mainly adipocytes and its microvasculature consists of a well-developed capillary system [74,75]. Red bone marrow contains hematopoietic cells and is found in the skull, clavicles, vertebrae, ribs, sternum, pelvis and at the ends of long bones [73]. The microvasculature consists of sinusoids [74,75]. In long bones, one or two nutrient arteries perforate the bone shaft cortex roughly at mid-shaft level [76] and split off into ascending and descending arterial branches that run longitudinally close to the endosteum [73,74]. From these, small radial arteries branch off regularly and end up as arterioles near the endosteum. These arterioles become sinusoids in red bone marrow [77] or capillaries in yellow bone marrow [50] that run towards the centre of the bone marrow cavity where they join the central longitudinal vein [50], or nutrient vein, which leaves the bone marrow cavity through the same nutrient foramen where the nutrient artery entered [74,76].

Since Schofield formulated the HSC niche hypothesis in 1978 [9], an avalanche of HSC niche studies has been published in which the vasculature of red bone marrow in combination with hypoxia generally played an important role. However, the exact anatomy of the vasculature was never taken into consideration. Because most studies on HSC niches have been performed in mice, the relationship between HSC niches and the vasculature of bone marrow remains largely unknown, particularly in humans. This relationship requires thorough investigations for the exact understanding of the functioning of HSC niches in health and disease, and, in particular, in relation to hypoxia.

In the diaphysis of mouse long bone, almost all arteriolar blood vessels are present within 40 μm of the endosteum, whereas sinusoids were mainly found at larger distances from the endosteum [78,79]. The arterial blood vessels comprise a volumetric fraction of 1.2% of the bone marrow whereas the sinusoidal vessels occupy 30% of the bone marrow volume, excluding the central vein [80]. The marrow cavity of sternum contains trabecular bone. Arterioles were not specifically found adjacent to bone but a statistically significant spatial association between HSCs and arterioles was observed, whereas a significant association between HSCs and sinusoids was not found [77,81]. Nombela-Arrieta et al. [77] found that HSCs are mainly localized in close vicinity of the endosteum. We investigated the bone marrow vasculature in human and murine bone marrow samples and our histological analysis shows that arterioles are localized adjacent to trabecular bone, whereas sinusoids are present at a larger distance from trabecular bone in human (Fig. 2A) and murine (Fig. 2B) bone marrow, in agreement with earlier studies [77].

In conclusion, arterial blood vessels in the bone marrow cavity are predominantly localized in close vicinity of endosteum of the bone shaft of long bones and trabeculae of trabecular bone. The arteriolar blood vessels become wide sinusoidal vessels that run towards a vein in which they drain. Therefore, we conclude that endosteal niches are located in close vicinity of arterioles adjacent to the endosteum whereas perivascular niches are located in close vicinity of sinusoids at larger distances from the endosteum and reticular niches do not have a specific localization in the red bone marrow.

2.5. Hypoxia in HSC niches

Hypoxia is an important regulator of HSC stemness and this is mainly orchestrated by HIF-1α [82]. Under hypoxic conditions, HIF-1α is stabilized and enters the nucleus to complex with the β-subunit which is constitutively expressed and induces transcription of target genes [83]. HIF-1α is a major regulator of HSC quiescence, because defective HIF-1α or overstabilization of HIF-1α both result in deregulation of HSC quiescence [82,84]. HIF-1α upregulates the expression of multiple factors that facilitate binding of HSCs in the niches, such as stromal derived factor-1α (SDF-1α), C-X-C receptor type 4 (CXCR4), stem cell factor (SCF), osteopontin (OPN), thrombopoietin (TPO), TPO receptor MPL, angiopoietin-1 (ANG-1) and tyrosine kinase receptor TIE2. Most healthy normal tissues are not generally believed to contain hypoxic areas [85]. Recently, light has been cast on the issue whether HSC niches are hypoxic and whether there is a difference between the oxygen concentration in endosteal niches and in perivascular niches. The situation seems to be more complex than generally assumed. First of all, the issue of the seemingly contradictory situation of hypoxia around arteriolar vessels has to be addressed as arterioles carry oxygen-rich blood. This phenomenon was recently explained in a study that investigated HSC niches [79] and in a study on glioma stem-like cell niches around arterioles in glioblastoma [86]. The former authors mention that “less permeable arteriolar blood vessels” may contribute to this phenomenon and this is supported by the latter authors. Hira et al. [86] elaborate that arterial blood vessels as well as larger venous vessels are transport vessels and not exchange vessels, whereas capillaries, sinusoids and the smallest venules are the exchange vessels where oxygen, carbon dioxide, nutrients, waste products, cells, signalling molecules are exchanged between the blood in the vessels and the surrounding extracellular interstitial fluid [87]. Because of the lack of oxygen supply to tissues around arterial blood vessels from their lumen, it is perfectly well possible that compartments of HSC niches around arterial blood vessels are hypoxic [86].

Second, direct measurement of local oxygen levels in red bone marrow revealed a surprising situation [78]. Oxygen levels (in mm Hg) were 23 in the lumen of arterioles, 20 in the lumen of sinusoids close to endosteum and 18 in the lumen of sinusoids away from the endosteum. The oxygen level was 14 in the tissue around arterioles and 10 around sinusoids. Thus surprisingly, hypoxia seems to be less severe near the endosteum than away from the endosteum. In comparison, periosteum on the outside of the shaft of long bones and cortical bone showed intravascular oxygen levels of 50 and 30, respectively [78].

There are indications that maintenance of HSCs in endosteal niches is not only mediated by low oxygen levels, but also by specific cellular metabolic mechanisms, such as low oxygen consumption and/or limited mitochondrial activity in HSCs [79,88]. For example, high intracellular levels of ROS are caused by mitochondrial activity and they are major inducers of differentiation, proliferation, migration and depletion of the pool of quiescent HSCs [89]. Cells with high intracellular ROS levels are relatively rare around arteriolar blood vessels but they are abundant around sinusoids [79]. Furthermore, high levels of HIF-1α are positively associated with anabolic glycolysis and negatively associated with mitochondrial respiration [90]. Therefore, HSCs are best described as cells low in oxidative phosphorylation [79,91]. These findings stress that analysis of metabolic profiles of individual HSCs in niches is an interesting next step in the elucidation of the functioning of HSC niches in relation to quiescence of HSCs.

The endosteal niches likely protect HSCs against ROS. Stress or tissue injury inhibits this protection and ROS activate the P38-MAPK pathway, which reduces cell adhesion and increases cell-cycle progression [92–94]. Perivascular niches with higher ROS levels promote proliferation of HSCs to generate progenitor cells that can migrate out of HSC niches into the circulation. This concept is supported by the finding that hypoxia limits ROS production and maintains the stemness of HSCs [88,95]. Conversely, HSCs that were exposed to elevated oxygen levels produced more ROS and lost their phenotype [95].

Collectively, these data support the hypothesis that the low oxygen levels in endosteal niches protect HSCs against ROS and create a pool of quiescent HSCs as a reserve. The perivascular niches with higher ROS levels are the environments where HSCs can differentiate into progenitor cells for hematopoiesis.

2.6. The compartmentalized continuous HSC niche

On the basis of recent studies on HSC niches and of our analysis of the vasculature of human bone marrow, we rebut the current concept that there are three separate types of HSC niches in human red bone marrow [21,22].

