Molecular mechanisms for stemness maintenance of acute myeloid leukemia stem cells

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1. INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive and fatal hematologic malignancy characterized by clonal expansion of myeloid blasts with blocked differentiation into mature cells. The general therapeutic strategy in patients with AML has not changed substantially in more than three decades, and for most newly diagnosed patients chemotherapy is the standard treatment that is usually divided into induction therapy and consolidation therapy. Although a complete response is typically achieved in around 30% to 40% of younger patients, long-term outcomes have not significantly improved with 5-year overall survival rates being less than 15% for patients over 60 years old. Therefore, it is necessary to further understand the pathogenesis of AML and explore potential therapeutic strategies for AML treatment.

The development of AML is associated with accumulation of acquired genetic and epigenetic alterations mainly occurred in hematopoietic stem and progenitor cells (HSPCs), which aberrantly alter HSPC state at both the molecular and cellular levels, and transform them into leukemia stem cells (LSCs). LSCs play critical roles in leukemia initiation, progression, and relapse, and need to be eradicated to achieve a cure in clinic. Key to successfully targeting LSCs is to fully understand the unique cellular and molecular mechanisms for maintaining their stemness. Here, we discuss LSCs in AML with a focus on identification of unique biological features of these stem cells to decipher the molecular mechanisms of LSC maintenance.

Abstract

Human acute myeloid leukemia (AML) is a fatal hematologic malignancy characterized with accumulation of myeloid blasts and differentiation arrest. The development of AML is associated with a serial of genetic and epigenetic alterations mainly occurred in hematopoietic stem and progenitor cells (HSPCs), which change HSPC state at the molecular and cellular levels and transform them into leukemia stem cells (LSCs) . LSCs play critical roles in leukemia initiation, progression, and relapse, and need to be eradicated to achieve a cure in clinic. Key to successfully targeting LSCs is to fully understand the unique cellular and molecular mechanisms for maintaining their stemness. Here, we discuss LSCs in AML with a focus on identification of unique biological features of these stem cells to decipher the molecular mechanisms of LSC maintenance.

Keywords: Acute myeloid leukemia, Leukemia stem cell, Maintenance, Molecular mechanisms, Stemness

2. IDENTIFICATION OF AML LSCs

Efficiently identifying functional stem cell populations that give rise to AML is a key step in the field of AML LSCs (Fig. 1). The...
hypothesis of cancer stem cells (CSCs) was first proposed over 50 years ago, however, the real progress of CSC study should attribute to the development of new technologies in the past two decades, including xenotransplant assay, quantitative stem cell assays and fluorescence activated cell sorting technique (FACS). Dick and colleagues’ study provided the first direct evidence for the existence of LSCs. Bone marrow CD34+CD38- cells from patients with AML not only initiate AML in vivo but also differentiate into leukemic blasts after transplanting into NOD/SCID mice; more importantly, serial transplantation demonstrated that these cells have a capacity to self-renew and transfer AML disease into secondary recipients. Therefore, this study showed for the first time that LSCs of human AML are exclusively CD34+CD38- cells, which have the same cell-surface phenotype and cellular hierarchy as normal human primitive hematopoietic cells.

Due to the intrinsic heterogeneity of AML, characteristics of LSCs including the immunophenotype are heterogeneous. Although LSCs are enriched in CD34+CD38- fraction in most AML patients, recent studies demonstrated that LSCs also reside in CD34+CD38+ or CD34- fractions in some AML patients. Moreover, the abundances and different cell types of primitive cells across AML patients are also determined by the underlying genetic alterations. It is worth to note that, from the experimental perspective, the variations on characteristics of LSCs are not only because of the intrinsic heterogeneity of AML patients, but also due to the xenograftent mice models, as the frequency and leukemic engraftment efficiency of LSCs are highly dependent on the model used. In addition, oncogenic transduction and transplantation AML mice models have been extensively used for studying LSCs. For instance, MLL-AF9 retrovirus-transformed granulocyte macrophage progenitors (GMP, IL7R "Lin" Sca-1- c-Kit+CD34+FcγRII/III+), called L-GMP, can function as LSCs and initiate AML development after transplanting into syngeneic recipient mice. Retrovirus-mediated transduction and transplantation model is also used for other oncogenic events in studying AML pathogenesis and AML LSCs.

