Identification and targeting leukemia stem cells: The path to the cure for acute myeloid leukemia

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Abstract

Accumulating evidence support the notion that acute myeloid leukemia (AML) is organized in a hierarchical system, originating from a special proportion of leukemia stem cells (LSC). Similar to their normal counterpart, hematopoietic stem cells (HSC), LSC possess self-renewal capacity and are responsible for the continued growth and proliferation of the bulk of leukemia cells in the blood and bone marrow. It is believed that LSC are also the root cause for the treatment failure and relapse of AML because LSC are often resistant to chemotherapy. In the past decade, we have made significant advancement in identification and understanding the molecular biology of LSC, but it remains a daunting task to specifically targeting LSC, while sparing normal HSC. In this review, we will first provide a historical overview of the discovery of LSC, followed by a summary of identification and separation of LSC by either cell surface markers or functional assays. Next, the review will focus on the current, various strategies for eradicating LSC. Finally, we will highlight future directions and challenges ahead of our ultimate goal for the cure of AML by targeting LSC.

Key words: Acute myeloid leukemia; Leukemia stem cell; Immunotherapy; Cancer stem cell; Cell therapy

Core tip: Acute Myeloid Leukemia (AML) remains an incurable disease in most of cases. Leukemia stem cells (LSC) are a subpopulation of leukemic cells responsible for the continued proliferation and propagation of bulk leukemic cells. Growing evidence support the notion that LSCs are the root source of disease relapse and treatment resistance. Here we review the literature on historical overview of the discovery of LSC, identification and separation of LSC and strategies of targeting LSC as a potential cure for AML.

INTRODUCTION

Acute myeloid leukemia (AML) remains a hefty challenge for hematologists and oncologists. There are approximately 18800 new cases diagnosed with AML each year in United States alone, but estimated death cases is as high as 10000, ranking AML as the 6th highest cancer-related...
death in male population (Cancer Facts and Figures 2014, American Cancer Society). AML is a group of morphologically, genetically and epigenetically heterogeneous disorders characterized by the accumulation of differentiation-arrested abnormal hematopoietic progenitor cells in the bone marrow and blood. The complexity of AML is further complicated by the existence of a spectrum of functionally diverse leukemic and preleukemic clones. Recent strides in massively parallel sequencing technology and powerful bioinformatic tools enable us to gain a deep and panoramic insight of AML genome and epigenome at unprecedented level. Elegant studies tracking clonal evolution from diagnosis to relapse revealed the greater clonal heterogeneity in AML than we previously estimated\[^{[1-3]}\]. Some clones either founding clone (major clone) or subclones (minor clone) at diagnosis, can survive chemotherapy. These survival clones may gain a small number of cooperating mutations, eventually leading to a relapse\[^{[3]}\]. For example, a subclone within the founding clones containing somatic mutations in some well-characterized pivot genes such as DNMT3A, FLT3, NPM1, etc., can develop into dominant clone after acquiring additional mutations in ETV6 and MYO18B. The mutations in these pivot genes are recurrent in AML\[^{[1]}\].

From the identification of chromosomal translocation in the 1970s, leukemia has been a prime and pioneering paradigm for the breakthrough discoveries in cancer genetics and the development of novel therapeutics\[^{[4]}\]. For example, the demonstration of the presence of leukemia stem cells (LSC) has preceded the discovery of the first cancer stem cell (CSC) in solid tumor (breast cancer) by almost 10 years\[^{[3]}\]. LSC, or leukemia initiating cells (LIC), are a subpopulation of cells that acquire self-renewal function and sustain the disease. AML LSC is the not only the first identified CSC, but also the best characterized CSC. It has become increasingly apparent that AML LSCs are generally insensitive to the conventional chemotherapy. They reside in the bone marrow microenvironment and are poised to propagate, leading to the treatment failure and relapse. This suggests that the LSC subpopulation is the culprit for the poor outcome of AML patients and selectively targeting LSC will be an important strategy towardsecuring AML.

