Cancer Cell
Article

Malignant Transformation Initiated by *Mll-AF9*: Gene Dosage and Critical Target Cells

Weili Chen,[1,5] Ashish R. Kumar,[1,2,5] Wendy A. Hudson,[1] Quanzhi Li,[1] Baolin Wu,[4] Rodney A. Staggs,[1] Erik A. Lund,[1] Thien N. Sam,[1] and John H. Kersey[1,2,3,*]
[1]University of Minnesota Cancer Center
[2]Department of Pediatrics
[3]Department of Laboratory Medicine and Pathology
[4]Division of Biostatistics, School of Public Health
University of Minnesota, 420 Delaware Street S.E., Minneapolis, MN 55455, USA
[5]These authors contributed equally to this work.
*Correspondence: kerse001@umn.edu
DOI 10.1016/j.ccr.2008.03.005

SUMMARY

The pathways by which oncogenes, such as *MLL-AF9*, initiate transformation and leukemia in humans and mice are incompletely defined. In a study of target cells and oncogene dosage, we found that *Mll-AF9*, when under endogenous regulatory control, efficiently transformed LSK ($Lin^-Sca1^+c\text{-}kit^+$) stem cells, while committed granulocyte-monocyte progenitors (GMPs) were transformation resistant and did not cause leukemia. *Mll-AF9* was expressed at higher levels in hematopoietic stem (HSC) than GMP cells. *Mll-AF9* gene dosage effects were directly shown in experiments where GMPs were efficiently transformed by the high dosage of *Mll-AF9* resulting from retroviral transduction. *Mll-AF9* upregulated expression of 192 genes in both LSK and progenitor cells, but to higher levels in LSKs than in committed myeloid progenitors.

INTRODUCTION

Cellular development proceeds in a hierarchical fashion from rare self-renewing stem cells to committed progenitor (transit-amplifying) cells to differentiated postmitotic cells (Jordan et al., 2006). Currently, little is known as to whether naturally occurring cancers arise from normal stem cells or from committed progenitor cells, either of which could potentially acquire oncogenic mutations.

Research on translocations involving *MLL* fusion oncogenes has been extremely productive for more than 20 years and has revealed important information about the biology of leukemia including the role of *HOX* gene expression, histone modifications, and leukemia stem cells (Krivtsov and Armstrong, 2007). The well-studied *MLL-AF9* oncogene initiates myeloid leukemia in both humans and mice (Dobson et al., 1999; Iida et al., 1993). An *Mll-AF9* transgenic murine model that results in myeloid leukemia has been described and studied in some detail (Corral et al., 1996; Johnson et al., 2003; Kumar et al., 2004). In this model, the *Mll-AF9* oncogene, introduced by homologous recombination, is under control of the endogenous *Mll* promoter and, thus, expressed at physiologic levels. This model is potentially informative because it permits the study of well characterized mammalian hematopoietic stem and progenitor cells (Akashi et al., 2000; Spangrude et al., 1988).

A study of the *MLL-ENL* fusion gene introduced by retrovirus showed that both hematopoietic stem cells (HSCs) and committed myeloid progenitor cells were transformed by the fusion oncogene with highest efficiency in HSC population (Cozzio et al., 2003). More recent studies showed that *MLL-AF9* introduced by retrovirus could transform both early hematopoietic progenitors (Somervaille and Cleary, 2006) and committed myeloid progenitors (Krivtsov et al., 2006). A potential limiting factor in these previous studies comes from the utilization of retroviruses to introduce the oncogene. Retroviral introduction can result in noncontrolled and potentially nonphysiologic levels of oncogene

SIGNIFICANCE

In a comparison of *Mll-AF9* oncogene expression in retroviral and knockin models, we showed a direct relationship between transformation susceptibility and oncogene dosage in committed progenitor cells. In the knockin model where oncogene expression is under endogenous regulatory control, we found high *Mll-AF9* gene expression levels and high expression levels of downstream target genes in stem compared to committed progenitor cells. These results encourage further analysis of physiologically regulated oncogene dosage effects on genes that are critical to cell-specific transformation susceptibility. Studies of cell-specific effects are increasingly important with the recognition that certain oncogenes, such as the *MLL* fusion genes, are globally active.

expression, depending on the numbers of viral integrations and the type of promoters. The transforming effects of cellular oncogenes, including *MLL* fusions, *MYC*, *BCR-ABL*, and *CEBPA*, may differ significantly depending on oncogene expression levels (Caslini et al., 2004; Chapiro et al., 2006; Ren, 2004). To circumvent these limitations, we studied the knockin *Mll-AF9* murine model, which permits a direct comparison of the susceptibility to transformation of LSK ($Lin^-c\text{-}kit^+Sca\text{-}1^+$, including hematopoietic stem cell HSC and common lymphoid progenitor CLP) stem and committed myeloid progenitor (common myeloid progenitor CMP and granulocyte-monocyte progenitor GMP) cells. The knockin model also permits expression of *Mll-AF9* at physiologic gene dosages, which we postulate should differ across the hematopoietic stem and various progenitor cells populations based on studies of wild-type *Mll* expression (Jude et al., 2007; McMahon et al., 2007).

We report differences in transformable cells (LSKs > CMPs > GMPs) when the *MLL* fusion oncogene is expressed at physiologic gene doses. We describe the importance of oncogene dosage which is suggested by (1) differences in *Mll-AF9* expression in HSCs and GMPs and (2) biologic differences between retrovirally introduced *MLL-AF9* and endogenous *Mll-AF9* expression.

RESULTS

Mll-AF9 Mice Show Increased HSCs, CLPs, and CMPs

Bone marrow cells from 8- to 10-week-old *Mll-AF9* knockin mice or their wild-type (WT) siblings were used in this study. At this age, *Mll-AF9* mice show myeloid cell proliferation but do not develop leukemia until 6 months of age (Chen et al., 2006; Corral et al., 1996). We sorted bone marrow cells into previously well-defined progenitor or stem cell populations. $Lin^-Sca1^+c\text{-}kit^+$ (LSK) (Ikuta and Weissman, 1992; Spangrude et al., 1988) markers were used to sort the closely related self-renewal hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) (Kondo et al., 1997; So et al., 2003; Terskikh et al., 2003). The comparison groups of committed myeloid progenitors included common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs). Analysis of the sorting profiles of HSCs, CLPs, CMPs, and GMPs revealed that *Mll-AF9* resulted in increased percentages of $c\text{-}kit^+Sca1^+$ cells in both the HSC and CLP populations (Figure 1A). We also saw an increased number of $Fc\gamma RII/III^{lo}$ (CMP) but not $Fc\gamma RII/III^{hi}$ (GMP) cells. In multiple experiments, sorted cell populations from *Mll-AF9* mice showed a consistently higher percentage of HSCs, CLPs, and CMPs, but not GMPs (Figure 1B), when compared to the ones from WT mice (Figure 1B).