Identifying cell surface markers that can distinguish LSCs from normal HSPCs has been of great interest, as the ability to prospectively separate LSCs from residual HSPCs would enable important scientific and clinical implications. In other words, an ideal marker should be uniquely expressed in LSCs rather than HSPCs. Although no LSC-specific markers have been identified so far, a number of them have been found that are upregulated in AML LSCs, such as CD123, TIM3, CD44, CD47, CD93, CD96, and CD99. For instance, CD123 is the α chain of interleukin-3 receptor (IL3R), which forms the functional heterodimeric high-affinity IL3R with CD131. The binding of IL3 to CD123 leads to activation of the receptor and promotes cell survival and proliferation. Previous studies demonstrated that CD123 is overexpressed in AML blasts, CD34+ leukemic progenitors, LSCs, when compared to normal HSCs. Increased expression of CD123 contributes to the activation of STAT5, cell cycling, maintenance of primitive cell-surface phenotype, and resistance to apoptosis. Recent studies identified T cell immunoglobulin mucin-3 (TIM-3) as another AML LSC specific marker, as TIM-3 is more highly expressed on multiple specimens of AML LSCs than on normal bone marrow HSCs. Engraftment assay using NOD/SCID/IL2Rγ-null mice showed that LSC function from multiple AML specimens resides predominantly in the TIM3- compartment, whereas HSC function resides predominantly in the TIM3+ fraction of normal bone marrow. The serum levels of TIM3 ligand, Gal-9, were significantly elevated in AML patients and in mice engrafted with primary human AML samples. Interestingly, TIM-3 and Gal-9 constitute an autocrine loop critical for LSC self-renewal by activating NF-κB and β-catenin signaling pathways, and neutralization of Gal-9 inhibited xenogeneic reconstitution of human AML. Importantly, these specific surface markers might provide potential therapeutic targets for treating AML. For instance, an anti-CD123-neutralizing antibody 7G3 clearly targets AML LSCs by inhibiting IL3-mediated intracellular signaling and impairing the engraftment ability of LSCs. Targeting TIM3-Gal9 is another useful strategy for specific eliminating AML LSCs. Anti-human TIM-3 mouse IgG2a antibody could block engraftment of AML after xenotransplantation, and significantly reduce leukemic burden and eliminate

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**Figure 1.** Identification of human AML leukemia stem cells. Similar to normal hematopoietic stem cells (HSCs), leukemia stem cells (LSCs) are defined as a rare population that have self-renewal capacity and can initiate leukemogenesis. Based on these features, xenograftment assay is used for efficiently identifying functional leukemia stem cells. Surface markers CD34+CD38-CD123+ or CD34+CD38+ TIM3+ are used to specifically isolate AML patient-derived LSCs by fluorescence activated cell sorting (FACS), and these cells are transplanted into immune deficient recipient mice. LSCs but not non-LSCs can reconstitute leukemia. Alternative strategies, such as the levels of ROS or sensor for NAD+/NADH (SoNar), are also used to identify functional LSCs.
LSCs capable of reconstituting human AML in secondary recipients. Moreover, this antibody does not impair the function of normal human HSCs.19 Thus, identifying specific surface markers of AML LSCs remains an important direction in the future research of this field.

Relative levels of reactive oxygen species (ROS) have been employing as an alternative strategy to define functional LSCs. This method can effectively enrich phenotypic LSC population (CD34+CD38−/CD123+) in the ROSlow subset in most AML patients regardless of heterogeneous markers.22 Similar strategy reported recently that SoNar, genetic sensor for NAD+/NADH that can evaluate the glycolysis and mitochondrial respiration activities in live cells by sensing the NAD+ and NADH redox states in the cytoplasm in real time, can be exploited to isolate functional LICs, and SoNarhigh cells are enriched for higher LIC frequency.23

Together, identification of LSCs in human AML and murine models has been greatly deepening our understanding of CSC biology, such as the cellular state of LSCs, and molecular mechanisms for maintaining LSC self-renewal and limited differentiation capacity.

3. MOLECULAR MECHANISMS FOR MAINTAINING THE FUNCTION OF LSCs

Normal HSCs are largely in a state of quiescence with glycolytic, autophagy-dependent, tightly controlled levels of protein synthesis, and intact stress response system.24–27 While LSCs inherit common stem cell characteristics of normal HSCs, they also have some unique functional changes (Fig. 2). In term of human AML, its pathogenesis is highly complex and controlled by multilayer genetic and epigenetic dynamics,28,29 which not only endow the inherent complexity and heterogeneity of LSCs, but also pose an obstacle to our understanding of the biology of LSCs.

