Recent advances in acute myeloid leukemia stem cell biology

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ABSTRACT

The existence of cancer stem cells has long been postulated, but was proven less than 20 years ago following the demonstration that only a small sub-fraction of leukemic cells from acute myeloid leukemia patients were able to propagate the disease in xenografts. These cells were termed leukemic stem cells since they exist at the apex of a loose hierarchy, possess extensive self-renewal and the ability to undergo limited differentiation into leukemic blasts. Acute myeloid leukemia is a heterogeneous condition at both the phenotypic and molecular level with a variety of distinct genetic alterations giving rise to the disease. Recent studies have highlighted that this heterogeneity extends to the leukemic stem cell, with this dynamic compartment evolving to overcome various selection pressures imposed upon it during disease progression. The result is a complex situation in which multiple pools of leukemic stem cells may exist within individual patients which differ both phenotypically and molecularly. Since leukemic stem cells are thought to be resistant to current chemotherapeutic regimens and mediate disease relapse, their study also has potentially profound clinical implications. Numerous studies have generated important recent advances in the field, including the identification of novel leukemic stem cell-specific cell surface antigens and gene expression signatures. These tools will no doubt prove invaluable for the rational design of targeted therapies in the future.

Key words: acute myeloid leukemia, leukemic stem cells, cellular, molecular.

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Introduction

The existence of cancer stem cells (CSC) was first demonstrated over a decade ago in acute myeloid leukemia (AML) using xenogeneic transplant models.[1,2] Since then CSC have been identified in a variety of solid tumors,[3] although their existence in some malignancies remains contentious.[4,5] AML is a heterogeneous disease, both biologically and clinically, in which a number of distinct genetic abnormalities have been described. However, despite this heterogeneity, early pioneering studies demonstrated that only the most primitive $Lin^-CD34^+CD38^-$ fraction of AML cells and not the more mature $Lin^-CD34^+CD38^+$ or $CD34^-$ populations were capable of transferring disease to NOD/SCID mice.[2] In the recipient mice, the $CD34^+CD38^-$ cells differentiated into leukemic blasts and recapitulated the phenotype of the disease observed in the patient. Furthermore, these cells were able to reconstitute and give rise to AML in secondary recipients, indicating self-renewal of the LSC in the primary recipients.[2] Thus, in a similar way to normal hematopoiesis, it was demonstrated that AML is arranged as a loose hierarchy in which a small population of self-renewing leukemic stem cells (LSC) give rise to a large population of more mature leukemic blasts which lack self-renewal capacity.

This organization helps to explain the all too frequently observed clinical scenario in AML whereby current chemotherapeutic regimens frequently induce remission but relapses, often fatal, are commonly observed. The hierarchical organization of AML suggests that this may relate to current therapeutics targeting only the rapidly proliferating leukemic progenitors, and not the more chemoresistant LSC.[6] A thorough understanding of how LSCs differ from their normal counterparts at both the phenotypic and molecular level is, therefore, pivotal to developing targeted therapies, with acceptable toxicities, for AML. This review will aim to describe recent advances in the LSC field with regard primarily to AML, although the concepts described will extend to LSC/CSC in other tumors. The cellular and molecular (both genetic and epigenetic) properties of LSC, and how these might differ from normal hematopoietic stem cells (HSC), will be summarized and potential therapeutic targets explored.

Phenotypic characterization of leukemic stem cells

Initial studies suggested that LSC were restricted to the $Lin^-CD34^+CD38^-$ population, a phenotype shared by the HSC capable of reconstituting normal hematopoiesis in NOD/SCID mice.[7] However, since these initial studies, xenograft transplant models have been further refined through the administration of anti-CD122, to suppress Natural Killer cell function, as well as through the use of more

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immune deficient mouse strains such as NOD/SCID/$\beta_2m^{-/-}$ or NOD/SCID/IL2R$\gamma^{-/-}$ mice. Although these refinements did not facilitate engraftment of AML samples which failed to engraft in the original NOD/SCID model,[8] engraftment efficiency was increased in the newer models. Furthermore, they also allowed LSC activity to be demonstrated in the more mature $Lin^-CD34^+CD38^+$ progenitor population of some AML patients.[9] In addition, intra-femoral, rather than standard tail vein, injection of the transplanted cell innoculum was also found to increase the efficiency of disease transfer.[10] Using these refinements LSC were found in more than one compartment and the efficiency of disease transfer followed a hierarchy, with limiting dilution experiments demonstrating that the frequency of LSC was higher in the $Lin^-CD34^+CD38^-$ population compared to the $Lin^-CD34^+CD38^+$ population. However, in both compartments LSC were rare and varied in frequency from 1 in 1.6 $\times10^5$ to 1 in 1.1×10^6 cells.[11] These studies suggest that the LSC compartment in AML is more heterogeneous than previously anticipated and includes cells with the surface phenotype of committed progenitors. Importantly, normal $Lin^-CD34^+CD38^+$ progenitor cells are only able to transiently reconstitute NOD/SCID mice and cannot repopulate secondary recipients.[12] In fact, studies in ALL may suggest an even greater degree of plasticity within the LSC compartment. In these studies, LSC potential was also demonstrated in populations with phenotypic characteristics of progenitors ($CD19^+$ and $CD34^-$).[13] However, not only were these populations able to transfer leukemia to recipient mice, but $CD34^-$ populations were also able to regenerate $CD34^+$ progeny within the transplanted leukemia. Similar results within a recently described murine model of AML suggest that AML LSC may also have a comparable developmental plasticity.[14]

