Recent progress on targeting leukemia stem cells

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Leukemia is a type of malignant clonal disease of hematopoietic stem cells (HSCs). A small population of leukemic stem cells (LSCs) are responsible for the initiation, drug resistance, and relapse of leukemia. LSCs have the ability to form tumors after xenotransplantation in immunodeficient mice and appear to be common in most human leukemias. Therefore, the eradication of LSCs is an approach with the potential to improve survival or even to cure leukemia. Using recent research in the field of LSCs, we summarize the targeted therapy approaches for the removal of LSCs through surface markers including immune checkpoint molecules, pathways influencing LSC survival, or the survival microenvironment of LSCs. In addition, we introduce the survival microenvironment and survival regulation of LSCs.

Keywords: Leukemia; Leukemia stem cells (LSCs); Targeted therapy; LSC eradication; Survival microenvironment

Leukemia is a type of malignant clonal disease of HSCs. According to the differentiation degree and natural course of leukemia, it can be divided into acute and chronic disease. Clonal leukemia cells proliferate and accumulate in bone marrow (BM) and other hematopoietic tissues because of mechanisms such as uncontrolled proliferation, blocked differentiation, and decreased apoptosis. These leukemia cells also infiltrate other tissues and organs, while normal hematopoiesis is inhibited [1]. The differentiation of acute myeloid leukemia (AML) cells stagnates during the early stages, producing mainly primitive and early cells, and the disease develops rapidly over the course of only a few months [2]. Thus, AML is characterized by a block in mature myeloid differentiation that is sustained by LSC self-renewal. Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of LSCs, characterized by generation of the BCR-ABL oncogene [3]. Hence, LSC-derived AML and CML are characterized by uncontrolled expansion of BM progenitor cells, showing more or less serious defects in LSC maturity, respectively [1,4]. The treatment outcome of patients with AML depends on several factors, including karyotype and molecular alterations in the leukemic cell bulk. Combination chemotherapy leads to complete remission (CR) in most patients with AML [5]. However, ~50% of patients achieving CR relapse within 5 years of their initial diagnosis. This recurrence of the disease is believed to be caused by chemotherapy-resistant LSCs [6–8]. The introduction of BCR/ABL-specific tyrosine kinase inhibitors (TKIs) approximately two decades ago dramatically improved the outcome of patients with CML. However, disease-initiating LSCs in CML are resistant to TKIs despite BCR-ABL inhibition. Consequently, an important area of unmet clinical need in CML and AML is to directly target LSCs to overcome drug resistance.

The concept of LSCs is based on several studies, which showed that only a small number of leukemic cells can proliferate widely *in vitro* and *in vivo* [8–11]. LSCs are thought to originate from

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normal hematopoietic stem/precursor cells, which are characterized by self-renewal and differentiation potential [12–16]. Meanwhile, LSCs rely on other tissues and the microenvironment in which they live to avoid being killed by chemotherapeutic drugs or radiotherapy, resulting in drug resistance [17]. Moreover, related research has found that LSCs use adipose tissue to avoid killing by chemotherapeutic drugs [18]. Based on their leukemia-initiating and propagating capacity, LSCs are regarded as a major, clinically relevant therapeutic cell targets. Therefore, identifying new therapeutic approaches to eradicating LSCs is crucial [11]. In this review, we explore research on targeting LSCs from three aspects: surface markers, related pathways, and the microenvironment.

Survival microenvironment of LSCs

LSCs are regulated by the cellular and molecular components of the microenvironment, and microenvironmental interactions can protect LSCs from conventional and targeted therapy [19]. LSCs occupy endosteal and sinusoidal niches in the BM as well as HSCs [20]. Endosteal niches of HSCs are formed and regulated by osteoblasts, which result in HSC homing, promoting self-renewal of HSCs, affecting both the canonical and noncanonical wingless-type (WNT) signaling pathways, mesenchymal stromal cells (MSCs, regulating the survival and maintenance of HSCs), osteoclasts (retaining HSCs in endosteal niches, regulation of HSC quiescence, and inducing migration of HSCs out of niches), reticular cells, macrophages, and regulatory T cells (Tregs). By contrast, the endosteal niche of LSCs is associated with MSCs and osteoblasts, in which LSCs surviving in a quiescent state are resistant to therapy. The sinusoidal niche of HSCs is formed with sinus endothelial cells and perivascular stromal cells expressing leptin receptor (lepR +), whereas LSCs are related to sinusoidal endothelial cells (Fig. 1). BM homing is an important biological process for HSC function, and is governed by a cascade of molecular interactions [21]. First, migrating cells are captured from the fluid stream onto BM tissue endothelium. After intimate contact with the endothelium, cells are exposed to chemical signals (principally chemokines, but also other cytokines and inflammatory agents), resulting in the upregulation of integrin adhesiveness, which leads to firm adhesion, followed by endothelial transmigration. Finally, HSCs home and locate to BM niches. Besides regulating HSC functions, the BM niche might similarly regulate the functions of LSCs. Studies have demonstrated that both endosteal and sinusoidal niches are crucial for LSC survival, proliferation, differentiation, and drug resistance [16,22–24]. Moreover, this research indicates that the interaction of human LSCs with the hematopoietic microenvironment, mediated by prosurvival cell adhesion, is an underly-