3.1. AML LSCs exhibit dysregulated molecular programs

AML is characterized by clonal expansion of myeloid blasts with differentiation arrest, and a series of genetic alterations successively occurred during leukemogenesis confer LSCs heterogeneous molecular programs through regulating signaling pathways and affecting the function of transcriptional factors. Single-cell RNA-seq revealed that human AML primitive cells exhibit dysregulated transcriptional programs with co-expression of stemness and myeloid priming genes, and suggest a prominent role for genotype in determining the cell-type composition and hierarchy of AML primitive cells.5 It is known that chromosomal translocations found in AML generate fusion oncoproteins that transform normal HSPCs into LSCs by reprogramming transcriptional regulatory properties during leukemogenesis. For instance, recurrent rearrangements of the mixed lineage leukemia gene (MLL1) result in MLL fusions with over 70 translocation partners, such as AF9, AF4, AF10, and ENL.30 Previous study showed that during transformation of LSCs from committed progenitors, MLL-AF9 re-activates a subset of genes highly expressed in normal HSCs, which is essential for LSC self-renewal.11 AML1-ETO is another fusion oncoprotein that plays an important role in inducing self-renewal of HSPCs, leading the development of LSCs through a COX/β-catenin signaling pathway.31 In addition, AML patients carry somatic mutations, such as FLT3-ITD, CEBPA, GATA2, and RUNX1, and over 30% AML patients have FLT3-ITD.
Compared to normal HSCs, CD34+CD38− LSCs also expresses high levels of FLT3 that up-regulates MCL1 to promote survival of AML LSCs via FLT3-ITD-specific STAT5 activation.32,33 CCAAT-enhancer binding protein α (C/EBPα) is the main regulator of myeloid differentiation during hematopoiesis.34 C/EBPα mutated in approximately 11% AML patients increases proliferation of HSCs and pre-leukemia expansion of HSPCs, and functions as a platform for transduction of myeloid leukemia.35–37 GATA2 is highly expressed in immature cells and essential for maintenance of HSCs.38 and its somatic mutation in about 11% intermediate-risk karyotype AML is important for self-renewal of LSCs and differentiation arrest of blasts. RUNX1 is another major transcription factor in hematopoiesis.10,41 Being mutated in approximately 10% of AML patients, mutant RUNX1 promotes the transformation of HSPCs into LSCs by impairing proliferation and differentiation.42 shRNA-mediated knockdown of its mutant form inhibits AML cell growth and survival after transplanted into immune-depleted mice.53

In addition to these founding mutations in AML, LSCs are hypersensitive to perturbation of transcriptional regulatory machinery. HOXA9, playing an essential role in embryonic development, is frequently overexpressed in MLL-rearranged leukemia. Interfering HOXA9 expression in human MLL-rearranged leukemia cells inhibits survival in vitro, and reduces leukemia burden in mouse xenograft.44 Notably, HOXA9 cooperates with MEIS1 and sufficiently immortalizes HSPCs in vitro and propagates leukemia in vivo.53,47 Additionally, it was suggested that PBX3 is another important cofactor of HOXA9 in leukemogenesis. PBx3 is also co-overexpressed with Hoxa9 in MLL-rearranged murine leukemia and essential for LSC maintenance.47,48 Similar to Meis1, co-expression of PBX3 and HOXA9 causes leukemia rapidly in mice.47 Transcriptional factor IKZF2 is a target of RNA-binding protein MS2 that is essential for LSC function.49 Recent study demonstrates that IKZF2 is highly expressed in LSCs, and its deficiency impairs LSC function showing reduced colony formation, increased differentiation and apoptosis, and delayed leukemogenesis. Further, IKZF2 regulates a HOXA9 self-renewal gene expression program and inhibits C/EBP-driven differentiation program.50 Together, LSCs display unique molecular programs in maintaining their stem cell function, and further exploring more regulators that are specific for AML LSCs is necessary for fully understanding the pathogenesis of AML. Interestingly, by performing CRISPR-mediated genome wide screening, researchers successfully identified ZFP64 as an essential transcription factor in MLL-rearranged leukemia. Mechanistically, ZFP64 sustains expression of MLL via binding to the MLL promoter.51 This study highlights the importance of oncogene cis-regulatory elements that can construct the basis for transcription factors addiction.