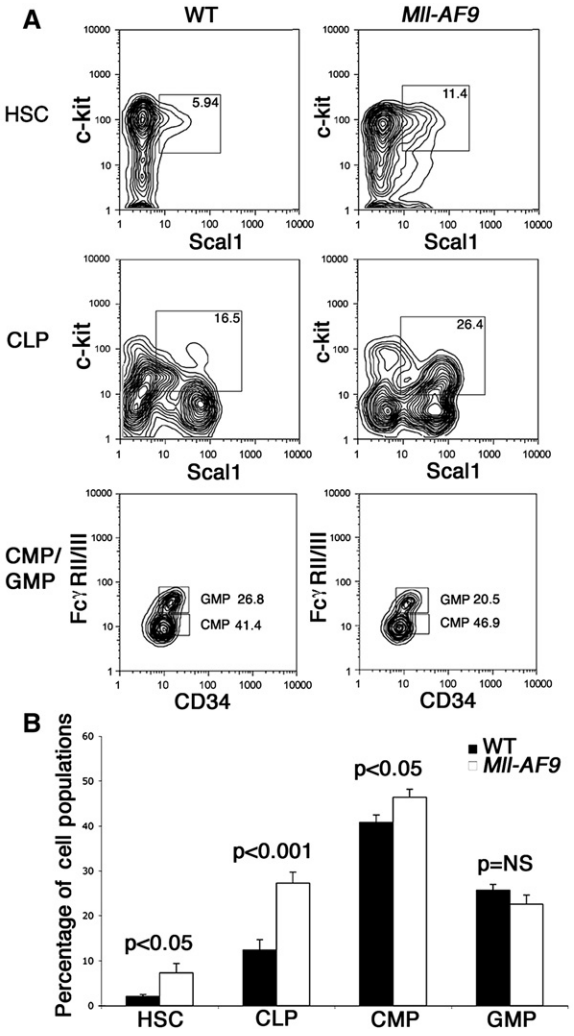

Figure 1. *Mll-AF9* Results in the Expansion of HSC, CLP, and CMP Populations

(A) Sorting profiles of HSCs, CLPs, CMPs, and GMPs showing the expansion of $c\text{-}kit^+Sca1^+$ HSCs (in $Lin^-IL\text{-}7R^-$ population) and CLP (in $Lin^-IL\text{-}7R^+$ population) in *Mll-AF9* mice. Expansion of the CMP population is also shown.

(B) Significantly higher percentages of HSCs, CLPs, and CMPs were found in lineage negative marrow cells of *Mll-AF9* than those of wild-type mice. Error bars represent the standard error of the means.

Leukemia Risk Is Dependent on the Type of Transplanted Cells that Express Physiologic Levels of *Mll-AF9*

MLL-ENL, when introduced into stem and progenitor cell populations (including HSCs, CMPs, and GMPs) by retrovirus and under the control of the retroviral promoter, transformed those populations and produced leukemia in transplanted mice with additional events (Cozzio et al., 2003). Similarly, retrovirally transduced *MLL-AF9* transformed GMP cells and produced leukemia (Krivtsov et al., 2006). In this study, we tested the ability of *Mll-AF9*, expressed under the control of the endogenous *Mll* promoter, to transform stem and progenitor cell populations and to produce leukemia in transplanted mice.

Lethally irradiated WT mice received 25–2500 sorted *Mll-AF9* HSC, CLP, CMP, or GMP cells. Results are shown in Figure 2A and with more details in Table 1. A hierarchy in the ability to produce leukemia was found: The progeny of only 100 HSCs were sufficient to produce fatal leukemia in 90% of animals. However, only the higher dose of 2500 (but not 250) CMPs caused disease and with a longer latency than the recipients of LSKs ($p < 0.0001$, log-rank test). The relatively long latency to leukemia even with LSKs (HSCs/CLPs) suggests that additional events are required to develop fatal leukemia. In repeated experiments, none of the animals receiving GMPs developed leukemia even at the maximum of 2500 GMP cells. To determine the minimum number of

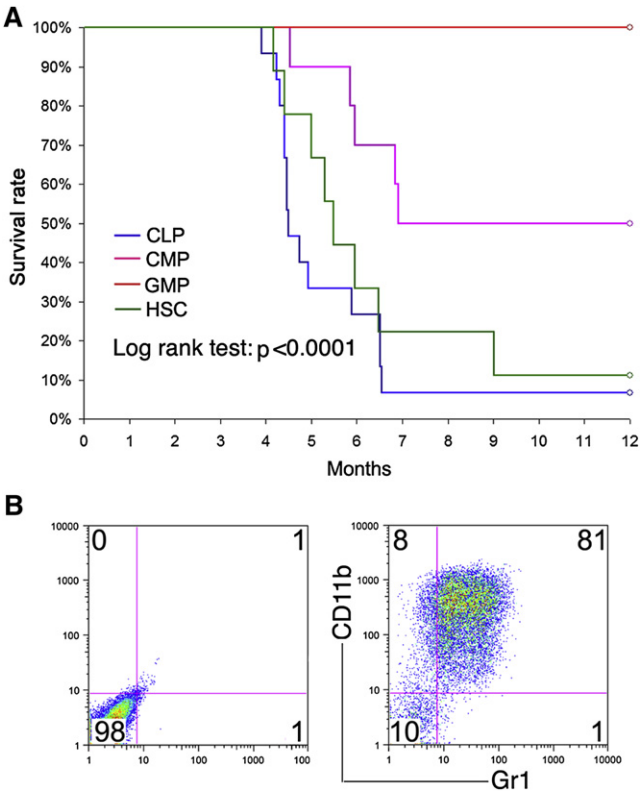

Figure 2. HSCs and CLPs Are More Efficient than CMPs and GMPs in Producing Leukemia In Vivo

(A) Survival of irradiated mice receiving 100–2500 cells. The survival rate was calculated using Kaplan-Meier analysis. The HSC and CLP groups had significantly poorer survival than the CMP and GMP groups ($p < 0.0001$, log-rank test). (B) Recipient mice developed myeloid leukemia after transplantation. All recipient mice showed the same high $CD11b^{+}Gr1^{+}$ profile in spleen previously described in *Mll-AF9* leukemic mice. FACS on the spleen of a representative recipient is shown on the right while the isotype control is shown on the left.

cells required to produce leukemia, five animals received only 25 HSCs each, two of which developed leukemia (Table 1). By limiting dilution analysis, the frequency of "transformable hematopoietic cells" (THCs) was 1:45 in HSCs and 1:57 in CLPs, which was significantly higher than the 1:1043 in CMPs ($p < 0.0001$) (Table 1).

Although the different cell populations exhibited varying ability to cause disease, the type of leukemia caused by those cells was similar. Immunophenotyping revealed that the majority of cells from the enlarged spleens of recipient animals were myeloid ($CD11b^{+}Gr1^{+}$) in phenotype (Figure 2B). Leukemias could be transferred to secondary recipients and the immunophenotype remained the same as the transplanted cells from the primary recipients (data not shown). The demonstration that the relatively mature "downstream" myeloid leukemia cells are independent of the cell types transplanted is similar to that reported in the retroviral *MLL-ENL* and *MLL-AF9* models (Cozzio et al., 2003; Somervaille and Cleary, 2006).

