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Review

Leukemia stem cells

Steven W. Lane[a,b], D. Gary Gilliland[c,d,*]

[a] Department of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA
[b] University of Queensland, Brisbane, Australia
[c] Department of Hematology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
[d] Merck Research Laboratories, North Wales, PA, USA

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ABSTRACT

Leukemia stem cells (LSC) reside within a hierarchy of malignant hematopoiesis and possess the ability to instigate, maintain and serially propagate leukemia *in vivo*, while retaining the capacity to differentiate into committed progeny that lack these properties. In most cases, LSC appear to share immunophenotypic characteristics with committed hematopoietic progenitors, however have pathologically enhanced self-renewal, mediated through the activation of certain cellular pathways. The presence of a LSC that solely possesses the ability to initiate and sustain leukemia has implications for the treatment of patients with this disease. In this review, we will discuss these issues as well as some of the recent controversies regarding LSC frequency and alternative theories of leukemogenesis.

1. Definition of LSC

Longstanding observations have implicated functional heterogeneity within different cell types derived from tumors of the hematopoietic system [1,2]. In the last two decades, the widespread utilization of fluorescence activated cell sorting (FACS) and the application of this technology to identify hematopoietic stem (HSC) and progenitor cell populations [3,4], has enabled the prospective isolation and characterization of distinct cell populations that possess the ability to initiate acute myeloid leukemia (AML) *in vivo*. These leukemia initiating, or leukemia stem cells (LSC) reside at the apex of a hierarchy of malignant cells that is analogous to the hierarchy found in normal hematopoiesis [5]. LSC may be identified by various surface markers and other characteristics such as limitless self-renewal *in vitro*. However, by analogy to normal primitive cells of the hematopoietic system, they are stringently defined by functional attributes including the ability to instigate, maintain and serially propagate leukemia *in vivo* while retaining capacity to differentiate into committed progeny that lack these properties [5–7].

The first descriptions of LSC in human AML by Lapidot et al. [8] and Bonnet and Dick [5] from John Dick's laboratory identified a subpopulation of $CD34^+$ $CD38^-$ human AML cells that were able to serially transplant leukemia in a mouse xenograft model. In contrast, the more committed progenitors (expressing CD38) lacked this potential. These reports demonstrated that LSC were rare, however the frequency of these LSC varied greatly between different AML samples, ranging from 1 in 10^4 to 10^7 cells [5]. In this xenograft model, LSC were not limited to their ability to cause leukemia, but also gave rise to progeny that lost leukemia initiating activity, leading to the hypothesis that AML is arranged in a hierarchy with the LSC at the apex and the more "differentiated" blasts representing the bulk, non-transplantable tumor population. This deterministic model differs from the original stochastic model based on observations that only rare cells within tumors randomly possessed or acquired the ability to form colonies and transplant disease [1,2]. Subsequent murine models have supported the hierarchical model of leukemia. For example, either whole bone marrow or committed progenitors that have been transformed by the fusion oncogene *MLLT3-MLL* (also known as *MLL-AF9*) gave rise to a transplantable AML *in vivo* [9,10]. In these leukemias, leukemia initiating activity was predominantly contained within the $cKit^{high}$ population, whereas the bulk tumor population expressed low cKit levels and had markedly reduced leukemia initiating activity [9,10]. In addition to cKit (the receptor for stem cell factor), AML LSC may be marked by aberrant lineage marking (for example, the lymphoid antigen B220) that is not present in the bulk tumor population [11]. In retroviral models of chronic myelogenous leukemia (CML) $lineage^{low}cKit^+Sca1^+$ cells, an immunophenotypic compartment containing HSC and progenitors in normal hematopoietic ontogeny, efficiently transplant disease [12].