First of all, the finding that almost all HSCs are in contact with

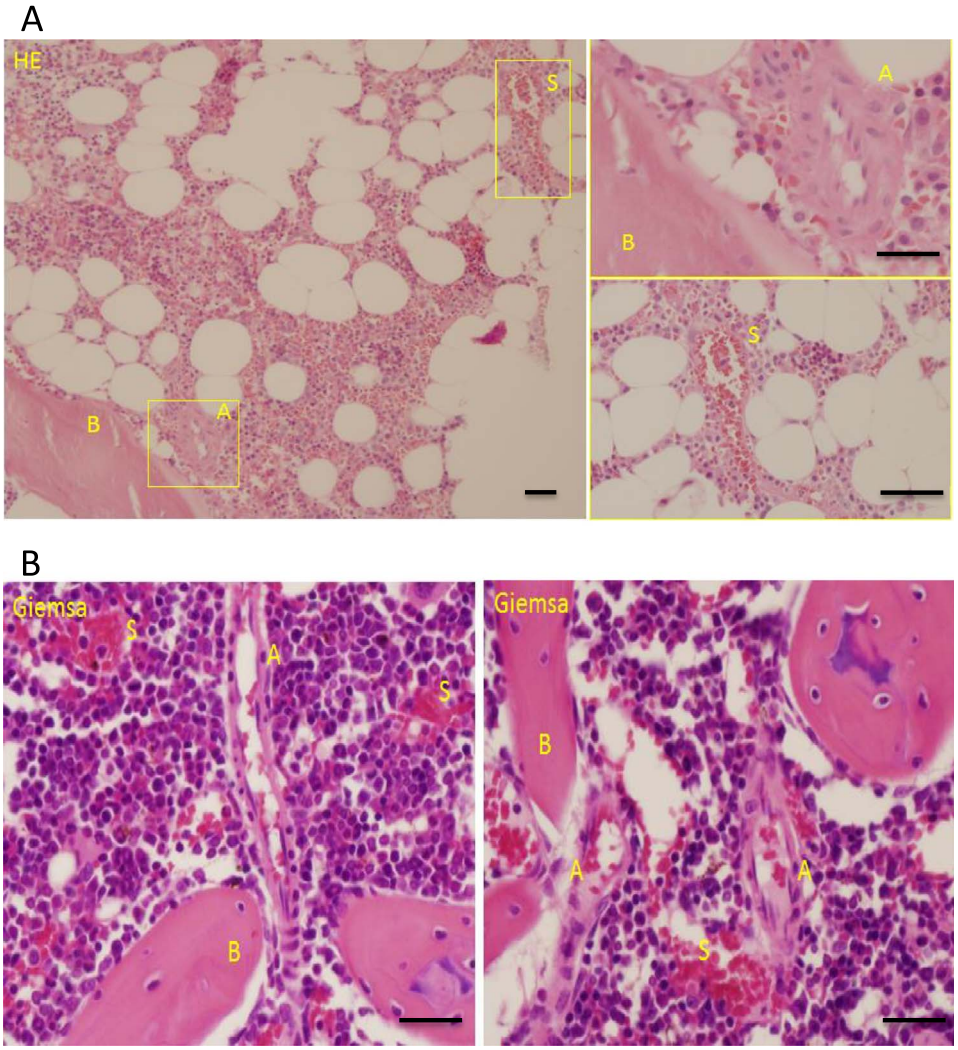

Fig. 2. Vasculature of human and murine bone marrow. Hematoxylin and eosin staining of human trabecular bone (A) and Giemsa staining of murine trabecular bone (B) demonstrate arterioles (indicated in yellow with A) adjacent to bone (indicated in yellow with B) and sinusoids (indicated in yellow with S) at larger distance from bone. Scale bars, 50 μm.

processes of CAR cells throughout the bone marrow [21,61] raises the question whether the reticular niche is actually a separate niche, or rather a crucial compartment of the endosteal niche and the perivascular niche. If the reticular niche would indeed be a separate niche, not all HSCs in the endosteal niche and perivascular niche would be in contact with CAR cells, but only a subset of the HSCs at most. Therefore, we propose that separate reticular niches do not exist and that the cells that make up the reticular niche have no or limited stand-alone function and mostly function in cooperation with the cells that make up the endosteal niche or the perivascular niche.

Secondly, studies of the bone marrow vasculature revealed that arterioles are mainly present near the endosteum and trabecular bone, whereas sinusoids are mainly present at distance of the endosteum and trabecular bone. HSC niches are closely associated with the arterioles near the endosteum [77,80,81]. These niches are hypoxic and the intracellular ROS levels in HSCs in niches close to the endosteum are low, whereas further away from the endosteum around sinusoids intracellular ROS levels are higher [77,94,96–98].

These phenomena enable the formulation of a novel concept of a single hypoxic HSC niche that comprises two compartments that are continuous, the periarteriolar compartment with osteoblasts, osteoclasts, macrophages, reticular cells, MSCs and low intracellular ROS levels which maintains the HSC pool, and the perisinusoidal compartment with the endothelial cells of sinusoids, reticular cells and MSCs and elevated ROS levels enabling differentiation of HSCs into progenitor cells that proliferate, further differentiate and migrate out of HSC niches. CAR cells are ubiquitously present in both parts of this single HSC niche (Fig. 3). Table 1 summarizes the functional elements in the periarteriolar and perisinusoidal compartments and shows that there are striking similarities in functional elements in both compartments, which strengthens our hypothesis that there is only one type of HSC niche consisting of 2 continuous compartments.

In AML and ALL, leukemic cells home into both compartments of HSC niches to become LSCs using the same molecular mechanisms as HSCs. This opens therapeutic venues to expel LSCs out of the niches, which will be discussed in the next chapters.

3. Leukemic cells hijack HSC niches

Leukemias are hematologic malignancies that are classified as either lymphoid or myeloid and can be chronic or acute [99]. This review is focused on the acute leukemia types AML and ALL, because AML and ALL cells have been shown to be present in HSC niches as LSCs.

AML is a heterogeneous disease that is characterized by expansion of HSCs and progenitor cells with subclonal genotypes [1–3,70,100]. The subclonal evolution results in refractory clones that are able to resist chemotherapy, survive and repopulate. Although various types of chemotherapy are available for treatment of AML, therapy resistance is a major problem. Complete remission is achieved in 70–80% of patients younger than 60 years, but ultimately relapse occurs in the majority of

