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Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia

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Summary

Using a mouse model of human acute myeloid leukemia (AML) induced by the MLL-AF9 oncogene, we demonstrate that colony-forming cells (CFCs) in the bone marrow and spleen of leukemic mice are also leukemia stem cells (LSCs). These self-renewing cells (1) are frequent, accounting for 25%–30% of myeloid lineage cells at late-stage disease; (2) generate a phenotypic, morphologic, and functional leukemia cell hierarchy; (3) express mature myeloid lineage-specific antigens; and (4) exhibit altered microenvironmental interactions by comparison with the oncogene-immortalized CFCs that initiated the disease. Therefore, the LSCs responsible for sustaining, expanding, and regenerating MLL-AF9 AML are downstream myeloid lineage cells, which have acquired an aberrant Hox-associated self-renewal program as well as other biologic features of hematopoietic stem cells.

Introduction

Acute myeloid leukemia (AML) is a clonal neoplastic disorder that originates from a single transformed cell, which has progressively acquired critical genetic or epigenetic changes that disrupt key growth-regulatory pathways (Hanahan and Weinberg, 2000). Within the leukemia clone, there is significant cellular morphologic, phenotypic, and functional heterogeneity analogous to the hierarchical organization of normal hematopoiesis. Notably, only a subfraction of cells are proposed to be leukemia stem cells (LSCs) with the ability to self-renew extensively, and to initiate, sustain, or regenerate the disease. Conversely, the majority of cells are either transitional cells with limited proliferative capacity or more differentiated end cells (Mackillop et al., 1983; Kummermehr, 2001). Evidence for a hierarchical cellular organization of human AML derives from studies showing that only a small proportion of AML cells are clonogenic in in vitro culture (Buick et al., 1977), and that an even smaller fraction of AML blood blasts, defined by a $CD34^+$ $CD38^-$ surface phenotype, can transfer disease to immune-deficient mice (Lapidot et al., 1994; Bonnet and Dick, 1997). Since normal human hematopoietic stem cells (HSCs) are also $CD34^+$ $CD38^-$, these and other observations (Miyamoto et al., 2000; Hope et al., 2003) have been taken to suggest that AML LSCs originate from and reside exclusively within the most immature bone marrow (BM) progenitor compartment. However, this lineal relationship, which has important pathogenic and clinical implications, may not always hold true. For example, a recent study of blastic transformation of chronic myeloid leukemia proposed that cells with a granulocyte-macrophage progenitor (GMP) cell phenotype were candidate LSCs (Jamieson et al., 2004).

Elimination of the LSC compartment within the leukemia clone is likely to be essential, and probably sufficient, for cure of disease, and thus increasing efforts have focused on attempting to define the unique biological properties of AML LSCs by comparison with the transit and end cell compartments within the leukemia, as well as with normal hematopoietic stem and progenitor cells. However, an essential prerequisite for defining these unique properties, so that targeted chemo- and immunotherapeutic strategies may be developed, is an accurate determination of the phenotype as well as the frequency of LSCs within the AML clone. To date, such efforts have primarily focused on xenografting human leukemia cells into immune-deficient mice. A complementary, but currently unexploited, approach is to characterize LSCs in mouse genetic models of human leukemia, which have proven to be invaluable tools in defining the mechanisms of disease pathogenesis. Two recent studies have demonstrated that AML may be initiated by expression of MLL-ENL or MOZ-TIF2 in progenitor cells with limited self-renewal capacity, as well as in HSCs (Cozzio et al., 2003; Huntly et al., 2004). However, these studies did not address the critical issue of whether the respective murine leukemias

SIGNIFICANCE

An essential prerequisite for the development of more effective targeted therapies in AML is a characterization of the frequency and biological properties of LSCs. In a mouse model of human MLL-AF9 AML, we have defined the size and lineal derivation of the LSC compartment, the extent of which was significantly underestimated by limit dilution analysis. Our findings support a revision of the prevailing hypothesis that AML LSCs are always rare and solely located within the most immature bone marrow stem/progenitor compartment. Furthermore, LSCs exhibit markedly different microenvironmental interactions, compared with cells simply immortalized by MLL-AF9, indicating that acquisition of sensitivity to stromal cell-derived survival and proliferative signals is a critical feature of LSCs, in addition to their extensive self-renewal capabilities.

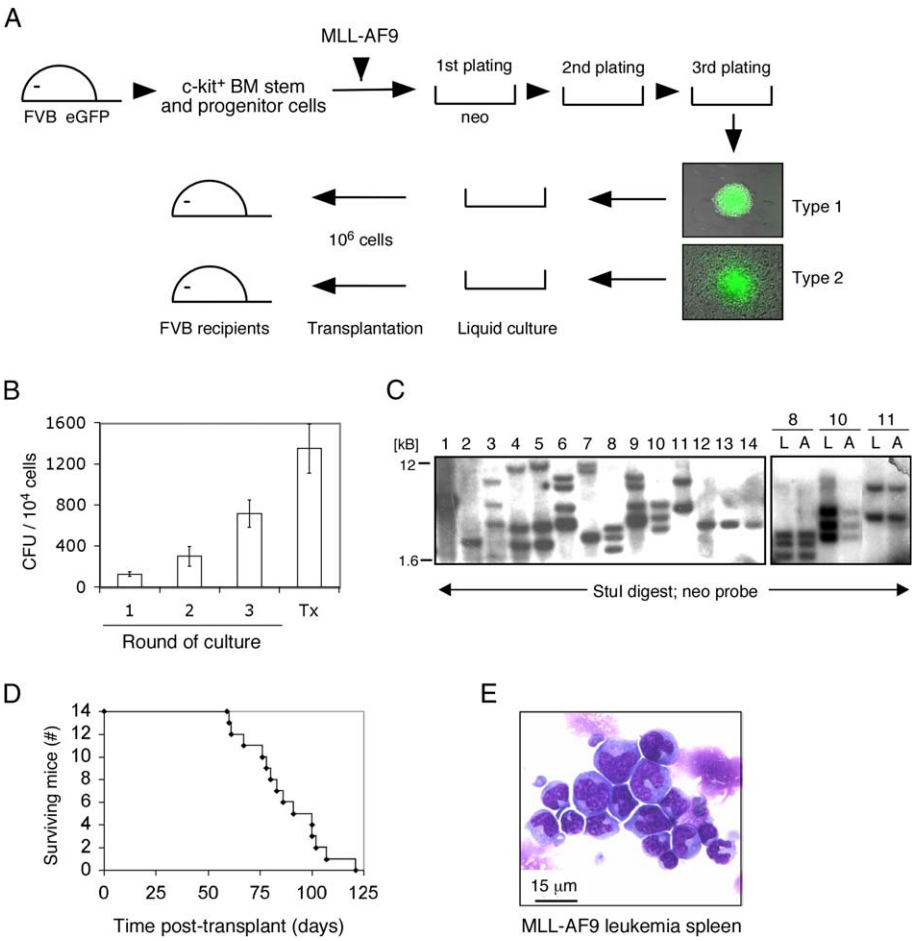

Figure 1. MLL-AF9-immortalized CFCs have high leukemogenic potential

A: Schematic illustration of the experimental approach employed to assess the correlation of leukemogenic potential with CFC activity of MLL-AF9-immortalized cells. BM stem and progenitor cells (c-kit$^+$) were transduced with MLL-AF9 and then serially replated in methylcellulose medium every 5 days, with G418 drug selection in the first round. Single colonies (33 total over five experiments) with either type I (16 each) or type II (17 each) morphology (Lavau et al., 1997) were plucked and individually expanded in liquid (29) or semisolid (4) medium, and 10^6 cells were then transplanted into syngeneic recipient mice. Single CFCs routinely expanded to 10^6 progeny cells within 12–15 days.

B: The mean (±SEM) number of colonies (≥1000 cells) per 10,000 cells plated in each round is indicated. The clonogenic potential of progenitors transduced with empty vector was exhausted by the end of round two (data not shown). The mean (±SEM) frequency of CFCs at the time of transplant in the 14 lines derived from single plucked colonies is indicated in the last column (Tx).

C: Southern blot analysis (left panel) demonstrates the integration sites in 14 separate transplanted lines derived from single immortalized CFCs (neo probe of Stu1-digested genomic DNA). A median of three integration sites per clone is observed. Comparison of the integration sites (right panel) in three representative paired transplanted lines (L) with their respective AML cells (A) confirmed that they were clonally identical.

D: Survival curve of animals transplanted with cells (10^6) derived from single MLL-AF9-immortalized CFCs.