2. LSC cell of origin

The term "leukemia stem cell" may initially suggest that a hematopoietic *stem cell* has undergone transformation to become

* Corresponding author at: 1 Merck Dr., P.O. Box 100, Whitehouse Station, NJ 08889-0100, USA. Tel.: +1 908 423 1000; fax: +1 617 730 0934.
E-mail address: ggilliland@rics.bwh.harvard.edu (D.G. Gilliland).

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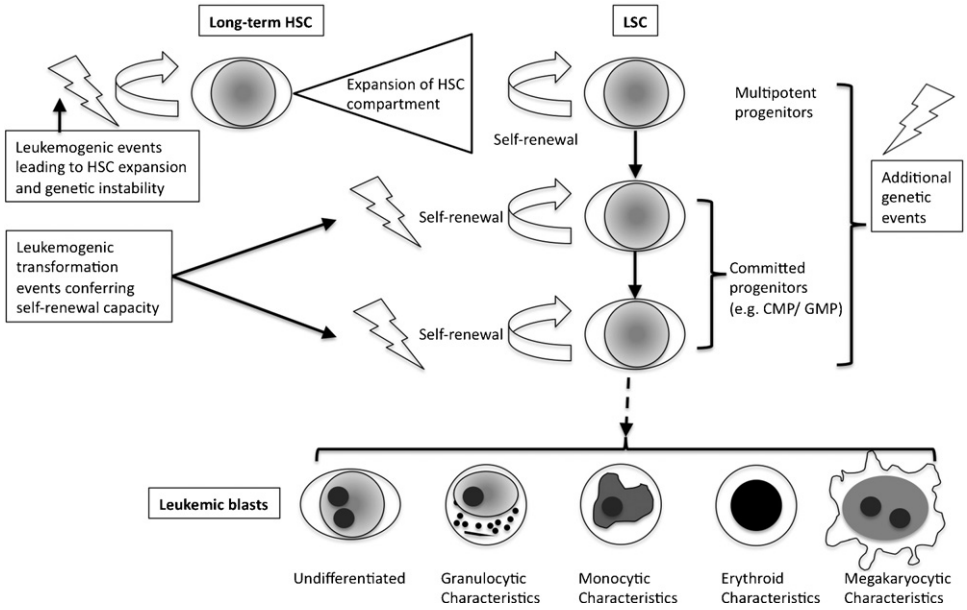

Fig. 1. Hierarchy of leukemia stem cells (LSC). Oncogenic mutations may occur within long-term hematopoietic stem cells (HSC) or in committed downstream progenitors. Mutations within long-term HSC may give rise to a pre-leukemia state with expanded HSC numbers and genetic instability leading to additional genetic events. Conversely, leukemogenic events that confer limitless self-renewal, such as expression of the *MLL-AF9* fusion oncogene, can transform HSC or committed progenitors. A hierarchical structure exists with the LSC at the apex and the leukemic blasts (that lack the ability to initiate leukemia *in vivo*) representing the bulk tumor population.

its leukemic counterpart. Indeed, certain early models of LSC biology appeared to support this theory [5], however it has become apparent that this is not always the case prompting vigorous debate regarding the most appropriate terminology [13]. Recent work has provided further insight into the likely cell that has undergone transformation to initiate the leukemia and it appears that LSC do not need to be derived from their corresponding tissue stem cell (i.e. HSC) although they may share functional characteristics and components of the self-renewal gene expression program seen in HSC [10].

