

## REVIEW

# Unique molecular and cellular features of acute myelogenous leukemia stem cells

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It is well known in the field of acute myelogenous leukemia (AML) that many different translocations and genetic aberrancies are found with the various forms of the disease. Indeed, specific translocations are often associated with disease subtypes that manifest themselves through the accumulation of immature myeloid cells at varying stages of differentiation. Moreover, the differentiation state of myeloid blast populations has been utilized as a means of categorizing different AML subtypes (French, American, British, or FAB classification system). Thus, the notion that AML is a family of related but distinct diseases is a common view. Interestingly, however, studies in recent years that have formalized the concept of a leukemic stem cell (LSC) have also begun to define shared developmental, cellular and molecular features amongst the malignant stem cells that give rise to different AML subtypes. Moreover, some of these conserved features appear to be unique to the leukemia stem/progenitor cell population, and are not found in normal hematopoietic stem cells (HSCs). This article will summarize data emerging from the study of LSCs and suggest how distinct molecular and cellular characteristics of the LSC population may provide new opportunities for AML therapy.

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## Introduction

A broad range of studies over many years has indicated that AML arises from mutation(s) at the level of the hematopoietic stem/progenitor cell. These malignant stem cells are capable of limited differentiation, but undergo developmental arrest prior to terminal differentiation.<sup>1,2</sup> Consequently, as is observed for normal hematopoiesis, LSCs lie at the root of clonal growth and are therefore thought to be responsible for perpetuation of the leukemic population. While the general concept of LSCs is well-established,<sup>3–9</sup> a formal description of stem cells in AML has only come about as the result of relatively recent studies. Primarily through the efforts of John Dick and colleagues, a specific initial phenotype (CD34<sup>+</sup>/CD38<sup>-</sup>) was assigned to the AML stem cell, and the characteristics of this population were defined by transplantation studies using the xenogeneic NOD/SCID mouse model system.<sup>10,11</sup> Importantly, it was shown that LSCs possess the hallmark stem cell characteristic of self-renewal, as well as extensive proliferative capacity. In addition, a series of studies from other investigators have further elaborated the phenotype of LSCs and demonstrated their capacity to proliferate *in vivo* and in long-term culture assays.<sup>12–16</sup> Thus, using the same strategies that were originally used to describe normal HSCs, stem cells in human AML specimens have also been defined. In so doing,

such studies have demonstrated a hierarchical structure to AML populations, which in turn highlights an important parallel to normal hematopoiesis. In the same way that normal stem cells are biologically distinct from their more differentiated progeny, with specific cellular and molecular mechanisms that control their behavior, one is similarly forced to conclude that LSCs possess unique features and are quite different from more mature leukemic blasts. Consequently, drug regimens designed to kill AML blasts may not be effective for destruction of the LSC population. Given the central role LSCs are likely to play in the development and pathogenesis of leukemic disease, failure to target this population is a possible cause of relapse. Further, considering the potential biological similarity between LSCs and normal HSCs, it may be difficult to identify drugs that will preferentially target the LSC population and thereby yield a significant therapeutic index. Thus, obtaining a detailed understanding of LSC-specific molecular characteristics is a high priority for the future development of leukemic drugs. Importantly, while some phenotypic features of LSCs are similar to HSCs, recent studies have also described features that are unique to AML (see below). Consequently, researchers now have the ability to prospectively isolate highly enriched samples of AML stem cells and to analyze their cellular and molecular features. In the same way that purification of normal stem cells has been a powerful tool for research, isolation of LSCs will certainly provide unique opportunities to elucidate leukemia-specific stem cell properties.

## Features of AML stem cells

### Phenotype

Despite giving rise to blast cells of markedly different phenotypes, the cell surface characteristics of LSCs from AML subtypes M0, M1, M2, M4 and M5 appear to be quite similar. Using *in vitro* culture assays and transplantation into NOD/SCID mice, researchers have shown that LSCs are typically: CD34<sup>+</sup>, CD38<sup>-</sup>, CD71<sup>-</sup>, HLA-DR<sup>-</sup>, CD90<sup>-</sup>, CD117<sup>-</sup> and CD123<sup>+</sup>.<sup>10–14,16</sup> While expression of several of these markers is the same as on normal HSCs (CD34, CD38, CD71 and HLA-DR), some antigens display leukemia-specific characteristics (CD90, CD117 and CD123). To date, no studies have reported an antigenic difference between LSCs derived from distinct AML subtypes. Thus, while molecular aberrancies that segregate to specific subtypes may generate blast cells with unique features, at the level of primitive cells such differences are not readily detected. This observation would seem to suggest that stem cells destined to contribute to leukemic blast populations share a certain degree of biological commonality. If so, such features may represent conserved consequences of the leukemogenic process, and therefore might provide useful targets for therapy. Indeed, given the recent success of mono-

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clonal antibody-based therapies,<sup>17,18</sup> developing antibodies to LSC-specific antigens may be an exciting approach to AML therapy. CD123 is one such potential target antigen, and it is conceivable that many others await discovery. Application of modern means for antibody selection, such as the use of phage-based antibody libraries, represents one potential strategy for identifying new LSC-specific antigens.