E: Representative cytospin of splenocytes (May Grunwald Giemsa stain) from a mouse with MLL-AF9 AML.

were sustained by a small fraction of self-renewing Lin$^-$ stem and/or progenitor cells, as predicted by current models of AML based on NOD/SCID transplantation assays, or alternatively, whether the LSC compartment is larger and altogether distinct from the normal stem and progenitor compartment.

We report here the identification and characterization of LSCs in a mouse model of AML initiated by MLL-AF9, a frequently occurring MLL fusion oncogene typically associated with the FAB-M4 or M5 subtypes of human AML (Swansbury et al., 1998). Our studies indicate that LSCs are neither rare nor synonymous with the stem and progenitor cells targeted by initiating MLL mutations. Rather, LSCs in this model are frequent, located almost exclusively downstream of the normal progenitor compartment by immunophenotype, and constitute myeloid lineage cells that have acquired an aberrant self-renewal program as well as other biologic features of HSCs, including substantially altered microenvironmental interactions.

Results

MLL-AF9-immortalized colony-forming cells display high leukemia-initiating potential

The leukemogenic potential of single MLL-AF9-immortalized colony-forming cells (CFCs) was evaluated using a retroviral transduction/transplantation assay that reproducibly reads out the properties of MLL fusion oncogenes (Lavau et al., 1997). BM stem and progenitor cells (c-kit$^+$) from EGFP transgenic mice were transduced with MLL-AF9 and serially replated in semisolid medium (Figures 1A and 1B). At the end of the third round of culture, individual colonies were isolated and expanded further in either liquid or semisolid media (Figure 1A). Fourteen lines (representative of ten unique clones) (Figure 1C) were randomly selected and injected (10^6 cells) into separate sublethally irradiated, wild-type recipients. All mice developed AML, with a median latency of 84.5 days (range 60–121) (Figures 1D and 1E; Tables S1 and S2 in the Supplemental Data available with this article online). Southern blot analysis demonstrated that each leukemia was clonally identical to its respective injected cell population (Figure 1C). Therefore, immortalized colony-forming cells (ICs) transformed by MLL-AF9 in vitro consistently possess the potential to initiate leukemias in vivo, demonstrating a high correlation of CFC activity with leukemogenic potential in this model of AML.

LSCs are frequent in mice with MLL-AF9 myeloid leukemia

A similar approach was employed to analyze the leukemogenic potential of CFCs derived from leukemic mice (Figure 2A). Using semisolid culture assays, the CFC frequencies in the BM and spleens of leukemic mice were 29.8% ± 4.1% and

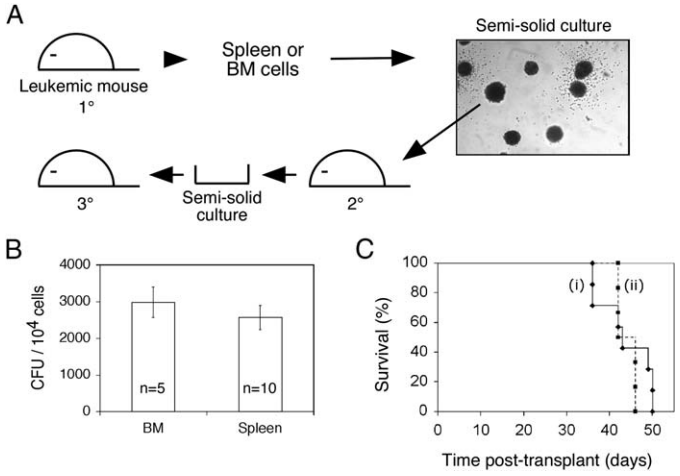

Figure 2. LSCs are frequent in mice with MLL-AF9 leukemia

A: Schematic illustration of the experimental approach for determining the correlation between clonogenic and leukemogenic potentials of single MLL-AF9 myeloid leukemia CFCs. Spleen or BM cells from leukemic mice (1°) were plated in semisolid medium. After culture for 7 days, single colonies (typically 10^4 cells) were plucked, washed once in PBS/EDTA, and then directly injected into sublethally irradiated, syngeneic recipients (2°) (16 colonies in total transplanted from three separate donors). The process was repeated using tertiary recipients (3°) (six colonies from one donor).

B: Bar graph indicates the mean (±SEM) number of colonies (≥1000 cells) after 5 days of culture per 10,000 plated spleen or BM myeloid lineage cells explanted from leukemic mice. The proportion of myeloid lineage cells in the spleen and BM was determined by examination of MGG-stained cytospins and confirmed by flow cytometry.

C: Representative survival curves of sublethally irradiated mice transplanted with single AML colonies. Single AML colonies (n = 7) from an animal with disease latency of 60 days were plucked after 7 days semisolid culture and directly injected into secondary recipients (i). Similarly, single AML CFCs (n = 6) from one of the secondarily transplanted mice (disease latency 36 days; splenic AML CFC frequency 27.2%) were plucked and injected to confirm the ability of AML CFCs to serially transplant disease (ii).

25.7% ± 3.3%, respectively, as a proportion of all myeloid lineage cells (Figure 2B). To confirm that individual CFCs from leukemic mice were also LSCs, defined here as cells with the potential to transplant leukemia from one syngeneic host to another, single colonies (16 total derived from three separate donors) were randomly selected, plucked from culture, and directly injected into syngeneic recipients without further in vitro manipulation. All recipient mice succumbed to short latency AML (Figure 2C), and the resultant disease could be serially transplanted to tertiary recipients using identical methodology (Figure 2C). Similar results regarding leukemic CFC frequency, and the cells' potential to transplant disease, were obtained regardless of whether AML was initiated by transplantation of cells derived from serially replated CFCs (Figure 1A) or transplantation of c-kit$^+$ stem and progenitor cells immediately following retroviral transduction (data not shown). Therefore, the CFCs derived from mice with MLL-AF9 leukemia are LSCs. Based on these criteria, LSCs are present at a frequency of approximately 25%–30% of myeloid lineage cells in the BM and spleen of leukemic mice.

MLL-AF9 LSCs express myeloid lineage-specific antigens and are downstream of the normal progenitor compartment by immunophenotype

To more precisely define LSCs with respect to their phenotype and lineal origin, FACS analyses were performed on splenocytes from leukemic mice. Almost all EGFP$^+$ donor cells expressed Mac1 and/or Gr1, while somewhat lower percentages of cells expressed c-kit, FcγII/IIIR, CD24, Pecam1, CD34, and/or F4/80 (Figure 3A and Table 1). Leukemia cells consistently lacked expression of the stem cell antigen Sca1, the cytokine receptor Flt3, or markers specific for the lymphoid (B220, IgMa, CD3, TCRβ), megakaryocytic (CD41), or erythroid (TER119) lineages (Table 1). A similar phenotype was displayed by the expanded, immortalized cell populations, which originated from single MLL-AF9 ICs in vitro (data not shown). Since approximately 26% of EGFP$^+$ splenocytes were LSCs, the near-uniform expression of Mac1 indicated that LSCs express myeloid lineage-specific antigens. Further support for this conclusion was provided by FACS sorting of leukemic splenocytes based on surface marker coexpression, which revealed that 91.3% ± 3.8% of splenic LSC activity was present in the Mac1$^+$ Gr1$^+$ fraction (Figure 3B) and 51.9% ± 29.0% was present in the Mac1$^+$ F4/80$^+$ fraction (Table 1). Furthermore, animals secondarily transplanted with leukemic splenocytes (20–100,000) sorted for Gr1 expression died of AML with a median latency of 28 days (range 24–38 days; n = 5). Similarly, all mice secondarily transplanted with single plucked colonies derived from splenocytes sorted for coexpression of c-kit, Gr1, and F4/80 died of AML with a median latency of 44 days (range 44–52; n = 5). Thus, MLL-AF9 LSCs express lineage-specific antigens most consistent with a relatively late-stage myeloid phenotype, suggestive of maturational arrest distal to the GMP.