Mll-AF9-Induced Transformation Is Highest in Stem Cells and Lowest in GMPs in *Mll-AF9* Knockin Mice

In a series of experiments, we studied the mechanisms for the differences in leukemia outcomes based on the critical cell types. We first compared the self-renewal effects of *Mll-AF9* knockin stem and progenitor cells using a myeloid colony forming assay (Johnson et al., 2003). Sorted cells were cultured in methylcellulose medium containing IL-3, IL-6, SCF, and GM-CSF, replated every 7 days, and colonies were studied at day 21. Figure 3A shows the significant increase in colony numbers from all *Mll-AF9* stem/progenitor cells compared to wild-type. Notably, LSKs formed the greatest number of colonies, with CMPs and GMPs forming significantly fewer colonies. We and others have previously shown that in addition to increased colony numbers, *MLL* fusion genes induce the formation of compact colonies, which are composed predominantly of immature myeloid cells (Johnson et al., 2003; Somervaille and Cleary, 2006). As shown in Figure 3B, significantly more compact colonies were found in *Mll-AF9* LSK cultures than in those from CMP and GMP cultures. No compact colonies were found in any wild-type cultures. Overall, colony assays showed that enhanced self-renewal induced by *Mll-AF9* was greatest in LSKs (HSCs/CLPs) compared to the committed myeloid progenitor populations (CMPs and GMPs). Immunophenotyping revealed that cells from colonies in all *Mll-AF9* groups were $CD11b^{+}Gr1^{+/-}$ myeloid (Figure S1).

Retrovirus-Induced Expression of *Mll-AF9* in GMPs Results in Increased Myeloid Colonies and Long-Term Self-Renewal In Vitro; These Changes Are Not Found in *Mll-AF9* Knockin GMPs

In contrast to the GMPs transformed by knockin *Mll-AF9* that did not produce leukemia, GMPs transformed by the *MLL-AF9* retrovirus were capable of producing leukemia in transplanted animals (Krivtsov et al., 2006). Thus, we compared the effects of *MLL-AF9* in GMPs transduced by retrovirus to those in GMPs from *Mll-AF9* knockin mice. Wild-type GMPs were transduced with MSCV-MLL-AF9-GFP retrovirus as previously described (Krivtsov et al., 2006), while *Mll-AF9* knockin GMPs were transduced with the MSCV-GFP retrovirus as controls. The reagents and protocols for these studies were identical to those used by Krivtsov et al. In the first series of experiments, we compared myeloid colonies from both methods of fusion gene introduction. GFP^{+} cells were selected as previously described and myeloid colonies were counted after three sequential platings on day 21. Results in Figure 3C, left panel, show that total myeloid colonies were more than four times higher in the GMPs transduced by *MLL-AF9* retrovirus than in *Mll-AF9* knockin GMPs transduced with the MSCV-GFP control virus. Similarly, when colony types were examined, more compact immature colonies were found in the *MLL-AF9* transduced cells than in the knockin cells that constitutively express *Mll-AF9* (Figure 3C, right panel). These data show enhanced self-renewal of *MLL-AF9* retrovirally transformed cells in vitro; enhanced self-renewal of these cells was further shown in cytokine (IL-3, IL-6, SCF, and GM-CSF)-enriched liquid culture where *Mll-AF9* knockin cells did not survive beyond 20 days, while retroviral *MLL-AF9* cells continued to grow in long-term liquid culture (Figure S2).

MLL-AF9 Expression Is Significantly Higher in Retrovirally Transduced GMPs Than in *Mll-AF9* Knockin GMPs

The known strength of the retroviral promoter, combined with data from the colony assays and Southern blotting, all suggested

Table 1. Summary Data of Transplantation Experiments

Starting *Mll-AF9* Population	No. of Cells Transplanted	No. of Animals Transplanted	No. of Animals with AML (%)	Latency of AML (Median and 95% CI in Days)	Frequency of Transformable Hematopoietic Cells (THCs)
HSC	25	5	2 (40%)	-[a] (167, -)	1:45
	100	10	9 (90%)	165 (152, 197)	
CLP	100	5	4 (80%)	198 (134, NA)	1:57
	250	5	5 (100%)	136 (119, 179)	
	2500	5	5 (100%)	137 (131, 199)	
CMP	250	5	0 (0%)	- (-, -)	1:1043
	2500	5	5 (100%)	181 (138, 210)	
GMP	250	5	0 (0%)	- (-, -)	–
	2500	6	0 (0%)	- (-, -)	

Five negative control mice injected with 2.5×10^5 WT bone marrow cells were all alive for the duration of the experiment.
[a] Inestimable.

that expression of *MLL-AF9* will be higher in retrovirally transduced GMPs than in *Mll-AF9* knockin GMPs. Using primers that detected a sequence present in both retroviral *MLL-AF9* and knockin *Mll-AF9* constructs (but not in wild-type mice), we compared the expression levels of the fusion gene in the *MLL-AF9* transduced GMPs to those in MSCV-GFP transduced *Mll-AF9* GMPs by real-time quantitative RT-PCR. Figure 3D, left panel, shows that GFP^+ *MLL-AF9* retrovirally transduced cells had 170-fold higher expression of *MLL-AF9* than knockin GMPs with virus control. In long term culture, expression levels of *MLL-AF9* in the subclones from *MLL-AF9* retrovirally transduced GMPs remained very high (Figure 3D, right panel). Results from Southern blotting with GFP as a probe on the genomic DNA from these cultured cells showed more than one band; these results provide evidence that multiple *MLL-AF9* integrations were likely (Figure S3).

Gene Expression Profiles Induced by *Mll-AF9* Expressed at Physiologic Levels

A goal of our study was to define the molecular pathways that would explain the differences between *Mll-AF9* HSCs and GMPs. We compared the early (preleukemic) in vivo effects of the *Mll-AF9* fusion gene on gene expression levels in the cells from *Mll-AF9* knockin mice. RNA was extracted from sorted HSCs, CLPs, CMPs, and GMPs and amplified for analysis by Affymetrix murine 430 2.0 microarrays. To identify genes differentially expressed as a result of *Mll-AF9* expression, we performed a two-way ANOVA using a stratified permutation test (See Supplemental Experimental Procedures). Allowing for a false discovery rate (FDR) of 10% (Benjamini and Hochberg, 1995), this analysis yielded 446 genes that were differentially expressed in *Mll-AF9* compared to WT cells (Figure S4). A clustering analysis was performed using this 446 gene set, with results shown in Figure 4A. The expected clustering of CMPs with GMPs and HSCs with CLPs was found. *Mll-AF9* HSCs and CLPs were clustered with each other instead of their wild-type counterparts, suggesting very similar downstream effects of the fusion gene in the two related populations. Of the 446 genes selected by the two-way ANOVA, 192 were expressed at high levels in all four *Mll-AF9* cell types compared to wild-type, while 179 genes displayed lower expression in the *Mll-AF9* populations (Tables S1 and S2). The top 50 genes upregulated in all four cell types are shown in the heat map in Figure 4B. These genes are ranked in decreasing order of fold changes in HSCs.