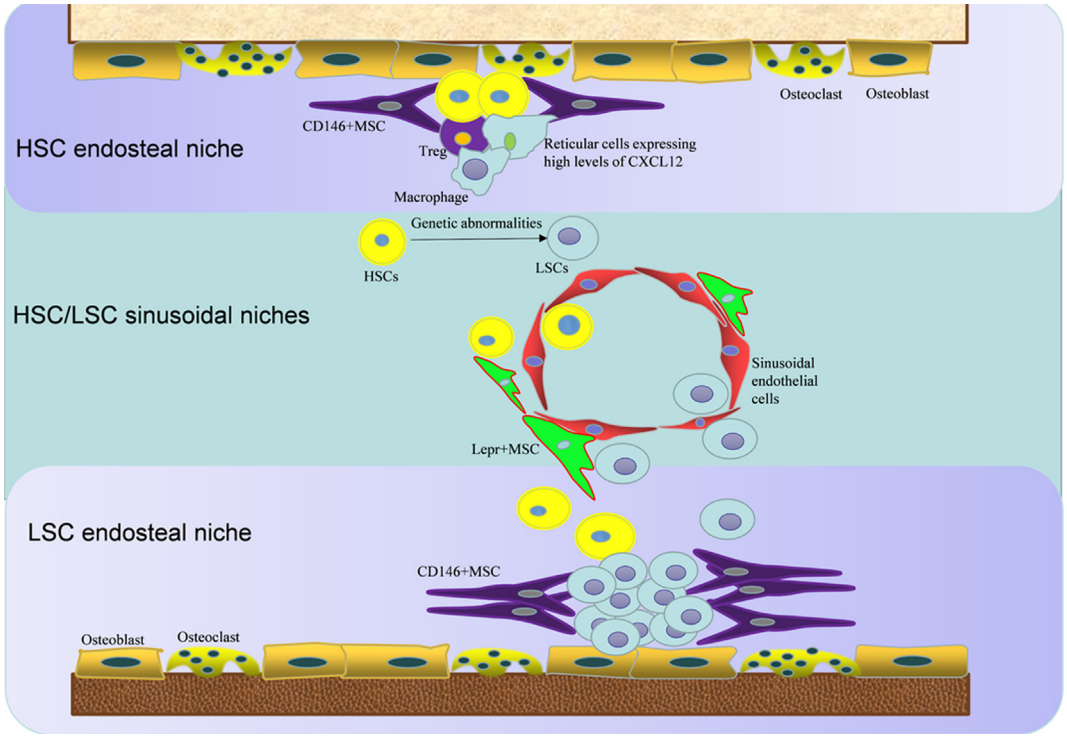

FIGURE 1

The bone marrow (BM) endosteum and sinusoids as two crucial niches for hematopoietic stem cells (HSCs) and leukemic stem cells (LSCs). Endosteal niches of HSCs are formed and regulated by osteoblasts, mesenchymal stromal cells (MSCs), osteoclasts, reticular cells, macrophages, and regulatory T cells (Tregs). By contrast, the endosteal niche of LSCs is associated with MSCs and osteoblasts, in which LSCs surviving in a quiescent state are resistant to therapy. HSCs form a sinusoidal niche with sinus endothelial cells and perivascular stromal cells expressing leptin receptor (LepR), whereas LSCs are related to sinusoidal endothelial cells.

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ing mechanism for resistance to cell cycle-dependent cytotoxic chemotherapy [25]. Therefore, the microenvironment of LSCs could be a suitable therapeutic target.

