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Exploiting Unique Biological Features of Leukemia Stem Cells for Therapeutic Benefit

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ABSTRACT

Cancer stem cells play a critical role in disease initiation and insensitivity to chemotherapy in numerous hematologic malignancies and some solid tumors, and these stem cells need to be eradicated to achieve a cure. Key to successful targeting of cancer stem cells is to identify and functionally test critical target genes and to fully understand their associated molecular network in these stem cells. Human chronic myeloid leukemia (CML) is well accepted as one of the typical types of hematopoietic malignancies that are derived from leukemia stem cells (LSCs), serving as an excellent model disease for understanding the biology of LSCs and developing effective, selective, and curative strategies through targeting LSCs. Here, we discuss LSCs in CML with a focus on identification of unique biological features of these stem cells to emphasize the feasibility and significance of specific targeting of LSCs while sparing normal stem cell counterparts in leukemia therapy. STEM CELLS TRANSLATIONAL MEDICINE 2019;00:1–7

SIGNIFICANCE STATEMENT

Molecular mechanisms by which leukemia stem cells (LSCs) survive and self-renew are poorly understood, and an effective anti-LSC therapeutic strategy for chronic myeloid leukemia is yet to be developed. In discussing the establishment of anti-LSC methods in the present study, much attention has been paid to the identification of fundamental biological differences between LSCs and normal hematopoietic stem cells (HSCs) with a goal of eradicating LSCs specifically to avoid or minimize unwanted cytotoxic side effects on normal HSCs. The authors hope to provide convincing arguments to emphasize that it is feasible to specifically target LSCs while sparing normal HSCs.

INTRODUCTION

Cancer stem cells are believed to be associated with cancer initiation and insensitivity to chemotherapy in numerous hematologic malignancies and some solid tumors involving the breast, brain, pancreas, colon, lung, and prostate, and need to be eradicated for achieving a cure [1–9]. Although the cancer stem cell theory cannot be used to explain the pathological features of all types of cancers, it has become clear that some major forms of human hematopoietic malignancies such as chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) are derived from leukemia stem cells (LSCs) that are responsible for leukemia initiation, progression, and relapse [10]. To develop effective and curative anti-stem-cell strategies, CML and AML are good model diseases for understanding the molecular biology of LSCs, and a key initial step is to identify and functionally test critical target genes and the molecular pathways they communicate with in LSCs. In this article, we intend to focus on CML because we have more direct evidence showing the biology of LSCs and their insensitivity to tyrosine kinase inhibitors (TKIs), the first-line treatments for CML patients.

LSCs are leukemia-initiating cells with the capacity to self-renew, differentiate, and remain in a state of quiescence [1, 2]. In CML, a myeloproliferative disease that originates from an abnormal hematopoietic stem cell (HSC) harboring the Philadelphia chromosome (Ph$^+$) [11], functional LSCs in mice reside in a cell population that does not express cell lineage markers but express both c-Kit and Sca-1 (Lin$^-$c-Kit$^+$Sca-1$^+$, LSK) [12], recapitulating the cell surface markers expressed on normal HSCs. LSCs in human CML also reside in the HSC population [13], displaying phenotypically Lin$^-$CD34$^+$CD38$^-$CD90$^+$ with some specific surface markers such as interleukin-1 receptor accessory protein (IL1RAP) and CD26 [14, 15].

At a molecular level, gene expression profiling studies using leukemia mice and human patient samples have shown some dramatic changes in

gene expression of LSCs [16]. These findings help to lay a foundation for characterizing LSCs for the treatment of hematopoietic malignancies. However, a challenging question still remains: are there fundamental differences between LSCs and their normal stem cell counterparts at a molecular level? In other words, can we specifically target LSCs while sparing normal stem cells when treating leukemias? To answer this question, we need to identify and test key target molecules/genes that are solely or more specifically required for survival and proliferation by LSCs in CML. Although eradication of LSCs in the treatment of CML patients is yet to be achieved, we believe that for therapeutic benefit, it is critical to identify unique biological features of LSCs for developing effective strategies aiming to kill LSCs while protecting normal HSCs with a hope of curing CML. In this article, we will pay much attention to discussing the potential strategies for targeting LSCs more specifically.