A more detailed comparison of the LSC phenotype with normal myeloid stem and progenitor ontogeny has revealed that in the vast majority of $CD34^+$ AML patients, $Lin^-CD34^+CD38^-CD90^-CD45RA^+$ and $Lin^-CD34^+CD38^+CD123^+CD45RA^+$ LSC compartments coexist. These respective populations resemble the normal lymphoid-primed multipotent progenitor (LMPP) and granuloctye-monocyte progenitor (GMP) populations at both the phenotypic and molecular level.[15] Although the majority of AML cases express the CD34 marker, in some patients, including those with Nucleophosmin (NPM1) mutations, the $CD34^+$ percentage is very low. In patients with less than 0.5% $CD34^+$ cells, LSC activity was exclusively restricted to the $CD34^-$ population, whereas in other patients LSC were present in both the $CD34^+$ and $CD34^-$ populations.[16] Together, these studies confirm that the LSC population is phenotypically diverse and can vary markedly between patient subgroups, and even between individual patients within these subgroups. However, how this might reflect the heterogeneity of the initial target cell transformed or the combinations of collaborating mutations is, as yet, unknown.

In addition to CD34 and CD38, LSC have been shown to express a variety of other markers including the myeloid antigens CD33, CD123 and CD13.[17] More recently several novel markers that are more highly expressed on $CD34^+CD38^-$ LSC than normal $CD34^+CD38^-$ HSC have been described. These include CLL-1, CD96, TIM3, CD47, CD32 and CD25 (Table 1). C-type lectin-like molecule-1 (CLL-1) was expressed by leukemic blasts at diagnosis from 92% of AML patients analyzed.[19] Moreover, although this antigen was expressed on normal $CD34^+CD38^+$ myeloid progenitors, it was absent on normal HSC. However, as with many LSC selective antigens, it is not expressed on every LSC. Within the $CD34^+CD38^-$ LSC compartment, a median expression of 33% CLL-1^+ cells was observed when the data from 29 AML patients were combined.[20] CD96 (also known as Tactile) is a member of the Ig gene family. It is also expressed at higher levels in normal progenitors than HSC. Expression was elevated in the $CD34^+CD38^-$ LSC compartment when compared to normal HSC in 65% of AML patients.[21] TIM3 is a negative regulator of Th-1-T cell immunity. In addition, the low level of TIM3 expression by HSC compared to LSC enabled the prospective separation of LSC in a variety of AML patients.[22] The transmembrane protein CD47 is the ligand for signal regulatory protein α (SIRPα). SIRPα is expressed on phagocytic cells and its interaction with CD47 results in inhibition of phagocytosis. Expression of CD47 by LSC was found to protect them from being phagocytozed by macrophages and dendritic cells and its presence contributed to poor overall survival in patients.[23] Although CD47 was consistently more highly expressed by LSC than HSC, there was a large degree of variation across patients in terms of the percentage of LSC that expressed CD47.[23] This was also true for the recently identified LSC-specific markers CD25 and CD32, which were found on 34.4% and 24.6%, respectively, of LSC from 61 AML patients analyzed.[24] Thus, despite the identification of novel LSC-specific markers, there is a large degree of heterogeneity in expression of these markers among patients. Thus, patient-specific targeting of LSC surface antigens may be necessary.

Molecular characterization of leukemic stem cells

LSC share several properties with normal HSC, including a generally quiescent cell cycle status, apoptotic resistance and the capacity to self-renew. Several pathways have been shown to mediate self-renewal in both LSCs and HSCs including WNT/β-catenin, NOTCH and

Table 1. Summary of cell surface marker expression on hematopoietic stem cells and acute myeloid leukemia LSC.

Marker	Expression on HSC	Expression on LSC	Reference
CD34	+	+/-	1, 2, 16
CD38	-	+/-	1, 2, 9, 15
CD90	+	-/+	15
CD123	+	++	15, 17
CD45RA	-	+	15
CD33	++	++	17
CD13	+	++	17
CD44	+	++	18
CLL-1	-	+	19
CD96	+	++	20
TIM3	+	++	21
CD47	+	++	22
CD32	-	+	23
CD25	-	+	23

+ indicates expression of the marker on some or all of the cells. ++ indicates that the marker is expressed at high levels and – indicates that the marker is not expressed in this compartment.

Hedgehog signaling, as well as several members of the clustered HOX gene family and the polycomb group protein Bmi1.[25,26] However, in AML and other malignancies, these genes or pathways are often mutated, activated or aberrantly expressed. Thus, a therapeutic window may exist, whereby interfering with these pathways might ablate LSC while sparing the normal HSC compartment.