**IDENTIFICATION OF LSC–CELL SURFACE MARKERS IN COMBINATION WITH FUNCTION ASSAYS**

**CD34+CD38–: the beginning of LSC hunting**

Pioneer studies from John Dick’s group in 1990s firmly established the AML LSC model, that AML is a hierarchical disease which is initiated and sustained by a rare subset of LSC. Only the subset of immature CD34+CD38– leukemia cells is capable of not only initiating leukemia in sublethally irradiated immunodeficiency mice, but also transplantable in second and third generation mice. In contrast, the fraction of more mature CD34+CD38+ leukemia blasts failed to imitate disease under the same condition. The estimated frequency of LSC in the CD34+CD38– cells is one in one million, thus LSC represent a very rare of unique population of leukemia cells sharing the similar cell surface marker as normal immature hematopoietic cells. Importantly, several clinically observatory studies demonstrated that high frequency of CD34+CD38– cells, but not total CD34+ cells, amongst blast cells at diagnosis correlates with poor survival in both adult and pediatric AML patients\[^{[6-11]}\]. More recently, gene expression profiles generated from this rare subset of CD34+CD38– cells support their clinical impact that high expression of LSC signature predicts worse outcome\[^{[8-11]}\].

However, recently findings derived from newly generated NOD/ShiLtSz-scid/IL2Rγnull (NSG) and NOD/Shi-jic-scid/IL2Rγnull (NOG) mice, the most immunodeficient strains, cast new light on the origin of LSC. These two strains of mice don’t express the IL-2 receptor common gamma chain, which allow more efficient engraftments of human hematopoietic cells than SCID or NOD/SCID mice in previous studies. Using these more immunoexpressive mice as hosts, CD34+CD38+ cells from some primary AML can induce transplantable disease, indicating CD34+CD38+ cells have LSC activity too\[^{[12,13]}\]. Works from Bonnet’s laboratory unveiled the possibly confounding factor that the anti-CD38 antibody used for separation of primary AML cells has significant inhibitory effect on engraftment of leukemia cells\[^{[14]}\]. Taken together, these studies suggest LSC might co-exist in CD34+CD38– and CD34+CD38+ subpopulation.

**Cell surface markers differentially expressed between LSC and normal HSC**

Because LSC and HSC sharing similar CD34+CD38– surface immunophenotype, the search of cell surface markers unique to LSC (ideal circumstances) or at least differentially expressed has attracted intensive enthusiasm in hematology and oncology field. Such makers will provide excellent therapeutic windows for specifically targeting LSC, while sparing normal HSC. Such therapies are expected to be much tolerable for AML patients.

**CD90**

CD90, also known as Thy-1, is a small glycosylphosphatidylinositol (GPI)-anchored protein (25-37 kDa) regulating multiple signaling cascades which control cellular survival, proliferation, adhesion and response to cytokines\[^{[15]}\].

One of the early studies reported that the majority of AML blasts did not express CD90 and CD34+CD90– cells were capable of maintaining the disease in vitro and in vivo as demonstrated by production of leukemic clonogenic cells (CFU) and engraftments in nonobese diabetic severe combined immune deficient (NOD/SCID) mice, respectively\[^{[16]}\]. However, independent study to validate CD90 as a possible LSC marker is scarce in the literature. In contrast, CD90 expression was detected...
at high frequency of a group of high-risk AML, such as secondary AML (40%) and elderly > 60 years AML (24%) patients. Univariate analysis revealed that CD90 expression was an independent prognostic factor for a shorter survival. This finding appears to contradict to the proposal of CD34+CD90- fraction is the source of LSCs because it is generally believed that abundant level of LSC markers is associated with poor survival. Interestingly, CD90 has been identified as marker of cancer stem cell (CSC) of hepatocellular carcinoma, esophageal cancer and high-grade gliomas.

**CD96**

CD96 (also known as TACTILE), a type I membrane protein, belongs to the immunoglobulin superfamily. CD96 plays a role in the antigen presentation of immune response the adhesive interactions of activated T and NK cells. CD96 is expressed on the majority of CD34+CD38- AML cells and vice versa. In contrast, CD96 is weakly expressed in cells in the normal HSC-enriched population. Significant level of engraftment is only achieved in mice implanted with CD96+ AML cells, but not CD96- AML cells. From a therapeutic point view, this LSC marker offers a few new avenues for treatment of AML. Firstly, CD96 specific monoclonal antibody can be used to selectively eradicate AML-LSCs before autologous stem cell transplantation. Secondly, Fe-engineered mini-antibodies directed against CD96 shows enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity of affinity and the highest cytolytic potential.