MLL-AF9 LSCs are not enriched in the normal stem and progenitor compartments

Rare EGFP$^+$ splenocytes (0.11% ± 0.06%; n = 9) lacked expression of lineage-specific antigens (Figure 3C). Since the Lin$^-$ compartment harbors normal stem and progenitor cells, Lin$^-$ EGFP$^+$ cells were analyzed for expression of antigens that mark normal HSCs. None of the cells displayed a c-kit$^+$ Sca1$^+$ Lin$^-$ (KSL) immunophenotype (Figure 3C; n = 6), indicating that no donor-derived cells were present in the normal HSC compartment. By contrast, cells with a KSL immunophenotype were clearly detected in the EGFP$^-$ Lin$^-$ splenocyte population, consistent with the presence of residual, recipient origin, normal hematopoiesis in the leukemic mice (Figure 3C). FACS sorting and in vitro culture of the rare EGFP$^+$ c-kit$^+$ Sca1$^-$ Lin$^-$ cells from leukemic mice produced a lower mean CFC frequency (8.5% ± 8.3%; n = 3) than that found in the Lin$^+$ fraction. Thus, there was no enrichment of LSCs within the Lin$^-$ compartment, and indeed, when the absolute numbers of CFCs with a Lin$^+$ phenotype were considered, it was clear that the overwhelming majority (>99.8%) of CFCs, and therefore LSCs, resided by immunophenotype downstream of the normal progenitor hierarchy (Figure 3B). Similar AML CFC frequencies (4.4% ± 4.2%; n = 4) were found in FACS-sorted Lin$^-$ splenocytes from leukemic mice where disease was initiated using c-kit$^+$ stem and progenitor cells transplanted immediately following retroviral transduction, and likewise 99.9% of the CFC/LSCs had a Lin$^+$ phenotype.

MLL-AF9 LSCs establish a leukemia cell hierarchy

A subset of cells in each leukemia expressed c-kit, a surface antigen expressed highly by normal BM stem and progenitor cells. To determine if c-kit expression specifically marks the LSC compartment in MLL-AF9 AML, EGFP$^+$ spleen cells from leukemic

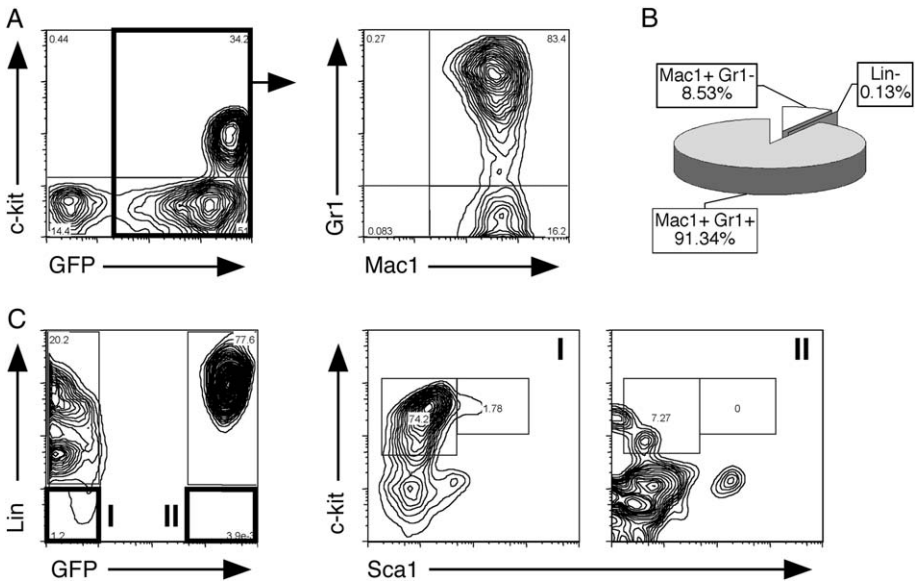

Figure 3. MLL-AF9 LSCs and ICs express myeloid lineage-specific antigens

A: Representative FACS profiles of splenocytes from a mouse with MLL-AF9 AML. Almost all of the donor ($EGFP^+$)-derived cells (left panel) express the myeloid antigens Gr1 and/or Mac1 (right panel).

B: Pie chart shows the percentage of LSCs in the indicated immunophenotypic fractions. Clonogenic cell frequencies for each immunophenotype were determined by culture of FACS-sorted $EGFP^+$ AML splenocytes (n = 4 animals). The proportion of total LSCs located in each fraction was determined as the percentage of cells with a specific immunophenotype times the CFC frequency for that immunophenotype.

C: Representative $c\text{-}kit^+/Sca1^+/Lin^-$ (KSL) FACS profiles of splenocytes from a mouse with MLL-AF9 AML. A very small fraction (less than 4 in 100,000) of donor ($EGFP^+$) cells in this example are Lin^-, and all lack a KSL immunophenotype (II). Rare cells are present in the progenitor gate ($c\text{-}kit^+ Sca1^-$). In contrast, $EGFP^- Lin^-$ recipient splenocytes demonstrate a typical progenitor cell and KSL immunophenotype (I).

mice were double flow-sorted into c-kit-positive and -negative fractions (Figure 4A). The $c\text{-}kit^+$ populations contained a higher proportion of cells with cytologic features of blasts (Figure 4B), displayed a 14.2-fold higher proportion of CFCs (Figure 4C), and induced death from AML significantly more rapidly in secondarily transplanted recipients (Figure 4D). LSC-enriched ($c\text{-}kit^+$) cells induced AML that fully recapitulated the pathologic, morphologic, and phenotypic features of the original disease (data not shown). Quantitative PCR analysis demonstrated significant downregulation of several *Hoxa* genes and upregulation of the cell cycle inhibitors *Cdkn2a* and *Cdkn2c* in $c\text{-}kit^-$ versus $c\text{-}kit^+$ populations (Figure 4E). Further cell sorting experiments demonstrated no substantial differences in the clonogenic potentials of cells based on the presence or absence of expression of Pecam1, CD24, CD34, FcγII/IIIR, Gr1, or F4/80 (Table 1). These results indicated that LSCs were highly enriched, but not exclusively contained, within the $c\text{-}kit^+$ fraction. LSCs in this model of AML are not distinguished by a specific immunophenotype. However, the distinctive morphologic, clonogenic, transcriptional, and transplant properties of cells differentially expressing c-kit demonstrate a loose hierarchical organization of the AML clone. Furthermore, the LSC-containing apex of the hierarchy is broad (based on 25%–30% AML CFC frequency), but almost entirely outside of the normal progenitor compartment, and extends into the c-kit-negative leukemia cell population.

MLL-AF9 LSCs are aberrantly self-renewing myeloid lineage cells, as opposed to HSCs, which ectopically express myeloid lineage antigens

Further comparative transcriptional profiling analyses were performed on $c\text{-}kit^+$ and $c\text{-}kit^-$ leukemia cell populations as well as sorted normal stem and progenitor cell populations. $c\text{-}kit^+$ leukemia cell populations containing a high proportion of LSCs expressed moderate to high levels of genes (*Ela2*, *Prtn3*, and *Gstm2*) normally expressed in committed myeloid lineage cells, versus low levels of genes (*Flt3* and *Mpl*) normally expressed highly by stem cells or multipotent progenitors (Figure 5) (Terskikh et al., 2003). Consistent with the presence of an aberrant MLL-AF9-driven self-renewal program in LSCs, most *Hoxa* genes were expressed at levels equal to or greater than those in normal stem and multipotent progenitor cells (Figure 5). Thus, the transcriptional profiles and immunophenotypes of MLL-AF9 LSCs, in addition to their lack of multilineage differentiation potential (Table 1), are characteristic of aberrantly self-renewing downstream myeloid lineage cells and not HSCs ectopically expressing myeloid lineage antigens.

Enhanced engraftment potential qualitatively distinguishes MLL-AF9 LSCs from ICs

The foregoing studies demonstrated a high correlation of CFC and leukemia-initiating potential for both ICs and LSCs (Figures 1 and 2). However, further transplant studies indicated that, at an equivalent cell dose (10^6), cells derived from single ICs induced AML with a 3- to 4-fold longer latency than cells derived from single LSCs (median 27 versus 100 days, respectively; survival curves not shown). The differences in leukemic potential were confirmed by limit-dilution transplant studies, which revealed that the predicted mean dose of ICs required to initiate AML was 66,300, whereas the required number of AML splenocytes was 121 (Figure 6A). Since CFC frequencies were broadly similar in the IC and LSC populations, the results strongly suggested that ICs are qualitatively different and must acquire additional functional properties in vivo to become fully competent LSCs.