Gene expression profiling studies in mouse and human LSC or pre-leukemia initiating cells have attempted to identify the molecular drivers of LSC function. In particular, mouse models have proven particularly useful in identifying LSC-specific gene expression profiles. Analysis of leukemia models initiated by a range of different MLL-fusion proteins revealed that LSC were enriched within the c-Kit$^+$ compartment.[27] These cells were able to transfer disease to secondary recipients with greater efficiency than c-Kit$^-$ cells. Comparison of the gene expression profiles of the c-Kit$^+$ LSC-enriched populations *versus* the c-Kit- cells, revealed that LSC in these models expressed a gene signature more akin to embryonic stem cells than adult HSC.[28] Another group purified LSC to near homogeneity from leukemias induced by expression of MLL-AF9 in the GMP compartment.[29] In this model LSC resembled GMP at the phenotypic and molecular level but expressed a set of genes normally restricted to HSC, designated the *self-renewal signature*. This signature included various *Hox* genes, including *Hoxa9*, *Hoxa10* and *Meis1*. These genes are highly expressed in human AML with MLL-translocations and have been shown to regulate the survival and self-renewal of LSC.[30,31] This self-renewal signature was partially shared by LSC generated from a completely different initiating mutation: loss of the CEBPA p42 isoform.[32] The overlap in gene expression profiles of MLL-AF9 and *Cebpa* deficient LSC suggests the existence of common mechanisms of progenitor transformation. This idea was further extended to assess gene expression changes in pre-leukemia and leukemia stem cells following expression of a number of disparate AML-associated initiating oncogenes (AML1-ETO, NUP98-HOXA9 and MOZ-TIF2). Despite heterogeneity with regard to the initiating mutation, common and overlapping downstream genes were identified including *Bmi1*, *Meis1*, *Sox4*, *Tcf4*, *Hoxa9* and *Smad7*.[33] Interestingly, some of these genes were able to partially phenocopy the original mutation when over-expressed in murine bone marrow cells. Taken together, these findings suggest that common pathways which facilitate leukemic transformation exist downstream of a variety of different initiating mutations and identifies these pathways as potential therapeutic targets.

Although gene expression analysis of bulk primary human samples has greatly informed the classification and biology of AML, few studies have been performed in human LSC. Nonetheless, a small number of recently performed studies have reported LSC-specific gene expression profiles generated from patient samples.[11,15,34-36] All these studies compared gene expression in populations enriched for LSC, as either demonstrated by function or inferred by surface phenotype, with either AML populations which lack LSC properties and/or normal hematopoietic stem and progenitor cells (HSPC) of identical surface phenotype. The studies demonstrate that LSC populations retain similarities of gene expression with the equivalent phenotypic normal HSPC compartment, again reinforcing the possibility that both HSC and progenitors may be the targets of transformation in AML (see below). In addition, they demonstrate that differential gene expression distinguishes LSC populations from those that lack LSC activity. Utilizing the differential LSC *signatures*, these studies were also able to identify *a priori* poor risk cases of AML from bulk gene expression profiles, with their predictive value independent of other known prognostic markers, including karyotype and mutational status for *FLT3*, *NPM1* and *CEBPA*.[11,34,35] It is hoped that these signatures may also identify potential drivers of LSC function and putative molecular LSC targets. In support of this concept, in one study, pathways involved in adherens junction, actin cytoskeleton regulation, apoptosis, MAPK and WNT signaling were dysregulated in LSC.[36] The identity of these genes contained within the signatures, which include *ERG*, *MEIS1*, *MECOM* (*EVI1*), *HOXA5*, *MEF2C* and *SETBP1*, is also supportive of their role in leukemogenesis. However, the signatures from the three studies[11,34,35] contained, at best, a modest overlap, although *FLT3* and *HLF* were within this overlap. This may reflect molecular heterogeneity within the LSC compartment but likely also reflects the small numbers of profiles assessed and differences in methodology and bioinformatic analysis. It is hoped that an increase in numbers of LSC gene expression profiles and standardization of their analysis will deconvolute these signatures further, allowing critical pathways to be revealed.

The cell of origin in acute myeloid leukemia

Although it is tempting to infer information about the cell of origin in AML based on the cellular phenotype of the LSC, it may be misleading to do so. It is entirely possible that the initial transforming event results in aberrant surface marker expression on this pre-leukemic LSC, such that it is phenotypically uncoupled from its normal counterpart. Despite this, LSC have been isolated which share the cellular and molecular phenotype of HSC and more committed myeloid progenitors,[15,29] demonstrating that, at least to some extent, cell surface marker expression on the LSC is suggestive of the cell type initially transformed.

