MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors

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Summary

To better understand the origin of leukemic stem cells, we tested the hypothesis that all leukemia oncogenes could transform committed myeloid progenitor cells lacking the capacity for self-renewal, as has recently been reported for *MLL-ENL*. Flow-sorted populations of common myeloid progenitors and granulocyte-monocyte progenitors were transduced with the oncogenes *MOZ-TIF2* and *BCR-ABL*, respectively. *MOZ-TIF2*-transduced progenitors could be serially replated in methylcellulose cultures and continuously propagated in liquid culture, and resulted in an acute myeloid leukemia in vivo that could be serially transplanted. In contrast, *BCR-ABL* transduction conferred none of these properties to hematopoietic progenitors. These data demonstrate that some, but not all, leukemia oncogenes can confer properties of leukemic stem cells to hematopoietic progenitors destined to undergo apoptotic cell death.

Introduction

Limitless self-renewal potential is one of the hallmarks of all cancers (Hanahan and Weinberg, 2000). It had been thought that all cells of a tumor contained such potential. However, recent evidence from human malignancies, including acute myeloid leukemia (AML) (Bonnet and Dick, 1997), acute lymphoblastic leukemia (ALL) (Cobaleda et al., 2000), and breast (Al-Hajj et al., 2003) and CNS (Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003) cancer has demonstrated the existence of subpopulations of cells, delineated by differential expression of surface markers, that exclusively contain the ability to recapitulate the human disease upon transplantation into recipient NOD-SCID (non-obese diabetic/severe combined immunodeficient) mice. The tumors that develop are phenotypically very similar to the original human tumor, and serial transplantation of the tumor to secondary recipients confirms the true stem cell nature of these subpopulations of malignant cells.

It has been suggested that the target cells for malignant transformation are adult somatic stem cells (Reya et al., 2001), and that oncogenic mutations appropriate the capacity for self-renewal inherent in these cells. Stem cells persist throughout adult life, undergo an increased number of cell divisions, and therefore may have a greater opportunity than more short-lived cells to acquire the minimum number of mutations required for malignant transformation (Bradford et al., 1997; Chesier et al., 1999; Lansdorp, 1997). Phenotypic evidence also implicates somatic stem cells as the target cells for transformation. Both stem cells and tumor cells express similar levels of telomerase (Morrison et al., 1996) required to maintain telomere length and prevent replicative senescence. In human AML, the distinctive leukemia "stem cell" that is sufficient to generate AML in the NOD-SCID mouse model (the SCID leukemia initiating cell, SL-IC) has a similar phenotype (CD 34+/CD 38−) to that of normal SCID-repopulating cells (Lapidot et al., 1994) and to the cell compartment enriched for true human hematopoietic stem cells (HSC) (Manz et al., 2002). Furthermore, recurrent cytogenetic abnormalities associated with leukemia have also been detected in this cell compartment in patients with AML, chronic myeloid leukemia (CML), and ALL (Deininger et al., 2000; Haase et al., 1995; Mehrotra et al., 1995; Quijano et al., 1997). Indeed, the Philadelphia chromosome, the cytogenetic hallmark of CML, can be detected in cells of the myeloid, erythroid, megakaryocytic, and B-lymphoid lineages, suggesting transformation of a

SIGNIFICANCE

The existence of cancer stem cells has been demonstrated in leukemias and solid-organ cancers. It has been suggested that they result from transformation of adult somatic stem cells with inherent self-renewal capacity. We report that some leukemia-associated oncogenes, but not others, can confer properties of leukemic stem cells to committed murine myeloid progenitors, and exclude retroviral insertional events as the sole explanation for these findings. These data provide tools for the identification of cellular programs that confer self-renewal properties to somatic cells previously destined to undergo terminal differentiation and apoptotic cell death. Characterization of these programs may identify new targets for therapeutic intervention in leukemia, and genes that confer properties of self-renewal to adult somatic cells for regenerative therapeutic purposes.

cell with multilineage differentiation potential such as an HSC (Deininger et al., 2000; Martin et al., 1980).