Further transplant studies were conducted to investigate whether the qualitative differences in leukemic potential resulted from differential homing and/or engraftment. Paired IC and LSC populations clonally derived from a single IC were directly compared for these and all subsequent analyses. Twenty-four hours following transplantation of syngeneic mice, comparably small proportions of the injected ICs (0.34% ± 0.16%) and LSCs (0.05% ± 0.006%) were located in the BM ($p = 0.16$; $n = 4$) and spleen (0.03% ± 0.008% versus 0.005% ± 0.002%, respectively; $p = 0.10$; $n = 4$) (Figure 6B). These data indicated that both cell populations were markedly inefficient in homing following transplantation and thus did not account for the enhanced leukemic potential of LSCs compared to ICs. However,

Table 1. Phenotypic features correlated with clonogenic properties of MLL-AF9 LSC populations

	Acute myeloid leukemia cells		
Antigen	Positively stained cells (%) (n = 13)[a]	% CFCs in positive fraction[b]	% CFCs in negative fraction[b]
Mac1/CD11b	97.6 ± 0.6	ND	ND
Gr1/Ly6G	85.8 ± 2.8	91.3 ± 3.8 (n = 4)	8.7 ± 3.8
c-kit/CD117	54.1 ± 6.4	93.4 ± 3.0 (n = 6)	6.6 ± 3.0
FcγRII/III/CD16/32	76.2 ± 3.1	67.7 (n = 1)	32.3
CD34	56.2 ± 10.6	52.3 ± 43.4 (n = 2)	47.7 ± 43.3
CD24	53.3 ± 8.4	13.1 (n = 1)	86.9
Pecam1/CD31	70.7 ± 5.8	64.6 ± 1.2 (n = 2)	35.4 ± 1.2
F4/80	23.8 ± 8.2	51.9 ± 29.0 (n = 2)	48.1 ± 29.0
Flt3/CD135	0	NA	NA
Sca1	0	NA	NA
B220	0	NA	NA
IgMa	0	NA	NA
CD3	0	NA	NA
TCRβ	0	NA	NA
CD41	0	NA	NA
TER119	0	NA	NA

NA, not applicable. ND, not determined, because infrequent Mac1-negative cells were almost always Gr1 positive (see Figure 3A). See text for data on Lin$^-$ cells.

[a]EGFP$^+$ splenocytes from mice with AML were analyzed for the indicated antigens by FACS. Dead cells were excluded from analysis using propidium iodide staining.

[b]EGFP$^+$ splenocytes from a subset of animals with AML were additionally FACS sorted for the presence or absence of expression of the indicated antigens, and then cultured for 6 days in methylcellulose to determine the CFC frequency (data not shown) in each fraction. The percentage of clonogenic cells in the positive (column 3) versus negative (column 4) immunophenotypic fractions for each marker was then determined by multiplying the CFC frequency for each immunophenotype by the percentage of cells with that immunophenotype (column 2).

at day 8 following transplantation, the number of LSC progeny present in the BM had increased 5900-fold, versus only a 5-fold increase for IC progeny (Figure 6B). Similar divergent fold increases were observed in the spleen (895 versus 0.5). At day 20 posttransplant, mice that received LSC-derived cells contained approximately 200- and 3700-fold more EGFP$^+$ cells in their BM and spleens, respectively, compared with those that received IC-derived cells. Therefore, despite similarly poor BM homing of the respective populations, LSCs were much more efficient at proliferating and generating progeny (i.e., engrafting) after homing. Taken together with the limit dilution analyses, these data confirm that acquisition of enhanced engraftment potential is a key step in the progression of ICs to LSCs.

MLL-AF9 LSCs display increased sensitivity to stroma-derived proliferation signals

To investigate whether growth factor sensitivities may underlie the engraftment differences of MLL-AF9 LSCs versus ICs, paired cell populations were cultured in medium containing single cytokines. No significant differences in proliferation between the populations were observed when cells were cultured in either interleukin-3 (IL-3) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Figure 6C). However, when cultured in interleukin-6 (IL-6), interleukin-11 (IL-11), stem cell factor (SCF), or Flt ligand (FL) alone, LSC-containing populations frequently displayed an acquired ability to proliferate by comparison with their corresponding IC-containing populations (Figure 6D). Additionally, LSC-containing populations tolerated growth factor withdrawal for 5 days in significantly higher numbers (Figure 6E, columns 1 and 4; $p < 0.05$).

The observed growth factor sensitivities acquired by LSCs suggested an enhanced ability to derive survival and proliferation signals from stromal cells, which secrete IL-6 and IL-11. When cultured on an S17 stromal cell layer, LSC-containing populations proliferated significantly more rapidly than IC populations in a 5 day assay (3.0- ± 0.4-fold expansion compared with 1.4 ± 0.2; $p = 0.005$) (Figure 6E). Similarly, LSC populations cocultured for 5 days on OP9 stroma were on average 7.2-fold more abundant than IC populations ($p = 0.003$) (Figure 6E). Experiments using stroma-conditioned medium indicated that secreted factors accounted for only a portion of the stroma-derived survival/proliferation signal (Figure 6E). Therefore, an improved ability to survive/proliferate in response to stroma-derived signals, both secreted and contact-mediated, is a critical feature that distinguishes MLL-AF9 LSC from IC populations.

AML LSCs demonstrate increased chemotaxis to SDF1 and express higher levels of activated Rac and Cdc42

Normal HSC engraftment is critically dependent on SDF1/CXCR4 and Rac1 signaling (Peled et al., 1999; Gu et al., 2003) and correlates with the ability to undergo SDF1-induced chemotaxis (Peled et al., 1999). LSC populations exhibited significantly increased Cxcl12 (SDF1)-induced chemotaxis by comparison with their paired IC populations (Figure 7A), and this was not due to differential adhesion properties (Figure 7A), nor increased expression of Cxcr4, as estimated by FACS mean fluorescence intensity (Figure 7B). Expression levels of small GTPases, which are critical mediators of cell migration and engraftment, showed a small, but consistent, significant increase in Cdc42 (Figure 7C) and a greater, but more variable, increase in Rac in LSC by comparison with paired IC populations. Levels of Cdc42-GTP and Rac-GTP were also elevated. Therefore, progression of MLL-immortalized cells to LSCs is accompanied by several features associated with enhanced engraftment potential, including increased survival/proliferative abilities in contact with stromal cells, increased SDF1 induced chemotaxis, and elevated expression of small GTPases.

Discussion

We have identified and characterized LSCs in a somatic, genetic mouse model of leukemia that faithfully recapitulates many of the pathologic and clinical attributes of human AML initiated by MLL oncogenes. Unexpectedly, we found that the LSC compartment is relatively large, lineage-positive, and positioned phenotypically almost entirely downstream of the GMP. Therefore, the LSCs responsible for sustaining AML in this model are not synonymous with the stem and progenitor cells initially targeted by MLL oncogenes. Nevertheless, LSCs share several genetic and functional features of HSCs, including a self-renewal transcriptional program of high *Hox* gene expression. They also display a wide range of hematopoietic growth factor sensitivities, increased SDF1-induced chemotaxis, and an enhanced ability to interact with BM stromal cells, all of which are also biologic attributes shared with HSCs. These features likely explain the observed substantial enhancement of LSC engraftment potential in transplantation assays by comparison with ICs. Thus, LSCs in MLL-AF9 AML are downstream myeloid

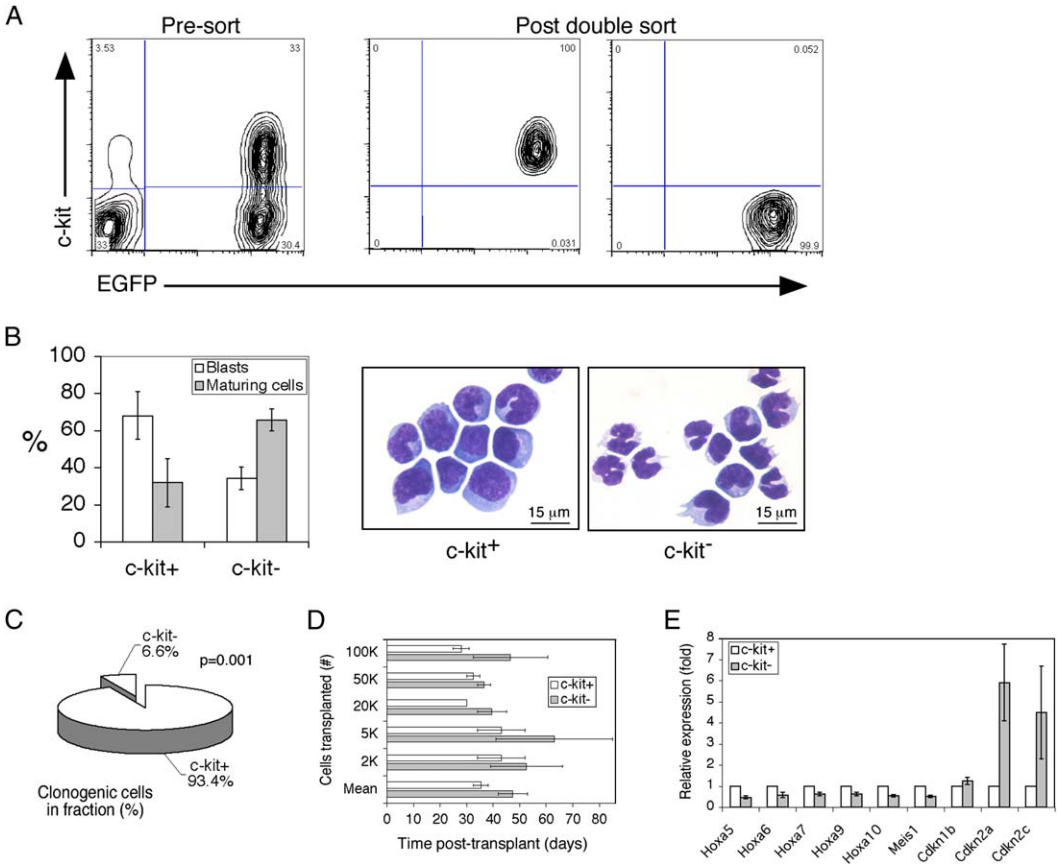

Figure 4. Phenotypic, morphologic, and functional evidence of a cellular hierarchy in MLL-AF9 leukemia