It remains unclear as to whether the initiating mutation responsible for generating the leukemic clone occurs in an HSC, downstream progenitor cell, or both. Murine retroviral models have demonstrated that certain leukemia associated fusion oncogenes including MLL-ENL, MOZ-TIF2 and MLL-AF9 are able to transform committed progenitors into LSC.[29,37,38] However, when under the control of the endogenous MLL promoter, MLL-AF9 was unable to transform GMP, suggesting that gene dosage may play an important role.[19] In addition to MOZ-TIF2 and MLL-AF9, NUP98-HOXA9 and AML-ETO were also able to confer self-renewal properties to committed progenitors, although the latter was unable to transform these progenitors *in vivo*.[33] Interestingly, other oncogenes, including BCR-ABL, FLT3-ITD and co-expression of Hoxa9 and Meis1, were not able to alter the self-renewal properties of progenitors.[33,38,40] Instead, Hoxa9 and Meis1 or BCR-ABL were oncogenic only when expressed in HSC.[38,40] In accordance with this, in chronic myeloid leukemia (CML), the initiating chromosomal translocation t(9;22) leading to formation of the BCR-ABL fusion gene occurs in an HSC.[41] However, transition of the disease to myeloid blast crisis occurs as a result of additional events accumulated in GMP, including activation of β-catenin, which confer self-renewal activity to this compartment.[42] CML can also

progress to lymphoid blast crisis in a minority of patients. Although it was unclear whether the leukemias studied represented lymphoid blast crisis or *de novo* acute lymphoblastic leukemia (ALL), the initial cell transformed in P210 BCR-ABL1 ALL was demonstrated to be an HSC, in that the chromosomal rearrangement was present in this phenotypic compartment.[43] This contrasts with other cases of ALL, including those with P190 BCR-ABL1 and *ETV6-RUNX1* rearrangements in which a committed B-cell progenitor was demonstrated to be the likely origin of disease. In addition, in elegant experiments in primary human ALL cells, *ETV6-RUNX1* expression conferred self-renewal activity to the B-cell progenitor compartment.[43,44] As has been previously mentioned, a similar situation occurs in *de novo* AML, where many patients demonstrate functionally defined LSC with the surface phenotype of committed myeloid progenitors.[9,15] Thus, it seems likely that in AML and ALL both HSC and committed progenitors may serve as the cell of origin (Figure 1). A prerequisite for this argument is that, in the case of progenitor transformation, the initiating mutation must confer self-renewal activity to that compartment in order for the mutation to be propagated.

Evolution of the leukemic stem cell compartment

The finding that multiple populations of LSC may exist within a single patient suggests that the LSC population is neither uniform nor static and may evolve from one cellular phenotype to another depending upon the acquisition of additional genetic or epigenetic alterations (Figure 2). Genetic evolution of the LSC compartment has been most convincingly demonstrated in ALL. The *ETV6-RUNX1* translocation is a known initiating event that occurs prenatally in a subset of childhood B-ALL cases.[46] Acquisition of this initiating event results in the generation of a pre-leukemic clone that requires subsequent additional alterations for the development of overt leukemia.[47] Studies in twins harboring the *ETV6-RUNX1* translocation and non-concordant for the development of leukemia, revealed the presence of a $CD34^{+}CD38^{-}CD19^{+}$ cancer propagating cell in the leukemic twin which was ancestrally related to a pre-leukemic stem cell found to be clonally expanded in the healthy twin.[44] Furthermore, elegant studies in which known copy number alterations (CNAs) were examined by FISH at the single cell level in $ETV6\text{-}RUNX1^{+}$ cells, revealed considerable complexity in both the structure and hierarchical organization of multiple independent