Alternatively, it has been suggested that committed hematopoietic progenitors without self-renewal capacity may also be potential targets for transformation, and that this could explain phenotypic differences between leukemias associated with the same characteristic molecular abnormality (Fialkow et al., 1981; Griffin and Lowenberg, 1986). However, committed progenitors have engaged programs for terminal differentiation and apoptotic cell death, and do not have the properties of leukemic stem cells. It has been recently reported that retroviral transduction with *MLL-ENL* confers properties of self-renewal to committed myeloid progenitors and demonstrates induction of AML following their transplantation into irradiated mice (Cozzio et al., 2003). However, although progenitors transduced with transformation-disabled mutants of *MLL-ENL* did not engender this phenotype, these findings did not address whether this is a capability conferred by all leukemia-associated oncogenes, either alone or in concert with possible retroviral integration effects.

In this study, we tested the hypothesis that all leukemia-associated oncogenes have the capacity to confer self-renewal properties of leukemic stem cells to hematopoietic progenitors that inherently lack the ability to self-renew. We used multiparameter flow cytometry to isolate purified populations of committed myeloid progenitors that lack self-renewal capacity (Akashi et al., 1999; Na Nakorn et al., 2002). These populations were then transduced with either the *MOZ-TIF2* (Carapeti et al., 1988; Deguchi et al., 2003) or *BCR-ABL* oncogenes (Deininger et al., 2000; Sattler and Griffin, 2003; Wong and Witte, 2001), as representative members of the transcription factor/cofactor or tyrosine kinase fusion oncogene families, respectively, and their self-renewal and transformation properties were analyzed with in vitro and in vivo systems and compared with similarly transduced HSC and whole bone marrow. The contributions of the initial cell of transduction to the eventual phenotype of the disease were also assessed utilizing the same experimental design.

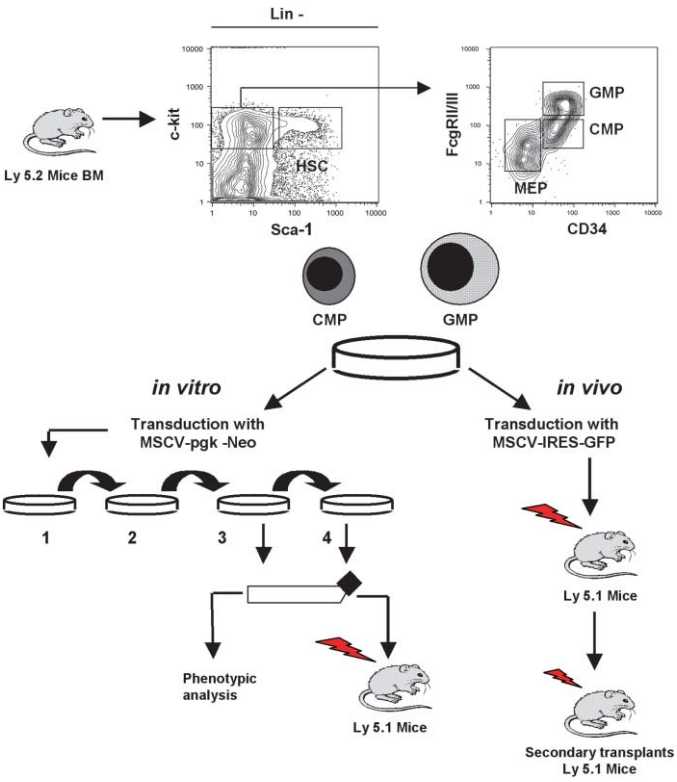

Figure 1. Schema of in vitro and in vivo experiments used to test the ability of *MOZ-TIF2* and *BCR-ABL* to confer properties of self-renewal to committed myeloid progenitors