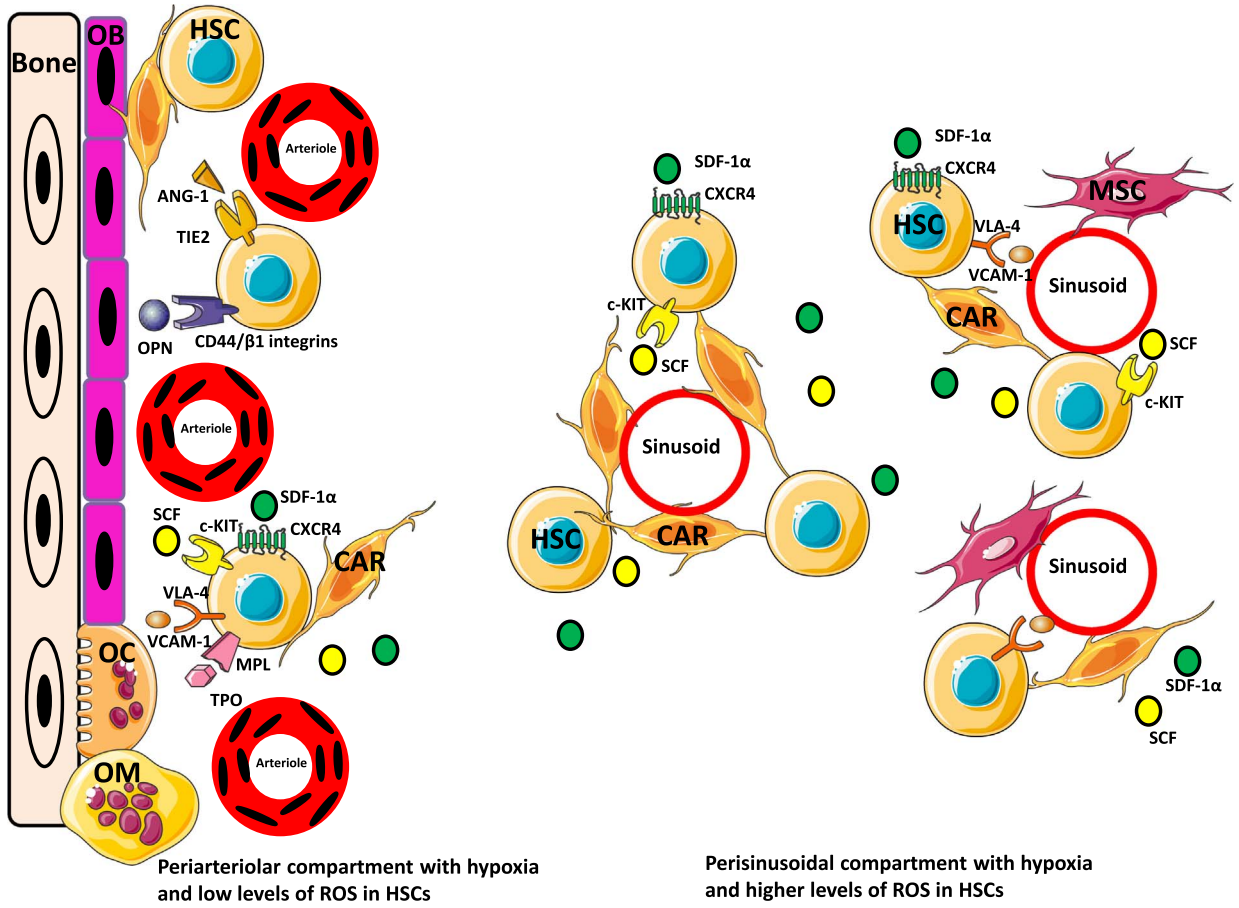

Fig. 3. Compartmentalized continuous HSC niches. Our novel concept of the HSC niche is that there is one hypoxic continuous HSC niche with two compartments: periarteriolar compartments adjacent to bone with low intracellular ROS levels in HSCs and perisinusoidal compartments at a larger distance from bone with higher ROS levels. Low ROS levels in HSCs in periarteriolar compartments maintain the stemness of HSCs, whereas the higher intracellular ROS levels in HSCs in perisinusoidal compartments enable differentiation of HSCs into progenitor cells and their migration out of the HSC niche into the peripheral blood. Abbreviations: HSC, hematopoietic stem cell; ROS, reactive oxygen species.

patients and the overall 5-year survival is 40–45%. The prognosis of patients who are 60 years or older is worse with a median survival of less than one year [101]. ALL is characterized by monoclonal and/or oligoclonal proliferation of lymphoid progenitor cells [102] and despite intensive chemotherapy regimens, relapse occurs in the majority of adult patients and the overall 5-year survival after relapse is only 7%, even after HSC transplantation, which emphasizes the need for novel therapies [103].

The rationale to explore the manipulative actions of AML cells and ALL cells in normal HSC niches is to develop novel approaches and strategies to improve current therapies. Because chemotherapy is not specific for leukemic cells but for rapidly-dividing cells, LSCs can evade the effects of chemotherapy as they are quiescent and protected in HSC niches [2,10,104]. Accordingly, clinical trials are performed in leukemia patients with agents that disrupt interactions between LSCs and HSC niches to increase their sensitivity to chemotherapy (Table 2). Of note, the neoplastic cells that initiate and drive leukemogenesis and leukemic recurrences may be not literally be LSCs, but rather HSCs that are pre-leukemic [2]. However, this distinction is not clear yet [3,100] and also not relevant for the present review, because the existence of a leukemogenic population of cells that provides a disproportional contribution to leukemia recurrences is undisputed like the relevance of targeting this cell population in effective therapies against leukemia. We refer to this cell population as LSCs throughout this review while we do not negate the fact that the discussion of the origins of this cell population has not been settled. To the best of our knowledge, it has not been shown that the specific origin of this cell population (*e.g.* HSC or LSC) has implications for how critical it is to therapeutically target this cell population and the interactions between this cell population and HSC niches. This renders our review future proof in the context of the aforementioned debate, regardless of its outcome.

3.1. Therapeutic targeting of AML cells hijacking HSC niches

AML cells hijack HSC niches which leads to the acquisition of stem cell characteristics, such as quiescence and resistance to chemotherapy [70,105]. After this transformation of AML cells into LSCs, HSC niches protect LSCs from cell-cycle targeting chemotherapeutic agents, such as Ara-C, azacitidine and decitabine [70]. In the next sections, we describe therapies in clinical trials based on disruption of interactions between LSCs and HSC niches.

3.1.1. Therapies that target the transformation of AML cells into LSCs

3.1.1.1. Angiopoietin-1/TIE2 interactions. AML cell adhesion to the microenvironment of niches is a crucial component of the hijacking process. The TIE2 receptor is almost always overexpressed in AML cells, which facilitates adhesion to HSC niches via interactions with ANG-1 [106,107]. Similarly to HSCs (see Section 2.1.1.4) ANG-1/TIE2 interactions maintain the LSCs in a quiescent and anti-apoptotic state in HSC niches [108] and prevention of these interactions increases the proliferation rate of AML cells [109]. A phase II monotherapy study with trebananib (AMG 386), a peptide that disrupts ANG-1/TIE2 interactions, showed encouraging results [109]. Presently, a phase I clinical trial of a combinational therapy with AMG 386 and Ara-C chemotherapy is in progress (Table 2).

Table 2
An overview of clinical trials of compounds that target LCSs in HSC niches in AML and ALL. Abbreviations: AXL, tyrosine protein-kinase receptor; BH3, BCL-2 homology 3; CBP, competitive binding protein; CXCR4, C-X-C receptor type 4; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; TIE2, receptor tyrosine kinase; VLA-4, very late antigen-4; XIAP, x-lined inhibitor of apoptosis protein.