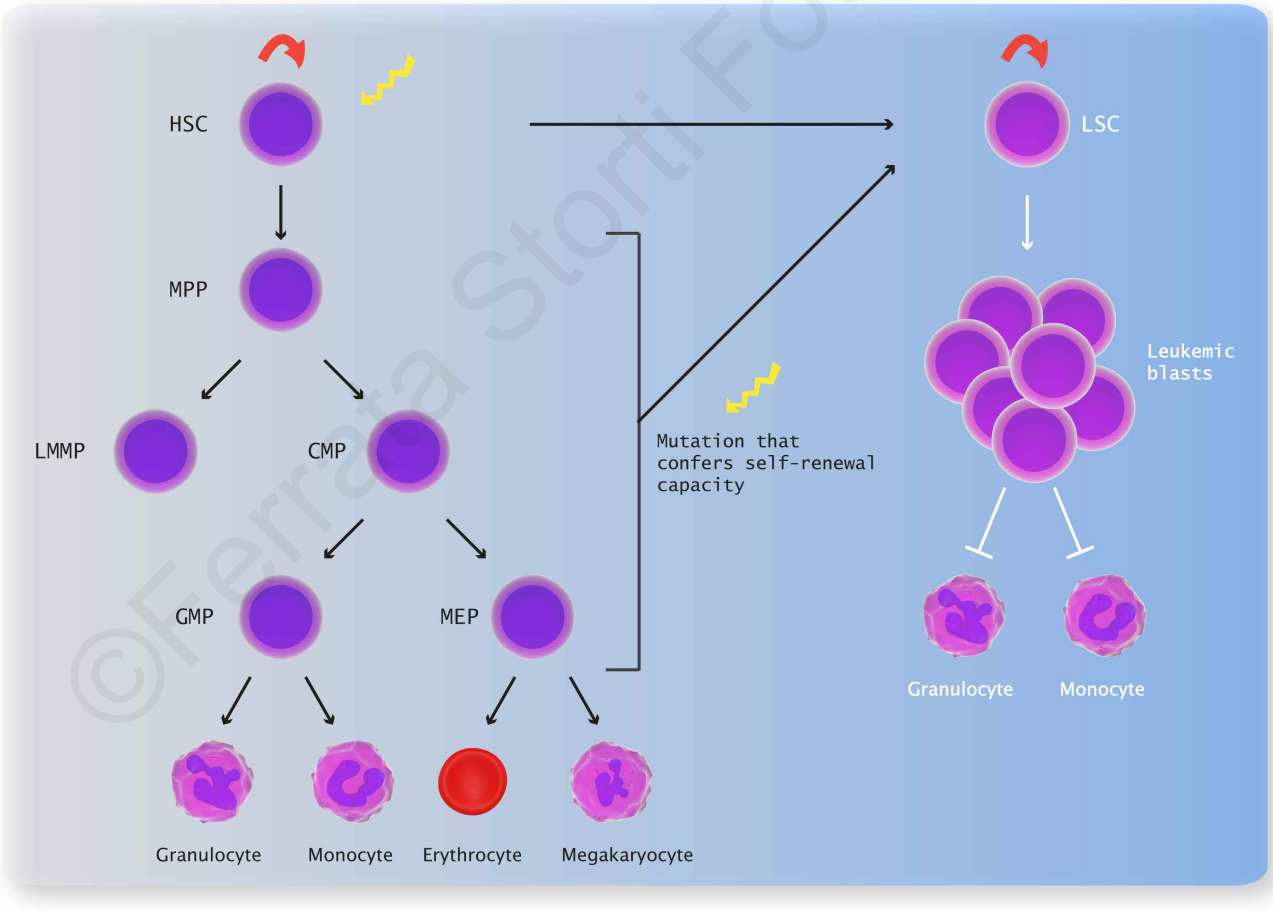

Figure 1 The cell of origin in AML. During normal myelopoiesis, HSC differentiate into mature blood cells via progenitor populations in a series of lineage restriction steps. They first give rise to multipotent progenitors (MPP) that in turn differentiate into lymphoid-primed multipotent progenitors (LMPP) and common myeloid progenitors (CMP). Granulocyte-monocyte progenitors (GMP) are formed from either LMPP or CMP whereas only CMP give rise to megakaryocyte-erythroid progenitors (MEP). Mutations may accumulate in the long-lived HSC population that has inherent self-renewal capacity, resulting in the generation of a (pre)-LSC. Alternatively mutations may occur in the aforementioned progenitor populations. However, since these cells inherently lack self-renewal activity, the mutation must confer this capacity to the progenitors in order for the mutation to be propagated in a self-renewing (pre)-LSC.

leukemic sub-clones present within individual patients.[48] Another study has posited a similar multi-branching model of clonal evolution of BCR-ABL$^+$ ALL LSC.[49]

Studies such as these are rare in AML patient samples. However, the continued presence of the AML1-ETO rearrangement in phenotypic HSC from patients in long-term remission has been demonstrated, suggesting the existence of a pre-leukemic stem cell in certain forms of *de novo* AML.[50] In addition, a recent study, presented in abstract form, has extended this work in residual HSC isolated from presentation AML tumor samples that were fully genotyped by next generation sequencing. In small numbers of patients, this study demonstrated the presence of founder mutations in this apparently normal cellular compartment, identifying the residual 'normal' HSC compartment as a reservoir of pre-leukemic stem cells which lacked the complete mutational spectrum necessary for full transformation.[51] In addition, the demonstration that some AML patients harbor specific mutations at diagnosis (such as FLT3-ITD) which are not present at relapse, or vice versa, further suggests the presence of a pre-leukemic stem cell or of clonal evolution in the LSC compartment.[52] Recent work, in which the genomes of AML patients at diagnosis and relapse were sequenced, has provided further insight into the clonal evolution of LSC during disease relapse. Additional mutations were acquired in either the dominant clone at diagnosis or a minor sub-clone that presumably enabled the cells to survive the selective pressures of chemotherapy and contribute to relapse.[53] This study demonstrates that elimination of not only the founding clone but also sub-clones derived from it are required for long-term remission and highlights the role that chemotherapy may play in contributing to disease relapse.

We have provided evidence that both the surface phenotype and mutational genotype of LSC can evolve with