Molecular mechanisms controlling LSC growth and survival

It is well known that LSCs originate from genetic abnormalities of HSCs. Hence, LSCs share many characteristics with HSCs, such as capacity for self-renewal, the ability to give rise to new hematopoietic tissues, and an immunophenotype similar to HSCs ($CD34^+$, $CD38^-$, $CD71^-$, and $HLA\text{-}DR^-$). In addition, LSCs can be phenotypically distinguished from HSCs with aberrant expression of several cell surface antigens, such as CD123; CD25; CD9; CD93; CD96; and immune checkpoint antigens, such as CD274 (PD-L1), CTLA4, and TIM-3 [26] (Table 1). In certain types of leukemia, some LSCs might even reside in a CD34-negative compartment. Several studies have described pathways that might influence LSC survival, such as Wnt/β-catenin, canonical circadian pathway, *trans*-chromatin regulatory pathway, and the PTEN-C/EPBα-CTNNA1, PI3K/AKT/MTOR, JAK2-STAT3 and WNT/JAK2 pathways [22,27–31]. For example, increasing evidence suggests that Wnt/β-catenin signaling has an important role in the development of AML and CML [32,33]. In mouse models of AML induced by co-expression of Hoxa9 and Meis1a oncogenes or by fusion oncoprotein MLL/AF9, the Wnt/β-catenin signaling pathway is required for self-renewal of LSCs that are derived from either HSC or granulocyte–macrophage progenitors (GMPs). During leukemogenesis, β-catenin is essential for AML initiation from HSCs or GMPs [34]. Similarly, it has been demonstrated that β-catenin and Wnt pathway-associated genes are highly expressed in LSCs compared with normal HSCs in CML [35–38]. BCR-ABL stimulates transcription of arachidonate 5-lipoxygenase (Alox5) to jointly form a unique pathway that is crucial for survival regulation of LSCs of CML, with a minimal role in normal HSCs [39,40]. Alox5 has been shown to have important roles in many signaling pathways, such as p53, NF-κB, and PI3K [41–43].

In addition, Park et al. reported that Krüppel-like factor 4 (KLF4) represses the dual-specificity-tyrosine-phosphorylation-regulated kinase 2 (DYRK2) gene in LSCs to prevent abrogation of self-renewal and survival [44]. In summary, the survival of LSCs is inseparable from the regulation of genes, and their self-renewal requires special pathways (Fig. 2). These pathways and genes could provide us with more appropriate therapeutic targets.

Targeting surface markers of LSCs

CD123

CD123, or interleukin 3 receptor (IL-3Rα), is a glycoprotein on the cell membrane with a unique α chain paired with a β subunit, and belongs to the type I cytokine receptor family. CD123 can be detected on the surface of blasts in most patients with AML with significantly higher expression levels compared with normal $CD34^+$ hematopoietic progenitors [45]. Many studies have explored CD123 expression at the level of leukemic progenitor/

TABLE 1

Expression of cell surface markers (CD25, CD96, and CD123) of LSCs in CML and AML compared with normal BM stem cells.[a]

Target/Marker	CD	CML LSCs	AML LSCs	Normal stem cells
IL-2RA	CD25	+++++	+++	++
Tactile	CD96	+	++	+
IL-3RA	CD123	+++++	+++++	++++

[a] Expression score: +++++, expressed on LSCs in >90% of all patients/donors; ++++, expressed on LSCs in 75–90% of all donors; +++, expressed on LSCs in 50–75% of all donors; ++, expressed on LSCs in 15–50% of donors; +, expressed on LSCs in <15% of donors.

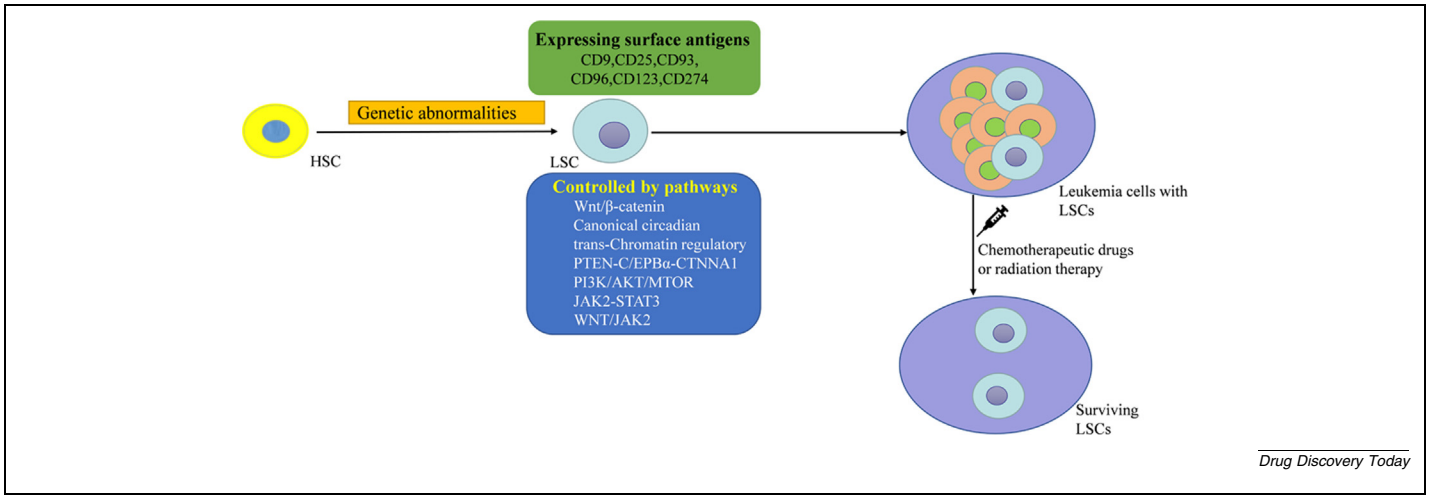

FIGURE 2

Leukemic stem cells (LSCs) originate from genetic abnormalities of hematopoietic stem cells (HSCs). The figure shows the genes and pathways involved in the survival and self-regulation of LSCs.