3.2. Deregulation of the epigenetic properties in LSCs

Driver mutations in human AML often occur in genes involved in epigenetic regulation, implying their crucial roles in LSCs. Remarkably, the mutated DNA methylase DNMT3A is found frequently in de novo AML with a frequency approaching to 25%.52 DNA methylation plays a critical role in defining cellular identity and gene expression of HSCs.53 DNMT3A mutation amplifies HSC pool, causes clone hematopoiesis, and creates a pre-leukemia state from which AML evolves.4,54,56 Mechanistically, the founding DNMT3A mutation provides a context that is susceptible to other genetic mutations. For example, DNMT3AΔR82H coordinated with other mutations, such as FLT3, NPM1, and NRAS, can directly bind and enhance transactivation of stemness genes critical for leukemogenesis.57–59 In addition, loss-of-function mutation of DNA demethylase Ten-eleven-translocation 2 (TET2) is also found in nearly 10% de novo AML. TET2 deletion causes increased HSC self-renewal and myeloid lineage deregulation while restoration of TET2 function blocks aberrant self-renewal, induces cellular differentiation, and represses leukemia progression.60

Histone modification is another aspect of epigenetic regulation. MLL1, encoding a chromatin modifying protein with histone methyltransferase activity, was recurrently found in chromosomal rearrangements involving 11q23 in AML, highlighting the significance of this epigenetic modifying enzyme in AML development, although it is argued recently that wide-type MLL1 is dispensable for MLL-rearranged AML. Surprisingly, the parallel research demonstrated that MLL2 is essential for leukemogenesis and maintenance.61 Notably, an earlier identification of MLL-specific histone modification enzyme is DOT1L in MLL-AF10-mediated leukemogenesis. Fusion of hDOT1L with MLL results in leukemic transformation, which phenocopies MLL-AF10 in a methyltransferase activity-dependent manner and leads to the up-regulation of a program of stemness-associated genes, such as HOX9.62 Interestingly, recent study provides a theoretical basis for the development of targeted drugs (e.g., EPZ4777) against DOT1L.63 Other histone modification enzymes, such as KDM1A,64 JMJD1C,65 STEAD1A,66 SETD2,67 KDM2B,68 and EZH2,59 have also been shown involving in leukemogenesis and stemness maintenance via the enzyme activity-dependent or -independent ways.

In contrast to epigenetic modifications of DNA and histone, epitranscriptomics emerges as an infant field, and its role in LSCs remains to be further understood. The well-known modification in eucharyotic mRNA is N6-methyladenosine (m6A) catalyzed by m6A modifiers, including METTL3-METTL14-WTAP methyltransferase complex, and demethylases ALKBH5 and FTO. The first m6A modifiers established to play a role in leukemia is FTO. Overexpression of FTO promotes leukemogenesis while inhibits all-trans-retinoic acid-induced leukemia cell differentiation by removing m6A modifications of ASB2 and RARA transcripts.70 Subsequently, METTL3 was found overexpressed in human AML cells, causing cell differentiation and apoptosis and repressing leukemia progression in vivo.71 Another methyltransferase METTL14 also displays an important role in AML. Deletion of Mettl14 in mice bone marrow cells delays leukemia development, impairs LSC function by altering MYC and MYB methylation.72 Collectively, these findings highlight the critical roles of m6A modification in AML development and LSC maintenance.

3.3. Reprogrammed cellular metabolism of LSCs

LSCs undergo unique reprogrammed cellular metabolism, an important hallmark of cancers.73 Given that recurrent somatic mutations in IDH1 and IDH2 occur in 10% to 20% of AML patients,42 it is reasonable to think that metabolism alteration is critical in leukemogenesis. IDH1/2 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) in a NADP+-dependent manner. Mutations in IDH could produce an oncometabolite, 2-hydroxyglutarate (R-2HG). Previous study showed that reinforce expression of IDH2R140Q in murine HSCs synergistically induces AML with Hoxa9 and Meis1, and is essential for LSC maintenance.74 Notably, inhibitor Enasidenib (AG-221) has been approved for the treatment of relapsed or
refractory AML with IDH mutations. Controversially, a recent work showed that regardless as an oncometabolite R-2HG exhibits broad and variable anti-proliferation effects in IDH wide-type leukemia rather than functions as an oncometabo-

lili. This might result from, to some extent, the heterogeneous genetic foundation of AML patients.