A: FACS analyses demonstrate c-kit expression on fresh splenocytes from mice with AML prior to (Pre-sort) and after (Post double sort) double flow sorting into c-kit-positive and c-kit-negative fractions.

B: Splenocytes from leukemic mice were sorted based on their expression of c-kit and analyzed for the proportion of cells with morphologic features of blasts versus differentiation following May Grunwald Giemsa staining of cytospin preparations. Bar graph indicates the mean (±SEM) number of cells with the indicated morphologic and phenotypic features (n = 4).

C: Pie chart shows the percentage of clonogenic cells residing within the c-kit$^+$ versus c-kit$^-$ fractions of EGFP$^+$ leukemic cells (n = 6). FACS-sorted leukemic splenocytes were cultured in methylcellulose medium for 6 days to determine the CFC frequencies in each fraction (29.2% ± 3.5% and 5.4% ± 2.0%, respectively; $p < 0.001$).

D: Bar graph indicates an average 25% shorter survival across varying cell doses for mice secondarily transplanted with c-kit$^+$ AML cells versus recipients of equivalent numbers of c-kit$^-$ cells. Disease latencies shown are the means of two separate experiments using sorted cells from separate donor animals with AML, one recipient animal per indicated cell dose. The numbers of transplanted cells are indicated on the left (k = 1000).

E: Bar graph indicates the change in expression levels of the indicated transcripts in sorted populations of EGFP$^+$ c-kit$^-$ leukemic splenocytes by comparison with EGFP$^+$ c-kit$^+$ leukemic splenocytes (mean ± SEM; n = 5). All transcript levels were normalized to levels of *Actb* expression using ΔCt methodology.

lineage cells that have aberrantly acquired both genetic and biologic features of HSCs.

The high frequency of LSCs in MLL-AF9 AML is significantly underestimated by assays dependent on engraftment

The estimated frequency of cancer stem cells in murine tumors, as determined by limit dilution experiments, has been reported to vary widely depending on the tumor type (Hewitt et al., 1976; Hill and Milas, 1989). We employed a different approach to estimate LSC frequency based on the observations that single CFCs from the spleens of leukemic mice consistently induced short-latency AML following transplantation (of single plucked colonies) into secondary recipients. When this approach was used, the proportion of cells that fulfilled the criteria for AML LSCs (i.e., a single cell that can self-renew extensively and possesses the potential to initiate, sustain, or regenerate AML) was approximately 26%–30% of the myeloid lineage cells in the BMs and spleens of mice with AML, substantially higher than previous estimates of LSC frequencies in human AML. Conversely, a conventional measure of LSC frequency based on limit dilution analysis indicated that 0.8% of AML cells (1 in 121) had the operational features of LSCs. Thus, by comparison with our single colony isolation and transplantation experiments, limit dilution analysis appeared to underestimate the number of cells with LSC potential by approximately 30-fold. The difference strongly suggests that clonogenic LSCs in this model of AML engraft with low efficiency (estimated to be about 3%), substantially lower than normal murine HSCs; most studies concur that HSCs engraft in syngeneic transplant recipients with approximately 30% efficiency (Camargo et al., 2006). Likely contributing to this phenomenon, LSCs in our study were markedly inefficient at homing, as indicated by the extremely low percentage (0.05%) of secondarily transplanted AML splenocytes

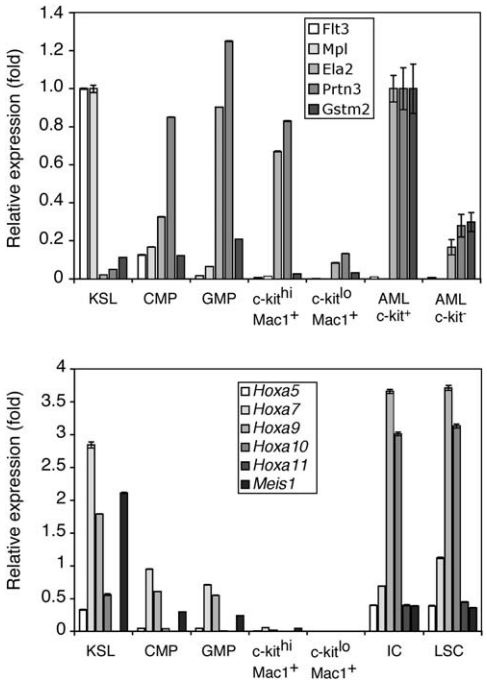

Figure 5. Quantitative transcriptional profiling of MLL-AF9 LSCs and FACS-sorted normal BM cell subsets

Bar charts show the relative expression levels of the indicated genes in FACS-sorted normal BM cell subsets, and double-sorted c-kit$^+$ and c-kit$^-$ leukemia cell populations (upper panel), or IC and LSC populations (lower panel), normalized to *Actb* expression, using ΔCt methodology. Error bars for normal BM populations represent SEMs of three replicate analyses. Error bars for leukemia cell populations represent SEMs for expression analyses from five separate leukemic animals (upper panel) and for eight separate paired populations (lower panel). Transcript levels (lower panel) were further normalized to one another using ΔCt methodology to show the relative expression levels of different *Hoxa* genes. KSL, c-kit$^+$, Sca1$^+$, Lin$^-$; CMP, common myeloid progenitors; GMP, granulocyte-macrophage progenitors; c-kit^{hi} Mac1$^+$, normal myeloblasts; c-kit^{lo} Mac1$^+$, mostly normal granulocytes and myelocytes.

present in the BM and spleen of syngeneic recipients 24 hr post-transplant. By comparison, normal murine HSCs typically exhibit 24 hr homing efficiencies of around 10% (Szilvassy et al., 2003).

Our results are consistent with previous observations suggesting that transplantation assays can significantly underestimate the frequency of cancer stem cells. For example, the number of murine adenocarcinoma cells required to initiate a subcutaneous tumor was substantially reduced from 6900 to four (Hewitt et al., 1973) by coinjection of lethally irradiated cells from the same tumor (presumably containing stromal elements), suggesting that as many as a quarter of the tumor cells in this instance have cancer stem cell potential given an appropriate microenvironment. Thus, in our experiments, LSCs may be relatively deficient in locating supportive niches, by comparison with normal HSCs, accounting for why many cells with LSC potential failed to read out in a transplant assay. The results of our studies are therefore most consistent with a high frequency of LSCs in MLL-AF9 AML that are relatively inefficient in their ability to home, engraft, and read out in conventional transplantation assays. Similar interpretations regarding cancer stem cell frequencies in murine tumors have been suggested but not previously proven (Trott, 1994).