BIOLOGICAL FEATURES OF LSCS

With self-renewal and multipotency at the hub of what defines a LSC (Fig. 1), the major focus of current and future research should be on studying the biology of LSCs with a goal of fully understanding the underlying molecular and cellular processes.

Leukemia Stem Cells Display Unique Cellular State

The developmental processes and biological characteristics of normal HSCs have been extensively investigated in the past decades. It is commonly accepted that normal HSCs are largely in a state of quiescence with autophagy-dependent, glycolytic, and tightly controlled levels of protein synthesis [17–20]. Leukemogenesis occurs because of the serial genetic and epigenetic alterations that transform normal HSC/progenitor cell into LSCs [21, 22]. This transformation changes the steady
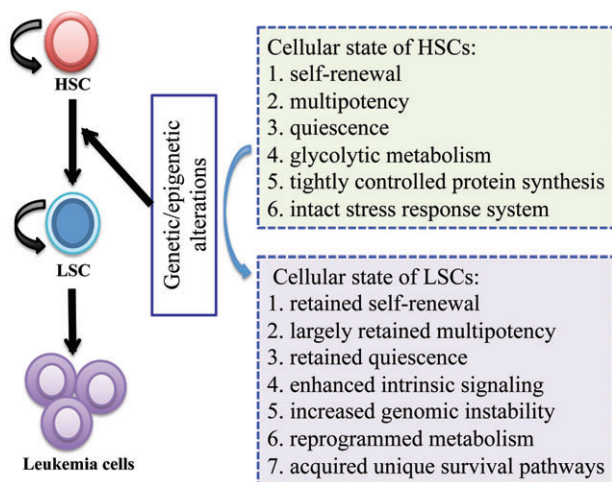

Figure 1. Biological properties of normal HSCs and LSCs. Normal HSCs have three major biological features or stem cell properties: self-renewal, multipotency, and quiescence. After acquiring genetic lesions, HSCs undergo cellular transformation to become LSCs that retain the major stem cell properties of HSCs with enhanced signaling activities and also acquire some unique biological features. These biological features define the cellular states of HSCs and LSCs, and provide opportunities to develop strategies for specifically targeting LSCs while sparing normal HSCs. Abbreviations: HSC, hematopoietic stem cell; LSC, leukemia stem cell.

cellular state of normal HSCs. Using CML as an example, the molecular evolution of CML LSCs initiates from the formation of reciprocal translocation between chromosomes 9 and 22, leading to generation of the BCR-ABL oncogene in a HSC and subsequent expansion of myeloid progenitors [23]. As a result, kinase activity of BCR-ABL tyrosine kinase is constitutively activated, causing uncontrolled activation of some growth-related signaling pathways such as Wnt/β-catenin [24], hedgehog [25], JAK/STAT [26], Hif1a [27, 28], TGFβ-FOXO [29], etc. These intrinsic genetic and signaling changes increase the abilities of CML LSCs in self-renewal, resistance to apoptosis, and genomic instability [30]. However, these pathways also play important roles in normal development, and when searching for potential therapeutic targets in LSCs, we suggest that we should pay more attention to the genes that are more specifically required for survival, self-renewal, and proliferation of LSCs.