Murine models provide perhaps the strongest direct evidence that the leukemia initiating cell may be a progenitor, more mature in ontogeny than the primitive HSC. For example, using some fusion oncogenes capable of engendering limitless self-renewal, committed progenitors may be transformed leading to LSC that are more immunophenotypically mature than HSC. This is in contrast to tyrosine kinase related oncogenes (such as *BCR-ABL1* associated with t(9;22)(q34.1;q11.23) and found in patients with CML, AML and acute lymphoblastic leukemia) that provide proliferative advantage but are unable to generate a self-renewal program in committed progenitors [7]. The LSC in certain murine models of AML generated by retroviral transformation of committed progenitors with oncogenes such as *MLL-AF9*, *MLLT1-MLL* or *MYST3-NCOA2* (also known as *MLL-ENL* and *MOZ-TIF2*, respectively) did not express the stem cell antigen Sca1 [10,14] and had an immunophenotype similar to normal granulocyte-macrophage progenitors (GMP), that is lineage^{low}cKit^{+}Sca1^{-}FcγRII^{+}CD34^{+} [7,10,15]. In some situations, these LSC may also express a limited array of lineage markers such as CD11b (Mac1) in MLL-induced AML [9,15] or in a transgenic model of AML induced by mutated CCAAT/enhancer binding protein alpha (*C/EBPα*) [16]. Furthermore, in a transgenic model of acute promyelocytic leukemia, the most phenotypically mature form of AML usually associated with the t(15;17) translocation leading to *PML-RARA* fusion, the LSC compartment is enriched in promyelocytic cells that express surface Gr1, a marker of relatively mature myeloid cells (promyelocytes co-express CD34^{+}cKit^{+}FcγRII^{+}Gr1^{+}) [17,18]. The LSC found in other transgenic models do not provide direct evidence to the transformation of a committed downstream progenitor, however also offer relevant biological observations to human disease. For example, in contrast to the retroviral model, the putative LSC in a *MLL-AF9* transgenic model of AML was found within the lineage^{low}cKit^{+}Sca1^{+} compartment, enriched for HSC in normal hematopoiesis [19]. This was presumably due to the use of the endogenous promoter of *Mll1* that drives expression at low levels in downstream progenitor cells in contrast to the high levels achieved with retroviral over-expression of *MLL-AF9*. This *MLL-AF9* transgenic model, along with a CML model in which LSC were found to be lineage^{low}cKit$^{\pm}$Sca-1^{+}CD150^{-} [20] appears to most faithfully reflect the LSC immunophenotype observed in patient samples.

Most correlative studies have documented leukemia initiating activity in xenograft studies within the CD34^{+}CD38^{-} fraction of AML bone marrow, thereby demonstrating a similar immunophenotypic profile as normal HSC [5,21]. Although phenotypic and morphologic studies may link LSC to HSC, human LSC do not express CD90 (Thy1) [22], an immunophenotype more consistent with the multipotent progenitor (MPP) compartment that is more mature and normally lacking in the limitless self-renewal capability of long-term HSC. Despite this, leukemogenic oncogenes can have effects on long-term HSC. Pathogenic fusion oncogenes, such as *RUNX1-RUNX1T1* (also known as *AML1-ETO*) may be found in pre-leukemic samples in the CD90^{+} HSC enriched compartment [23]. This documents the presence of a primitive pre-leukemic clone that can presumably predispose to subsequent mutations engendering the full LSC phenotype in downstream hematopoietic precursors. This hypothesis is supported by a murine transgenic model of AML (induced by expressing a mutated form of *C/EBPα* from the endogenous promoter) in which there was pre-leukemic expansion of normal HSC prior to the development of leukemia arising from a downstream progenitor [24]. Further support for this model has also been provided from observations in acute lymphoblastic leukemia (Fig. 1) [25,26].

Although a full discussion of solid organ tumor cancer stem cells (CSC) is beyond this review, a hierarchical model has also been proposed to exist in solid organ tumors. For example, putative CSC have been identified for breast cancer (CD44^{+}CD24$^{-/low}$), brain (CD133^{+}), colorectal (EpCAM^{high}CD44^{+}) and prostate cancers (Hoechst$^{side\ population}$ CD44^{+}) by using a combination of surface

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marker expression and *in vivo* functional validation [13,27,28]. The importance of the prospective identification and isolation of cell populations enriched for leukemia or cancer initiating activity cannot be over-emphasized if we are to successfully incorporate new therapeutic modalities to specifically target the LSC or CSC population.

