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

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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−/C0−/Sca1+c-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; lida 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-kit+Sca-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- c-kit+Sca-1+, including hematopoietic stem cell HSC (Kondo et al., 1997; Ikuta and Weissman, 1992; Spangrud 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-kit+Sca1+ cells in both the HSC and CLP populations (Figure 1A). We also saw an increased number of FcγRII/IIIb (CMP) but not FcγRII/IIIa (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).

**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, retroviral 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
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 MCV-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...
that expression of MLL-AF9 will be higher in retrovirally transduced GMPs than in MII-AF9 knockin GMPs. Using primers that detected a sequence present in both retroviral MLL-AF9 and knockin MII-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 MII-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 MII-AF9 Expressed at Physiologic Levels
A goal of our study was to define the molecular pathways that would explain the differences between MII-AF9 HSCs and GMPs. We compared the early (preleukemic) in vivo effects of the MII-AF9 fusion gene on gene expression levels in the cells from MII-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 MII-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 MII-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. MII-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 MII-AF9 cell types compared to wild-type, while 179 genes displayed lower expression in the MII-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 MII-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 MII-AF9 LSK overexpressed genes are shown in the heat map of MII-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 MII and MII-fusion proteins—Hoxa5, Hoxa9, and Meis1. Also included is Evi1, not currently known to be a direct target of MII or MII 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, Hox9, Meis1, and Evi1 in each population of cells with results shown in Figure 5B. Importantly, these four genes were most highly expressed in MII-AF9 LSKs, expressed at intermediate levels in wild-type LSKs and MII-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 MII-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 MII-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 MII and MII-AF9 Is Higher in HSCs Than in GMPs
We next evaluated the hypothesis that the upregulated expression of genes known to be downstream of MII, such as Hoxa genes in HSCs, could be a result of higher expression levels of the MII-AF9 fusion gene in HSCs. The expression of wild-type

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### Table 1. Summary Data of Transplantation Experiments

<table>
<thead>
<tr>
<th>Starting MII-AF9 Population</th>
<th>No. of Cells Transplanted</th>
<th>No. of Animals Transplanted</th>
<th>No. of Animals with AML (%)</th>
<th>Latency of AML (Median and 95% CI in Days)</th>
<th>Frequency of Transformable Hematopoietic Cells (THCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC</td>
<td>25</td>
<td>5</td>
<td>2 (40%)</td>
<td>-&lt;sup&gt;4&lt;/sup&gt; (167, -)</td>
<td>1:45</td>
</tr>
<tr>
<td>CLP</td>
<td>100</td>
<td>10</td>
<td>9 (90%)</td>
<td>165 (152, 197)</td>
<td></td>
</tr>
<tr>
<td>CMP</td>
<td>250</td>
<td>5</td>
<td>4 (80%)</td>
<td>198 (134, NA)</td>
<td>1:57</td>
</tr>
<tr>
<td>GMP</td>
<td>250</td>
<td>5</td>
<td>5 (100%)</td>
<td>136 (119, 179)</td>
<td></td>
</tr>
</tbody>
</table>

Five negative control mice injected with 2.5 x 10<sup>5</sup> WT bone marrow cells were all alive for the duration of the experiment.

<sup>4</sup> Inestimable.
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. 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...
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 \textit{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 \textit{Mll} fusion gene expression in the different cell types are recent data with conditional knockout mice: Wild-type \textit{Mll} showed highest expression in HSCs compared to other cell types (Jude et al., 2007). Wild-type \textit{MLL} and likely \textit{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 \textit{Mll} target genes appear to be further enhanced by \textit{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 \textit{Mll-AF9} gene dosage and a more receptive cellular environment may be responsible for the superior transformation of LSKs.

Previously published studies with \textit{Mll} fusion genes (\textit{MLL-ENL} or \textit{MLL-AF9}) have utilized retroviruses with strong promoters and multiple virus insertions resulting in noncontrolled and potentially nonphysiologic expression levels of oncogene. Non-physiologic expression could mask important cell-type-specific effects on the promoters of target cells. One advantage of the \textit{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 \textit{Mll-AF9} GMPs contrasts with

![Figure 5. A Set of Genes Are Upregulated by \textit{Mll-AF9} to Highest Levels in HSCs/CLPs Compared to Both to \textit{Mll-AF9} CMPs/GMPs and to Wild-Type HSCs/CLPs](image)

(A) \textit{Mll-AF9} upregulated genes are highly expressed in HSCs and CLPs compared to CMPs and GMPs. Of the 192 genes overexpressed in \textit{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 \textit{Mll-AF9} upregulated genes Hoxa5, Hoxa9, Meis1, and Evi1 is highest in \textit{Mll-AF9} HSCs/CLPs. Data represent average expression relative to levels in wild-type GMPs.

![Figure 6. \textit{Mll-AF9} Expression Is Higher in HSC than GMP Populations, and Retrovirally Transduced \textit{MLL-AF9} Results in Very High Expression Levels of the Oncogene](image)

(A) \textit{Mll} expression in the HSCs and GMPs from WT and \textit{Mll-AF9} mice by microarray.

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

(C) \textit{Mll-AF9} expression in the HSCs and GMPs from \textit{Mll-AF9} mice by real-time RT-PCR. Error bars represent standard error of the means.
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-kit+sca-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 (Barghest van WaaLwik 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 Rabbits (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−c-kit+sca-1−) 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 Gladbach, Germany) according to manufacturer’s instructions. Lin− cells were partially removed by magnetic beads (MACS, Miltenyi, Bergisch Gladbach, 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-Thy-1.1 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 Sca1+ c-kit+ (Kondo et al., 1997; So et al., 2003) using a similar method. CMPs (Lin-IL-7R Sca-1- c-kit-CD34+FcγRil/IIl) and GMPs (Lin-IL-7R Sca-1- c-kit-CD34+FcγRil/IlII) were separated as described previously (Mantz 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+h 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/Ill cells in Lin-Sca1 -c-kit- population; and % GMPs is the percentage of CD34+ FcγRil/Ill 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 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 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+h 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+ FcγRil/Ill cells in Lin-Sca1 -c-kit- population; and % GMPs is the percentage of CD34+ FcγRil/Ill 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).

Gene Expression Studies

For quantitative real-time RT-PCR, reverse transcription was performed using the SuperScript 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, using methocult 3534 (StemCell Technologies). Methylcellulose Culture

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 geno- mic 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.

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