Phase of the clinical trial	Molecular target	Compound	ClinicalTrials.gov identifier	Monotherapy or combinational therapy
Phase II	PI3K/mTOR pathway	PF-05212384 (PKI-587)	NCT02438761	Monotherapy
Phase I/II	XIAP	AEG35156	NCT02438761	Monotherapy
Phase I	Hypoxia (hypoxia-activated prodrug)	TH-302	NCT01149915	Monotherapy
Phase I	TIE2	Trebananib (AMG 386)	NCT01555268	In combination with Ara-C
Phase I	CXCR4	Plerixafor/(AMD3100)	NCT01120457	Monotherapy
Phase I/II	CXCR4	Plerixafor/(AMD3100)	NCT01236144 (Uy et al. [110])	In combination with MEC
Phase II	VLA-4	AS101	NCT01010373	Monotherapy
Phase I	BH3	ABT-199	(Souers et al. [147])	Monotherapy
Phase I/II	Notch signalling	LY3039478	NCT02518113	Monotherapy
Phase I	AXL	BGB324	NCT02488408	Monotherapy
Phase I	Hedgehog pathway	PF-04449913	NCT00953758	Monotherapy
Phase I	Hedgehog pathway	PF-04449913	NCT02038777	Monotherapy
Phase I/II	Hedgehog pathway	PF-04449913	NCT01546038	Monotherapy
Phase I	Hedgehog pathway	PF-04449913	NCT01841333	Monotherapy
Phase II	Hedgehog pathway	PF-04449913	NCT01842646	Monotherapy
Phase I/II	CBP/β-catenin	PRI-724	NCT01606579	Monotherapy
Phase II	CBP/β-catenin	PRI-724	NCT01302405	Monotherapy

3.1.1.2. Stromal derived factor-1α/C-X-C receptor type 4. Adhesion of AML cells in HSC niches is also dependent on expression of the receptors CXCR4, VLA-4 and CD44 on the cell surface of AML cells and binding to their ligands SDF-1α, VCAM-1 and HA and/or OPN, respectively. This binding results in retention of LSCs in HSC niches which induces LSC therapy resistance, and ultimately relapse of the disease (Fig. 4) [70]. Similarly to the role of the SDF-1α/CXCR4 axis in HSC retention in HSC niches (see Section 2.1.1.1), the SDF-1α/CXCR4 axis is involved in homing and retention of LSCs in HSC niches (Fig. 4). Treatment of AML cells with plerixafor/AMD3100 inhibits the activity of SDF-1α as chemoattractant by blocking its receptor CXCR4 which leads to migration of CXCR4-positive LSCs out of HSC niches. An uncontrolled phase I/II clinical trial was performed in AML patients with plerixafor in combination with mitoxantrone, etoposide and Ara-C (MEC; Table 2). Plerixafor caused a two-fold increased mobilization of AML cells into the peripheral blood and the overall complete response rate was 46% in this clinical trial, which is higher than the overall complete response rate to MEC chemotherapy alone of 21% [110]. It is assumed that the underlying mechanism is the forced migration of AML cells out of the protective HSC niches that induces their sensitization for chemotherapeutic agents.

3.1.1.3. Hypoxia-induced factor-1α and vascular endothelial growth factor. In response to hypoxia, HIF-1α and vascular endothelial growth factor (VEGF) overexpression causes excessive formation of sinusoidal microvessels in the bone marrow of AML patients [111]. HIF-1α induces expression of its transcription targets SDF-1α and CXCR4 in endothelial cells and AML cells, resulting in hijacking of HSC niches by AML cells, their transformation into LSCs, LSC survival and expansion (Fig. 4). Accordingly, increased HIF-1α/VEGF expression is associated with reduced AML patient survival [112]. We propose that this poor patient survival is caused by the increased homing of therapy-resistant LSCs in perisinusoidal compartments of HSC niches, mediated by SDF-1α/CXCR4 interactions (Fig. 4). Therefore, VEGF blockade therapies, such as with bevacizumab, may effectively complement the use of CXCR4 antagonists and/or drugs that interfere with SDF-1α/CXCR4 interactions to mobilize LSCs out of HSC niches to render them sensitive to chemotherapy.

3.1.1.4. CD44/hyaluronic acid and CD44/osteopontin. A monoclonal antibody against the adhesion molecule CD44 eradicates LSCs in mouse models of AML by disrupting the interactions between CD44 and the ECM component HA [113] which facilitates homing of HSCs into the bone marrow [113–115]. This homing can be blocked by an anti-CD44 antibody or by soluble HA [113,114]. It was argued that CD44 and HA play a key role in SDF-1α-dependent transendothelial migration of HSCs and their final anchorage within HSC niches in the bone marrow [113]. In support of this concept, treatment with the HA synthesis inhibitor 4-MU is effective against leukemic cell proliferation and survival [116]. This mechanism is likely SDF-1α-dependent since SDF-1α stimulates adhesion of HSCs to HA *in vitro*, whereas adhesion to HA did not occur when HSCs were pre-treated with anti-CD44 antibodies. However, further investigations are required to understand the molecular link between CD44, HA and SDF-1α [116]. OPN is another ligand of CD44, which facilitates anchorage of LSCs in the HSC niche [115,117] so besides the CD44-HA interactions, anti-CD44 antibodies may target CD44-OPN interactions as well. OPN expression was increased in bone marrow blasts and in bone marrow serum of AML patients as compared with healthy controls and OPN overexpression was associated with a reduced overall survival in a multivariate analysis [118].

3.1.1.5. Very late antigen-4/vascular cell-adhesion molecule-1. Interactions of AML cells with stromal cells render AML cells resistant against chemotherapy via the VLA-4/VCAM-1 axis, which triggers activation of the anti-apoptosis factor NF-κB in stromal cells. Inhibition of the VLA-4/VCAM-1 axis and its downstream protein NF-κB sensitizes AML cells to chemotherapy [119]. Currently, a phase II clinical trial is ongoing in AML patients with the compound AS101 that targets VLA-4 (Table 2).

Collectively, these data indicate that AML cells hijack HSC niches by upregulating receptors on their cell surface, such as TIE2, CXCR4, CD44 and VLA-4, resulting in retention of LSCs in HSC niches via interactions with adhesion molecules ANG-1, SDF-1α, OPN/HA and VCAM-1, respectively. All these molecular interactions are potential targets for therapy, since retention of LSCs in HSC niches results in quiescence and survival of LSCs and subsequently protection against chemotherapy. It would be interesting to determine the effect of cocktails of inhibitors against the ANG-1/TIE2, SDF-1α/CXCR4, VLA-4/VCAM-1, CD44/HA and CD44/OPN axes in combination with Ara-C chemotherapy to target the LSCs effectively.

3.1.1.6. Notch signalling. AML cells bind to the ECM in bone marrow via β1 and β2 integrins [120–122]. This binding promotes leukemogenesis by increasing proliferation and suppressing apoptosis of AML cells [120,122]. Increased Notch signalling was found in AML [123–125] and nuclear localization of β-catenin was observed in 38% of human AML/myelodysplastic syndromes (MDS) bone marrow samples. Expression of a constitutively activated β-catenin protein (Ctnnb1^{CAosb})