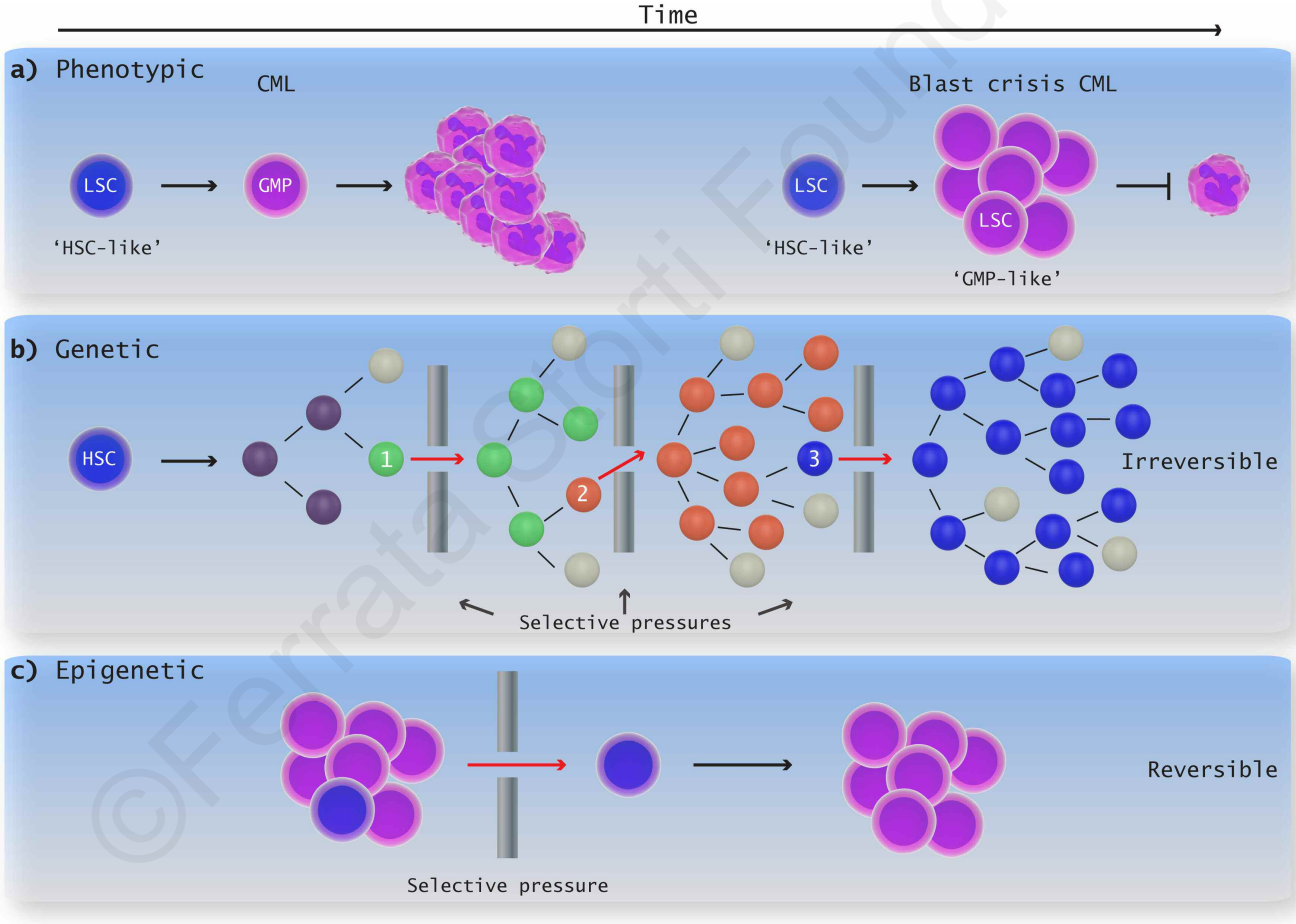

Figure 2 The heterogeneity of AML. (A) During disease progression the hierarchical organization of the leukemia and phenotype of the predominant LSC population may change. For example, in CML, LSC possess a Lin$^-$CD34$^+$CD38$^-$ *HSC*-like phenotype during the chronic phase. As the disease progresses towards blast crisis, mutations conferring self-renewal activity to the downstream GMP population occur, such that the predominant LSC population at this stage of disease is CD34$^+$CD38$^+$CD123$^+$CD45RA$^+$ GMP-like. (B) The genetic repertoire of the LSC is also subject to change during disease progression. Various selection pressures, such as nutrient deprivation, space limitations, anoxia and, most importantly, chemotherapy may select for cells with mutations that enable them to overcome these *bottlenecks*. Thus various subsets of genetically distinct LSCs may exist within individual patients. Each colored circle represents a cell and a change in color represents the acquisition of an additional mutation. The number of mutations acquired is shown within the cell prior to progression through the bottleneck. Gray cells represent apoptotic cells. Adapted from Greaves.[45] (C) In addition to genetic diversity, there is also likely to be epigenetic diversity within the LSC population (as indicated by the cell with the blue nucleus). However, unlike the acquisition of genetic mutations that is an irreversible process, epigenetic modification is dynamic and the LSC may revert to its original epigenetic status after selective pressures have been overcome.

time, particularly under selective pressures such as chemotherapy. It has recently been demonstrated in solid organ tumors that altered epigenetic states may also provide sufficient survival/resistance signals for CSC to negotiate these *bottlenecks*. In the first of these studies, the self-renewal of melanoma cells was demonstrated to be dependent upon dynamic, rather than hierarchical, regulation of the histone H3K4 demethylase JARID1B.[54] The second study extended the role of dynamic chromatin regulation in cancer stem cell adaption, demonstrating that the resistance of lung cancer cells bearing the stem cell markers CD24 and CD133 to erlotinib was mediated not by acquired mutation but by global gene expression changes in association with upregulation of signaling via IGF-1R.[55] Furthermore, they demonstrated that IGF-1R signaling upregulated the histone demethylase KDM5A/JARID1A and that this axis altered global H3K4 methylation and H3K14 acetylation patterns. Taking therapeutic advantage of these findings, the authors were able to restore sensitivity in these cells via the addition of histone deacetylase inhibitors or selective inhibitors of the IGF-1 receptor. Thus dynamic epigenetic changes in CSC may mediate self-renewal and survival.