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stem cells, mainly contained in the $CD34^+/CD38^-$ cell fraction. Guzman and coworkers provided evidence that $CD34^+/CD38^-/CD123^+$cells can initiate a leukemic process when inoculated into immunodeficient mice [46]. $CD34^+/CD38^-/CD123^+$cells were clearly detectable in ~75% of patients with AML [47] and their number is predictive of clinical outcome [48]. In addition, several membrane markers, including CD33, CLL1, TIM3, CD244, CD47, CD96, CD157, and CD7, were ubiquitously expressed on AML bulk cells at diagnosis and relapse, irrespective of genetic features [49]. Haubner et al. explored the expression of these membrane antigens in a large set of patients with AML at diagnosis and at relapse. They found that CD33 and CD123 were homogenously expressed at relapse and CD123 was expressed at a higher level compared with CD33 in LSCs [50]. In addition, it was hypothesized that CD123 overexpression through CXCR4 downregulation induces the egress of LSCs in AML from the BM into the circulation [51,52].

Meanwhile, emerging studies have shown that CD123 is highly expressed on the membrane of LSCs and is related to the initiation and evolution of leukemia. *In vivo* studies confirmed that transplanted $CD34^+/CD123^+$ AML LSCs into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice initiated leukemia, which suggested that CD123 is a specific marker of LSCs. Using flow cytometry, CD123 expression was likewise detected in LSCs of patients with AML but absent in normal HSCs [53].

Preclinical and clinical research into monoclonal antibodies (mAbs) and IL-3-conjugated diphtheria toxin, Bi-specific T cell engager (BiTE), CD3 × CD123 dual-affinity re-targeting (DART), and anti-CD123 CAR-T therapies is undeway [54]. Another study successfully developed anti-CD123 mAbs capable of specifically binding to both the commercial and native antigens. The CD123-targeting mAbs can selectively target the CD123 antigen in patients with AML [55]. At present, there are three types of immunotherapy-based CD123-targeting drug that have been developed and evaluated in clinical trials: mAbs (such as CSL362 and KHK2823), antibody-dependent cellular cytotoxicity (ADCC) (e.g., SGN-CD123A and IMGN63) and antibody-toxin conjugates (e.g., SL-401) [1]. Therefore, CD123 has great potential as a target for clearance of LSCs.

Immune checkpoint molecules

Interesting checkpoint molecules have been detected on leukemic cells, such as PD-L1, CTLA4, and TIM-3. They are defined as ligand–receptor pairs with stimulatory or inhibitory effects on immune responses. Combinations of PD-L1 or PD1 inhibitors with hypomethylating agents are currently being tested in clinical trials in patients with AML [56]. However, AML LSC responses to these antibodies via the hypomethylation-induced upregulation of PD-L1 in all patients are variable. Moreover, most BRD4/MYC blockers, especially BRD4 degraders, also have strong direct antineoplastic effects on AML LSCs by the same mechanism [57]. TIM-3 and its ligand Galectin-9 have been shown to be crucial for the survival of AML LSCs. Blocking of the Galactin-9–TIM-3 interaction alone or in combination with other targeted drugs is currently being explored in clinical trials in AML [58]. CTLA-4 and CD28 are structurally related and both bind to CD80 and CD86. CTLA4 blockade is useful in the post-HSCT setting in AML, although it remains unknown whether this immune checkpoint molecule also has a role in LSC resistance in AML [1].

CD25

CD25, also known as the α chain of interleukin-2 receptor, is strongly expressed on activated T cells and regulatory T cells [59]. CD25 was reported as a potential target for LSC specific therapy because it is highly expressed in LSCs but not in normal HSCs. The safety of targeting CD25 was also demonstrated by the long-term multilineage hematopoietic reconstitution capacity of normal HSCs depleted of $CD25^+$ cells [60]. Additionally, DR5-specific TNF-related apoptosis-inducing ligand (TRAIL) peptide can be used in CD25-targeted therapy of chemotherapy-resistant LSCs [61].