Further analysis of the 192 upregulated genes in the *Mll-AF9* populations revealed 96 genes more highly expressed in transformation-sensitive LSKs compared to transformation-resistant CMPs/GMPs (FDR < 0.1, Significance Analysis of Microarrays; Tusher et al., 2001). The top 50 of the 96 *Mll-AF9* LSK overexpressed genes are shown in the heat map of *Mll-AF9* transformed cells in Figure 5A. Representatives of the genes more highly expressed in LSK than in CMP/GMP group are well-known targets of *Mll* and *Mll*-fusion proteins—*Hoxa5*, *Hoxa9*, and *Meis1*. Also included is *Evi1*, not currently known to be a direct target of *Mll* or *Mll* fusion genes. *Evi1* overexpression was confirmed by quantitative RT-PCR shown in Figure S5. We analyzed in more detail the relative levels of known targets *Hox5*, *Hoxa9*, *Meis1*, and *Evi1* in each population of cells with results shown in Figure 5B. Importantly, these four genes were most highly expressed in *Mll-AF9* LSKs, expressed at intermediate levels in wild-type LSKs and *Mll-AF9* CMPs/GMPs, and expressed at the lowest levels in wild-type CMP/GMPs. Several of the 192 genes that we found to be upregulated by *Mll-AF9* in all four cell populations, including *Hoxa5*, *Hoxa9*, *Hoxa10*, and *Meis1*, were previously found to be highly "immediately" expressed in GMP cells transformed by the *MLL-AF9* retrovirus (Krivtsov et al., 2006).

We also carried out a comparative analysis of the previously reported leukemias resulting from retrovirus transduced GMPs (Krivtsov et al., 2006) and our knockin preleukemia cells. This analysis showed 20 genes that were upregulated in both groups (Table S3). Included were the expected *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10*, and *Meis1* genes plus novel genes, such as *IL31 receptor A* and *Chemokine-like factor super family 6* genes. Since the previous study used only GMPs rather than the four cell types of this study and a different Affymetrix probe set, a complete comparison of the two is difficult, and it is likely that the number of genes in common is actually greater than 20.

Expression of *Mll* and *Mll-AF9* Is Higher in HSCs Than in GMPs

We next evaluated the hypothesis that the upregulated expression of genes known to be downstream of *Mll*, such as *Hoxa* genes in HSCs, could be a result of higher expression levels of the *Mll-AF9* fusion gene in HSCs. The expression of wild-type

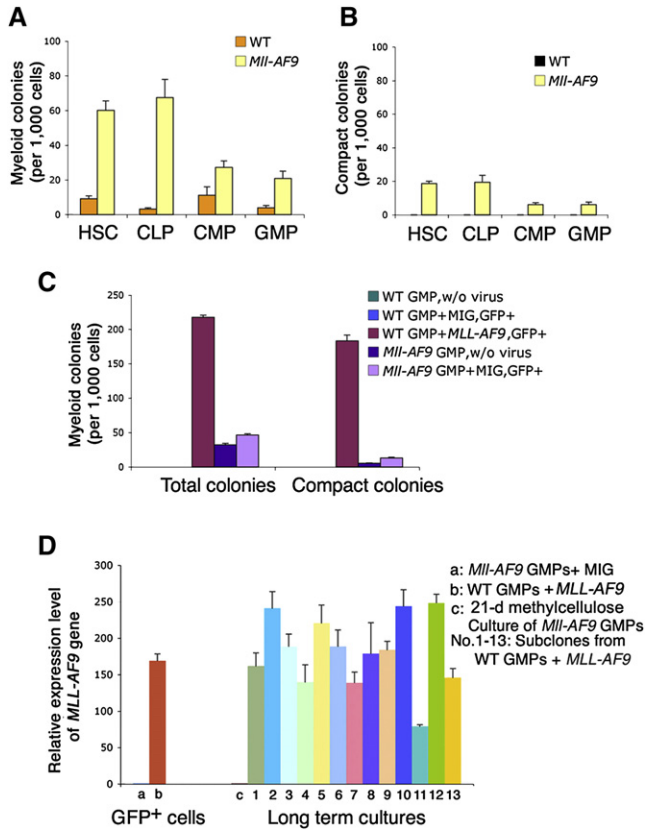

Figure 3. In *Mll-AF9* Knockin Mice, HSCs and CLPs Produce More Total and Compact Myeloid Colonies with Enhanced Self-Renewal In Vitro Than CMPs and GMPs, and *MLL-AF9* Retrovirally Transduced GMPs Produce the Most Total and Compact Myeloid Colonies

(A) Total myeloid colony numbers were higher in HSCs and CLPs than in CMPs and GMPs in *Mll-AF9* mice after 21 day culture under myeloid conditions. All the *Mll-AF9* populations had significantly increased colonies compared to WT.

(B) Compact colony numbers were higher in HSCs/CLPs than in CMPs/GMPs in *Mll-AF9* mice after 21 day culture. No compact colonies were detected in WT mice. Error bars represent standard error of the means.

(C) Total myeloid colony and compact colony numbers were the highest in *MLL-AF9* retrovirally transduced GMP cells compared to the MIG (MSCV-IRES-GFP) vector transduced knockin *Mll-AF9* GMPs and other knockin *Mll-AF9* GMP controls as labeled.

(D) *MLL-AF9* expression in retrovirally transduced GMPs and knockin *Mll-AF9* GMPs. Error bars represent standard error of the means.

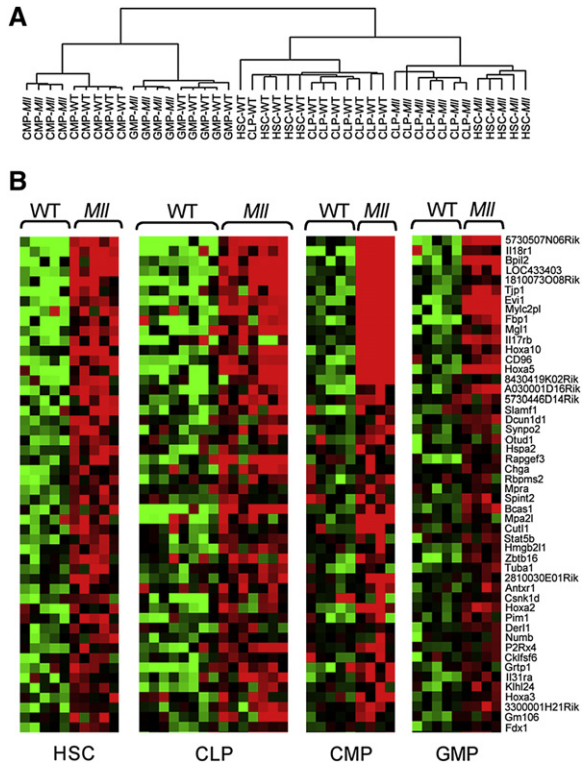

Figure 4. Hierarchical Clustering of *Mll-AF9* HSCs/CLPs and CMP/GMP Populations, and Genes Overexpressed in HSC, CLP, CMP, and GMP Populations

(A) A two-way ANOVA with stratified permutation testing was performed to select genes differentially expressed in *Mll-AF9* compared to wild-type cells in each of the four populations. Hierarchical clustering performed with the 446 genes selected by the two-way ANOVA (FDR < 0.1) separates the HSCs/CLPs from the CMPs/GMPs. WT, wild-type; *Mll*, *Mll-AF9*.