Besides inheriting common stem cell characteristics, LSCs also have some unique functional changes, as exemplified by LSCs that undergo reprogrammed cellular metabolism, a hallmark of cancers [31]. Fatty acid metabolism enzyme stearoyl-CoA desaturase (Scd1) is an endoplasmic reticulum enzyme in a family of Δ9-fatty acid desaturase isoforms and catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids, which are the most abundant fatty acids present in mammalian organisms [32]. Fatty acid synthesis has been found to be associated with tumorigenesis and tumor progression [33]. However, we found that Scd1 is downregulated in LSCs and plays a tumor-suppressive role in LSCs with no notable inhibitory effect on normal HSCs [34], suggesting a cell-content-dependent role of fatty acid in cancer. In addition, BCAT1, a cytosolic aminotransferase for branched-chain amino acids is aberrantly activated and functionally required for AML LSCs [35]. It also plays an essential role in the progression of CML chronic phase to blast crisis through induction of cell differentiation arrest [36]. Furthermore, a metabolic analysis on both stem-cell-enriched (CD34$^+$ and CD34$^+$CD38$^-$) and differentiated cells (CD34$^-$) derived from CML patients reveals that CML LSCs rely on upregulated oxidative metabolism for their survival [37]. Compared with differentiated CML cells, LSCs show an increase in glycerol-3-phosphate, carnitine, acylcarnitine derivatives, and a decrease in free fatty acid such as oleic and stearic acids. Another example for the functional changes in LSCs is that Alox5, a lipid-metabolic gene encoding the arachidonate 5-lipoxygenase, is required for survival of CML LSCs and essential for CML development [38].

Heterogeneity of LSCs

Cellular heterogeneity is one of well-recognized characteristics of both normal HSCs and LSCs. With respect to the clonal heterogeneity of differentiation and self-renewal properties in normal HSCs, two distinct subtypes of HSCs (lymphoid-deficient and lymphoid-myeloid-balanced) have been identified and distinguished by assessing the contributions of individual HSCs to the circulating cell lineages in serial transplantation experiments [39, 40]. Also, the post-transplant clonal analysis of HSC expansion suggests that both HSC subtypes display an extensive but variable self-renewal activity with occasional interconversion [40]. Similarly, heterogeneity of LSCs has been recognized. Using the SCL-tTA/BCR-ABL mouse model of CML, a recent study reveals that long-term repopulation and leukemia-initiating capacity of LSCs after transplantation is restricted to

BCR-ABL-expressing long-term HSCs (LT-HSCs) with remarkable heterogeneity [41]. This heterogeneity of BCR-ABL-expressing LT-HSCs is determined based on comparing the global gene expression between leukemic and nonleukemic LT-HSCs by RNA sequencing. A higher level of MPL expression is found in some leukemic LT-HSCs with enhanced JAK/STAT signaling and cell proliferation in response to stimulation of the thrombopopoietin (TPO) receptor MPL [41]. In contrast, BCR-ABL-expressing LT-HSCs with low MPL expression show a reduced response to TPO-induced JAK/STAT signaling and decreased leukemogenic potential, suggesting that this subtype of LSCs may be insensitive to inhibition by JAK inhibitors. Therefore, this study identifies MPL expression levels as a key determinant of heterogeneous leukemia-initiating capacity of LSCs in CML [41]. Importantly, the heterogeneity of LSCs is thought to contribute to leukemia initiation, progression, and relapse. It has been reported that residual BCR-ABL^{+} stem cells persist in some CML patients who have maintained long-term remission, and after discontinuing the treatment with a TKI, molecular relapse occurs in a significant number of CML patients [12, 13, 42–45]. The discrepancies in leukemogenic potential between MPL-high and MPL-low LSCs could be explained by the heterogeneity of CML LSCs, which likely reflects uniqueness of LSCs determined by the intrinsic molecular machinery or extrinsic microenvironment.