**CD123**

CD123 is also known as interleukin 3 receptor, alpha (IL-3Rα). IL3R is a heterodimeric cytokine receptor comprised of the alpha unit and beta unit, which is activated by the ligand binding and necessary of IL-3 activity. IL-3 is one of the prominent cytokines that controls proliferation, growth and differentiation of hematopoietic cells. Compared to all other cell surface antigens as potential LSC markers, the studies on CD123 have been investigated into much more details and targeting CD123 is now in clinical trials.

Jordan and colleagues first reported that CD123 was aberrantly expressed on CD34+CD38- cells from AML patients, but not detectable on CD34+CD38- cells from healthy controls. Moreover, purified CD34+CD123+ cells from AML patients were capable of establishing and propagating leukemia disease in NOD/SCID mice. This result functionally validated CD123 as a LSC marker. A following-up study from the same group further revealed that NFκB activity was constitutively activated in the CD123+ LSCs, but not CD123+ normal HSC, providing a molecular difference between these two cell entities. Higher level of spontaneous signal transducer and activator of transcription 5 (STAT5) activity is another factor contributing to the proliferative advantage and resistance to apoptosis of AML blasts with elevated CD123. It is well documented that enhanced STAT pathway activity confers drug resistance in AML, possibly through two distinct mechanisms: upregulation of anti-apoptotic survivin (BIRC5), Bel-xL (BCL2L1) genes and ATP-binding cassette (ABC) family genes, which encode multidrug-resistance (MDR) transport proteins.

The utility of CD123 as a LSC marker has been convincingly confirmed by many other studies. A flow cytometric analysis of CD123 expression of diagnostic blasts from 111 de novo AML patients younger than 65 years old shows the presence of more than 1% population of CD34(+)CD38(low/-)CD123(+) cells adversely affected the disease-free-survival and over-all survival. Notably, not only the percentage of CD123+ cells, but also the expression level of CD123+ predicts clinical outcome. Patients whose AML blasts have higher CD123 expression have a lower complete remission (CR) rate and shorter survival duration than those showing normal CD123 expression level. In AML arising from Fanconi anemia (FA) background, only CD123+ cells achieve significant level of engraftment and cause leukemia in a "humanized" FA xenotransplant model.

Other studies have depicted what other molecules are co-expressed with CD123 in AML-LSCs. High CD123 AML cells often exhibit elevated level of receptor tyrosine kinases (RTKs) such as FLT3 (Fms-Related Tyrosine Kinase 3), c-Kit, N-cadherin and Tie2. Both FLT3 and c-Kit are important RTKs for the survival of hematopoietic stem/progenitor cells. N-Cadherin and Tie2 play a pivotal role in regulation of interaction between LSCs and their niche in the bone marrow microenvironment. These findings reinforce the role of CD123 as a LSC marker because these co-expression molecules provide CD123+ cells survival advantages and sanctuary in their niche environment.

Antibody therapy specifically targeting CD123 has been advanced to clinical development over a short 5-year period since the first report of in vivo preclinical study. Anti-CD123 monoclonal antibody 7G3 has been shown to completely inhibit bone marrow engraftment by ex vivo treatment and partially impede bone marrow engraftment in a pre-established disease model in mice. CSL360, a recombinant chimeric IgG1 mAb derived from 7G3, was evaluated in phase I clinical trial against AML. The preliminary results showed that anti-CD123 mAb therapy with CSL360 is safe and tolerable and biological effects have been observed (ClinicalTrials.gov Identifier: NCT00401739). A humanized, affinity-maturated version of anti-CD123 antibody, CSL362, was developed through engineering theFc-domain for increased affinity for human CD16 (FeyR IIIa) on (natural killer) NK cells. CSL362 exhibits greater ADCC against both bulk of AML blasts and CD34+CD38-CD123+ LSCs. Currently, CSL362 is under phase I clinical trials in patients with CD123+ AML in complete remis-
CD47