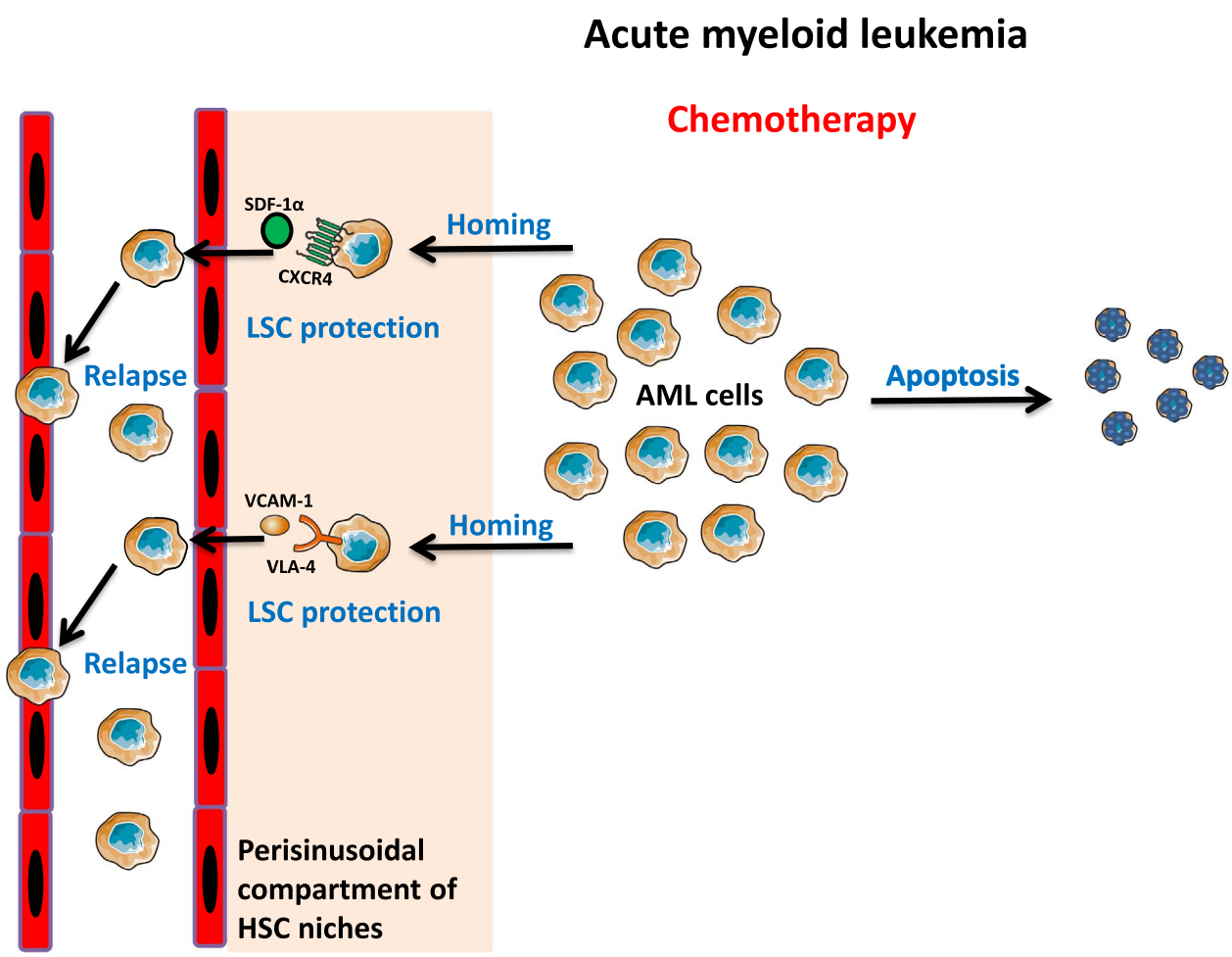

Fig. 4. Therapy-resistance of LSCs in HSC niches. Chemotherapy induces apoptosis of the majority of AML cells. However, a fraction of AML cells is resistant after hijacking perisinusoidal compartments of HSC niches via the SDF-1α/CXCR4 and VCAM-1/VLA-4 axes and transformation into LSCs. LSCs are able to fuse with sinusoidal endothelial cells and integrate in the endothelium, which results in survival of LSCs and protection against chemotherapy and stress, causing relapse of the disease. Abbreviations: AML, acute myeloid leukemia; CXCR4, C-X-C receptor type 4; HSC, hematopoietic stem cell; LSC, leukemic stem cell; SDF-1α, stromal derived factor-1α; VCAM-1, vascular cell-adhesion molecule-1; VLA-4, very late antigen-4.

by osteoblasts in the bone marrow induces AML via Notch signalling. Ctnnb1^{CAosb} promotes expression of the Notch ligand Jagged, which induces expression of forkhead homeobox type O-1 and ɣ-secretase, a protease that cleaves transmembrane proteins [121,126]. Inhibition of increased Notch signalling using the ɣ-secretase inhibitor dibenzazepine (DBZ) normalizes blood cell counts, rescues hematopoietic defects and reverses the induction of AML in Ctnnb1^{CAosb}-expressing mice [121]. Several clinical trials with inhibitors of Notch signalling, such as LY3039478, are conducted in patients with T-cell leukemias (Table 2).

3.1.1.7. Growth-arrest specific gene 6/AXL/MER paracrine signalling. Growth-arrest specific gene 6 (GAS6) is expressed by bone marrow-derived stromal cells [127]. In co-cultures of these stromal cells and AML cells, the secretion of IL-10 and granulocyte-macrophage colony-stimulating factor by AML cells is upregulated, which in turn promote the expression and secretion of GAS6 by the stromal cells. In this paracrine loop, GAS6 binds to the tyrosine-protein kinase receptor AXL on AML cells which increases proliferation and suppresses apoptosis [128–131]. This is reversed by AXL blockade with the small-molecule AXL inhibitor BGB324 via inhibition of the AKT and ERK signalling pathways and activation of the mitochondrial apoptosis pathway [130,131]. It is associated with activation of B-cell lymphoma 2 (BCL-2) and p53-upregulated modulator of apoptosis, also known as BCL-2-binding component 3. In addition to reactivated apoptosis, AXL blockade also contributes to increased chemosensitivity of AML cells in a mouse model, as a combination regimen of AXL blockade by BGB324 and the chemotherapeutic doxorubicin had a synergistic effect on AML cell death [130]. In concordance with these results, AXL expression was found in ~50% of AML patients and high AXL and high GAS6 expression predicted worse prognosis in 2 independent cohorts of AML patients [130,131]. BGB324 is now studied in AML patients as a stand-alone therapy or in combination with Ara-C in a phase 1 clinical trial (Table 2). In addition, the proto-oncogene tyrosine-protein kinase MER is an alternative GAS6-binding protein that is expressed in ~60% of AML patients [130,131] and stimulates leukemogenesis [132]. MER activation by GAS6 results in activation of the intracellular ERK, p38, AKT, cAMP regulatory element-binding protein and STAT6 signalling pathways, which all contribute to cellular proliferation, survival and suppression of apoptosis. Consequently, MER knockdown using short hairpin RNA resulted in decreased AML colony-forming potential *in vitro* and prolonged survival in mice transplanted with AML xenografts [130,131]. This suggests that besides inhibition of AXL using agents such as BGB324, inhibition of MER may also have therapeutic efficacy.

3.1.1.8. Hedgehog signalling. Hedgehog signalling is important in the regulation of stem cell and progenitor cell proliferation and lymphocyte differentiation [133]. More specifically, the ligand Indian Hedgehog (IHH), its receptor Patched and the constitutively active signaling molecule Smoothened are expressed in HSCs. HSCs secrete IHH which in turn changes cytokine expression profiles of HSCs and this ultimately induces their proliferation [133]. Inappropriate activation of Hedgehog signaling has been described in a variety of cancer types and cancer

stem cells (CSCs), including leukemia and LSCs. For instance, proliferation of LSCs containing the Breakpoint Cluster Region-Abelson (BCR-ABL) fusion gene is dependent on activation of the Hedgehog pathway [134]. Paracrine loops between leukemic cells and the microenvironment contribute to activation of Hedgehog signaling especially via blocking of Human Hedgehog-Interacting Protein (HHIP), which is an intrinsic Hedgehog-signaling inhibitor. HHIP expression is decreased in AML/MDS-derived stromal cells compared with healthy donor-derived stromal cells. Consequently, IHH and Smoothened expression is found in AML/MDS cells but not in HSCs. Furthermore, HHIP knockdown in HSCs results in increased proliferation of primary AML/MDS cells in co-cultures. HHIP expression is regulated by methylation of the HHIP promoter. In primary AML cells, this methylation is reversed by 5-azacytidine treatment to reactivate HHIP expression and reduce proliferation of AML cells [135].