### Cell cycle

In addition to conserved antigenic features, LSCs also appear to share an important cellular characteristic in that they often exist in a quiescent state. Evidence for quiescent LSCs was reported by Terpstra *et al*,<sup>19</sup> who showed that treatment with the cycle active drug 5-fluorouracil was not effective in ablating AML cells in SCID mice transplanted with primary leukemic specimens. Further support has recently come from elegant studies of CML stem cells in which flow cytometric and functional assays demonstrated the presence of a quiescent stem cell population.<sup>20</sup> This observation indicates that at least a subset of CML stem cells are dormant *in vivo* and will therefore be refractory to standard chemotherapeutic agents. Finally, our own recent studies have used flow cytometry to examine primary AML populations. The large majority of human specimens show phenotypically primitive cells with ~95% G<sub>0</sub> status.<sup>21</sup> Thus, while malignant AML cells may proliferate rapidly *in vivo*, the originating stem cell (like its normal stem cell counterpart), appears to be predominantly quiescent. This observation highlights an important difference between LSCs and more mature blast cells, and provides at least one explanation for why AML patients commonly relapse after treatment with cycle-active drugs. Further, if the LSC population is quiescent, there is no reason a priori to believe that a significant therapeutic index would exist between normal and leukemic stem cells. While a minority of AML patients clearly do respond to chemotherapy, it is tempting to speculate that at least some patients may relapse because standard agents do not effectively target quiescent LSCs.

### Molecular biology

Two recent studies have begun to analyze the molecular features unique to primitive AML cells. In the first report, cDNA array studies were performed on CD34<sup>+</sup>/CD38<sup>-</sup> cells from primary human specimens in an effort to identify genes associated with leukemogenesis.<sup>22</sup> Interestingly, these experiments showed that two tumor suppressor genes, interferon regulatory factor 1 (IRF-1), and death associated protein kinase (DAPK) were consistently up-regulated in primitive AML cells, but not in CD34<sup>+</sup>/CD38<sup>-</sup> cells from normal specimens. While the underlying biological importance of this finding remains to be determined, it may suggest that one mechanism of drug resistance for AML stem cells is to suppress proliferation. Clearly, the leukemic clone must ultimately have a growth or survival advantage to become malignant; however, given the cell cycle data described above, it appears that differences in the growth rate of normal vs malignant stem cells may be quite subtle. Thus, expression of tumor suppressor genes may be related to limiting the growth of LSCs, at least until they have differentiated to a later stage.

A second study has shown that primitive AML cells express an active form of the transcription factor NF- $\kappa$ B.<sup>21</sup> While activation of NF- $\kappa$ B has been documented in many malignant cell

types, the situation in AML appears unique in that NF- $\kappa$ B is evident in quiescent populations. In the context of tumor cells, NF- $\kappa$ B is typically associated with anti-apoptotic functions, which are thought to confer a survival advantage to the malignant population.<sup>23</sup> Evidence has been reported for several solid tumors, as well as lymphoma and lymphoid leukemia, indicating an anti-apoptotic function for NF- $\kappa$ B.<sup>24–28</sup> Thus, expression in the LSC population implies that inhibition of NF- $\kappa$ B may be an effective strategy for selectively killing AML stem cells. This is a particularly exciting option given the lack of detectable NF- $\kappa$ B activity in normal HSCs. Consequently, the therapeutic index for drugs that target NF- $\kappa$ B might be quite favorable. Direct evidence in support of this theory has recently been reported through use of the proteasome inhibitor MG-132. Like other agents of this nature, MG-132 strongly inhibits NF- $\kappa$ B by blocking degradation of the negative regulator I $\kappa$ B.<sup>29,30</sup> A 12 h treatment of primitive AML cells with MG-132 was shown to result in rapid down-regulation of NF- $\kappa$ B and subsequent apoptosis of leukemic CD34<sup>+</sup>/CD38<sup>-</sup> cells. However, the same treatment of normal CD34<sup>+</sup>/CD38<sup>-</sup> cells induced almost no cell death.<sup>21</sup> Further studies have demonstrated that primitive AML cells treated in this fashion show a dramatic loss of NOD/SCID repopulating ability, while normal cord blood cells are unimpaired (MLG and CTJ, manuscript in preparation). Thus, both *in vitro* and *in vivo* functional data indicate that proteasome inhibition is a potentially useful strategy for specific ablation of the LSC population.