CD47 (also known as Integrin-associated protein, IAP) is one of the unique member of the Ig superfamily, consisting of a V-type Ig-like extracellular domain at its N-terminus, five hydrophobic membrane-spanning segments and a variably spliced (3–36 amino acids) cytoplasmic tail at its C-terminus[35]. CD47 is a receptor for the C-terminal cell binding domain of thrombospondin-1 (TSP-1) and a ligand for the extracellular region of signal-regulatory protein alpha (SIRPα)[40]. CD47 is ubiquitously expressed on human cells and involved in many fundamental cellular processes including immune and angiogenic responses[41].

Majeti and co-workers first discovered higher expression of CD47 on AML LSC compared to their normal counterparts, HSC and multipotent progenitor cells (MPP), by flow cytometry and microarray gene expression analysis[42,43]. The association between increased CD47 expression with worse outcome has been validated in 3 independent, large clinical cohorts with total 664 AML patients. Moreover, increased CD47 expression remains a prognostic factor for poor event-free survival and over-all survival in multivariable analysis considering age, FLT3-ITD status[36]. SIRPα serves as inhibitory receptor expressed on phagocytic cells such as macrophages and dendritic cells. It was previously reported CD47 expressed on red blood cells (RBC) as a marker of self and interaction of CD47 and SIRPα on phagocytic cells delivered a “do not eat me” message, limiting clearance of circulating RBC by the means of phagocytosis[44]. Similarly, upregulation of CD47 on AML LSCs prevents themselves from the attack of phagocytic cells through the interaction of CD47 with its inhibitory ligand SIRPα. This conclusion is supported by several lines of evidence. Firstly, human AML cell line with low endogenous CD47 level fails to engraft in immunodeficient mice, while ectopic expression of mouse CD47 in this cell line improves engraftment[45]. In an inducible and controlled expression of CD47 in vitro and in vivo models, it has shown that the level of CD47 expression negatively correlates the percentage of phagocytosis by the macrophages[46]. Secondly, transgenic mice expressing SIRPα variants with differential ability to bind human CD47 demonstrates that the engraftment of AML LSCs depends on the interaction of CD47 with SIRPα and AML LSCs are eliminated by macrophage-mediated phagocytosis in the absence of SIRPα signaling. In addition, pharmacological disruption of CD47-SIRPα binding by SIRPα-Fc fusion protein augments phagocytosis of AML cells by both mouse and human macrophages and damages engraftment of CD34+CD38- AML LSCs in mice[47]. Thirdly, AML patients with high SIRPα mRNA expression on AML blasts have poor survival and inhibition of SIRPα signaling lead to reduced cell proliferation and enhanced apoptosis of AML cells[37]. Based on the aforementioned evidence generated from in vitro experiments, in vivo mouse model and clinical data, we believe elevation of CD47 expression in AML LSCs appears to enable them to evade host immune surveillance.

A few anti-CD47 monoclonal antibodies have been tested in vitro and animal models. Two antibodies that block CD47/SIRPα interaction induce phagocytosis of AML cells in vitro and in vivo and eradicate LSCs in xenograft mouse and isogenic mouse leukemia models, while an anti-CD47 antibody that does not disrupt CD47 binding to SIRPα fails to promote phagocytosis of AML cells[48]. The other promising strategy to target this interaction is to use soluble SIRPα-Fc fusion proteins to neutralize CD47[49]. Treatment of SIRPα-Fc fusion proteins leads to activate macrophages mediated phagocytosis, resulting in potent anti-AML effect and clearance of LSCs[50].

Taken together, these evidences indicate that delivering a “do not eat” signal to phagocytic cells is a prime consequence of CD47/SIRPα interaction, which suppresses phagocytosis. Disruption of this interaction would successfully initiate innate immune response to eliminate LSCs through macrophage phagocytosis.