(B) *Mll-AF9* upregulates expression of genes in multiple cell types (FDR < 0.1, two-way ANOVA with permutation testing). Heat-maps show the expression level of the top 50 genes upregulated in the *Mll-AF9* samples compared to WT ranked in decreasing order of fold change upregulation in HSCs. Expression levels are represented by colors: black = median; red > median; green < median. Gene identifiers are at right.

Mll has been shown to be highest in HSCs compared to more mature progenitors (Jude et al., 2007). Since the *Mll-AF9* fusion gene is present in all cells of knockin mice, we studied expression levels of the oncogene in the various hematopoietic cells with the expectation that expression of *Mll-AF9* would parallel that of *Mll*. The microarray results showed that the expression level of *Mll* was higher in WT than *Mll-AF9* cells and was higher in HSCs than GMPs (Figure 6A). Quantitative real-time RT-PCR interrogating the 3′ end of *Mll* showed that *Mll* expression level in HSCs is 4- to 5-fold higher than in GMPs from *Mll-AF9* mice (Figure 6B). Similarly, using a 5′ *Mll* primer/probe set, higher expression was found in HSCs compared to GMPs (data not shown). Quantitative real-time RT-PCR analysis was also performed to evaluate *Mll-AF9* levels in HSCs and GMPs. Results in Figure 6C show 4- to 5-fold higher levels of *Mll-AF9* in HSCs compared to GMPs.

To summarize, the higher expression levels of *Mll-AF9* in HSCs compared to GMPs in the physiologic model suggests the importance of *Mll-AF9* gene dosage in producing downstream effects in the *Hoxa* family and other genes, although other cell context-specific differences are also likely to be important and deserve further research.

DISCUSSION

This study focused on the malignant transformation initiated by the fusion gene *Mll-AF9* when expressed at physiologic levels in the knockin model or at supraphysiologic levels in the retroviral model. The data from the physiologic model showed highest levels of *Mll* and *Mll-AF9* in the most transformable HSCs and lower levels in the more resistant committed myeloid progenitor GMPs. Complementary data showed high *Mll-AF9* gene dosage

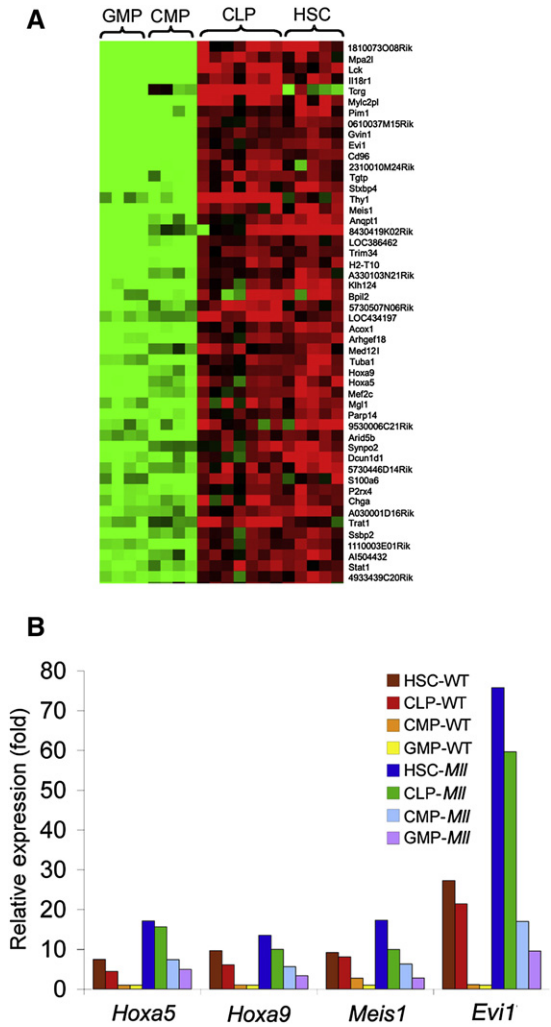

Figure 5. A Set of Genes Are Upregulated by *Mll-AF9* to Highest Levels in HSCs/CLPs Compared to Both to *Mll-AF9* CMPs/GMPs and to Wild-Type HSCs/CLPs

(A) *Mll-AF9* upregulated genes are highly expressed in HSCs and CLPs compared to CMPs and GMPs. Of the 192 genes overexpressed in *Mll-AF9* cells, 96 genes are expressed at higher levels in HSCs and CLPs compared to CMPs and GMPs (FDR < 0.1, SAM). The top 50 genes in this subset are shown. Expression levels are represented by colors: black = median; red > median; green < median.

(B) Expression of *Mll-AF9* upregulated genes *Hoxa5*, *Hoxa9*, *Meis1*, and *Evi1* is highest in *Mll-AF9* HSCs/CLPs. Data represent average expression relative to levels in wild-type GMPs.

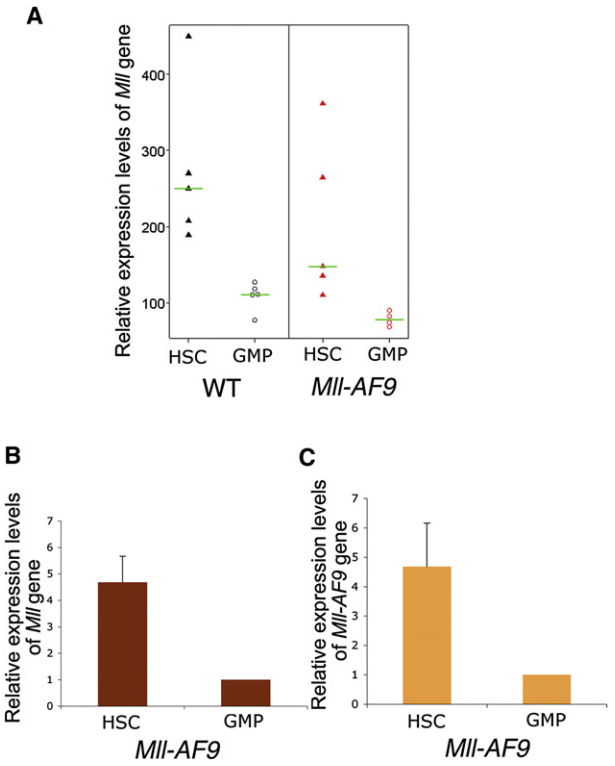

Figure 6. *Mll-AF9* Expression Is Higher in HSC than GMP Populations, and Retrovirally Transduced *MLL-AF9* Results in Very High Expression Levels of the Oncogene

(A) *Mll* expression in the HSCs and GMPs from WT and *Mll-AF9* mice by microarray.