3. Properties of LSC

LSC, like their normal HSC counterparts, possess a range of characteristics that enable their long-term survival and some of these also facilitate their escape from the cytotoxic effects of chemotherapy. For example, LSC express the p-glycoprotein multidrug resistance efflux pump, ABCB1 (also known as MDR1) that can remove potentially cytotoxic chemotherapeutic agents from the cell [29]. By reducing cytotoxic stress, LSC may become a reservoir for the selection of mutants that are resistant to targeted or conventional therapy [30].

LSC are characterized by limitless self-renewal and experimental evidence implicates the primacy of key, developmentally conserved self-renewal pathways such as Bmi-1 [31], Wnt/β-catenin [12,32] and Hedgehog [33,34] in this process. Increased expression of *Hox* genes, such as *HoxA9*, has been implicated in the pathogenesis of *MLL-AF9*-induced AML [10,35]. However, not surprisingly, this dependence may be context and oncogene dependent as evidenced by the importance of Hedgehog pathway signaling in CML LSC [33,34] but not in *MLL-AF9*-induced AML [36]. LSC may also evade apoptosis by up-regulation of the pro-survival factor NF-κB [37,38] or evasion of programmed cell death mediated by Fas/CD95 interactions [39].

In distinction to the shared mechanisms of self-renewal and cytoprotection found in HSC, telomerase activity may be lost or attenuated in LSC. Telomerase activity is high in HSC contributing to long-term maintenance of self-renewal in a tightly regulated manner [40]. In contrast, telomerase activity (in particular the hTERT catalytic subunit) appears to be reduced in CML [41,42] leading to shortened telomeres. In support of this observation, shortened telomere length has been described in the BCR-ABL1 negative myeloproliferative neoplasms (MPN) and this length inversely correlates with allelic disease burden [43]. Furthermore, rare familial phenotypes with a predisposition to AML have been linked to *TERT* mutations [44]. Based on these observations it has been hypothesized that accelerated telomere shortening may predispose to disease progression to AML- or therapy-induced myelodysplastic syndrome [45].

LSC evidently possess ample cell-intrinsic mechanisms that aid malignant progression and protect from the effects of chemotherapy. In addition to these, LSC may use novel mechanisms to evade immune-surveillance as evidenced by recent work from the laboratory of Irving Weissman. LSC were shown to express CD47, a surface protein that interacts with the macrophage receptor signal regulatory protein alpha (SIRPα) to inhibit phagocytosis. Under normal homeostatic conditions, this molecule was selectively expressed on migratory HSC upon exit from the bone marrow niche, presumably to protect against immune clearance of HSC [46]. LSC were shown to up-regulate surface expression of CD47 to evade the host's innate immune response *in vivo*. Moreover, patients that had high levels of CD47 on their leukemic cells were demonstrated to have an inferior outcome after conventional therapy [46,47].

There is substantial emerging evidence however, that LSC do not exist primarily in the blood circulation. Rather, LSC reside within and utilize the normal bone marrow microenvironment, taking refuge in the sanctuary of this niche during chemotherapy and consequently re-emerging to initiate disease relapse (for a recent comprehensive review, see Lane et al.) [48]. LSC home to and engraft the bone marrow niche and within this niche, they are somewhat protected from chemotherapy-induced apoptosis, potentially through niche-induced LSC quiescence [21]. These LSC–niche interactions are essential for the proper engraftment of LSC and formation of AML in xenograft models [49]. Using these xenograft models, it has been demonstrated that LSC cells that reside adjacent to endosteal bone marrow niches are resistant to the effects of conventional chemotherapy (cytarabine) [21]. The exact mechanism for this cytoprotection remains unclear, however bone marrow stromal cells can secrete enzymes such as asparagine synthetase resulting in a novel paracrine interaction that may directly induce resistance in acute lymphoblastic leukemia (ALL) cells to a commonly used chemotherapeutic agent, L-asparaginase [50].