In brief, flow-sorted CMP and GMP from Ly5.2 mice were transduced with retroviral vectors expressing the MOZ-TIF2 or BCR-ABL oncogenes along with either the neomycin resistance gene or GFP. For the in vitro studies, serial replating assays were performed following the initial selection of neomycin-resistant transduced cells in the first methylcellulose plate. Colonies which formed in the 3rd or 4th plates were subsequently tested for the ability to grow in IL-3-supplemented liquid culture, and these cell lines were subsequently transplanted into irradiated mice. In the in vivo studies, CMP and GMP transduced with vectors expressing MOZ-TIF2 or BCR-ABL and GFP were directly injected into lethally irradiated congenic Ly5.1 mice and assessed for the development of leukemia. Where leukemia developed in primary recipients, the long-term self-renewal of the initially transduced progenitors was further tested in serial transplant of leukemic cells to sublethally irradiated secondary recipients.

Results

MOZ-TIF2, but not *BCR-ABL*, confers properties of self-renewal to committed myeloid progenitors in vitro

Unmanipulated murine bone marrow has a finite ability to serially replate in methylcellulose cultures supplemented with cytokines (Lavau et al., 1997). Transduction of whole bone marrow with certain leukemia oncogenes confers the ability to serially replate in vitro, although the target population(s) of cells that acquire the ability to serially replate is unknown (Lavau et al., 1997). The ability of CMP and GMP transduced with *MOZ-TIF2* or *BCR-ABL* to serially replate was tested as shown in the schema in Figure 1. CMP or GMP were transduced with MSCV-pgk-Neo retroviral constructs and selected for G418-resistant transduced cells in the first plating. In control experiments, untransduced CMP and GMP did not serially replate (Figure 2A). We also observed that CMP or GMP transduced with *BCR-ABL* did not serially replate, indicating that BCR-ABL lacks the ability to alter the programs for terminal differentiation and apoptosis in committed myeloid progenitors. However, in striking contrast, CMP or GMP transduced with *MOZ-TIF2* did serially replate (Figure 2A). After 4 replatings, the *MOZ-TIF2*-transduced colonies were small and tightly packed, with morphology consistent with small CFU-blast (Figure 2C). Cytospins of single colonies derived from either CMP or GMP demonstrated primitive myelomonocytic morphology and hemophagocytosis, features evident in the human disease associated with *MOZ* rearrangements (Borrow et al., 1996; Carapeti et al., 1988) (Figure 2D), as well as in murine models of leukemia induced by *MOZ-TIF2* (Deguchi et al., 2003).

Mutational analysis of *MOZ-TIF2* demonstrates the same domain requirement for self-renewal as for leukemic transformation

Leukemia induction by *MOZ-TIF2* has been shown to minimally require the nucleosome binding motif of *MOZ* (abrogated in the C543G mutant) and the *CBP* interaction domain of *TIF2* (abrogated by the LXXLL mutant), but histone acetyltransferase activity of *MOZ* is not required (Deguchi et al., 2003). A proposed mechanism of transformation by *MOZ-TIF2*, therefore, involves