The finding of inefficient LSC engraftment and underestimation of LSC frequency in a syngeneic mouse model has potential implications for the interpretation of human LSC quantitation and characterization using NOD/SCID mice. The reported frequency of NOD/SCID leukemia-initiating cells (SL-ICs) in the blood of humans with AML ranges from 1 in 10^4 to 1 in 10^7 (Bonnet and Dick, 1997). However, as many as one-half of human AMLs fail to engraft recipient mice at a transplanted dose of 10^7 cells (Pearce et al., 2006), suggesting the possibility that xenogeneic differences create a high barrier to AML LSC engraftment. Furthermore, the percentage of human cells detected in the BM of engrafted mice 8 weeks following transplantation of 10^7 AML cells is typically remarkably low (median 4%, n = 43) (Ailles et al., 1999). Evidence that conventional NOD/SCID mouse assays fail to detect at least a proportion of LSCs, due in part to immunological mechanisms, is further provided by the observation that 17%–25% of AMLs that fail to engraft in conventional NOD/SCID mice are able to engraft NOD/SCID mice, which are additionally deficient for β2-microglobulin (Feuring-Buske et al., 2003; Pearce et al., 2006). Furthermore, the efficiency of engraftment of normal human HSCs in even the most permissive immune-deficient mouse model (NOD/SCID *Il2rg* $^{-/-}$) remains markedly inferior to engraftment efficiencies observed for normal murine HSCs in syngeneic settings (Bonnet et al., 1999; Shultz et al., 2005; Camargo et al., 2006). Taken together, these studies strongly suggest that xenogeneic transplantation approaches are likely to underestimate LSC frequency in human AML analogous to, and perhaps even more profoundly than, the underestimation observed in our syngeneic model of AML. Direct evidence that human LSCs might be significantly less efficient at engrafting NOD/SCID mice by comparison with normal HSCs comes from studies of human p210 BCR-ABL acute lymphoblastic leukemia (Castor et al., 2005). Transplantation of FACS-sorted CD34$^+$, CB38$^-$, CD19$^-$ cells from the blood of leukemic patients invariably resulted in exclusively normal human hematopoietic engraftment in NOD/SCID mice, even though FISH analysis of transplanted cells demonstrated that >65% were BCR-ABL positive.

MLL-AF9 establishes an AML hierarchy that is outside and downstream of the normal progenitor compartment

Our studies define the architecture and antigen expression repertoire of the LSC compartment in a model of human MLL-AF9 AML. The LSC compartment has a mature myeloid immunophenotype, lying almost entirely downstream of the normal BM stem and progenitor compartment. The hierarchical organization of murine MLL-AF9 leukemia was loosely defined by c-kit expression, although a proportion of c-kit-negative cells nevertheless possessed the ability to induce AML. Expression of *Hoxa* transcripts was lower in the c-kit$^-$ fraction, and cell cycle inhibitor transcripts were higher, suggesting the possibility that this pattern of gene expression might be associated with loss of LSC potential. Expression of Cdk inhibitors has previously been suggested to be inversely associated with self-renewal divisions in HSCs (Ito et al., 2004; Yuan et al., 2004).

A recent study of murine MLL-AF9 leukemia initiated in GMPs (Krivtsov et al., 2006) concluded that LSCs were uncommon cells (approximately 1:150 at late-stage disease) with a Lin$^-$ c-kit$^+$ phenotype, findings that are in marked contrast to ours. These differences might be explained by alternative experimental methodology, for example, the reliance on limit dilution

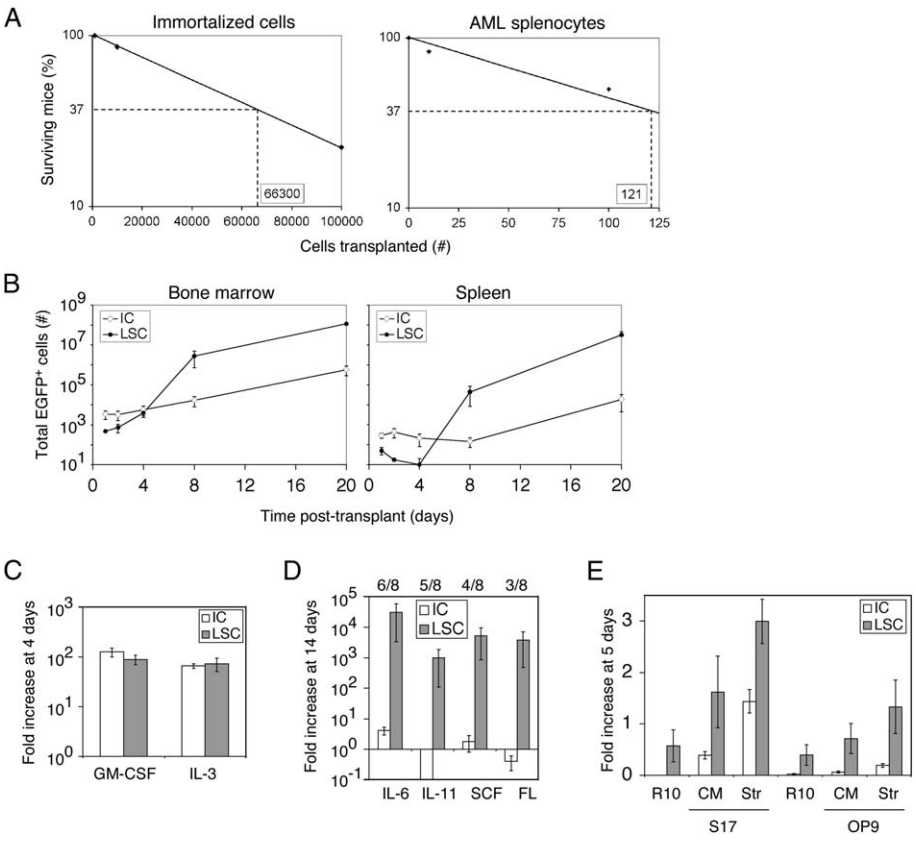

Figure 6. LSCs from mice with MLL-AF9 AML engraft with greater efficiency than ICs and possess altered sensitivities to stromal cell-derived survival and proliferation signals

A: Limit dilution analyses were performed using the method of maximum likelihood (Miller and Eaves, 1997) to determine the number of transplanted immortalized cells or leukemic splenocytes required to induce AML in sublethally irradiated syngeneic recipient mice. Between six and nine mice were transplanted for each indicated cell dose. Mice were followed for 9 months (left panel) and 7 months (right panel), and disease latencies increased with decreasing cell dose to a maximum of 6 months (left panel) and 14 weeks (right panel). FACS analysis of at least 10^5 nucleated cells obtained from BM aspirates (Verlinden et al., 1998) at the end of the follow-up period confirmed the absence of $EGFP^+$ donor leukemia cells and subclinical disease in surviving mice. No mice transplanted with 10^3 or fewer ICs developed AML, whereas all mice transplanted with 10^3 leukemic splenocytes developed AML.

B: Cohorts of mice transplanted with either ICs or leukemic splenocytes derived from the same original IC (10^6 cells/recipient) were euthanized on the indicated days, and the numbers of $EGFP^+$ cells in the femurs and spleen were determined by FACS analysis (mean ± SEM for three experiments, two different clones). The calculations of total $EGFP^+$ cells in the respective anatomic compartments assumed that each femur contains 7% of the BM of a mouse (Boggs, 1984) and that 70% of cells were recovered intact following splenic disaggregation or BM flushing.

C: Bar graph indicates the fold proliferation for ICs or leukemic splenocytes cultured for 4 days in IL-3 or GM-CSF alone (mean ± SEM; n = 8 pairs derived from the same single cell and representative of 7 separate clones).

D: Bar graph indicates the fold proliferation for ICs or leukemic splenocytes after 14 days culture in liquid medium supplemented with IL-6, IL-11, SCF, or FL alone (mean ± SEM). The number of leukemic splenocyte populations (out of a total of eight pairs) that acquired the ability to proliferate in the indicated cytokine alone is shown above the graph.

E: ICs or leukemic splenocytes were cultured in medium with FCS but no growth factors (R10) or medium conditioned by stromal cells (CM), or on a stromal cell layer (S17 or OP9). Bar graph indicates the fold change in cell number (based on trypan blue exclusion) after 5 days of incubation (mean ± SEM; n = 8 pairs).

analysis rather than AML colony analysis for estimating LSC frequency, and different flow cytometry methodology, including the exclusion of anti-Mac1 from the cocktail of lineage antibodies and the use of an unconjugated anti-Gr1 antibody.

An important question is whether our model accurately reflects the cellular organization of human MLL-AF9 AML. Evidence that this is indeed the case is suggested by studies demonstrating that $CD34^-$ blood blasts from patients with AML-M5 harboring a t(9;11), the cytogenetic hallmark of an MLL-AF9 fusion gene, were able to engraft NOD/SCID recipients (Blair et al., 1997; Blair and Sutherland, 2000). This raises the possibility that human and mouse MLL-AF9 AML share similarities in their hierarchical organizations outside of the normal progenitor compartment. Thus, the observed differences in the architecture of the leukemia in our model by comparison with that presumed for the majority of human AMLs, where SL-ICs mostly have a $CD34^+$ $CD38^-$ Lin^- phenotype, may be due at least in part to oncogene-specific effects, and not fundamental differences in mouse and human AMLs, or limitations of our experimental model, which involves the use of a retroviral promoter to drive expression of MLL-AF9. The hyperexpression of *Hox* genes maintained by MLL oncoproteins (Ayton and Cleary, 2001) may confer specific architectural features on the leukemia cell hierarchy at a critical stage downstream of the normal progenitor compartment. Our findings therefore suggest that, particularly in murine models of leukemia, and possibly in human AML associated with MLL fusion oncogenes, the assumption that all LSCs reside in the lineage-negative compartment should be revised. Furthermore, the phenotype of cells targeted for AML initiation cannot necessarily be deduced by the phenotype of the LSC. Our studies also raise the possibility that targeting rare leukemia cells with a stem cell phenotype may not necessarily result in improved therapeutic outcomes for certain types of AML, since disease relapse may be mediated by more frequent cells with immunophenotypes that are distinct from normal HSCs.