It is unclear whether manipulation of the microenvironment would be effective in the eradication of LSC as an adjunct to normal chemotherapy. In support of this tenet, LSC receive vital cues from the bone marrow microenvironment that dictate their behavior and eventual disease phenotype. For example, the leukemia phenotype in a human xenograft model of MLL-AF9 leukemia could be altered between lymphoid, biphenotypic or myeloid by the recipient microenvironment expression of human cytokines *KITLG*, *CSF-2* and *IL3* (stem cell factor (*SCF*), granulocyte-macrophage colony stimulating factor (*GM-CSF*) and interleukin 3, respectively) [51]. *MLL-AF9* transformed LSC exhibit altered migration to the chemokine ligand CXCL-12 (stromal derived factor-1α, SDF1α), in part mediated through increased activity of the Rho GTPases CDC-42 and Rac [9]. The relationship between LSC and the niche need not be unidirectional and there is some evidence to suggest that normal HSC can be altered by signals within a pathological niche to cause hematopoietic dyscrasias [52,53]. In addition to this, LSC may circumvent normal constraints and create their own distinct niche at the expense of normal HSC leading to disproportionate impairment of HSC engraftment and hematopoietic function [54].

4. Implications for diagnosis, prognosis and therapy

The existence of LSC and their centrality within leukemogenesis has important implications for the diagnosis and management of patients with AML. LSC can be identified in diagnostic samples from patients with AML, and from these samples, we may be able to further characterize the underlying genetic events that causes the leukemia. Although there has been focus on LSC-directed therapy in preclinical studies, LSC may also be important in the determination of prognosis and monitoring of patients with AML. LSC may be identified through the expression of LSC specific antigens such as CD123 [55] or CD96 [56] that are not found on normal HSC. Alternatively, rare cell populations with aberrant surface marker expression may specify LSC populations [11]. Identification of these antigens in diagnostic samples from patients with AML may facilitate the longitudinal monitoring by highly sensitive means, such as multiparameter flow cytometry to detect low level AML burden much more efficiently than conventional morphological or cytogenetic methods. Although molecular genetic methods (real-time quantitative PCR of specific fusion genes) may be even more sensitive for detecting minimal residual disease (MRD), monitoring of LSC-associated antigens would provide a direct link between MRD detection and a crucial functional attribute of the leukemia.

LSC, defined either functionally or immunophenotypically, may also provide insights into disease prognosis after treatment with conventional chemotherapy. LSC frequency, defined as CD34+CD38− percentage at diagnosis, correlated with increased residual disease detection and was associated with inferior survival after treatment in patients with AML [57]. Furthermore, a worse prognosis was seen in patients whose AML samples engrafted

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NOD/SCID immunocompromised mice in xenograft transplantation assays [58]. These studies will need to be confirmed with prospective evaluation in consideration of other known adverse prognostic factors such as age, cytogenetics, treatment received and molecular markers [59]. For example, samples with higher LSC number and function also demonstrated fewer samples with favorable cytogenetic profiles [60].

Therapeutic targeting of LSC remains the hope for a silver bullet in AML where long-term survival has improved incrementally, but slowly over the preceding 40 years. A variety of compounds are in preclinical and early phase clinical trials, but have been limited thus far by moderate efficacy and/or dose-limiting toxicity. Monoclonal antibodies targeting the specific epitopes expressed on LSC, rather than HSC appear to be a logical avenue to target LSC *in vivo* and encouraging results in preclinical models have supported the development of these agents. Treatment with a monoclonal antibody to CD44 (the cell surface receptor for hyaluronic acid, osteopontin and other bone marrow niche components) can prevent engraftment of LSC *in vivo* in both AML and CML models [49,61]. Unfortunately, directly implanted LSC were able to retain the ability to form leukemia, limiting considerations of this type of therapy to the transplantation setting. As previously discussed, CD47 expression appears to be an important mechanism by which LSC evade the host's innate immune response. Furthermore, blockage of CD47 with a specific monoclonal antibody leads to increased phagocytosis and reduced engraftment of LSC with concomitant reduction in leukemic burden [47]. Finally, specific targeting of the LSC specific interleukin-3 receptor alpha (IL-3Rα) chain (CD123) with a monoclonal antibody can impair homing to the bone marrow and activate host innate immunity, leading to longer overall survival in recipient mice [55]. Clinical trials testing such agents are ongoing and the results of these trials are highly anticipated.