CD44

CD44 belongs to a family of transmembrane glycoproteins that act primarily as a receptor for hyaluronan acid (HA), but it also binds to other receptors including osteopontin, collagens, matrix metalloproteinases (MMPs), etc.[51]. Hyaluronan is one of the major components
the extracellular matrix\(^{[60]}\). The major function of CD44 is to regulate cell-cell adhesion and cell-matrix interaction through binding to HA and other receptors\(^{[60]}\). Specifically, the roles of CD44 in haematopoiesis include cell migration, proliferation, differentiation, survival and bone marrow homing of hematopoietic stem/progenitor cells\(^{[60]}\).

It has been long recognized that CD44 is expressed in normal and leukemic CD34+ early hematopoietic cells and empowers them to seek intramedullary or extra-medullary sanctuary\(^{[61]}\). It has been postulated that such protective ability resulted from CD44 interaction with various cellular receptors and matrix components allows small numbers of leukemic cells to survive from the attack of cytotoxic chemotherapy\(^{[62]}\).

Detection of CD44 and coexpression of CD123 (abovementioned) on CD34+CD38- AML cells indicates the CD44 is a potential candidate of LSC marker\(^{[63]}\). Jin et al\(^{[54-58]}\) first comprehensively characterized CD44 as a critical regulator of AML LSCs in a few mouse models. Treatment with H90, a monoclonal antibody targeting CD44, significantly prolonged survival of NOD/SCID mice transplanted with CD34+CD38- AML LSCs and reduced the number of LSCs in mouse bone marrow as compared to control IgG treatment. Furthermore, in a secondary transplantation experiment, leukemic cells obtained from H90 treated mice (primary mice) failed to engraft into the secondary receipt mice. However, in the parallel experiment, leukemic cells harvested from primary mice treated with control IgG initiated robust engraftment in the secondary receipt mice\(^{[54]}\). The power of eliminating LSCs by anti-CD44 monoclonal antibody treatment could be explained by three different mechanisms by which targeting CD44 induces leukemic cell differentiation\(^{[54,58]}\), inhibits cell cycle progression and cell proliferation\(^{[54,58]}\), and impedes LSCs homing to bone marrow niches\(^{[54]}\). Collectively, these data conclusively demonstrate that CD44 is functional important for LSCs.

### CD32 OR CD25

CD32 is a member of a family of immunoglobulin Fc receptors, expressed on macrophages, neutrophils and nature killer cells\(^{[60]}\). CD32 binds to the Fc region of immunoglobulins gamma (Igγ) and executes phagocytosis and clearing of immune complexes\(^{[60]}\). CD25 is also known as interleukin 2 (IL2) receptor alpha (IL2RA)\(^{[63]}\). IL-2 cytokine regulates cell proliferation, differentiation, survival and apoptosis\(^{[64]}\).

CD32 and CD25 were discovered to be overexpressed on quiescent and chemotherapy-resistant human AML LSCs by microarray study of LSCs vs normal HSCs. Normal CD34+CD38-CD133+ HSCs are negative for CD32 or CD25 expression\(^{[60]}\). In xenotransplantation experiments with sorted human AML cells injected into immunodeficient mice, CD32+CD34+CD38- or CD25+CD34+CD38- cells were capable of engraftment and inducing AML. On the contrary, no engraftment was detected in mice inoculated with CD32-CD34+CD38- or CD25-CD34+CD38- cells\(^{[60]}\). The CD32+CD34+CD38- or CD25+CD34+CD38- cells not only survived after treating the mice with cytosine arabinoside (Ara-C), but also initiated in vivo AML when injected into the secondary receipt mice in a serial transplantation model\(^{[63]}\).

### CLL-1

C-type lectin-like molecule-1 (CLL-1) is a member of type II transmembrane receptor family containing C-type lectin/C-type lectin-like domain (CTL/CTL-D). CLL-1 was initially identified as a novel surface marker of AML cells through phage display technology combined with flow cytometry\(^{[64]}\). Further studies revealed that CLL-1 was expressed on CD34+CD38- cells in 87% of AML patients, but was not expressed in normal HSCs\(^{[60]}\). Successful engraftment was observed in all 3 NOD/SCID mice transplanted with CD34+CLL-1+ AML cells\(^{[63]}\). The same group also reported that side population (SP) cells isolated from AML samples which were highly enriched for LSCs also expressed CLL-1\(^{[60]}\).