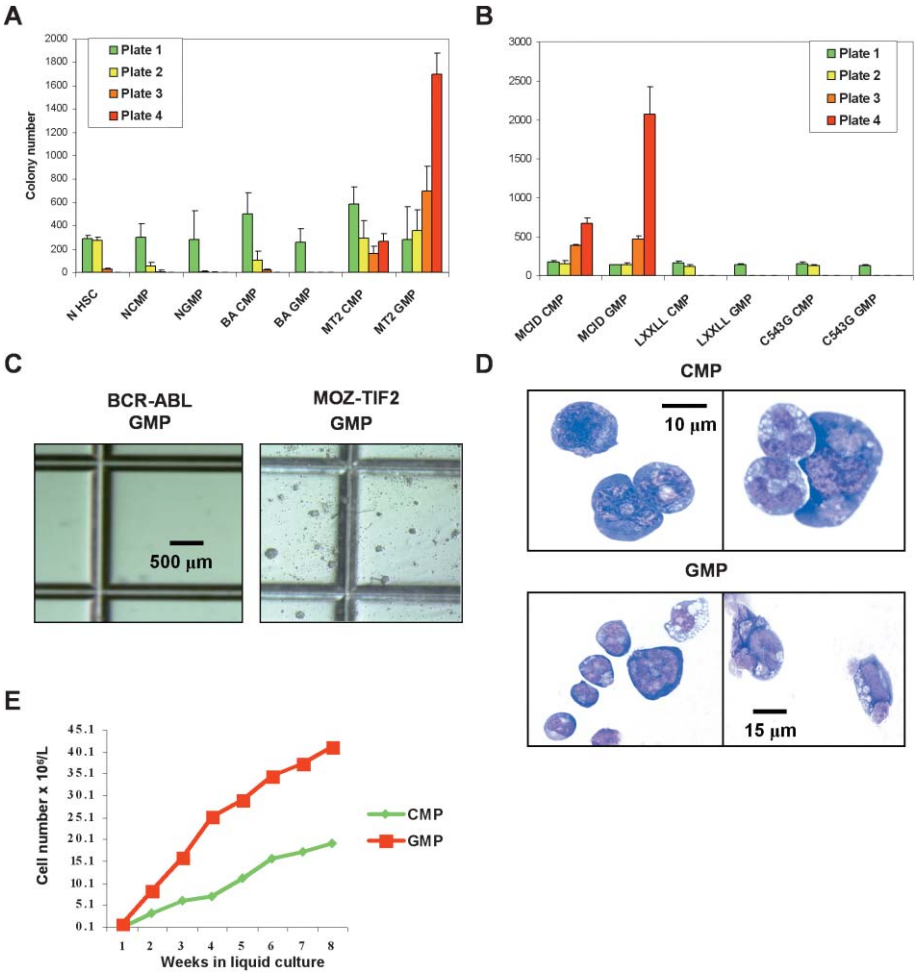

Figure 2. In vitro analysis demonstrates that *MOZ-TIF2*, but not *BCR-ABL*, can confer properties of self-renewal to committed myeloid progenitors

A: Bar chart of serial replating experiments. In contrast to untransduced progenitors (normal HSC, NHSC, NCMP, and NGMP) and progenitors transduced with BCR-ABL, CMP and GMP transduced with MOZ-TIF2 demonstrated replating potential to the 4th plating.

B: Serial replating experiments with *MOZ-TIF2* mutants, demonstrating that the nucleosomal binding site (mutated in C543G mutant) and *CBP* interaction domain (mutated in LXXLL mutant) necessary for transformation are also required to confer serial replating ability. Results presented are the mean (± SD) of 2 (untransduced HSC, CMP, GMP, and *MOZ-TIF2* mutants) or 3 experiments (*BCR-ABL*- and *MOZ-TIF2*-transduced CMP and GMP) performed in duplicate. The MCID mutant contains both the nucleosomal binding site and *CBP* interaction domain.

C: Representative photomicrographs of 4th round platings for *BCR-ABL*- and *MOZ-TIF*-transduced GMP. While no colonies were seen on the *BCR-ABL* plate, numerous small tightly clustered CFU-blast could be seen in the *MOZ-TIF2* plate.

D: Morphology of cells derived from colonies of the 4th round of plating for *MOZ-TIF2*-transduced CMP and GMP. Primitive myelomonocytic cells were demonstrated with prominent hemophagocytosis (right panels), features of human *MOZ-TIF2*-associated AML.

E: Line graph demonstrating the sustained growth, over 8 weeks, of *MOZ-TIF2*-transduced CMP and GMP in IL-3-supplemented liquid culture.

aberrant recruitment of *CBP* to *MOZ* nucleosome binding sites (Deguchi et al., 2003). To test the requirements for self-renewal in committed progenitors, CMP and GMP were transduced with either the C543G and LXXLL mutants, or the *MOZ-CID* (*MOZ-CBP* interacting domain) mutant containing the minimal domain requirement for transformation, and were serially replated in methylcellulose as above. The results (Figure 2B) show serial replating only for the *MOZ-CID*, demonstrating that the domain requirements for self-renewal are the same as for leukemic transformation, and link self-renewal to the aberrant transcriptional program initiated by *MOZ-TIF2* that contributes to leukemogenesis.