The nature of the MLL-AF9 LSC

The immortalized (premouse) and leukemic (postmouse) CFCs in this model display some features of both stem cells (the biological property of extensive self-renewal) and differentiated cells ($Sca1^-$ Lin^+ immunophenotype). An important question is whether the CFCs are more akin to HSCs that aberrantly express myeloid lineage markers due to the leukemogenic event, or more differentiated cells that have aberrantly acquired *Hoxa*-mediated self-renewal. The latter scenario is suggested

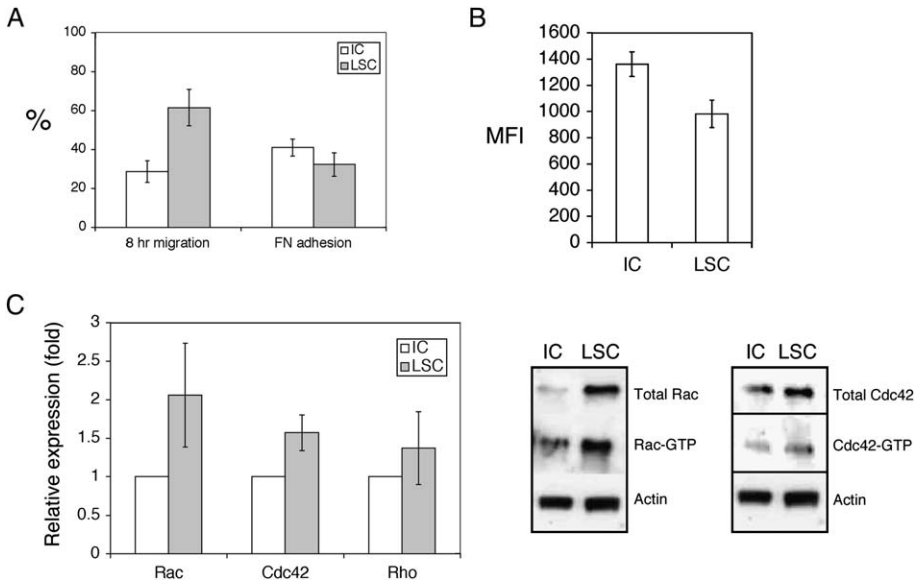

Figure 7. Enhanced SDF1 induced chemotaxis and increased small GTPase expression in MLL-AF9 LSC populations

A: Bar chart shows mean (±SEM; n = 8 pairs) percentage migrations of IC- or LSC-containing populations in fibronectin-coated Transwell chemotaxis assays toward a 100 ng/ml SDF1 gradient (left columns) and mean (±SEM; n = 8 pairs) adhesion of cells to fibronectin in adhesion assays (right columns). Cells did not migrate or adhere in the absence of SDF1 and fibronectin, respectively.

B: Bar chart shows mean (±SEM; n = 8 pairs) fluorescent intensity (MFI) of IC- or LSC-containing populations when analyzed by FACS for surface Cxcr4 expression. MFI of cell populations stained with streptavidin-PE alone, and no primary antibody, was <20.

C: Bar chart shows mean (±SEM; n = 7 pairs) protein levels of the indicated small GTPases, normalized to β-actin, as assessed by densitometry analysis of western blot bands using ImageJ software. The panels show western blots of cytoplasmic lysates from representative IC and LSC populations analyzed for Rac, Cdc42, and β-actin (top and bottom), and for cellular content of Rac-GTP and Cdc42-GTP (middle, determined by PAK1-PBD agarose bead pulldown).

by the observed transcriptional and immunophenotypic profiles of the c-kit$^+$ leukemia cell populations, which are enriched for LSC activity, by comparison with normal stem and progenitor cells, and the absence of detectable multilineage differentiation arising from the LSCs. A somewhat similar conclusion was reported by Krivtsov et al., (2006). Nevertheless, during the in vivo progression of immortalized CFCs to fully transformed LSCs in this model, it is interesting to note that the acquisition of (1) enhanced engraftment potential, (2) enhanced sensitivity to stromal cell-derived survival/proliferation signals, and (3) increased SDF1-induced chemotaxis could readily be characterized as the acquisition of a more stem-like biological phenotype, possibly permitting LSCs to take advantage of alternative hematopoietic niches to those available to either normal HSCs or MLL-AF9-immortalized CFCs.

Consistent with the observed enhanced migration and engraftment potential of LSC compared with IC populations, and the critical role of small GTPases in hematopoietic cell migration and engraftment (Yang et al., 2001; Gu et al., 2003; Cancelas et al., 2005), we found significantly increased expression levels of the small GTPase Cdc42 and more variable increases in Rac expression. Increased small GTPase protein levels were associated with increased activity. However, it is unlikely that these findings account for all of the biological differences between LSC and IC populations, which undoubtedly result from acquired heterogeneous genetic and/or epigenetic changes. This is suggested, for example, by the finding that the AML cell populations in this study displayed a range of cytokine sensitivity profiles. Furthermore, the observation that direct contact with stromal cells provided an optimal proliferative stimulus for LSCs, by comparison with stromal cell-conditioned medium, suggests that the stromal cell interaction with LSCs is complex, potentially involving multiple signaling pathways.

Nevertheless, the observation that LSCs consistently displayed an enhanced ability to survive/proliferate in contact with stromal cells by comparison with ICs emphasizes the likely biological importance of microenvironmental interactions for MLL-AF9-mediated leukemogenesis. Such interactions are crucial in solid tumors (Hewitt et al., 1973; Bhowmick et al., 2004; Orimo et al., 2005) and have previously been suggested to be important in AML (Giles et al., 2002). Although a stepwise progression in the pathogenesis of MLL-AF9 leukemia was reported previously in a knockin mouse model (Johnson et al., 2003), the critical biological observation was of progressive temporal increases in the frequency of aberrantly self-renewing stem and progenitor cells. Conditional activation of MLL-CBP resulted in increased stem and progenitor cell self-renewal, but this only induced a mild expansion of the myelomonocytic compartment (Wang et al., 2005). Uncharacterized secondary genetic and biological events induced by γ-irradiation or N-ethyl-N-nitrosurea were required to generate fatal myeloproliferative disease. By contrast, our finding that altered microenvironmental interactions are a consistent feature of LSCs by comparison with ICs provides important evidence that acquisition of sensitivity to stromal cell-derived proliferation signals is a critical step in MLL oncogene-mediated leukemogenesis and warrants further studies to establish the potential broader implications for AML in general.

Experimental procedures

Mice

FVB.Cg-Tg (GFPU) 5Nagy/J and wild-type FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on an inbred FVB background. All experiments on mice in this study were performed with the approval of and in accordance with Stanford's Administrative Panel on Laboratory Animal Care.

Retroviral constructs and BM progenitor cell transduction

A retroviral construct encoding MLL-AF9 was generated by fusion of the carboxy-terminal 91 amino acids of human AF9 (accession number BC036089) in-frame with the amino-terminal 1395 amino acids of human MLL in the retroviral vector MSCV-5′MLL-Neo (DiMartino et al., 2002). Prior to transduction, BM cells were flushed from the long bones of 4- to 8-week-old mice and immunomagnetically selected for c-kit expression using phycoerythrin (PE)-conjugated anti-CD117 (clone 2B8, BD Pharmingen, San Jose, CA), anti-PE

magnetic microbeads (Miltenyi Biotec, Auburn, CA), and an automated cell separator (AutoMACS, Miltenyi Biotec). c-kit$^+$ stem and progenitor cells were incubated overnight in RPMI 1640 with 20% fetal calf serum, 20% WEHI-conditioned medium (R20/20), 20 ng/ml SCF, and 10 ng/ml IL-6 (R&D Systems, Minneapolis, MN) to promote cell cycle entry. Cells were then spinoculated with retroviral supernatant in the presence of 5 μg/ml polybrene for 2 hr at 1350 × g and 32°C. Following spinoculation, cells were incubated overnight in the above medium to allow for expression of neomycin resistance in transduced cells prior to further manipulation.