INSENSITIVITY OF LEUKEMIA STEM CELLS TO DRUG THERAPY AND POSSIBLE MECHANISMS

BCR-ABL TKIs including imatinib mesylate (Gleevec, Novartis) are highly effective in controlling chronic phase CML, but they fail to eradicate leukemia-initiating cells or LSCs in CML mice [12] and patients [13, 46, 47]. Clinically, a complete and sustained molecular remission (undetectable levels of BCR-ABL transcripts) is difficult to attain even after a complete cytogenetic remission achieved through imatinib treatment [48–51], suggesting that imatinib and probably other BCR-ABL kinase inhibitors can effectively kill highly proliferating leukemia cells but are incapable of eradicating LSCs for cure. An anti-LSC strategy other than the use of a TKI alone needs to be developed to eradicate LSCs, and the success of this approach relies on uncovering the underlying mechanisms by which LSCs survive drug therapy (Fig. 2).

LSCs Are Insensitive to Inhibition by TKIs

TKIs have become first-line drugs in treating CML, and the majority of patients achieve a complete hematological response [52–55]. However, the fact that clinical relapse occurs in a significant number of CML patients once treatment is interrupted [56] indicates that CML LSCs are insensitive to drug therapy. In support of this idea, cells from CML patients in chronic phase
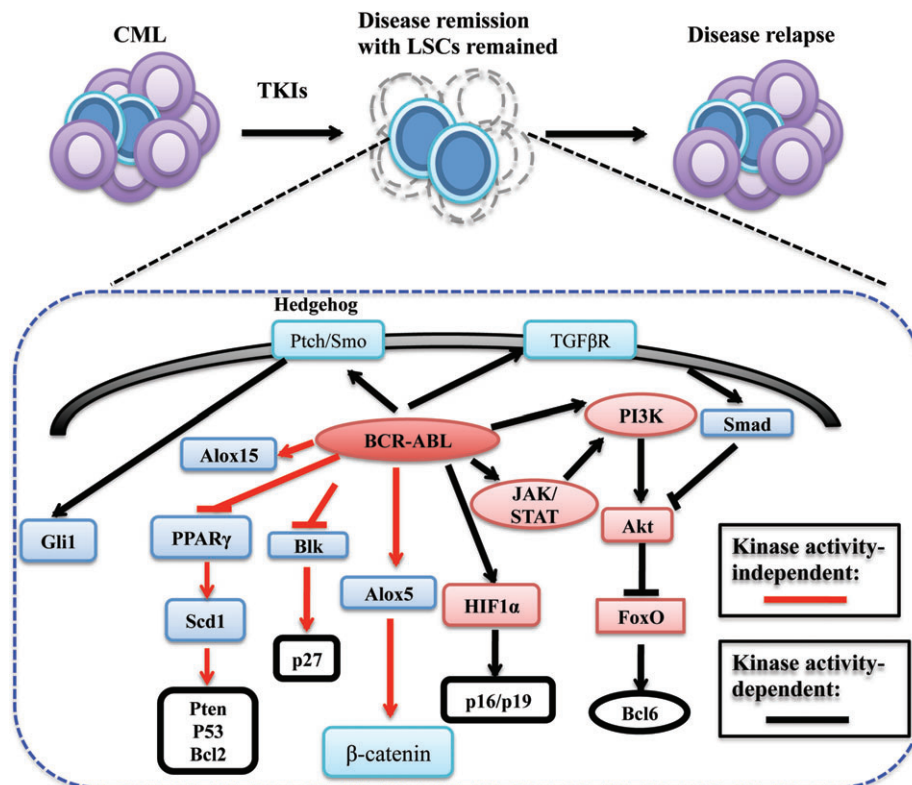

Figure 2. BCR-ABL kinase activity-dependent and kinase activity-independent pathways. TKIs are effective in controlling chronic phase CML, resulting in clinical remission in the majority of CML patients. However, LSCs are insensitive to inhibition by TKIs, as their survival is not dependent on BCR-ABL kinase activity. Besides altering signaling pathways in LSCs through its kinase activity, BCR-ABL activates or inhibits some survival- or growth-related pathways in a kinase-activity-independent manner. In other words, BCR-ABL kinase activity-independent pathways are not completely shut down by TKIs and must be targeted to inhibit or kill LSCs. In addition, some of these BCR-ABL kinase activity-independent pathways, including Alox5, Blk, and Scd1, are uniquely required by LSCs for survival and proliferation, serving as potential targets for eradicating LSCs. Abbreviations: Blk, B lymphocyte kinase; CML, chronic myeloid leukemia; LSC, leukemia stem cell; Scd1, stearoyl-CoA desaturase 1; TKI, tyrosine kinase inhibitor.