MOZ-TIF2-transduced committed progenitors can be propagated in liquid culture, and these cell lines can induce AML in mice

MOZ-TIF2-transduced CMP and GMP derived from the third and fourth rounds of replating were amalgamated and could be subsequently propagated in liquid culture containing IL-3 (Figure 2E), demonstrating sustained growth for at least 8 weeks. Morphological analysis of these cells at subsequent time points demonstrated a similar phenotype to the initiating colonies (data not shown). Flow cytometric analysis of the cell lines demonstrated a similar surface immunophenotype whether they were derived from either CMP or GMP, which was similar to the phenotype observed in *MOZ-TIF2* leukemic cells derived from transduction of whole bone marrow (Figure 5A, and data not shown). Cell lines derived from *MOZ-TIF2*-transduced progenitors were also capable of generating AML, with 1 out of 2 GMP cell lines and 2 of 3 CMP cell lines resulting in AML when transplanted into lethally irradiated recipient mice (latency 103–162 days) (data not shown).

Taken together, these data indicate that MOZ-TIF2, but not BCR-ABL, confers the ability to serially replate in vitro, one surrogate for self-renewal that is not present in normal committed hematopoietic progenitors, nor indeed in normal HSC (Figure 2A). Importantly, the capacity of *MOZ-TIF2* to confer the ability to serially replate cannot be attributed to retroviral insertional mutagenesis. Retroviral transduction with point mutants of *MOZ-TIF2* that are not leukemogenic, or with an unrelated but fully active leukemia oncogene, BCR-ABL, does not confer this ability.

MOZ-TIF2-, but not *BCR-ABL*-, transduced committed myeloid progenitors can generate AML in mice

We next tested the properties of transduced committed progenitors in vivo. CMP, GMP, or whole bone marrow mononuclear cells (BM MNC) derived from Ly5.2 mice were transduced with *MOZ-TIF2* or *BCR-ABL* and directly injected into lethally irradiated Ly5.1 congenic recipients according to the schema in Figure 1. The control experiments with unfractionated BM MNC showed that mice transplanted with BM MNC transduced with