Additionally, activated STAT5 is a major trigger of CD25 expression on various physiological and neoplastic cells, and BCR-ABL is known to induce STAT5 activation. The STAT5-targeting drug pimozide was found to decrease CD25 expression on $CD34^+/CD38^-$ CML LSCs [62]. A similar effect was obtained with BCR-ABL-targeting drugs. In particular, nilotinib and ponatinib were found to suppress the expression of CD25 on CML LSCs and KU812 cells [63]. By contrast, imatinib did not inhibit the expression of CD25 on CML LSCs. This discrepancy is best explained by intrinsic LSC resistance against imatinib, a phenomenon that might be associated with poor uptake of imatinib into LSCs or rapid efflux of the drug from LSCs after uptake [64]. In addition, STAT5 target genes are thought to have a role in CML disease evolution. Under physiological conditions, CD25 is only expressed on a few hematopoietic cell types, including activated T lymphocytes, activated B lymphocytes, and basophils. A remarkable observation is that, in patients with CML, abnormal expression of CD25 is restricted to LSCs [62]. In other words, although BCR-ABL and activated STAT5 are expressed in most clonal cells in patients with CML, the STAT5-dependent target gene *CD25* is only upregulated and expressed aberrantly on LSCs. Thus, CD25 could be used as an immunotherapy target using toxin conjugates. The elimination of preleukemic and LSCs could be an advantage of CD25-targeting therapy. Another approach would be to promote CD25 expression through inhibition of the PI3K-mTOR pathway [65].Therefore, LSCs can be eliminated through related pathways or in combination with CD25 inhibitors.

CD96

CD96 (also known as TACTILE) is a novel immune checkpoint receptor target with a crucial role in antitumor immune responses [66]. In addition, CD96 is presented on AML LSCs, which makes it a potential LSC-specific therapeutic target. Hosen et al. demonstrated that CD96 is expressed on most $CD34^+/CD38^-$ AML cells from diagnostic AML BM cells (74.0 ± 25.3% in 19 of 29 cases), whereas only a few (4.9 ± 1.6%) cells in the normal HSC-enriched population ($Lin^-CD34^+CD38^-CD90^+$) weakly expressed CD96. Moreover, by separating AML cells into $CD96^+$ and $CD96^-$ fractions and transplanting them into irradiated newborn $Rag2^{-/-}$ $\gamma c^{-/-}$ mice, the authors found that $CD96^+$ AML cells are enriched for LSC activity [67]. Collectively, these results indicate that AML LSCs can be distinguished from normal

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HSCs by the presence of CD96 expression. The engineering of hematopoietic progenitor cell (HPC) grafts by means of a CD96-specific antibody as well as its use in the clinic has the potential to improve the tolerability and efficacy of therapy in patients with AML [68].

In summary, CD96 is not expressed by the majority of cells in normal HSC-enriched populations, but is frequently expressed in AML LSC populations. Therefore, CD96 could be a specific target for eliminating LSCs (Table 1).

c-MPL

The receptor of thrombopoietin (TPO), c-MPL, is involved in regulating megakaryocyte development as well as the self-renewal and propagation of HSCs [69,70]. Yoshihara et al. identified c-MPL as a specific marker for long-term HSCs that has a role in the maintenance of HSC quiescence [71]. Chen et al. found that, during active Rac1-mediated leukemia initiation and maintenance, upregulation of c-MPL had an important role in the interaction of leukemia cells with the BM niche and contributed to quiescence and chemotherapy resistance in leukemia cells. In addition, analysis of c-MPL expression in primary human AML samples revealed that the expression of c-MPL in patients with AML, particularly CD34-positive AML, was significantly higher than that in normal donors [72].

In a study using the AML1-ETO9a (AE9a) mouse leukemia model that underwent chemotherapy, c-MPL$^+$ cells within Lin$^-$-c-Kit$^+$ leukemia cells were significantly enriched [73]. In addition, flow cytometry was used to detect the proportion of c-MPL-positive cells in BM samples from 29 patients with AML and seven controls. The results showed that the median percentage of c-MPL-positive cells in BM of patients with AML was 7.15 %, higher than that in control subjects (2.73%). In addition, the results showed that c-MPL is expressed in LSCs. It is speculated that, in the BM microenvironment of leukemia, TPO/c-MPL mediates the microenvironment, especially osteoblasts, to maintain the self-renewal and resting state of LSCs, thereby affecting their stemness [74]. Thus, targeting c-MPL could help eliminate LSCs.

Targeting related pathways

Canonical circadian pathway

Antileukemic effects are produced by targeting the *Bmal1* gene to destroy the canonical circadian pathway, causing impaired proliferation, enhanced myeloid differentiation, and LSC depletion [75]. In the hematopoietic system, the circadian clock regulates HSC egress from the BM microenvironment [76], hematopoietic engraftment [77], and BM mitotic activity [78]. Bmal1 is the core component of an autoregulatory loop that drives robust oscillations in gene expression to regulate circadian physiology [74,79]. Bmal1 and its heterodimeric partner Clock are functionally required for LSCs. This biological clock circuit has a role in both healthy HSCs and cancerous AML HSCs. Interestingly, however, disrupting this clock circuit effect impaired the activity of AML stem cells while leaving healthy HSCs relatively unharmed [80]. The researchers used a knockout model in which *Bmal1* was deleted only in blood cells. They found that, although AML stem cells required Bmal1 to grow, normal HSCs were able to survive even when *Bmal1* was not expressed [81]. Meanwhile, the presence of additional circadian regulators in leukemia cells raises the possibility that both positive and negative elements of the circadian transcriptional-translational feedback loop impact LSC function [80]. Thus, disrupting the circadian pathway by targeting of *Bmal1* might be an important way to treat leukemia.