These results have motivated several phase Ib and phase II clinical trials that investigate Hedgehog inhibitors such as PF-04449913 either as stand-alone therapy or in combination with low-dose cytarabine or demethylating agents (Table 2).

3.1.2. Intrinsic targeting of LSCs

In addition to therapies that target the hijacking of HSC niches by AML cells to become LSCs, it is also possible to target molecular pathways that are aberrant or constitutively active in LSCs after their hijacking of HSC niches. Since eradication of LSCs is considered to be essential for preventing AML relapses, therapies that specifically target key mechanisms in LSCs are expected to have the highest therapeutic index. After hijacking HSC niches, LSCs take over and alter the molecular pathways in HSC niches that are normally intended for the maintenance and quiescence of HSCs, such as the WNT pathway [38,39], NF-κB [136], the AKT pathway [137] and the anti-apoptosis protein BCL-2 homology 3 (BH3) [138].

The Wnt/β-catenin signaling pathway and NF-κB activation play crucial roles in the development of LSCs in mice. Wnt/β-catenin signaling promotes self-renewal of LSCs in mouse models of AML [112,139]. Wnt signaling takes place when β-catenin enters the nucleus and forms a complex with T-cell factor (TCF) and CREB-binding protein (CBP), which results in transcription of target genes [140]. Currently, several inhibitors of the Wnt/β-catenin pathway have been tested *in vivo*, such as PKF115-584 and CGP049090. These compounds inhibit the β-catenin/TCF complex formation and induce apoptosis of LSCs.

Another small molecular inhibitor of the Wnt/β-catenin pathway, ICG-001, binds to CBP and competes for binding with β-catenin, which results in elimination of drug-resistant clones in AML. ICG-001 has not been tested in clinical trials yet. PRI-724 is another CBP/β-catenin antagonist and has been tested in phase I clinical trials in AML patients and showed acceptable toxicity (Table 2). Phase II and phase I/II clinical trials with this inhibitor are ongoing in AML and chronic myeloid leukemia patients (Table 2) [141].

NF-κB is constitutively activated in LSCs and plays a significant role in survival of AML cells and LSCs. MG-132, an inhibitor of NF-κB, results in rapid induction of cell death *in vitro*, while leaving the healthy HSCs unharmed [142], but has not been tested in clinical trials yet.

AML cells exhibit constitutive activation of AKT via phosphorylation on either Thr308 or Ser473 (or both), which stimulates proliferation and downregulates apoptosis [143]. LY294002 is an inhibitor of PI3K, a kinase upstream of AKT, which induces apoptosis and decreases LSC survival *in vitro*. Besides, the downstream mediators of AKT signalling, p70 S6 kinase and 4-enhancer-binding-protein-1 were phosphorylated in LSCs. This phosphorylation was inhibited by mammalian target of rapamycin (mTOR) inhibitor everolimus (RAD001), which enhanced the efficacy of Ara-C [139,144]. These data show that the PI3K pathway is required for survival of LSCs and that inhibitors of this signaling pathway may be beneficial to improve AML treatment.

Aberrant overexpression of BH3 is associated with tumor progression and chemoresistance in AML. BCL-2 proteins are key regulators of apoptosis. ABT-737, a small-molecule BH3 mimetic, disrupts the dimerization of BCL-2 and BCL-2-associated X protein and induces apoptosis of LSCs via activation of the intrinsic apoptotic pathway. ABT-737 effectively killed AML blasts and LSCs without affecting normal HSCs *in vivo* with a similar efficacy as conventional chemotherapy [145]. There already are phase I clinical trials ongoing with BH3 mimetics, which demonstrated an overall response of 84% among 67 patients with relapsed CLL or small lymphocytic lymphoma [146]. Another BH3-mimetic, ABT-199/venetoclax, was recently FDA-approved for the treatment of 17p–deleted CLL [147].

3.2. Therapeutic targeting of interactions between MDS and myeloproliferative cells and HSC niches

Hijacking of HSC niches also occurs in a disease group called myeloproliferative neoplasms (MPN), which contains, among others, chronic myeloid leukemia, polycythemia vera, essential thrombocythemia and primary myelofibrosis [72,148]. In mice, *JAK2*-mutated MPN HSCs accelerate MPN progression by depletion of nestin-positive stromal cells in HSC niches, which was achieved by IL-1β inhibition of β3-adrenergic production by sympathetic nerves in the bone marrow. The mechanistic evidence that MPN HSCs hijack HSC niches is supported by the finding that β3-adrenergic agonists inhibited MPN progression [72,148]. These β3-adrenergic agonists, of which mirabegron is FDA-approved for the treatment of overactive bladder [149], thus represent attractive potential therapies in the treatment of MPN.

In another model, MPN formation in mice stimulated HSCs to increase the number of endosteal osteoblastic lineage cells that selectively supported LSCs over normal HSCs. These endosteal osteoblastic lineage cells have decreased expression of leptin receptor, SDF-1α, N-cadherin, SCF, ANG-1, and slit homology 2 protein. In addition, a shift in the expression pattern of TGF-β signaling molecules was observed in osteoblasts of MPN mice versus healthy mice, with downregulation of the quiescence-enforcing TGF-β1 and strong upregulation of the myeloid-promoting TGF-β2. These changes in expression of pivotal TGF-β proteins contribute to the decreased ability of MPN-expanded osteoblasts to maintain normal HSCs in favor of MPN development [150].

There is extensive crosstalk between HSC niches and cells of MDS, the benign lesion that can precede AML [151]. MDS is characterized by ineffective hematopoiesis, progressive bone marrow failure and variable risk of progression to AML. Although HSC niches in MDS harbor many aberrations, such as chromosomal aberrations and perturbed cytokine secretion by bone marrow MSCs, hijacking of HSC niches has never been found in MDS [151,152]. It would be interesting to determine whether hijacking of HSC niches by malignant cells is necessary for MDS to progress to AML. However, MSCs that are isolated from human MDS proliferate at a reduced rate, have increased senescence, a decreased efficiency to undergo osteogenic differentiation and an impaired ability to support normal HSCs in long-term *in vitro* culture [153]. Because it was not shown that these MSCs selectively support MDS cells, it has not been proven that MDS cells truly hijack HSC niches. Nonetheless, supportive and essential interactions between HSC niches and MDS cells exist and may be amenable for therapeutic targeting. For example, the successful engraftment of human MDS HSCs in xenografts depends on co-engraftment of MSCs from the same MDS patients. This supports the existence of HSC niche-MDS interactions that are essential for disease engraftment and that may contribute to disease progression [154]. Another argument is presented by the RNAse III endonuclease Dicer1, which is essential for correct processing of microRNAs and critical for the function of osteolineage cells from the bone marrow microenvironment in healthy mice. Gene targeting studies that deleted Dicer1 only from these osteolineage cells yielded a severe MDS-like syndrome in these animals [155]. This suggests that there may be differentially-expressed microRNAs in the bone marrow microenvironment that can be therapeutically targetable.