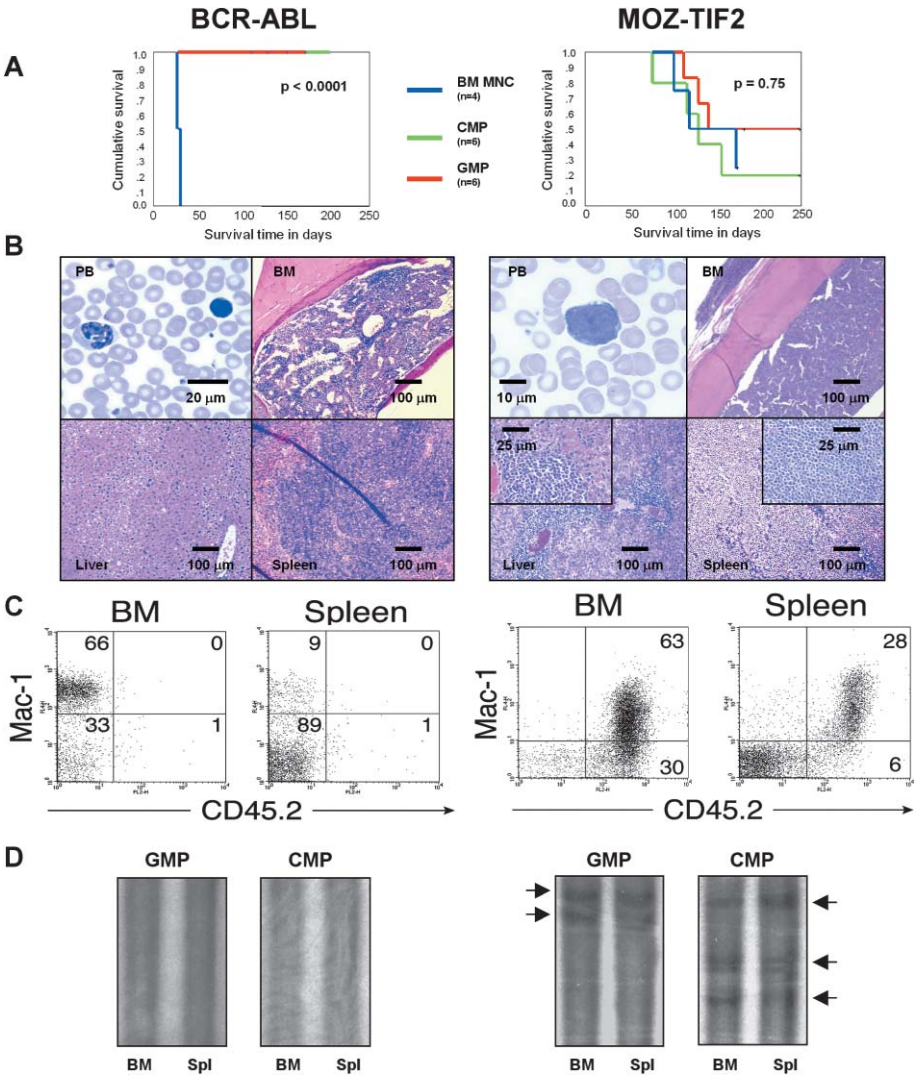

Figure 3. *MOZ-TIF2*, but not *BCR-ABL*, generates leukemia in transduced committed myeloid progenitors

A: Mice transplanted with *BCR-ABL*-transduced BM MNC succumb to a fatal myeloproliferative disease (MPD) within 30 days of transplantation, while mice transplanted with *BCR-ABL*-transduced CMP and GMP do not develop an MPD over a prolonged time period ($p < 0.0001$). In contrast, mice transplanted with *MOZ-TIF2*-transduced BM MNC, CMP, and GMP develop AML, with no significant difference in latency or penetrance ($p = 0.75$)

B: Histology of representative mice transplanted with *BCR-ABL*- (left panel) and *MOZ-TIF2*- (right panel) transduced GMP. No histological abnormality was detected in the *BCR-ABL* transplanted mice. In contrast, *MOZ-TIF2* transplanted mice demonstrated the presence of peripheral blood (PB) and bone marrow (BM) blasts and extensive tissue infiltration of organs including the liver, spleen, and lungs by leukemic blasts. Similar contrasting histology was demonstrated in mice transplanted with transduced CMP.

C: Flow cytometric analysis of spleen and bone marrow single cell suspensions from mice transplanted with *BCR-ABL* and *MOZ-TIF2*-transduced GMP. Relative fluorescence for the myeloid marker Mac-1 and the congenic marker Ly 5.2 are shown on a logarithmic scale. Quadrant markers segregate positive and negative cells and quadrant percentages are given. Abnormal Mac-1-positive Ly5.2 (CD 45.2) leukemic cells (upper right quadrant) are present in the bone marrow and spleen of mice transplanted with *MOZ-TIF2*- but not *BCR-ABL*-transduced cells.

D: Southern analysis of proviral integration in mice transplanted with *BCR-ABL*- and *MOZ-TIF2*-transduced progenitors. Oligoclonal integration is demonstrated for *MOZ-TIF2*-transplanted mice, but no integration is seen with *BCR-ABL* mice.

either *MOZ-TIF2* or *BCR-ABL* developed AML or a myeloproliferative disease, respectively, with latencies comparable to those previously described (Deguchi et al., 2003; Wong and Witte, 2001) (Figure 3A). In consonance with observations in the serial replating assays, mice transplanted with *MOZ-TIF2*-transduced CMP and GMP developed AML, with a similar long latency to mice transplanted with BM MNC (Figure 3A). In contrast, none of the mice transplanted with *BCR-ABL*-transduced CMP or GMP showed evidence of disease upon sacrifice at between 113 and 240 days, a time point at which all mice transplanted with *BCR-ABL*-transduced BM MNC had been sacrificed for development of myeloproliferative disease (Figure 3A).