Trans-chromatin regulatory pathway

Recent studies have found that only a subset of mutations contained in AML blasts were present in HSC-enriched cell fractions isolated from samples from patients with AML, and these cells were capable of nonleukemic differentiation [82]. Shlush et al. reported that the prehematopoietic stem cells of ancestral leukemia can regenerate the entire hematopoietic system and, at the same time, have a competitive multiplication advantage compared with nonleukemia HSCs, leading to clonal expansion [83]. These preleukemic HSCs are found in a high proportion of patients with AML who carry mutations in DNA methyltransferase 3A (DNMT3A) and isocitrate dehydrogenase 2 (IDH2), and unlike AML blasts, they survive induction chemotherapy and persist in the BM at remission, providing a potential reservoir for leukemic progression [83]. This finding is consistent with two mouse studies conducted by Challen et al. and Tadokoro et al., showing that HSCs lacking DNMT3A have a competitive growth advantage [82,84], and with a report by Kim et al. predicting that human DNMT3A mutations result in loss of function [85]. Some patients might have a reservoir from which relapse arises. If future phylogenetic single-cell lineage analysis establishes this possibility, then preleukemic HSCs should be directly targeted to prevent relapse. As new drugs are developed that effectively target mutations in *DNMT3A* or other genes that give rise to preleukemic HSCs (e.g., AG-221, an IDH2 inhibitor), there might be an opportunity to eradicate preleukemic HSC clones before the acquisition of additional mutations renders them more resistant to therapy. Duan et al.'s findings also support broadening the definition of minimal residual disease to include the posttherapy survival of not only AML blasts and LSCs, but also preleukemic HSCs [86]. In addition, Daniel et al. reported that the development of leukemia caused by *DNMT3A* mutation might be regulated by the *trans*-chromatin regulatory pathway [86]. Therefore, it might be possible to target DNMT3A BiTE via the *trans*-chromatin regulatory pathway.

PTEN-C/EPBα-CTNNA1 molecular pathway

The evolutionarily highly conserved PTEN-C/EPBα-CTNNA1 signal pathway controls the development of HSCs and the malignant transformation of LSCs [87]. There is some evidence that *CTNNA1* is a potential tumor suppressor gene in LSCs. One of its alleles is inactivated by the deletion of genome fragments, whereas the other is affected by epigenetic mechanisms (DNA methylation and histone deacetylation) inhibition. Given the limited therapeutic effects of DNA methylase inhibitors and histone deacetylase inhibitors, further searches for the epigenetic mechanism of *CTNNA1* inactivation and upstream signal regulation pathways will be necessary to reopen the genes in LSCs [88]. Fu et al. found that the PTEN-mTOR signal transduction pathway acts on the upstream region of the PTEN-C/EPBα-CTNNA1 axis; the authors also determined the ratio of wild-type p42C/EPBα

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to dominant negative p30C/EPBα at the translation level [87]. The low ratio of p42/p30 causes p30C/EPBα to bind preferentially to the proximal promoter of *CTNNA1*, and to recruit PRC2 protein complexes containing EZH2, EED, and SUZ12 to mediate the trimethylation of histone H3 at the 27th lysine residue (H3K27me3) and transcription inhibition [89]. By contrast, a high ratio of p42/p30 causes p42C/EPBα to bind to the proximal promoter element of *CTNNA1*, and activates CTNNA1 transcription by promoting H3K4 trimethylation (H3K4me3) modification. In addition, in the BM of PTEN-knockout mice and PTEN-knockout zebrafish embryos, the protein levels of wild-type C/EPBα and α-catenin were significantly downregulated [87]. More importantly, some studies found that ~20% of patients with myeloid leukemia have low expression of *CTNNA1* in LSCs and, within this LSC subpopulation, only PTEN or CEBPA frameshift mutations have been detected. These important leukemia suppressor genes regulate epigenetic mechanisms through a highly evolved signal transduction axis to control the development of HSCs and the malignant transformation of LSCs [90]. The discovery of the tumor suppressor axis of LSCs also provides important clues for targeted therapy of leukemia, especially for patients with low *CTNNA1* expression [89]. Thus, in summary, it might be possible to improve the inhibitory effect of LSCs via the PTEN-C/EPBα-CTNNA1 signaling pathway.