Aberrant epigenetic regulation is also likely to contribute to the heterogeneity of the LSC compartment in AML. Several epigenetic modifiers are mutated in AML either by chromosomal translocation (including MLL, MOZ and JARID1A[56]) or by point mutation / deletion (including DNMT3A, EZH2, TET2, IDH1 and ASXL1; for a further review see Fathi and Abdel-Wahab[57]). In addition, it has been demonstrated that DNA methylation patterns can classify AML patient samples and prognosticate outcome,[58] and that DNA methylation levels increased from diagnosis to relapse in 83% of AML patients.[59] Taken together these findings suggest that in AML altered epigenetic states play a role in mediating resistance of the LSC to chemotherapy.

Targeting the LSC compartment?

In order to effectively eradicate AML, drugs which selectively target the LSC compartment with minimum toxicity to the normal HSC compartment are required. Antigens which are selectively expressed by leukemic cells represent one such area of therapeutic intervention, and the use of rituximab, which targets the CD20 antigen, has revolutionized the treatment of lymphoproliferative disorders.[60] Initial studies in AML have focused on targeting CD33 which is highly expressed by LSCs and their progeny.[61] Gemtuzumab ozogamicin (Myelotarg), an antibody against CD33 conjugated to the toxin calicheamicin, is now in clinical trials.[62] Although this agent is effective at inducing remission in some patients, they are still prone to

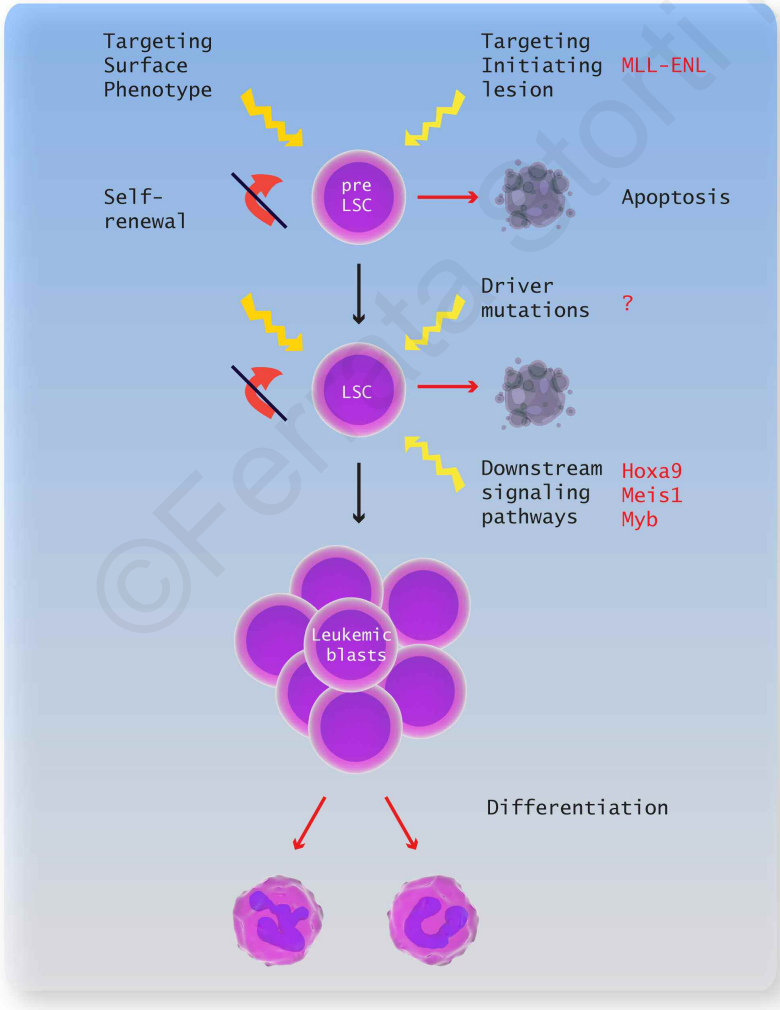

Figure 3 Targeting the LSC. Knowledge of the initiating genetic lesion and the downstream driver mutations will allow the delivery of specifically tailored targeted therapies. The same is true for identification of LSC-specific surface antigens. For example, MLL-ENL is a known initiating mutation in AML that results in the generation of a pre-LSC. Subsequent as yet poorly characterized (epi)genetic alterations which further drive the leukemic phenotype are thought to be acquired during the evolution of proper LSC. However, despite the acquisition of additional events, LSC continue to be dependent upon MLL-ENL expression. Therefore, interfering with MLL-ENL activity is a potential therapeutic approach. It is likely that targeting the subsequent driver mutations will also abrogate leukemia growth, although the tumors may be less dependent upon these lesions than initiating mutations. In addition, targeting pathways downstream of the initiating lesion, such as Hoxa9, Meis1 and Myb, is also a valid strategy. An alternate, and not mutually exclusive, strategy would be to target aberrant LSC-selective surface markers with antibodies or antibody conjugates. In reality, it is likely that such targeted therapies would need to be combined with each other and current chemotherapies for maximal effect. But it is hoped that efficient targeting of the LSC compartment could result in either loss of self-renewal, apoptosis or differentiation leading to improved outcomes in patients with AML.