In vitro culture of MLL-AF9-transformed progenitor cells

Postspinoculation c-kit$^+$ cells (10^4) were plated in methylcellulose medium (M3231, Stem Cell Technologies, Vancouver, BC) containing 20 ng/ml SCF, 10 ng/ml IL-6, 10 ng/ml GM-CSF, 10 ng/ml IL-3 (R&D Systems), and G418 (0.75 mg/ml) (Gibco, Carlsbad, CA). After 5 days of culture, colonies were counted and pooled, and then 10^4 cells were replated in the same medium but without G418. For the third round of culture, 5 × 10^3 cells were plated. At the end of the third round (or after one round of culture in the case of colonies derived from leukemic splenocytes), single colonies were plucked from methylcellulose and transferred to liquid culture initially in single wells in 96-well plates containing R20/20, 20 ng/ml SCF, and 10 ng/ml IL-6. Cultures derived from single colonies were subsequently transferred to 6-well plates on day 4 of culture, and the medium was changed every 5 days thereafter. Once individual cultures had expanded beyond 10^6 cells, they were maintained solely in R20/20. For colony assays of transplanted immortalized progenitor cells or leukemic splenocytes (sorted and unsorted), 2–5 × 10^3 cells were plated in methylcellulose medium with the above four growth factors but without G418.

For studies involving culture of cells in medium containing single growth factors, immortalized progenitor cells or cells derived from leukemic splenocytes (cultured in R20/20 for 6–19 days) were washed three times in RPMI 1640 and then plated at a concentration of 2 × 10^5/ml in medium containing RPMI 1640 with 20% fetal calf serum and either 20 ng/ml IL-3, 20 ng/ml GM-CSF, 20 ng/ml IL-6, 20 ng/ml SCF, 10 ng/ml human IL-11 (Peprotech, Rocky Hill, NJ), or 10 ng/ml human Flt3 ligand (R&D Systems). Viable cell counts using trypan blue dye exclusion were performed every 2–4 days, and medium was replaced when cultures were split, or every 4 days.

For studies involving stromal cell coculture, immortalized progenitor cells or cells derived from leukemic splenocytes (as above) were washed three times in RPMI 1640, and then 2 × 10^5 cells were added to either a confluent layer of stromal cells in one well of a 12-well plate containing 2 ml of medium, medium only, or stromal layer-conditioned medium. Either S17 stromal cells (cultured in RPMI 1640 with 10% fetal calf serum) (Collins and Dorshkind, 1987) or OP9 stromal cells (cultured in MEM α with 10% fetal calf serum) (Kodama et al., 1994) were used. Stromal layer-conditioned medium was made by combining medium harvested following 48 hr contact with the stromal layer with fresh medium in a 1:1 ratio. Following culture for 5 days, with medium being 50% replaced every 2 days, the number of viable hematopoietic cells was determined using trypan blue exclusion, following trypsinization in the case of the cells cultured on stromal layers. Hematopoietic cells were readily distinguished from stromal cells by both a marked difference in size and their green fluorescence.

Transplantation procedures, homing, and engraftment studies

Transplantation of immortalized cells, or secondary and tertiary transplantation of cells derived from leukemic animals, was performed by retro-orbital injection of sublethally irradiated syngeneic FVB mice (450 cGy). When c-kit$^+$ stem and progenitor cells were transplanted immediately following retroviral transduction (C57BL/6 background), mice were lethally irradiated (900 cGy), and 300,000 postspinoculation cells (representing 3600 transduced CFUs; CFU transduction efficiency 35%) were injected, together with 250,000 nucleated BM cells as radioprotection. When transplanted mice exhibited signs of ill health, they were euthanized. For homing and engraftment studies, cohorts of mice were transplanted with 10^6 EGFP$^+$ cells each, and then one mouse from each cohort was euthanized on days 1, 2, 4, 8, and 20 posttransplant. The BM was then flushed from both femurs, the spleen was disaggregated, and following red cell lysis, the numbers of EGFP$^+$ cells in the marrow and spleen were determined by flow cytometry.

Flow cytometry

Staining of cells for FACS analysis and sorting was performed using the following conjugated antibodies obtained from either BD Pharmingen (BD) or eBioscience (eB) (San Diego, CA), except where indicated, at a dilution of 1:100 in PBS containing 2% fetal calf serum and 2 mM EDTA (staining medium) for 20 min on ice: Mac1-PE (eB) or Cy7APC (BD) (clone M1/70), Gr1-PE (clone RB6-8C5, eB), c-kit-APC (clone 2B8, eB), Sca1-Cy5PE (eB) or FITC (BD) (clone D7), CD16/32-Cy7PE (clone 93, eB), Flt3-PE (clone AF2 10.1, BD), F4/80-PE (Caltag Laboratories, Burlingame, CA), biotinylated CD31 (clone 390, eB), biotinylated CD24 (clone 30-F1, BD), biotinylated or FITC-conjugated CD34 (1/20) (clone RAM34, BD), biotinylated Cxcr4 (clone 2B11, BD), streptavidin-conjugated Cy7PE (eB), or PE (BD) (1/400). To stain lineage-positive cells, a cocktail of the following PE-conjugated antibodies was used: Mac1, Gr1, B220 (clone RA3-6B2, BD), TER119 (clone TER-119, eB), CD3 (clone 145-2C11, BD), CD4 (clone RM4-5, BD), CD8 (clone53-6.7, eB). Normal progenitor cell populations were isolated by FACS sorting for the following immunophenotype (C57BL/6 mice used as donors): KSL, c-kit$^+$, Sca1$^+$, Lin$^-$; CMP, c-kit$^+$, Sca1$^-$, Lin$^-$, CD34$^+$, CD16/32^{lo}; GMP, c-kit$^+$, Sca1$^-$, Lin$^-$, CD34$^+$, CD16/32^{hi}; myeloblasts, c-kit^{hi}, Mac1$^+$; granulocytes/myelocytes, c-kit$^{lo/-}$ Mac1$^+$. Following staining, cells were washed once in staining medium and then analyzed using an LSR Model 1a flow cytometer (BD Biosciences, San Jose, CA). Some analyses and all cell sorts were performed using a dual laser FACS Vantage (BD Biosciences).

Real-time quantitative PCR analysis of gene expression

RNA from sorted cell populations was purified using Trizol, and 0.5 μg was reverse transcribed using an oligo-dT primer and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reaction products were diluted in TE, and 2 μl was subjected to real-time PCR, which was performed in triplicate using Taqman probes and the ABI Prism 7700 sequence detection system. Taqman probes for *Hoxa5* (Mm00439362_m1), *Hoxa7* (Mm00657963_m1), *Hoxa9* (Mm00439364_m1), *Hoxa10* (Mm00433966_m1), *Hoxa11* (Mm00439360_m1), *Meis1* (Mm00487664_m1), *Cdkn1b* (Mm00438167_g1), *Cdkn2a* (Mm00494449_m1), *Cdkn2c* (Mm00483243_m1), *Flt3* (Mm00438996_m1), *Mpl* (Mm00440310_m1), *Ela2* (Mm00469310_m1), *Prtn3* (Mm00478323_m1), *Gstm2* (Mm00725711_s1), and *β-Actin* (Mm00607939_m1) were purchased from Applied Biosystems (Foster City, CA). Expression levels of analyzed genes relative to that of *β-Actin* were calculated using the comparative Ct method, as described in ABI User Bulletin #2.

Transwell migration and adhesion assays, and GTPase expression and activity analyses

Cell migration assays were performed using fibronectin-coated Transwell polycarbonate membranes (5 μm pore size) in 24-well plates (Corning, Corning, NY). 2 × 10^5 cells, suspended in R20/20, were placed in the insert and allowed to migrate for 8 hr along a Cxcl12 (SDF1, Peprotech) gradient, created by addition of 100 nM Cxcl12 to R20/20 medium beneath the membrane. For adhesion assays, 5 × 10^5 cells suspended in R20/20 were placed in fibronectin-coated and BSA-blocked 12-well tissue culture plates for 1 hr to permit adhesion. Nonadherent cells were removed by gently adding, and then tipping out, PBS to the wells three times. Adherent cells were then trypsinized and counted.

Cdc42 and Rac GTPase activity assays, and western blot analyses, were performed using commercially prepared reagents according to the manufacturer's instructions (Cell Biolabs, San Diego, CA). Anti-actin (mouse monoclonal C4) was from Chemicon International (Temecula, CA).

Supplemental data

The Supplemental Data include two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/4/257/DC1/.

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