(B) *Mll* expression in the HSCs and GMPs from *Mll-AF9* mice by real-time RT-PCR.

(C) *Mll-AF9* expression in the HSCs and GMPs from *Mll-AF9* mice by real-time RT-PCR.

Error bars represent standard error of the means.

in retrovirally transformed GMPs, which were found to have enhanced self-renewal and ability to grow in long-term in vitro culture. While it is possible that the cell-type differences in transformation are unrelated to the expression levels of *Mll-AF9* at physiologic dosages, we favor the hypothesis that the "superactivation" of target genes observed in HSCs/CLPs (LSKs) is likely to be oncogene dose related. In support of the role of *Mll* fusion gene expression in the different cell types are recent data with conditional knockout mice: Wild-type *Mll* showed highest expression in HSCs compared to other cell types (Jude et al., 2007). Wild-type *MLL* and likely *MLL* fusion proteins bind to the promoters of many genes and serve as global regulators. As a result, a large number of downstream target genes are altered in regulation (Guenther et al., 2005; Milne et al., 2005; Scacheri et al., 2006). These cell-type differences in expression of *Mll* target genes appear to be further enhanced by *Mll* fusion genes, resulting in activation of cellular pathways, especially those that enhance self-renewal and block cellular differentiation. However, it is possible that additional molecular differences could result in increased sensitivity of HSCs to the fusion oncogene is possible. A combination of higher *Mll-AF9* gene dosage and a more receptive cellular environment may be responsible for the superior transformation of LSKs.

Previously published studies with *Mll* fusion genes (*MLL-ENL* or *MLL-AF9*) have utilized retroviruses with strong promoters and multiple virus insertions resulting in noncontrolled and potentially nonphysiologic expression levels of oncogene. Nonphysiologic expression could mask important cell-type-specific effects on the promoters of target cells. One advantage of the *Mll* fusion gene knockin model over retroviral or physical methods is that the oncogene should be expressed at physiologic levels with cell-type specificity. In the current study, the lack of leukemia in lethally irradiated recipients of *Mll-AF9* GMPs contrasts with

the experiments in which the fusion gene is introduced into GMPs by retroviral transduction (Krivtsov et al., 2006). These results are supported by our in vitro data which showed significantly enhanced cell growth in retrovirally transformed *MLL-AF9* cells, but not in physiologically expressed *Mll-AF9* cells. Enhanced self-renewal of retrovirally transformed GMPs was shown in the ability of these cells to grow in long term culture in vitro. In previous results from a *MLL-ENL* model, leukemia developed in animals that received 800–2490 retrovirally transduced GMPs (Cozzio et al., 2003). Also, a shorter latency to leukemia development is found in the retroviral models compared to our knockin model (Cozzio et al., 2003; Krivtsov et al., 2006; Somervaille and Cleary, 2006). We cannot rule out the possibility that the more rapid development of leukemia in retroviral models may in part or totally be due to retroviral enhancement of secondary cooperating events, but our short term myeloid colony data strongly suggest that some differences are immediate and very direct. Also, it is possible that the differences could result from the use of human *MLL* in the retroviral construct compared to the endogenous murine *Mll* in our studies. However, this is unlikely, as to date no differences in critical domains have been described for human and murine *MLL*, and the *AF9* portion of both models is identical. With these caveats, it is likely that the differences between the retroviral *MLL-AF9* and knockin *Mll-AF9* experiments are due to gene dosage effects. This conclusion is also supported by (1) the presence of multiple integration sites in the retrovirally transduced cells shown by Southern blotting and (2) the strong MSCV-based retroviral promoter in this study and others (Krivtsov et al., 2006; Somervaille and Cleary, 2006).

Our results showing that knockin *Mll-AF9* HSCs and CLPs, representing the relatively undifferentiated LSK ($Lin^-c\text{-}kit^+Sca\text{-}1^+$) hematopoietic cells, are most efficiently transformed and are similar to those reported for retrovirally introduced *MLL-ENL* (Cozzio et al., 2003). Also, similar to the retroviral *MLL-ENL* model and *MLL-AF9* model (Somervaille and Cleary, 2006), the bulk of cells of all the leukemias were relatively mature myeloid $CD11b^+Gr1^+$ in type, irrespective of the phenotype of the transplanted transformed cells. However, we did not determine the nature of the leukemia stem cells (LSCs) that initiate and maintain the leukemia in the animal. The long latency for development of the leukemias in animals suggests that there are important genetic and/or epigenetic events occurring during this latency period. These later events could also be important in determining the phenotype of the LSCs. The results presented have implications for therapy of both the early and later stages of leukemia.

In our knockin model, 192 genes were found to be upregulated by *Mll-AF9* in all four cell populations. Several, including *Hoxa3*, *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10*, and *Meis1*, were previously found to be highly "immediately" expressed in GMP cells transformed by the *MLL-AF9* retrovirus (Krivtsov et al., 2006). Also, as discussed in the Results, we found 20 genes in common in our knockin preleukemia data set and the leukemia data set described earlier (Krivtsov et al., 2006). Another report showed that *MLL-AF9* introduced by retrovirus resulted in upregulation of several critical genes when leukemia stem cells (LSCs) were compared to the transformed preleukemic "initiating" cells (Somervaille and Cleary, 2006). That study did not compare gene signatures in wild-type compared to "initiating" cells.

We found very high expression of *Evi1* in *Mll-AF9* cells compared to the corresponding wild-type cells. High levels of expression of *Evi1*, have been reported in human myeloid leukemias with *MLL*-rearrangements (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Valk et al., 2004). Also, *Evi1* overexpression is sufficient to immortalize murine hematopoietic cells (Du et al., 2005), which suggest that this gene should be studied further for its role in the pathogenesis of *MLL*-fusion leukemias.

The knockin murine *Mll-AF9* model is useful because the fusion gene is present and expressed in all progenitor and stem cells. The situation in humans is less clear, since the cell in which the human *MLL-AF9*-producing translocation develops is not defined. However, the human *MLL-AF9* gene will be present both in the cells with the initial "hit" plus all progenitor cells and cells at later stages of differentiation. While it is possible that the transforming human *MLL-AF9* translocation may take place at a maturation stage later than the HSC, murine studies suggest that this is much less likely to be functionally meaningful than a "hit" within the HSC population. Future studies will be necessary to further define this issue.

In conclusion, our results directly show that supraphysiologic oncogene doses of *Mll-AF9* produced biologically different effects from physiologic doses in the same cell type. We also show an association between oncogene dosage and cell type-specific transformation susceptibility; however, the oncogene dosage differences are less in the physiologic model compared to the retroviral model. While we favor the hypothesis that both *Mll-AF9* expression differences between cells types and other cell context differences are pathophysiologically important, direct evidence will need to be provided in future studies. Seminal earlier studies with *myc* and other oncogenes have shown that gene dosage effects are central to the pathophysiology of cancers that develop under natural conditions (Ren, 2004). Experimental studies that introduce oncogenes by viruses and other physical methods have been extremely important in cancer biology research. However, to the extent that they result in nonphysiologic oncogene expression levels, experimental results may differ from those in naturally occurring cancers.