Treatment with arsenic trioxide appears to specifically target the LSC in an experimental model of CML, an effect mediated through a reduction in PML levels [62]. This approach is particularly therapeutically relevant as arsenic is already used in the therapy of acute promyelocytic leukemia with considerable efficacy and generally acceptable toxicity [63,64]. The role of pro-survival pathways in LSC may also provide an opportunity to specifically target these cells. For example, drugs such as parthenolide (and its derivatives) or proteasome inhibitors are postulated to act by inhibiting the NF-κB anti-apoptotic pathway as well as other pathways (such as through reactive oxygen species) [37,65].

LSC–niche interactions represent a promising therapeutic modality and two recent reports have demonstrated proof of principle that interruption of CXCR4 mediated LSC adhesion can lead to LSC mobilization and sensitization to chemotherapy [66,67]. In many ways, this is analogous to the priming or mobilization of leukemia cells by granulocyte-colony stimulating factor (G-CSF) that led to improved disease free and overall survival in many patients with AML [68]. It remains unclear however, whether these priming agents act through synergistic cytotoxicity with chemotherapy, prevention of stromal cell-LSC interactions or the specific targeting of LSC by some other means (such as interference with a quiescent, long-term LSC population) [62]. Proteasome inhibitors, such as bortezomib can also inhibit AML blast cell migration in response to stromal cell derived CXCL-12 [69].

The nature of LSC precludes long-term *ex vivo* culture (without substantial loss of their pathognomonic leukemia initiating activity and changes in surface marker expression) [7,10] and this has complicated the rational and systematic search for agents with LSC specific activity. *In vitro* assays such as colony formation in semi-solid media may be used as a surrogate read-out, however these assays may not accurately reflect the most primitive LSC compartment, nor the LSC-microenvironment interactions. Heterotypic co-culture assays have been proposed and validated in HSC models [70,71]. Briefly, these assays comprise a supportive stromal cell layer (either cell lines or primary bone marrow derived stromal cells) with overlaid hematopoietic (or leukemic) cells. The frequency of long-term cells that survive and proliferate within these conditions (visualized as cobblestone area forming units or in methylcellulose colony forming assays) has been shown to mirror the frequency of long-term HSC *in vivo* [70]. Preliminary work supports the utility of this assay in LSC [62], although further validation is necessary. Other approaches to CSC screening have been recently described. A high-throughput screening approach for CSC was used in a breast cancer model, where the induction of an epithelial–mesenchymal transition was shown to greatly enhance CSC numbers. This approach identified salinomycin as a specific inhibitor of CSC activity while of great concern, the conventional chemotherapeutic agent paclitaxel was shown to increase CSC numbers [72]. Similar approaches have not yet been described for LSC, however the development of next-generation heterotypic cell culture assays with careful *in vivo* validation or RNA interference based *in vivo* models appears necessary for such progress.

5. Controversies in the field

LSC were originally described as rare cells that alone possess the ability to instigate and perpetuate the leukemia *in vivo* [5]. Recent evidence suggests that LSC are not always rare cells [73] and that the functional heterogeneity within hematopoietic tumors may also depend on a graded ability to contribute to tumor maintenance and respond to external cues [74]. This latter consideration has been referred to as the clonal evolution theory and implies that most of the malignant cells contribute to tumor growth and maintenance, albeit with varying proliferative potential leading to the outgrowth of more potent tumor subclones. The overwhelming and persuasive evidence supporting the LSC theory has been discussed in detail. However, there are some apparent exceptions regarding LSC frequency and the evolution of LSC clones over time that warrant further consideration.