Cancer cells generally rely on glycolysis for energy production instead of oxidative phosphorylation (OXPHOS), a more efficient ATP-producing process. Similarly, normal HSCs also prefer to utilize glycolysis to meet their energy demands, and functional LSCs have a much higher level of glycolysis in murine leukemia model and human AML cells. In contrast, recent studies suggest that LSCs are uniquely reliant on OXPHOS for their maintenance and survival. By interrogating the metabolism of human AML stem cells, Jordan et al found that the majority of functionally defined LSCs isolated from de novo AML patients are characterized by relatively low levels of ROS. These ROS-LO LSCs have higher levels of amino acids, which are metabolized in the tricarboxylic acid (TCA) cycle. Compared with more mature AML blasts, LSCs of AML are selectively dependent on amino acid metabolism for OXPHOS. Remarkably, they demonstrated that OXPHOS in LSCs can be targeted by BCL2 inhibition with a selective inhibitor—venetoclax, which has been approval for the treatment of AML. In addition, metabolism of branched-chain amino acids (BCAAs) is also important for LSC survival. BCAA transaminase 1 (BCAT1), a cytosolic aminotransferase for BCAAs, is activated in AML LSCs, important for LSC survival. BCAA transaminase 1 (BCAT1), a cytosolic aminotransferase for BCAAs, is activated in AML LSCs, and regulates intracellular α-KG homeostasis by transferring α-amino groups from BCAAs to α-KG. α-KG is an essential cofactor for α-KG-dependent dioxygenase, such as Egl-9 family hypoxia inducible factor 1 (EGLN1) and TET family. Elevated BCAT1 restrains intracellular α-KG and stabilizes HIF1α, which are required for LSC maintenance. In the upstream regulatory mechanism, the oncogenic RNA binding protein Musashi2 (MSI2) is physically associated with the BCAT1 transcript and positively regulates its protein expression in leukemia. Additionally, a recent work reported that AMP-activated protein kinase signaling sustains self-renewal of human AML LSCs by target mitophagy. Together, myeloid LSCs display unique cellular metabolic state, and more efforts are required for uncovering metabolic profiling of AML LSCs.

3.4. Bone marrow microenvironment of AML LSCs

Alterations in the microenvironment gain much attention recently for its role in leukemia progression and LSC maintenance. Bone marrow niche is generally considered as a complex ecological system composed of many cell types, and maintains and regulates stem cell function through cellular interactions and secreted factors. Comprehensive dissection of bone marrow stroma using single cell RNA-seq identified six broad cell types with 17 cell subsets with distinct interactions, differentiation continuums and HSC niche regulatory function. These studies provide a comprehensive and heterogeneous cellular architecture of bone marrow niche, and reveal a dynamic molecular landscape under certain contexts, such as leukemia. Infiltration of leukemic cells can cause profound bone marrow stem and progenitor dysfunction, and rebuild the niche that favors LSC rather than normal HSC maintenance, or a niche that protects leukemia propagating cells from chemotherapy. LSCs are presumed to reside in specific niches in the bone marrow. Disruption of the interactions between stromal-cell-derived factor 1 (SDF-1) and its receptor CXCR4 inhibits the homing of leukemia cells to the vascular area and results in a rapidly decrease of human AML cells, suggesting that specialized vascular structures might delineate a microenvironment with unique physiology that can be exploited by circulating malignant cells. More interestingly, signaling from the bone marrow niche might have differential impact on different myeloid leukemias. For instance, osteoblastic cell-specific activation of the parathyroid hormone (PTH) receptor promotes MLL-AF9 oncogene-induced AML but inhibits CML development. Increased TGF-β1 signaling mediates these opposing effects on LSCs of AML and CML respectively, suggesting that bone marrow microenvironment of CML and AML are distinct. Interestingly, adipose tissue could function as a reservoir pool for CD36+ LSCs, as lipolysis of gonadal adipose tissue fuels fatty acid oxidation for these LSCs, suggesting that bone marrow niche also provide a unique microenvironment to support the metabolic demands of LSCs. Overall, the crosstalk between LSCs and bone marrow stroma cells cooperatively construct a unique microenvironment that is essential for LSC maintenance.

4. CONCLUSIONS

Adequate understanding of LSC biology requires to further investigating the unique biological features of LSCs from the cellular and molecular level. These features will provide a keen insight into the dynamic state of LSCs, which is important in current leukemia research. Given that AML is a heterogeneous disease, the regulatory machinery for maintaining LSC function is extremely complicated, and involves multiple layers of alterations, such as transcription factors, metabolism, epigenetics, and microenvironments. Exploring the heterogeneity of LSCs could reveal the dynamic state of LSCs; further investigating the relationship of LSCs with bone marrow microenvironment might enable us fully understand the ecological system of LSC and its niche. From the pathogenesis perspective, these efforts help to fully uncover the process of leukemogenesis of human AML. From a clinical standpoint, studying these different aspects of LSCs will provide potential therapeutic targets. Overall, with the development of new technologies and methods, such as single cell technology, it is hopeful that we begin to understand how LSCs utilize unique cellular and molecular features to maintain their capability of survival and self-renewal, which will eventually contribute to the clinical outcomes in the future.

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