The PI3K/AKT/mTOR signaling pathway

The inhibition of the PI3K/AKT/mTOR pathway has been reported to have beneficial therapeutic effects in leukemia. This pathway involves many cellular functions, including protein synthesis, cell cycle progression, cell survival, apoptosis, angiogenesis, and drug resistance [91]. Related studies have shown that drugs targeting key molecules, such as PI3K, AKT, or mTOR, have a beneficial role in killing LSCs. The PI3K/mTOR inhibitor NVPBEZ235 can enhance the sensitivity of CML LSCs and progenitor cells to nilotinib and enhance their cytotoxicity in BCR/ABL mutant cells resistant to TKIs [92]. In some patients with CML, PTEN protein deletion and subsequent AKT activation are accompanied by a side population (SP) phenotype rather than by ATP-binding cassette subfamily G member 2 (ABCG2) expression during accelerated/blast crisis (AP/BP). These results suggest that PTEN regulates the expression of ABCG2 and SP via the PI3K/AKT pathway, which provides a new strategy for the targeted therapy of LSCs [93]. In addition, cyclin-dependent kinases (CDKs) are crucial regulators of cell cycle progression. Research has shown that CDK6 is involved in the activation of LSCs [94]. At the same time, LSCs are more dependent on CDK6 than are normal stem cells, and CDK6 might be related to the PI3K pathway [94]. In summary, the development of LSCs might be inhibited by targeting the PI3K/AKT/mTOR signaling pathway.

JAK2-STAT3 pathway

The JAK/STAT3 pathway can lead to aberrant cell survival [95]. In addition, some studies have shown that, after blocking YTHDF1 and 2, the expression of JAK2 and suppressor of cytokine signaling 3 protein (SOCS3) changes, leading to inhibition of the JAK2-STAT3 pathway [96].

The mRNA N6-methyladenosine (m^6A) modification reader YTHDF2 is highly expressed in a range of human AMLs. YTHDF2 reduces the half-life of various m6A transcripts, which contribute to the overall integrity of LSC function, including tumor necrosis factor receptor Tnfrsf2, which is upregulated in Ythdf2-deficient LSCs and initiates apoptosis. Interestingly, YTHDF2 is not necessary for normal HSC function, and lack of YTHDF2 increases the activity of HSCs [97]. Ng et al. found that the expression of YTHDF2 in AML samples with different cytogenetic abnormalities was significantly higher than that in nonleukemia control groups, and YTHDF2 protein was highly expressed in primary AML samples. They also found that the expression of YTHDF2 was related to LSC activity [96]. Wu et al. found that inhibition of YTHDF2 specifically impaired the development and reproduction of LSCs, and proved that targeting Ythdf2 protein can enlarge HSCs and enhance their myeloid remodeling [97]. These are the unique characteristics of YTHDF2, coupled with the permissible loss of YTHDF2 in adult mice, which highlights the therapeutic potential of inhibiting YTHDF2 as a therapeutic strategy for AML. This intervention has the dual advantages of eradicating malignant HSCs and giving normal HSCs a competitive advantage. Given that the isolation of a sufficient number of HSCs is a limiting factor for the use of HSC transplantation in a variety of diseases, this challenge can be avoided by inhibiting the expansion of HSCs by YTHDF2 *in vitro* or *in vivo*. To reveal why the loss of YTHDF2 is associated with weak leukemic potential, Paris et al. found that transcripts negatively associated with YTHDF2 expression were highly associated with the loss of leukemic potential [98]. In this way, when AML samples express a small amount of YTHDF2, transcripts associated with loss of leukemia potential are more expressed. By contrast, transcripts related to YTHDF2 expression are depleted from transcripts associated with weak LSC activity. Therefore, YTHDF2 negatively regulates its expression and restricts the transcription of LSC activity [99]. In conclusion, inhibition of YTHDF2 might have a key role in the treatment of leukemia.

Wnt/JAK2 signaling pathway

Adenosine deaminase acting on RNA1 (ADAR1) might participate in the proliferation of AML cells by regulating the Wnt or JAK2 signaling pathways [100]. In addition, in a humanized BC CML mouse model, combined JAK2 and BCR-ABL1 inhibition prevented LSC self-renewal commensurate with ADAR1 downregulation. Lentiviral ADAR1 wild-type, but not an editing-defective ADAR1E912A mutant, induced self-renewal gene expression and impaired the biogenesis of stem cell regulatory let-7 miRNAs. Combined RNA sequencing, qRT-PCR, CLIPA-DAR1, and pri-let-7 mutagenesis data suggest that ADAR1 promotes LSC generation via let-7 pri-miRNA editing and LIN28B upregulation [100]. A small-molecule tool compound antagonizes the effect of ADAR1 on LSC self-renewal in stromal co-cultures and restores let-7 biogenesis. Thus, ADAR1 activation represents a unique therapeutic vulnerability in LSCs with active JAK2 signaling. In addition, the highly activated ADAR1 editing system edited the let-7 miRNA. Ultimately, this activity increased the ability for cell regeneration or self-renewal, transforming leukocyte precursors into LSCs. LSCs promote the production of an aggressive drug-resistant CML during the acute