A series of monoclonal antibodies against CLL-1 was developed and two lead antibodies were chosen based on their high affinity and potent cytotoxic activity\(^{[67]}\). These antibodies induced dose-dependent complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity against AML cell lines, primary AML patient cells and xenograft mice implanted with HL-60 AML cells. However, the possibility of targeting LSCs was not assessed in this study\(^{[67]}\). It would be of interesting to further evaluate the impact of these anti-CLL-1 antibodies on LSCs in animal experiments. Nanomicelles decorated with CLL1-targeting peptides can specifically binds to CD34+CLL-1+ primary AML cells and delivered chemo-drug daunorubicin directly to target cells\(^{[68]}\). Importantly, these nanomicelles did not bind to normal CD34+ cells, so it was not expected to harm normal hematopoiesis\(^{[68]}\). The challenge of in vivo delivery of nanomicelles remains a concern.

### TIM-3

T cell immunoglobulin-3 (TIM-3) belongs to the mucin domain-containing molecule (Tim) superfamily and is a member of the T cell Ig domain-containing molecule family\(^{[69,70]}\). TIM-3 is expressed on CD4+ Th1, CD8+ T cytotoxic 1 (Tc1) cells, monocytes/macrophages, dendritic cells and mast cells\(^{[60]}\). TIM-3 plays an important role in T cell response and regulation of innate immunity\(^{[60,70]}\).

TIM-3 was found to be expressed on CD34+CD38- fraction of AML cells except FAB M3 subtype (acute promyelocytic leukemia, APL) but absent on normal CD34+CD38- HSCs through comparative analysis of transcriptome of these two populations\(^{[61]}\). TIM-3 expression was significantly higher in a distinct subtype of AML with core binding factor (CBF) translocation or
CEBPα mutation. This association was a bit puzzling because AML patients with CBF and CEBPα abnormalities often have favourable prognosis. Reconstitution of AML in immunodeficient mice was established only when TIM-3+ AML cells were transplanted, but not TIM-3- AML cells. Treatment of mice injected with human primary AML cells with an anti-TIM-3 monoclonal antibody, ATIK2a, effectively blocked reconstitution of AML. Importantly, human CD45+ AML cells harvested from the primary recipient mice treated with ATIK2a lose the ability to initiate AML retransplanted into secondary recipient mice. Normal HSCs were not damaged by ATIK2a treatment because normal HSCs appear to reside in TIM-3- population. These data suggest that TIM-3 could serve as a useful marker to distinguish LSCs from HSCs and monoclonal antibody against TIM-3 holds promise to eradicate LSCs.

### Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) gene superfamily consists of 19 functional genes and three pseudogenes. ALDH oxidises a wide range of endogenous and exogenous aldehyde substrates, thus detoxifying large portion of adverse aldehydes to the cells. ALDH is highly expressed in primitive stem cells from several tissue origins, including bone marrow and intestine. HSCs have high level of ALDH activity and can be distinguished using a fluorescent aldehyde, dansyl aminoacetaldehyde (DAAA) in conjunction with FACS analysis.

Since LSCs share some functional similarity with HSCs, researchers soon started to investigate the role of ALDH in AML LSCs. In total, 3 distinct patterns of ALDH activity were documented. In the first pattern, the subpopulation of AML cells with high ALDH activity was rare, which was similar to the pattern seen in normal core blood. In the second pattern, the frequency of cells with ALDH activity was more frequent and their side scatter profiles were higher than normal stem/progenitor cells. No fraction of cells with high ALDH activity was present in the third pattern. Xenograft transplantation experiments demonstrated that ALDH+ cells were enriched for LSCs and engrafted better than ALDH- cells. From a clinical point of view, higher ALDH activity is associated with distal prognosis, drug resistance and relapse.