were labeled with carboxy-fluorescein diacetate succinimidyl diester to track cell division, and imatinib treatment caused eradication of almost all dividing CD34$^+$ cells, but the nonproliferating quiescent cells remained [13]. In addition, BCR-ABL$^+$CD34$^+$ cells persisted in CML patients who achieved complete cytogenetic response with imatinib treatment [57]. Furthermore, although treatment with TKIs dramatically prolonged the survival of CML mice, the mice eventually died of this disease [12], indicating the failure of TKIs to completely eradicate leukemia cells. The incomplete therapeutic response of CML cells to TKI inhibition in mice is related to the inability of imatinib to eradicate LSCs [58]. Together, these studies indicate that CML LSCs are insensitive to TKI treatment, prompting us to provide a mechanistic explanation for TKI resistance of LSCs. It should be pointed out that the TKI resistance of LSCs we discuss here is not relevant to TKI-resistant BCR-ABL kinase domain mutations.

LSC Survival Is Not Dependent on BCR-ABL Kinase Activity

The failure of TKIs to completely eradicate CML LSCs suggests that survival of these LSCs is not dependent on BCR-ABL kinase activity. We provided a biochemical evidence showing that dasatinib, a second-generation TKI, inhibits BCR-ABL phosphorylation in BCR-ABL-expressing HSCs but fails to kill these stem cells [12], suggesting that LSCs likely use BCR-ABL kinase activity-independent pathways for survival. Similarly, BCR-ABL kinase activity is inhibited by TKIs in CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ cell populations from newly diagnosed CML patients, and phospho-CRKL, which is stimulated by BCR-ABL kinase activity, is reduced upon inhibition of BCR-ABL kinase activity as detected by immunoblots of sorted quiescent (Ki67$^-$) and cycling (Ki67$^+$) cells [46]. Additionally, in human CML CD34$^+$ cells cultured in serum-free media and treated with dasatinib, phospho-CRKL is completely inhibited by dasatinib, but the abilities of proliferation and self-renewal of the cells are retained [44]. These results demonstrate that the insensitivity of CML LSCs to inhibition by TKIs is not due to the inability of TKIs to inhibit BCR-ABL kinase activity in LSCs. It is likely that BCR-ABL also activates other signaling pathways in a kinase activity-independent manner, and it will be critical to identify and test these pathways in survival regulation of LSCs.

BCR-ABL Kinase Activity-Independent Pathways in LSCs

As described above, compared with proliferative leukemia cells, CML LSCs are much less sensitive to inhibition by TKIs even in the absence of BCR-ABL kinase domain mutations that cause TKI resistance. We believe that when its kinase activity is suppressed by TKIs, BCR-ABL can still activate some pathways that render CML LSCs insensitive to TKI inhibition. As a result, the cells continue to survive, whereas BCR-ABL kinase activity is inhibited by TKIs, indicating that this TKI-insensitive pathway activated by BCR-ABL must be targeted to lead to eradication of LSCs. This idea is supported by the essential role of Alox5 in survival regulation of CML LSCs [38]. We show that Alox5 is upregulated by BCR-ABL and essential for CML development, but this upregulation is not reduced by TKI treatment. These results provide a mechanistic explanation for why CML LSCs is insensitive to inhibition of BCR-ABL kinase activity by TKIs even in the absence of BCR-ABL kinase domain mutations. Thus, Alox5 represents a unique pathway that cannot be shut down upon kinase inhibition by TKIs in BCR-ABL signaling and plays a