EXPERIMENTAL PROCEDURES

Mice

The *Mll-AF9* mice were originally produced in the laboratory of Dr. Terence Rabbitts (Leeds, UK). Briefly, heterozygous mice were produced by fusing the human *AF9* short form (breakpoint to 3′ end) into exon 8 of the mouse *Mll* gene (Corral et al., 1996) and have been maintained on a C57BL/6 background. The wild-type mice used in the experiments were the littermates of *Mll-AF9* mice. All the mice were housed under specific pathogen-free conditions in an accredited facility at the University of Minnesota. All experiments were conducted after approval by the Institutional Animal Care and Use Committee (IACUC).

Cell Sorting and FACS Analysis

Single cell suspensions of bone marrow were obtained from 8-week-old WT or *Mll-AF9* mice. The purification of HSC population ($Lin^-Thy1.1^{lo}Sca\text{-}1^+c\text{-}kit^+$) was similar to the method described before (Kondo et al., 1997; Terskikh et al., 2003). Briefly, bone marrow cells were stained with biotin-conjugated lineage specific anti-IL-7R (PharMingen, San Diego, CA) and cocktail antibodies from the Lineage Cell Depletion Kit (Miltenyi, Bergisch Gladback, Germany) according to manufacturer's instructions. Lin^+ cells were partially removed by magnetic beads (MACS, Miltenyi, Bergisch Gladback, Germany). The remaining cells were stained with Streptavidin-PE-Cy5 conjugate and further

stained with APC-conjugated anti-c-kit, FITC-conjugated anti-Sca-1, and PE-conjugated anti-Thy1.1 antibodies (PharMingen, San Diego, CA). The HSC population was sorted by FACSAria (BD Biosciences Immunocytometry Systems, San Jose, CA).

The CLPs were sorted as Lin$^-$IL-7R$^+$Thy1.1$^-$Sca-1 ^{lo}c-kit ^{lo} (Kondo et al., 1997; So et al., 2003) using a similar method. CMPs (Lin$^-$IL-7R$^-$Sca-1$^-$c-kit$^+$CD34$^+$FcγRII/III^{lo}) and GMPs (Lin$^-$IL-7R$^-$Sca-1$^-$c-kit$^+$CD34$^+$FcγRII/III^{hi}) were separated as described previously (Manz et al., 2001; Terskikh et al., 2003). The purity of sorted cell populations was >95% by postsort analysis.

Relative percentage of HSCs, CLPs, CMPs, and GMPs from lineage negative marrow cells in wild-type and *Mll-AF9* marrow (Figure 1a, 1b) were calculated as follows: % HSCs is the percentage of Sca1$^+$c-kit$^+$Thy1^{lo} cells in Lin$^-$IL-7R$^-$ population; % CLPs is the percentage of Sca1$^+$c-kit $^+$ cells in Lin$^-$Thy1$^-$IL-7R$^+$ population; % CMPs is the percentage of CD34$^+$ FcγRII/III^{lo} cells in Lin$^-$Sca1$^-$c-kit $^+$ population; and % GMPs is the percentage of CD34$^+$ FcγRII/III^{hi} cells in Lin$^-$Sca1$^-$c-kit $^+$ population. Statistical comparisons were performed using the two-tailed t test.

For FACS analysis, single cell suspensions from either cultured cells or mouse hematopoietic organs (bone marrow or spleen) were stained with FITC or PE-labeled anti-mouse antibodies, CD11b and Gr1 (PharMingen or eBioscience, San Diego, CA), and acquired on a BD FACScalibur with Cell Quest software. Data were analyzed with FloJo software (Tree Star Inc., San Carlos, CA).

Methylcellulose Culture

Sorted cells were cultured in methylcellulose medium under myeloid conditions, using methocult 3534 (StemCell Technologies, Vancouver, Canada) and supplemented with 10 ng/ml GM-CSF (R&D, Minneapolis, MN) (Chen et al., 2006). Cells were cultured in triplicate for 21 days with transfers every 7 days. Colonies containing over 50 cells were counted and classified under the microscope as previously described (Jordan et al., 2006).

Mouse Transplantation with Sorted Cell Populations

Each sorted population was transplanted into mice at various doses: 25 and 100 sorted HSCs; 100, 250, and 2500 CLPs; and 250 and 2500 CMPs or GMPs from *Mll-AF9* were mixed with a radioprotective dose of 2.5×10^5 bone marrow cells from WT mice and injected into lethally irradiated (960 rad) recipients. Each group contained at least five mice. Five recipient mice injected with 2.5×10^5 WT bone marrow cells were used as negative controls. Mice were sacrificed when they became detectably ill. Necropsy, FACS, immunohistochemistry, and histopathology evaluations were performed at the time of sacrifice. The survival rate was calculated using the Kaplan-Meier method. The frequency of transformable hematopoietic cells was calculated by limiting dilution analyses using L-calc software (StemCell Technologies).

Retrovirus Transduction

Retrovirus constructs MSCV-MLL-AF9-GFP, MSCV-GFP, and package plasmid psi-Eco were used to produce retrovirus supernatant by cotransfection of 293T cells. Transduction of WT or *Mll-AF9* GMPs was performed as previously described (Krivtsov et al., 2006). After transduction, GFP-positive cells were sorted and put in methylcellulose culture for colony assays. RNA from these cells was extracted to detect *MLL-AF9* expression by quantitative RT-PCR. DNA was purified and digested by EcoRI for Southern blotting.

Gene Expression Studies

For quantitative real-time RT-PCR, reverse transcription was performed using the Supercript II reverse transcription kit (Invitrogen) and real-time PCR detection was performed using TaqMan primer/probe sets (Applied Biosystems Inc., Foster City, CA) and an ABI 7500 Real-Time PCR system. For *MLL-AF9*, real-time PCR detection was performed using SYBR Green. In all RT-PCR experiments, *Gapdh* was used as the housekeeping gene. Changes in expression calculations were performed by the $2^{\Delta\Delta CT}$ method using the Relative Expression Software Tool (REST, www.gene-quantification.info).

Microarray Analysis

For gene expression profiling, total RNA was extracted from sorted cells and amplified (Affymetrix). Labeled cRNA was hybridized to Mouse 430 2.0 genomic arrays. Normalization and analysis of chip data were performed using the Expressionist package (GeneData Inc., Supplemental Experimental Procedures). Heat maps were generated using Cluster and Treeview http://rana.lbl.gov/EisenSoftware.htm. See the Supplemental Data for detailed microarray analysis.

ACCESSION NUMBERS

All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE10627.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and three supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/5/432/DC1/.

ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (RO1-CA087053 to J.H.K., and K08-CA122191 to A.R.K.), the Children's Oncology Group and Minnesota Medical Foundation (A.R.K.), and by the Children's Cancer Research Fund (J.H.K. and A.R.K.). The authors thank Scott A. Armstrong and Andrei V. Krivtsov (Children's Hospital/Harvard Medical School) for providing the reagents and protocols for the retroviral studies, discussions of the microarray studies, and reading of the manuscript. Terry Rabbitts (Leeds, UK) originally produced the *Mll-AF9* knockin mouse. The University of Minnesota Cancer Center Biostatistical Core (Robin Bliss and Yan Zhang), Flow Cytometry Core (Tucker LeBien), the Histopathology Core (Dr. Gerald O'Sullivan), and the Biomedical Genomics Facility were critical to this project.

Received: January 22, 2007
Revised: December 28, 2007
Accepted: March 7, 2008
Published: May 5, 2008

REFERENCES

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *6774*, 193–197.

Barjesteh van Waalwijk van Doorn-Khosrovani, S., Erpelinck, C., Lowenberg, B., and Delwel, R. (2003). Low expression of MDS1–EVI1-like-1 (MEL1) and EVI1-like-1 (EL1) genes in favorable-risk acute myeloid leukemia. Exp. Hematol. *11*, 1066–1072.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. A Stat. Soc. *57*, 289–300.

Caslini, C., Serna, A., Rossi, V., Introna, M., and Biondi, A. (2004). Modulation of cell cycle by graded expression of MLL-AF4 fusion oncoprotein. Leukemia *6*, 1064–1071.

Chapiro, E., Russell, L., Radford-Weiss, I., Bastard, C., Lessard, M., Struski, S., Cave, H., Fert-Ferrer, S., Barin, C., Maarek, O., et al. (2006). Overexpression of CEBPA resulting from the translocation t(14;19)(q32;q13) of human precursor B acute lymphoblastic leukemia. Blood *10*, 3560–3563.

Chen, W., Li, Q., Hudson, W.A., Kumar, A., Kirchhof, N., and Kersey, J.H. (2006). A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. Blood *2*, 669–677.

Corral, J., Lavenir, I., Impey, H., Warren, A.J., Forster, A., Larson, T.A., Bell, S., McKenzie, A.N., King, G., and Rabbitts, T.H. (1996). An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: A method to create fusion oncogenes. Cell *6*, 853–861.

Cozzio, A., Passegue, E., Ayton, P.M., Karsunky, H., Cleary, M.L., and Weissman, I.L. (2003). Similar MLL-associated leukemias arising from

self-renewing stem cells and short-lived myeloid progenitors. Genes Dev. *24*, 3029–3035.

Dobson, C.L., Warren, A.J., Pannell, R., Forster, A., Lavenir, I., Corral, J., Smith, A.J., and Rabbitts, T.H. (1999). The mll-AF9 gene fusion in mice controls myeloproliferation and specifies acute myeloid leukaemogenesis. EMBO J. *13*, 3564–3574.

Du, Y., Jenkins, N.A., and Copeland, N.G. (2005). Insertional mutagenesis identifies genes that promote the immortalization of primary bone marrow progenitor cells. Blood *12*, 3932–3939.

Guenther, M.G., Jenner, R.G., Chevalier, B., Nakamura, T., Croce, C.M., Canaani, E., and Young, R.A. (2005). Global and Hox-specific roles for the MLL1 methyltransferase. Proc. Natl. Acad. Sci. USA *24*, 8603–8608.

Iida, S., Seto, M., Yamamoto, K., Komatsu, H., Tojo, A., Asano, S., Kamada, N., Ariyoshi, Y., Takahashi, T., and Ueda, R. (1993). MLLT3 gene on 9p22 involved in t(9;11) leukemia encodes a serine/proline rich protein homologous to MLLT1 on 19p13. Oncogene *11*, 3085–3092.

Ikuta, K., and Weissman, I.L. (1992). Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc. Natl. Acad. Sci. USA *4*, 1502–1506.

Johnson, J.J., Chen, W., Hudson, W., Yao, Q., Taylor, M., Rabbitts, T.H., and Kersey, J.H. (2003). Prenatal and postnatal myeloid cells demonstrate stepwise progression in the pathogenesis of MLL fusion gene leukemia. Blood *8*, 3229–3235.

Jordan, C.T., Guzman, M.L., and Noble, M. (2006). Cancer stem cells. N. Engl. J. Med. *12*, 1253–1261.

Jude, C.D., Climer, L., Xu, D., Artinger, E., Fisher, J.K., and Ernst, P. (2007). Unique and Independent Roles for MLL in Adult Hematopoietic Stem Cells and Progenitors. Cell Stem Cell *1*, 324–337.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *5*, 661–672.

Krivtsov, A.V., and Armstrong, S.A. (2007). MLL translocations, histone modifications and leukaemia stem-cell development. Nat. Rev. Cancer *11*, 823–833.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature *7104*, 818–822.

Kumar, A.R., Hudson, W.A., Chen, W., Nishiuchi, R., Yao, Q., and Kersey, J.H. (2004). Hoxa9 influences the phenotype but not the incidence of Mll-AF9 fusion gene leukemia. Blood *5*, 1823–1828.

Manz, M.G., Traver, D., Miyamoto, T., Weissman, I.L., and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood *11*, 3333–3341.

McMahon, K.A., Hiew, S.Y., Hadjur, S., Veiga-Fernandes, H., Menzel, U., Price, A.J., Kioussis, D., Williams, O., and Brady, H.J. (2007). Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell *1*, 338–345.

Milne, T.A., Martin, M.E., Brock, H.W., Slany, R.K., and Hess, J.L. (2005). Leukemogenic MLL fusion proteins bind across a broad region of the Hox a9 locus, promoting transcription and multiple histone modifications. Cancer Res. *24*, 11367–11374.

Ren, R. (2004). Modeling the dosage effect of oncogenes in leukemogenesis. Curr. Opin. Hematol. *1*, 25–34.

Scacheri, P.C., Davis, S., Odom, D.T., Crawford, G.E., Perkins, S., Halawi, M.J., Agarwal, S.K., Marx, S.J., Spiegel, A.M., Meltzer, P.S., and Collins, F.S. (2006). Genome-wide analysis of menin binding provides insights into MEN1 tumorigenesis. PLoS Genet. *4*, e51. 10.1371/journal.pgen.0020051.

So, C.W., Karsunky, H., Passegue, E., Cozzio, A., Weissman, I.L., and Cleary, M.L. (2003). MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. Cancer Cell *2*, 161–171.

Somervaille, T.C., and Cleary, M.L. (2006). Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. Cancer Cell *4*, 257–268.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *4861*, 58–62.

Terskikh, A.V., Miyamoto, T., Chang, C., Diatchenko, L., and Weissman, I.L. (2003). Gene expression analysis of purified hematopoietic stem cells and committed progenitors. Blood *1*, 94–101.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA *9*, 5116–5121.

Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B., and Delwel, R. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. N. Engl. J. Med. *16*, 1617–1628.