relapse, presumably because LSC are resistant to the toxin.[63] Furthermore, safety issues have been raised in at least one other trial and prolonged cytopenias have also been described despite effective clearing of the leukemic cells by the treatment.[62] This may reflect the expression of CD33 by normal HSC.[17]

Several antigens are more highly expressed by AML LSC than normal HSC. This raises the possibility of a *therapeutic window* whereby therapeutic antibodies could be used to selectively target LSC whilst sparing HSC (Table 1). Indeed, promising results were obtained when some of these antigens were targeted in xenografts. As an example, treatment of AML cells with a neutralizing antibody against CD123 prevented their engraftment in NOD/SCID mice and reduced leukemic cell burden in mice with established disease.[64] Similar results were obtained following treatment of AML xenografts with antibodies targeting CD44[18] and CD47.[23] Importantly, in all cases LSC were selectively targeted by the treatment and HSC were significantly less affected.

An alternative strategy is to target the initiating mutation or molecular pathways directly downstream. As direct proof of principle for this concept, several studies have ablated the initiating mutation in murine models of leukemia, leading to their regression. For example, the MLL-ENL fusion protein is sufficient for both the initiation and maintenance of disease in mouse models.[65,66] Ablation of MLL-ENL expression in mice with established AML resulted in disease regression, despite the acquisition of additional mutations.[66] Thus, despite evolution of the disease, the leukemic cells remained addicted to the initiating oncogenic lesion, indicating that therapies which interfere with MLL-fusion protein activity would be of therapeutic benefit.

MLL-fusion proteins aberrantly regulate gene expression through a number of interactions with multi-protein complexes including members of the super elongation complex (SEC)[67,68] and polymerase-associated factor complex (PAFc)[69,70] These associations are mediated, at least in part, through interaction with the bromodomain and extra-terminal (BET) chromatin adaptor proteins.[71] Displacement of BET family members from chromatin with a small molecule (I-BET151) which disrupts the protein-protein interaction between BET proteins and acetylated lysine residues in histone tails resulted in apoptosis of immortalized cell lines harboring MLL-fusion genes and enhanced the survival of mice with established leukemia.[71] Furthermore, I-BET151 treatment of human LSC from patients with MLL-rearranged AML completely ablated their clonogenic capacity *in vitro*, while normal HSC were relatively unaffected. Thus, indirect inhibition of MLL-fusion protein activity by preventing its association with chromatin is efficacious in pre-clinical studies.

A similar study demonstrated that knock down or inhibition of the BET family member BRD4 had a profound effect on the growth of human AML cells harboring a variety of mutations.[72] Furthermore, gene expression changes upon BRD4 inhibition were similar to those described by the same researchers upon Myb knock down in an MLL-AF9 AML mouse model. Ablation of Myb expression inhibited proliferation of leukemic cells *in vitro* and eradicated disease *in vivo* with limited effects on normal hematopoietic cells.[73] Thus, Myb appears to be an early and key player in oncogene addiction mediated by MLL-fusion proteins. This and other studies have shown that Myb expression may be a more general mediator of self-renewal in a variety of different sub-types of AML. As an example, Myb was also demonstrated to be part of an *immediate self-renewal program* downstream of other oncogenes including NUP98-HOXA9 and MOZ-TIF2.[33] Therefore, there are likely to be common and overlapping pathways mediating self-renewal downstream of a variety of different initiating mutations that may also offer avenues of therapeutic intervention in a wider group of AML patients.

In summary, it is apparent that the vast heterogeneity evident in AML extends to the LSC compartment at both the cellular and molecular level. However, our knowledge base for LSC biology and how this might differ from HSC biology continues to grow. The further identification of new LSC-specific markers represents a novel therapeutic avenue, although it is becoming apparent that no single marker is uniform for LSC between, and even within, individual patients. In addition, mouse models will continue to be invaluable tools for the study of mechanisms of leukemic transformation, LSC biology and therapy. Gene expression profiles of LSC enriched populations have also begun to provide much needed knowledge about the molecular mechanisms mediating self-renewal of human LSCs. However, the limited overlap in expression signatures between pioneering studies requires further scrutiny. Finally, proof-of-principle studies demonstrate that initiating lesions may be targeted, even those which require inhibition of a protein-protein interaction. Therefore, although targeted patient-specific therapies might still be a considerable way off for the majority of patients, therapies which target specific initiating mutations, their downstream pathways and LSC selective surface antigens are now a reality and clinical trials are underway for these as single agents. However, their long-term status in the treatment of AML will no doubt require their combination with standard chemotherapeutics. These studies are eagerly awaited and will hopefully yield exciting results.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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