critical role in mediating TKI resistance in LSCs. Another example is that B lymphocyte kinase is significantly downregulated by BCR-ABL in a kinase activity-independent manner, and this pathway plays a tumor-suppressive role in regulating the survival of CML LSCs [59]. Again, the abovementioned intrinsic mechanism provides one explanation for the insensitivity of LSCs to TKIs. It should be mentioned that some studies also suggest that TKI resistance of LSCs is related to receiving extrinsic signals from bone marrow niche with which LSCs interact [60]. Further research in this area will be beneficial for developing new strategies for eradicating LSCs.

STRATEGIES FOR TARGETING LSCs

It is obvious that one of the best strategies for inhibiting LSCs is to target the key genes that are required for survival regulation of LSCs but not normal HSCs. It may also be acceptable that as a potential anti-LSC target, a candidate gene is required more by LSCs than by normal HSCs, providing a therapeutic window for inhibiting LSCs more specifically. In other words, the unique biological features of LSCs provide better opportunities for specifically targeting LSCs while sparing normal stem cell counterparts.

Targeting Critical Molecular Pathways of LSCs

In CML, some genes have been shown to be involved in survival regulation of LSCs, including *Wnt/β-catenin* [24, 58], *Hedgehog* [25], *Bim-1* [61, 62], *p53* [63], *p16^{INK4a}* [64], *p19^{ARF}* [65], *Pten* [66], *PML* [67], *PP2A* [68], *TGF-β/FOXO* [29], *Musashi* [69], *Alox5* [38], *SIRT1* [70], *Alox15* [71], and *Hif1a* [27]. However, only some of these studies emphasize specific targeting of LSCs, although it is hoped that the target genes required for both LSCs and normal HSCs would only produce tolerable side effects after normal HSCs are inhibited to a certain degree. In fact, several chemical inhibitors against these targets have been developed and studied. For example, pharmacological blockade of Hedgehog signaling by clinical-grade SMO inhibitors (such as GDC-0449 and LDE225) [25, 72–74], inhibition of the TGFβ-FoxO pathway by Ly364947 [29], inactivation of BCL6 by the retro-inverso BCL6 peptide inhibitor RI-BPI [75], and suppression of autophagy by pharmacological inhibitors [76] have been shown to inhibit CML development by inhibiting LSCs. Inhibition of the HIF1α pathway by echinomycin is also effective in suppressing LSCs [27, 77]. It will be important to further evaluate these inhibitors for their clinical benefit in treating leukemia patients.

We have been focusing on identification of target genes uniquely or more specifically required for cellular functions by LSCs but not normal HSCs. In fact, we have identified Alox5 as a key gene that regulates the function of LSCs but not normal HSCs, because Alox5 deficiency or inhibition of function of this gene impairs survival and self-renewal of LSCs and prevents the initiation of BCR-ABL-induced CML with no significant inhibitory effect on normal HSC function [38]. Additionally, Scd1 plays a tumor-suppressive role specifically in LSCs, and we and others have tested and shown the inhibitory or apoptotic effect of PPARγ agonists on CML LSCs [34, 78]. Mechanistically, LSC apoptosis induced by the PPARγ agonist rosiglitazone is associated with an increased expression of Scd1, Pten, and p53 [34]. Furthermore, deficiency of Alox15 and inhibition of Alox15 function lead to remarkable inhibition

of LSCs with much less effect on normal HSCs in CML mice [71]. Finally, it has been recently shown that simultaneous targeting of P53 (by blocking its degradation) and c-MYC (by suppressing its transcription) has more dramatic inhibitory effect on CD34$^+$ cells from CML patients than on normal CD34$^+$ cells [79]. Taken together, these results support our belief that it is realistic and approachable to identify and target critical molecular pathways that play an essential role more specifically in LSCs. In other words, it is possible to develop new therapeutic strategies aiming to specifically eradicate LSCs while sparing normal HSCs.