MOZ-TIF2-associated leukemias are virtually indistinguishable regardless of the initial cell population transduced

MOZ-TIF2-associated leukemias arising from transduced committed progenitors populations (CMP and GMP) had a similar tissue distribution, histology, and immunophenotype to those arising from unfractionated bone marrow, and recapitulated many of the phenotypic characteristics of human *MOZ-TIF2* AML (Figures 3B [right panel], 4A, 4B, and 5A). These features included leukemic infiltration of the spleen, submandibular gland, and submental lymph nodes, liver, lung, and bone marrow. The presence of leukemic blasts was seen in the peripheral blood, and occasional mice demonstrated granulocytic sarcomata. Analysis of the leukemic cells from these mice showed expression of GFP and Ly 5.2 by FACS analysis and oligoclonal retroviral integration by Southern blotting (Figures 3C and 3D).

FACS analysis indicates that the hematopoietic compartment with properties of leukemic stem cells in *MOZ-TIF2*-associated leukemias lies downstream of GMP

FACS analysis, gated on the leukemic cell populations by GFP or Ly 5.2 expression, showed that cells derived from CMP, GMP, or whole bone marrow had very similar patterns of expression of surface markers, although the proportion of cells expressing some markers varied. The surface phenotype of the leukemic population was Sca-1-, CD-34-, CD 4-, CD8-, B220-, and CD19-negative, and expressed Mac-1 with varying degrees of Gr-1 and c-kit positivity (Figure 5A). There was uniform reduction of myeloid progenitor groups within this leukemia population, with