3.3. Therapeutic targeting of ALL cells hijacking HSC niches

ALL cells hijack HSC niches by various mechanisms. One of these is adherence to OPN [117,156], which is secreted by osteoblasts [7,24–26,117]. OPN provides anchorage of ALL blasts which results in homing into HSC niches and dormancy of ALL cells by forcing them out of the cell cycle [156]. This protects ALL cells against chemotherapy because these agents do not affect quiescent cells [16–18]. ALL cells specifically adhere to OPN via α4β1, α5β1, α9β1 integrins *in vitro* and secrete OPN in HSC niches *in vivo*. Intravital microscopy demonstrated that OPN is highly expressed adjacent to dormant ALL cells within the bone marrow of calvaria in ALL xenograft mice. Boyerinas et al. [117] developed an OPN neutralization strategy by using a cocktail of anti-mouse and anti-human OPN-neutralizing antibodies to inhibit the interactions between ALL cells and extracellular OPN in the models. Reduced interactions between OPN and ALL cells increased proliferation of ALL cells and increased their sensitivity to Ara-C chemotherapy, rendering OPN an important target for anti-ALL therapy [117,156].

The SDF-1α/CXCR4 pathway is highly activated in ALL, where CXCR4-positive LSCs cells are retained in HSC niches by high levels of SDF-1α. This is associated with increased homing of ALL cells into HSC niches and disruption of normal hematopoiesis. Since protection of leukemic cells by HSC niches facilitates LSC survival, increased SDF-1α and CXCR4 expression is considered to promote ALL expansion. There is emerging evidence that ALL cell survival is mediated by SDF-1α via the PI3K-AKT-NFκB and MAPK pathways, but the regulatory mechanisms of SDF-1α in ALL are unclear. In addition, several *in vivo* studies have shown that inhibiting CXCR4 using AMD3100/plerixafor sensitizes ALL to chemotherapy and CXCR4 antagonists have also shown promising results in clinical trials (Table 2) [102].

Dynamic *in vivo* imaging showed that ALL cells are able to disrupt normal HSC niches by altering the stromal bone marrow environment. ALL cells home into normal HSC niches in close vicinity of sinusoids and transplanted hematopoietic progenitor cells (HPCs) mobilize to the niches that are occupied by the ALL cells where SDF-1α and SCF are present as chemoattractant for ALL cells [157]. Treatment of ALL engrafted mice with neutralizing anti-SCF antibodies inhibited HPC migration into the ALL-occupied HSC niches and restored the HPC numbers in the ALL mice. This suggests that the ALL-occupied HSC niches cause dysfunction of HPCs and that ALL cells expel normal HSCs from the niches, because their numbers were reduced [157]. Real-time *in vivo* confocal and multiphoton microscopy imaging approaches revealed that SCF neutralization restored the number of HSCs. Thus, targeting the interactions between the ALL cells and the HSC niches, such as the SCF/c-KIT and SDF-1a/CXCR4 axes, may be interesting therapeutic strategies in order to maintain normal HSC and HPC function [157]. Thus, this study shows that ALL cells hijack the perisinusoidal compartments of HSC niches where they dedifferentiate into LSCs, while causing dysfunctions in the HPCs and that the SCF/c-KIT and SDF-1a/CXCR4 axes are involved in the hijacking of HSC niches.

Altogether, AML and ALL cells are able to hijack, occupy and manipulate HSC niches and outcompete normal HSCs, which protects LSCs while worsening patient survival. Promising therapeutic approaches to eradicate LSCs include strategies to disrupt multiple interactions between LSCs and HSC niches simultaneously, rather than just a single interaction, to prevent dedifferentiation of AML/ALL cells into LSCs or to induce differentiation and proliferation of LSCs and sensitize AML and ALL cells to chemotherapy.

3.4. Therapeutic targeting of hypoxia and LSCs in HSC niches

Because HSC niches that contain LSCs are hypoxic, HIF-1α is overexpressed by LSCs which upregulate VEGF secretion. VEGF affects both leukemic cells and endothelial cells and is a key factor in promoting proliferation of leukemic cells. Treatment with the HIF-1α inhibitor PX-478 lowers HIF-1α function by decreasing HIF-1α mRNA stability and translation, which decreases hypoxia-mediated VEGF expression in AML tumor xenografts and subsequently decreases AML activity [158]. This supports the notion that hypoxia supports tumor growth and AML survival. In addition, HIF-1α induces SDF-1α gene expression in endothelial cells, which increases migration and homing of CXCR4-positive leukemic cells, such as AML cells, into hypoxic HSC niches since HIF-1α and VEGF both upregulate CXCR4 expression on AML cells (see Section 3.1.1.3). Thus, HIF-1α seems to be an interesting therapeutic target [112]. However, this needs further investigation, since normal HSC niches are also hypoxic and dependent on HIF-1α and targeting HIF-1α may thus result in unfavorable toxic effects. Drolle et al. [159] demonstrated that bone marrow infiltrated with AML cells is not more hypoxic than healthy bone marrow, thus the level of hypoxia is not specific to leukemic HSC niches and targeting hypoxia may thus not have a therapeutic index. Physiological hypoxia of 1% oxygen results in cell-cycle arrest of AML blasts in the G_0/G_1 phase and decreased numbers of cells in the S phase. This may explain why AML blasts that reside in hypoxic HSC niches are not sensitive to Ara-C, because this drug is cytotoxic to cells in the S phase.

Exposure of AML cells to hypoxia upregulated VEGF expression, which subsequently activated the PI3K/AKT pathway and increased expression of anti-apoptotic X-linked inhibitor of apoptosis protein (XIAP). PI3K inhibition restored the sensitivity of AML cells to Ara-C under hypoxic conditions, suggesting that the PI3K pathway is responsible for maintaining AML cells in the G_0/G_1 phase under hypoxic conditions [159]. This implies that hypoxia contributes to chemoresistance of AML blasts. A phase II clinical trial is performed at present to investigate the dual inhibitor PF-05212384, which targets both PI3K and mTOR. mTOR is also upregulated in LSCs in AML, which results in increased proliferation and CXCR4 expression on LSCs, stimulating homing of LSCs into HSC niches [160] (Table 2). A phase I/II clinical trial with XIAP antisense was performed in AML patients to collect blood samples for the detection of XIAP levels and apoptosis. Treatment with XIAP antisense resulted in decreased XIAP mRNA and protein levels and a distinct induction of apoptosis in LSCs (Table 2) [161].

Since AML cells can survive low oxygen levels and hypoxia contributes to chemoresistance, the effectiveness of the hypoxia-activated prodrug TH-302 was investigated in xenografts. TH-302 is only activated under hypoxic conditions and releases bromo-isophosphoramide mustard, a potent DNA-alkylating agent which produces DNA double-strand breaks. Whereas human AML cells were less sensitive to Ara-C under chronic hypoxia (1% O_2, 72 h) relative to normoxia, treatment with TH-302 was more effective in hypoxic AML cells and reduced cell proliferation, increased numbers of DNA double-strand breaks and induced cell-cycle arrest and apoptosis. This translated to inhibited disease progression and prologned overall survival of human AML xenografts after TH-302 treatment [162]. A phase I clinical trial with TH-302 has been performed in patients with advanced leukemias (including AML and ALL; Table 2), but the final data of this clinical study have not yet been reported.