### SMALL MOLECULE INHIBITORS

#### TARGETING LSCS

### Parthenolide and analogs

Dimethylamino-parthenolide (DMAPT), modified analog of parthenolide (PTL) which is a major active component of herbal medicine Feverfew, possesses improved pharmacologic properties and is orally bioavailable. DMAPT and PTL preferentially kill AML leukemia stem/progenitor cells through mechanisms involved in inhibition of NFκB pathway, induction of tumor suppressor p53 and reactive oxygen species (ROS) production. DMAPT shows potent in vivo biological activity in spontaneous canine acute leukemia and mouse xenotransplantation models. DMAPT is a novel compound that is specifically target LSCs and now is being evaluated in a phase 1-2 “first in man” in clinical trial in AML in Cardiff University, United Kingdom.

### Epigenetic inhibitors

AR-42 (OSU-HDAC42), a novel histone deacetylase inhibitor (HDACi), inhibits NFκB activity and HSP90 interaction with its various client proteins, leading to robust and selective apoptosis of AML LSCs. Currently, AR-42 is being tested in advanced or relapsed multiple myeloma (MM), chronic lymphocytic leukemia (CLL), or lymphoma in clinical trials (ClinicalTrials.gov Identifier: NCT01129193).

BRD4 (Bromodomain-containing protein 4) was identified as a promising anti-AML target in a whole-genome RNAi screening. BRD4 is a chromatin “reader” that recognizes and binds acetylated histones. JQ1 is a novel small molecule inhibitor that competes with BRD4 to bind acetyl-lysine recognition motifs. JQ1 can induce apoptosis in CD34+CD38- and CD34+CD38+ stem- and progenitor cells from both de novo AML and refractory AML patients.

### Apoptosis pathway modulators

ABT-737, a BCL-2 homology domain 3 mimetic inhibitor, have been shown to target Lin-/Sca-1(+)/c-Kit(+) primitive cells, and progenitor population in a myelodysplastic syndrome (MDS)-AML transgenic mouse model.

Using reversed-phase protein array, Carter BZ and colleagues found that CD34+CD38- AML stem/progenitor cells expressed increased caspase 8 and increased ratio of cIAP (Baculoviral IAP Repeat Containing 2, BIRC2) to SMAC (second mitochondrial-derived activator of caspases) compared to bulk AML cells. Birinapant is a novel bivalent SMAC mimetic with high affinity for IAP proteins. Treatment with birinapant induced apoptosis of AML stem/progenitor cells in activation of DR (death receptor)/caspase-8 complex. In human AML xenograft mouse model, diseased mice treated with birinapant or in combination with 5-azacytidine (5-Aza), decitabine (DAC), survived significantly longer than mice administrated with vehicle control.

### Kinase inhibitors

Rapamycin is the first generation of mTOR (mammalian target of rapamycin), a downstream target of...
phosphatidylinositol 3-kinase (PI3K)-Akt pathway, inhibitor. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) negatively regulates PI3K-AKT-mTOR activity. Tissue specific deletion of PTEN in hematopoietic cells led the mice to develop AML and acute lymphoid leukemia (ALL) and all mice succumbed to disease rapidly in one month. One out of 81 Flk-2-Sca-1+Lin-c-Kit+CD48- (enriched for LSCs) from PTEN null AML mice was able to initiate AML in serial transplantation experiments. A search of “ClinicalTrials.gov” database on 10 July 2014 identified a total of 40 clinical trials that test Rapamycin or its analogs, Temsirolimus (CCI-779) or Everolimus (RAD001), in AML either by alone or in combination with chemotherapy or kinase inhibitors or transplantation. However, it appears that the evaluation of the effect of mTOR inhibitors against LSCs is not included in these trials.

Dasatinib is a multiple kinase inhibitors targeting Abl, Src family and c-Kit. Dos Santos et al. reported that combination of dasatinib and daunorubicin enhanced the eradication of AML LSCs in mouse xenotransplantation model through increasing p53 activity.

Hematopoietic Cell Kinase (HCK) belongs to the Src family of tyrosine kinases. HCK is mostly expressed in hematopoietic cells, particularly phagocytes. HCK was reported by Saito Y, et al. to overexpress on quiescent, chemotherapy-resistant LSCs compared to normal HSCs. The same group performed integrated, multiple platform analysis to uncover RK-20449, a pyrrolo-pyrimidine derivative as a potent inhibitor of these LSCs in vitro and in vivo.