### Emerging theories

In addition to the conserved molecular features of LSCs described above, it has also been recently proposed that conserved function among differing mutations in AML may be a critical aspect of pathogenesis. Gary Gilliland has described a two-hit model of AML in which the critical events are an activating mutation of a kinase combined with a mutation that alters the function of a hematopoietic transcription factor (in addition, similar models have recently been described by other investigators).<sup>2,31,32</sup> Importantly, this theory seeks to identify a unified molecular theme for AML which may help to explain how distinctly different mutations can generate similar biology among the various types of AML. In this model, the enhanced growth advantage provided by the constitutive kinase, along with the altered developmental function of the transcription factor are thought to provide the essential elements of acute leukemogenesis. This interesting theory is supported by a large amount of data from AML specimens in which these two types of mutations have been clearly documented. Given the recent studies describing leukemic stem cells, a prediction of the two-hit model is that both mutations must occur in a relatively primitive hematopoietic stem or progenitor cell in order for leukemogenesis to occur. One might imagine that the intrinsic self-renewal and proliferative characteristics of a stem cell, in combination with the molecular events suggested by Dr Gilliland, would provide the specific molecular environment necessary for onset of acute leukemia. Support for this idea has recently come from animal studies using retroviral gene transfer as a means to model primary human disease. Interestingly, two observations appear to be held in common among several transgenes tested. First, introduction of a single mutated gene can induce aberrant myeloid development of varying severity, but is generally insufficient to induce acute leukemia. This phenomenon has been observed for translocation-generated oncogenes such as MLL-ELL, MLL-

ENL, MLL-CBP, AML/MDS/EVI, NUP98/HoxA9, E2A-Pbx1 and AML-ETO.<sup>33–40</sup> However, with a sufficiently long latency period (often many months), second hits generally do occur in these animals, and leukemia ensues. Analysis of retroviral integration sites has shown that such AMLs tend to arise from one or a small number of clones. Moreover, limiting dilution studies show that the frequency of leukemia-initiating cells is typically in the range of 0.25–1.0%.<sup>37,41</sup> Collectively, these data strongly suggest that leukemic disease in the mouse model system arises from mutations in a stem/progenitor cell and that such a stem cell is required to perpetuate the disease.

## Summary

AML populations are comprised of a hierarchical structure and in recent years it has been possible to begin analyzing individual components of the leukemic clone. While varying AML subtypes clearly differentiate to differing degrees, it has become increasingly clear that important commonalities exist at the top of the developmental hierarchy. Since developmental aberrancies are not readily evident until stem cells have differentiated to later stages, it raises the question – do LSCs know they are leukemic? In other words, are leukemia-specific features manifested at the stem cell level, or is limited differentiation required in order for the consequence of mutations to be realized? While a variety of LSC characteristics (self-renewal, quiescence, cell surface phenotype, etc.) are virtually identical to normal HSCs, recent analyses of AML molecular biology suggest that some differences between normal and leukemic cells are apparent in the stem/progenitor cell pool. From a therapeutic perspective, this observation is extremely important because it suggests LSCs do have unique characteristics that may make them preferentially sensitive to apoptosis/ablation. These data also serve to emphasize the importance of better understanding LSCs and how they differ from normal HSCs.

Important directions for future research will be to determine the relevance of LSC-specific features. For example, is NF- $\kappa$ B a central regulator of LSC biology and if so what is the mechanism by which this prevalent transcription factor is activated? One intriguing possibility has been suggested by recent studies on the transmembrane tyrosine kinase, FLT3. Constitutive activation of FLT3 appears to be a common feature of AML cells<sup>42,43</sup> and may provide one explanation for how NF- $\kappa$ B becomes activated. In addition, recent studies indicate that inhibition of FLT3 can induce apoptosis in some primary AML specimens.<sup>44,45</sup> It will be interesting to determine whether NF- $\kappa$ B is associated with the FLT3 signal transduction pathway in primary AML cells. Another exciting direction that has been explored is the use of animal models of leukemia in conjunction with gene-targeting strategies. For example, a recent study examined the role of cytokines by using retroviral gene transfer of the BCR/ABL oncogene into HSCs from mouse strains deficient for IL-3 or GM-CSF.<sup>46</sup> A parallel study used the same strategy to analyze the pathogenesis of disease induced by several activated kinases.<sup>47</sup> This approach permits the analysis of a specific gene in the leukemogenic process. Importantly, such a strategy should be applicable to almost any gene that can be analyzed using gene-targeting methods. Further, molecular genetic strategies using conditional alleles of both the AML-ETO and BCR/ABL oncogenes have recently been employed as a means to induce *de novo* growth of preleukemic or leukemic cells.<sup>48,49</sup> Such approaches provide powerful tools for the analysis of AML and unique opportunities to

define the role of stem cells in the pathogenesis of leukemic disease.

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