Thus, hypoxia maintains LSCs in HSC niches, which results in propagation of the disease and resistance to therapy and is considered as a promising therapeutic target. Therefore, anti-hypoxia agents are tested in clinical trials in AML and ALL patients.

4. Therapeutic targeting of metabolism of AML cells under hypoxic conditions

AML cells that have hijacked a HSC niche enter a hypoxic environment [7,163,164] to which they adapt by switching their energy metabolism to a state which is specialized in providing a maximal support of biosynthesis and energy production in a hypoxic context while the generation of oxidative stress is kept at a minimum [88,164,165]. Therefore, therapies that preferentially attack the anaerobic energy metabolism in these hypoxic cancer cells may demonstrate selectivity for LSCs residing in HSC niches.

NB4 AML cells, which are dependent on glycolysis under normoxic conditions (21% O_2), demonstrated prominent apoptosis, growth suppression, and increased ROS production when cultured in hypoxic conditions (1% O_2). Treatment with the ROS scavenger N-acetyl cysteine prevented hypoxic NB4 AML cell death. The notion that ROS is produced under hypoxic conditions is still poorly understood [166].

It has been suggested that deficiencies in the mitochondrial oxidative phosphorylation (OXPHOS) cause loss of respirasomes [139,167]. Respirasomes are considered to be organizations of respiratory enzymes into supercomplexes that reduce oxidative damage in mitochondria [168,169]. Thus, loss of respirasome activity under hypoxic conditions may facilitate ROS generation in mitochondria. This may explain the paradox of reduced O_2 levels and enhanced ROS production under hypoxic conditions. Besides, hypoxia impairs the mitochondrial membrane potential, which in turn can cause disassembly of complexes of the respiratory chain and may eventually increase ROS production [166].

On the other hand, THP-1 AML cells, which are dependent on OXPHOS in normoxia, showed increased cell proliferation under hypoxic conditions by switching from OXPHOS to anaerobic glycolysis via upregulation of pyruvate dehydrogenase-1. Besides, THP-1 cells limited ROS production by switching the cytochrome c oxidase subunit 4 (COX4) from isoform COX4-1 to COX4-2. COX4-2 facilitates more efficient electron transport than COX4-1. This switch occurs via upregulation of the activity of mitochondrial ATP-dependent protease LON, which is required for COX4-1 degradation. Thus, increased activity of LON in THP-1 cells enables efficient respiration and protection against ROS under hypoxic conditions [170].

These findings show that AML cells are able to cope with environmental challenges such as hypoxia by avoiding ROS production. A potential therapeutic approach is to elevate ROS production by inhibiting the metabolic switch or inhibition of the LON protease. For that purpose, a study has started recently with AML patients to investigate whether inhibition of the activity of specific enzymes in the glycolysis pathway and OXPHOS pathway have therapeutic value. This is done by measuring metabolites in blood samples of patients (ClinicalTrials.gov NCT02581917).

5. Therapeutic targeting of CSC niches in solid tumors

The approaches that are discussed here to target CSCs may also be applicable in solid tumors, such as glioblastoma, in which CXCR4-positive therapy-resistant glioma stem-like cells (GSLCs) are retained in hypoxic GSLC niches by SDF-1α and OPN. In GBM, cathepsin K can degrade and inactivate SDF-1α which may result in release of GSLCs out of GSLC niches [86]. Furthermore, VCAM-1 facilitates binding of quiescent neural stem cells (NSCs) to ependymal cells in the NSC niches in the subventricular zone in the adult forebrain. Disruption of this binding causes a disrupted cytoarchitecture of the subventricular zone and proliferation and depletion of NSCs. Moreover, this VCAM-1 signalling functions via ROS [171]. In addition to these examples that illustrate the similarity between LSC niches and niches from other primary tumors, OPN is functional in metastatic niches. In melanoma, OPN expression facilitates metastasis to bone marrow and proliferation of melanoma cells in metastatic niches in bone marrow [172]. Furthermore, OPN expression promotes survival of metastatic breast cancer cells in the blood stream and mitigates immunosuppression in both primary breast cancer and metastatic niches in the lung to facilitate the successful metastasis of breast cancer to the lung [173]. Thus, there are striking similarities between HSC niches on one hand and other primary and metastatic niches on the other hand and future therapies in other types of cancer may also be based on the prevention and disruption of the interactions between CSCs or metastatic pioneer cells and their niches to sensitize them to chemotherapy and irradiation [86,174].

6. Conclusions

The ultimate goal in the field of CSC research in AML and ALL is to discover novel methods to effectively eradicate LSCs to prevent therapy relapses and, ultimately, offer a cure for these malignancies. We argue that targeting LSCs either by prevention to enter HSC niches or by forcing migration out of HSC niches will improve the clinical success of chemotherapy in AML and ALL.

The major findings described and discussed in this review are:

1) There is only one type of continuous HSC niches that consists of a periarteriolar compartment with hypoxia and low levels of ROS in HSCs and a perisinusoidal compartment with hypoxia and higher ROS levels in HSCs. Reticular cells interact with all HSCs in both compartments and have no specific localization in bone marrow;
2) Disruption of the interactions between LSCs and HSC niches is the most promising way to improve the clinical success of chemotherapy in AML and ALL since this approach prevents dedifferentiation of AML/ALL cells into LSCs or induces differentiation and proliferation of leukemic cells that already have become LSCs, resulting in sensitization to chemotherapy so that LSCs can be eradicated.

Retention and adhesion of LSCs in HSC niches are mediated via several interactions between receptors and ligands. The SDF-1α/CXCR4 axis [70,110,112], the VLA-4/VCAM-1 axis [119] and ANG/TIE2 [106,107], CD44/HA [113,114,116], CD44/OPN [113–115,117] and OPN/integrin interactions [117,156] are the most promising therapeutic targets in AML and ALL to block adhesion and thus retention of LSCs in HSC niches. Multiple clinical trials are currently being performed in AML and ALL patients with the ultimate goal to prevent entrance of AML/ALL cells into niches or to force LSCs to migrate out of niches to sensitize them to chemotherapy (Table 2). However, these clinical trials are early-phase and therefore only investigate single compounds that target one receptor-ligand interaction, in combination with chemotherapy (Table 2). In our opinion, the clinical response can be maximized when multiple receptor-ligand interactions are targeted simultaneously in combination with chemotherapy, since that may enforce a more complete migration of LSCs out of HSC niches. Thus, negative results of single-agent clinical trials should be interpreted with caution as only combination regimens that target multiple niche-anchoring interactions may yield significant clinical responses. However, a challenge is to find tolerable doses of combinations of drugs for AML and ALL patients, since the described interactions are also important for the normal functioning of HSC niches. In summary, LSCs are protected by HSC niches and receptor-ligand interactions which mediate retention of LSCs in HSC niches are promising therapeutic targets for leukemia.

Authorship contributions

Developed the concept of the review, designed its structure, wrote the manuscript and edited the manuscript: V.V.V. Hira. Wrote part of the manuscript, edited the entire manuscript and performed histological analysis of the bone marrow vasculature: C.J.F. van Noorden. Provided hematological expertise for editing of the manuscript: H.E. Carraway, and J.P. Maciejewski. Wrote the manuscript, edited the manuscript and supervised the writing of the review: R.J. Molenaar.

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