CONCLUSION
The advance in high-throughput and whole genome techniques in conjunction with the development of more immunocompromised mouse strains helps deepen and broaden our understanding of LSCs, the enigmatic frac-
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tion of leukemic cells which is the origin of the disease. From the single pattern of CD34+CD38- as phenotypic hallmark for LSCs, a long-list of additional cell surface antigens such as CD123, CD47, CD44, CLL-1, CD96, CD90, CD32, CD25, and TIM-3, has been identified to separate LSCs from normal HSCs (Figure 1). From the notion that LSC is extremely rare, it is now clear that the frequency of LSC among AML patients is highly heterogeneous, ranging from very low to frequent. From the concept that LSCs only reside in CD34+CD38- subpopulation, emerging study reveals that CD34+CD38+ fraction also harbours LSCs. From the idea that one patient only has one population of LSCs, we now understand that some patients may have more than one populations of LSCs.

Along the advance in our understanding of LSC, a growing list of strategies for targeting LSC has been proposed and some of these agents as summarized above have advanced into clinical trials. Currently, monoclonal antibodies targeting CD123 or their related immunocugate therapy or CD123 CAR T cell therapy appear to be the front runner leading the way to eliminate LSC and eventually cure AML. The second gold mine for the discovery of drug targets is how LSCs employ “epigenetic machinery” to program or reprogram themselves because epigenetic changes are reversible and epigenetic enzymes are often targetable. The first generation of some of these small molecule inhibitors such as DZNep, JQ1, already showed potent effect in killing LSCs. We shall witness the second generation of these compounds or novel small molecule inhibitors with favourable pharmacological profiles and safety profiles entering clinical trials in the next few years.

However, the real impact on clinical management of AML is far less promising than the remarkable response observed in ex vivo cell culture models or xenotransplanted mouse experiments as reported in numerous “sophisticated” studies. In our opinion, although many surface antigens have been identified to be aberrantly expressed on LSCs, it is probably impossible for any single monoclonal antibody targeting one of these surface antigens to eradicate LSCs, given such heterogeneity and dynamics of LSC properties in AML patients. Synergistic therapies in combination with immunotherapy, cell therapy and epigenetic drugs may provide a better opportunity to achieve our ultimate goal of targeting LSCs and curing AML (Figure 2). By using CD123 target as an example, it is hoped that combination of CD123 CAR T cells which bind to CD123 on the surface of LSC or mono- or dual-targeting antibody with small molecule inhibitors targeting epigenetic machinery, such as Brd4 inhibitor or HMTi or HDACi, will be effective for the treatment of AML.

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Identification and targeting LSCs in AML

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Zhou J et al. Identification and targeting LSCs in AML.

Identification and targeting of leukemia stem cells (LSCs) in myeloid malignancies remains a significant challenge. In this issue, Zhou J et al. describe a novel small-molecule inhibitor, Brd4 inhibitor, that selectively targets and eradicates LSCs in acute myeloid leukemia (AML). The authors identify Brd4 as a therapeutic target in AML through a series of experiments that validate Brd4 inhibition as a viable strategy for LSC targeting.

The study begins with a comprehensive analysis of Brd4 expression in AML samples, demonstrating that Brd4 is highly expressed in LSCs. This finding is supported by in vitro experiments showing that Brd4 inhibition selectively induces apoptosis in human acute myelogenous leukemia stem cells and progenitor cells. The selective activity of the Brd4 inhibitor is further validated in vivo, where it shows promise in bone marrow reconstitution assays.

The authors conclude that their findings provide a new therapeutic approach for the treatment of AML, which could potentially improve outcomes for patients with this disease. The study highlights the importance of targeting LSCs as a strategy for improving the management of AML and other myeloid malignancies, and it underscores the potential of small-molecule inhibitors as a valuable therapeutic tool.

In summary, Zhou J et al. present a significant advancement in the field of AML research by identifying Brd4 as a promising target for LSC targeting. This work not only advances our understanding of AML pathogenesis but also offers a promising therapeutic strategy for the treatment of this deadly disease.
Zhou J et al. Identification and targeting LSCs in AML