Clonal evolution theory proposes that the random development of mutations within individual tumor cells provides a sequential growth advantage to malignant cells. This theory may help to explain how downstream committed progenitors can acquire leukemia initiating properties through secondary mutations. One such example is the activation of canonical Wnt signaling in GMP by alternative splicing in GSK-3β [75,76] leading to LSC properties in GMP and transformation from chronic phase to blast crisis CML. In this case, the two theories are not mutually exclusive and the putative LSC may change in potency or identity with clonal evolution throughout the course of disease [13].

The other contention between the two theories relates to the frequency of cells within an individual tumor that are capable of tumor initiation. On one hand, the LSC theory suggests that LSC are rare (albeit of widely varying frequency) and the counter-argument asserts that tumor initiating activity may be found within most tumor cells. Indeed, there are a number of compelling models that exhibit a very high percentage of cells (10–20%) with cancer initiating activity such as Eμ-Nras T cell lymphoma, Eμ-Myc lymphoma, PU.1 deficient AML [73] or even some models of *MLL-AF9*-induced AML [9]. Furthermore, the engraftment of AML cells into xenograft models may be impaired by residual host innate immunity in NOD/SCID mice where limited NK-cell function remains. It has also been revealed that certain antibodies used to identify LSC (i.e. the HIT2 and AT13/5 clones of anti-CD38 but not their corresponding F(ab′)2 fragments) can encourage Fc-receptor mediated clearance of LSC, masking potential leukemia initiating activity within the $CD34^+CD38^+$ compartment [77].

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Some other potential divergences between these two models may also relate to the inherent limitations of xenograft transplantation as an assay of stem cell frequency. As previously discussed, LSC, in a fashion analogous to normal HSC, depend on the bone marrow microenvironment for long-term self-renewal, however in xenograft models the cytokine signals for LSC maintenance or chemotaxis signals to direct LSC to the niche may be incompatible between species [74]. While it remains possible, even likely, that we are underestimating the frequency of LSC by current xenograft assays it is also clear, however, that not all syngeneic models of leukemia are characterized by a high frequency of LSC [10,11,16–19,78] and therefore, that both models may provide complementary information dependent on the specific experimental model or context in which each is applied.

A number of steps may be considered to optimize our current xenograft assays of LSC frequency; the neonatal NOD/SCID/IL2Rγ^{-}/$^{-}$ [21] (carrying a complete null mutation for the interleukin-2 common gamma chain) appears to be a more permissive strain for LSC engraftment and phenotypic manifestation and this may be further optimized by humanization of cytokine/chemokine interactions or the immune system; pretreatment with intravenous gamma globulin or anti-CD122 to minimize the anti-engraftment effects of antibodies used to enrich for LSC [77]; direct intrafemoral injection [49] may reduce the inefficiency of LSC homing and minimize splenic or pulmonary sequestration of cells; finally, the use of co-transferred supporting cells (such as bone marrow stromal cells) or structures such as matrigel [13,79] may provide a species or tumor specific microenvironment that enhances the efficiency of engraftment of tumor initiating cells.

6. Future directions

The opportunity and challenge of LSC biology remains in the application of clinically meaningful LSC specific therapies to patients with leukemia and related disorders. Prospective screening platforms to interrogate LSC biology with rigorous *in vivo* validation will be required to realize this ambition and translate these biological insights into improved patient outcomes.

Conflicts of interest

S.W.L. has no relevant conflicts of interest to declare. D.G.G. is now an employee of Merck.

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Contributions. S.W.L. and D.G.G. wrote the paper.

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