Targeting Epigenetic Properties of LSCs

Besides acquiring genetic lesions, LSCs also undergo epigenetic changes. Targeting of epigenetic regulators has recently shown to be effective in eliminating CML LSCs. EZH2, the catalytic subunit of polycomb repressive complex 2, is overexpressed in CML LSCs [80, 81], which is associated with extensive reprogramming of H3K27me3 targets in the cells. Genetic inactivation of EZH2 in conventional conditional knockout mice and through CRISPR/Cas9-mediated gene editing reduces survival of LSCs and prolongs survival of CML mice [80]. An EZH2-specific inhibitor promotes apoptosis of LSCs from CML patients without impairing normal HSCs, which is more predominant when the combined treatment with an EZH2 inhibitor and a TKI is used [81]. These findings suggest a promising epigenetic-based therapeutic strategy for more specifically targeting LSCs.

Targeting LSCs Using Antibodies Against Cell Surface Antigens

Although cell surface markers expressed on CML LSCs and normal HSCs are similar, the levels of expression for some markers are much higher in LSCs than in HSCs, providing an opportunity for preferentially targeting LSCs using antibodies. For example, a gene-expression profiling study in CML CD34$^+$ cells and cord blood CD34$^+$ cells transduced with retroviral BCR-ABL showed that expression of IL1RAP is upregulated in the cells [82]. In this study, normal (Ph$^-$) and leukemic (Ph$^+$) cells within the CML CD34$^+$CD38$^-$ cell compartment were distinguished by fluorescence in situ hybridization, and the results showed that the CML CD34$^+$CD38$^-$ IL1RAP$^+$ cells were Ph$^+$, whereas CML CD34$^+$CD38$^-$IL1RAP$^-$ cells were almost exclusively Ph$^-$. Furthermore, a long-term culture-initiating cell assays showed that Ph$^+$ and Ph$^-$ candidate CML stem cells could be prospectively separated based on IL1RAP expression, and an anti-IL1RAP antibody could be used as a target on CML CD34$^+$CD38$^-$ cells to induce antibody-dependent cell-mediated cytotoxicity. Another example is CD33 that was found to have a much higher expression in CD34$^+$CD38$^-$CD123$^+$ cells from CML patients than in normal CD34$^+$CD38$^-$ stem cells [83]. Interestingly, colony formation and long-term culture-initiating cell assays showed that the CD33-targeting drug germtuzumab/ozogamicin produced growth inhibition of leukemic progenitor cells. These studies support a strong scientific premise for targeting CML LSCs using antibodies against cell surface antigens. Other examples include expression of cell surface molecules that are linked to signaling pathways in LSCs. In particular, CD25, a STAT5-dependent cell surface marker, regulates the growth of CML LSCs, which is associated with the PI3K/mTOR pathway [84, 85]. It is hopeful that CD25 could be a legitimate target for eradicating CML LSCs.

CONCLUSION

A full understanding of biology of LSCs allows exploiting the critical differences between LSCs and normal HSCs at a molecular level. This approach will subsequently lead to identification of unique biological features of LSCs for developing effective therapeutic strategies aiming to target LSCs specifically while sparing normal HSCs. Although there are still some difficult hurdles to cross, we believe that we are much closer to applying anti-LSC strategies for achieving durable disease remission or even a cure. However, the reality is that an effective anti-LSC therapy is yet to be developed, implying difficult challenges we are facing. Based on the recent scientific advances made in the LSC field, it is hopeful that we begin to understand how LSCs use unique molecular pathways to maintain their abilities of survival and self-renewal, which will lead to future clinical trails for testing new anti-LSC strategies.

AUTHOR CONTRIBUTIONS

H.Z., S.L.: manuscript writing. H.Z. and S.L.: contributed to the final revision and approval of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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