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transformation phase. This was the first mechanistic association between proinflammatory signals and RNA editing-driven reprogramming of precursor cells into LSCs. After understanding how the ADAR1 editing system works, the Jamieson team looked for a way to stop it. By using small-molecule compounds to inhibit sensitivity to inflammatory signals or to inhibit ADAR1, the researchers were able to counteract the effects of ADAR1 on the self-renewal of LSCs and, thus, restore the let-7 effect [101]. When a small molecule called 8-Aza was used to treat acute transformation CML stem cells, the self-renewal capacity of these cells decreased by ~40% compared with untreated CML stem cells [99,100]. Thus, ADAR1 could be used as an effective target for the clearance of LSCs.

Targeting the microenvironment of LSCs

CXCL12

C-X-C motif chemokine ligand 12 (CXCL12)-expressing cells include MSCs, endothelial cells, osteoprogenitors, and osteoblasts [102]. There is a key role for niche-specific effects of CXCL12 expression in maintaining the quiescence of TKI-resistant LSC populations of CML, given that CXCL12 is expressed in BM and controls the maintenance of HSCs. In a mouse experiment, Bhatia and colleagues [103] found that the removal of CXCL12 from MSCs, rather than CXCL12 from the other three types of BM microenvironment cell (CXCL12-abundant reticular cells, endothelial cells, and osteoblasts), promoted the development of leukemia and reduced the survival rate of mice. These results were related to the increase in cell cycle activity and expansion of CML LSCs. However, circulating LSCs become sensitive to TKI therapy, resulting in an increase in their elimination. BM imaging studies showed that the recombinant stromal cells colocated with LSCs in the discrete area of BM. When CXCL12 was deleted, the colocalized regions of these MSCs and LSCs disappeared, which further supported the importance of CXCL12 expression in maintaining the LSC niche. These results suggest that MSCs expressing CXCL12 have a role as specific regulatory regions in maintaining resting, treatment-resistant LSCs in BM. Compared with MSCs, the loss of CXCL12 expression of endothelial cells in the BM microenvironment led to a decrease in the number of CML LSCs and prolonged the survival rate of mice. This suggests that endothelial cells expressing CXCL12 contribute to the maintenance of LSCs. Therefore, the cellular regions with MSCs and endothelial cells expressing CXCL12 have different regulatory effects on LSCs. Given the diverse functions of different CXCL12-expressing cells, targeting of specific LSC interactions with CXCL12-expressing mesenchymal progenitor niches, or downstream effectors of these interactions, might be preferable [104]. In summary, CXCL12 regulates leukemic the microenvironment and, thus, has potential as a target for clearance of LSCs.

Niche

A study found that acute lymphoblastic leukemia LSCs construct a new BM microenvironment (niche) and build a 'temporary shelter' to escape chemotherapy [105]. Relapse easily occurs because chemotherapy is unable to fully remove LSCs from the BM. Therefore, understanding what exactly occurs in the BM following the start of chemotherapy is vital for the development of new strategies for leukemia treatment. Mendez-Ferrer et al. found that, after chemotherapy, LSCs secrete some cytokines, 'recruit' and transform BM MSCs, and establish a 'temporary shelter' to escape the chemotherapy [105]. They also found that this 'temporary shelter' initially comprised Nestin-positive MSCs, and later became α-SMA positive. Therefore, this newly built refuge was called the NSM microenvironment (NSM niche). Interfering with the formation or protective function of the NSM niche can significantly improve the effect of chemotherapy and remove the remaining LSCs in the BM. On this basis, the presence of the NSM niche has been detected in samples from patients with leukemia whose disease cannot be relieved or is only partially relieved by chemotherapy; they are not detected in the samples of patients with leukemia in complete remission, thus confirming the above research results from the disease model [106]. The researchers suggested that, clinically, the existence of the NSM niche can be detected to determine the prognosis of patients [106]. Given that the effect of chemotherapy can be improved by interfering with the formation and function of the NSM niche, the latter might be a suitable drug target for eliminating LSCs.

Concluding remarks and perspective

From targeting of the survival microenvironment of LSCs, to surface markers and related pathway controlling LSCs, targeted treatment of LSCs has produced several research hotspots with potential future development. Targeted treatment of LSCs can fundamentally reduce the recurrence rate and improve both survival and cure rates for patients. Targeted treatment of LSCs can also reduce the adverse effects of drugs while improving drug efficacy and patient compliance. Thus, we could achieve the targeted treatment of leukemia by preparing mAbs against specific surface antigens, toxin conjugates, small-molecule drugs and inhibitors targeting LSCs.

Declaration of interests

All authors declare that there is no conflict of interest.

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