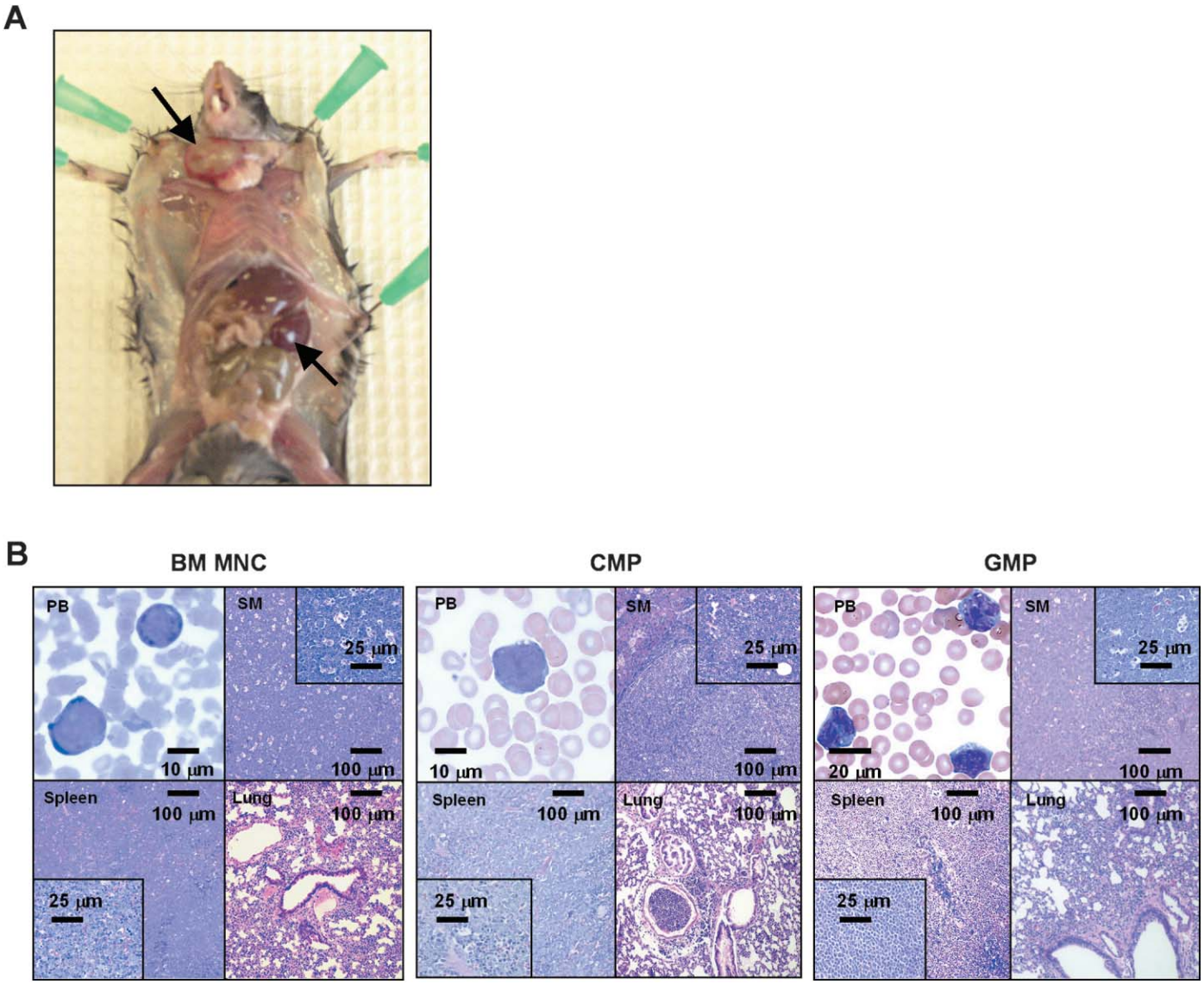

Figure 4. *MOZ-TIF2* leukemias are almost identical regardless of the initial cell population transduced

A: Tissue distribution of disease is similar for mice transplanted with *MOZ-TIF2*-transduced BM MNC, CMP, and GMP. Leukemic mice invariably demonstrated splenomegaly and neck masses resulting from infiltration of the submandibular glands and submental lymph nodes.
B: Representative morphology of peripheral blood (PB) and histology of the submandibular masses (SM mass), spleen, and lung are shown for leukemic mice transplanted with transduced BM MNC, CMP, and GMP. Primitive myelomonocytic cells were demonstrated in the peripheral blood, infiltrating the lung parenchyma and effacing the normal splenic and submental lymph node architecture.

undetectable HSC, CMP, or MEP (megakaryocyte erythroid precursors) and only residual numbers of GMP. However, there was a population of c-kit +/FcγRII+/CD34− cells that were not present in wild-type mice (Figure 5B). Taken together, these data indicate that there is impaired differentiation associated with the development of leukemia, and that the population of cells with properties of leukemic stem cells is downstream of the transduced CMP and GMP.

MOZ-TIF2 transduction of HSC also results in AML that is indistinguishable from other *MOZ-TIF2*-associated leukemias

We next tested whether *MOZ-TIF2* could also engender acute myeloid leukemia when transduced into the HSC compartment. Purified HSC were transduced with *MOZ-TIF2* using the same protocol, and serial replating and direct transplantation assays were performed as described above for *MOZ-TIF2*-transduced CMP or GMP. In consonance with results in CMP and GMP, HSC transduced with *MOZ-TIF2* demonstrated serial replating ability and were able to grow in liquid culture in an IL-3-dependent manner (data not shown). Direct transplantation of *MOZ-TIF2*-transduced HSC also led to the development of AML in mice with a similar penetrance but a slightly longer latency than in transduced CMP, GMP, or whole BM MNC, perhaps relating to their lower cell dose and transduction efficiency at transplantation (Figure 6A).

The leukemias that developed were indistinguishable macroscopically from other *MOZ-TIF2* associated leukemias, again

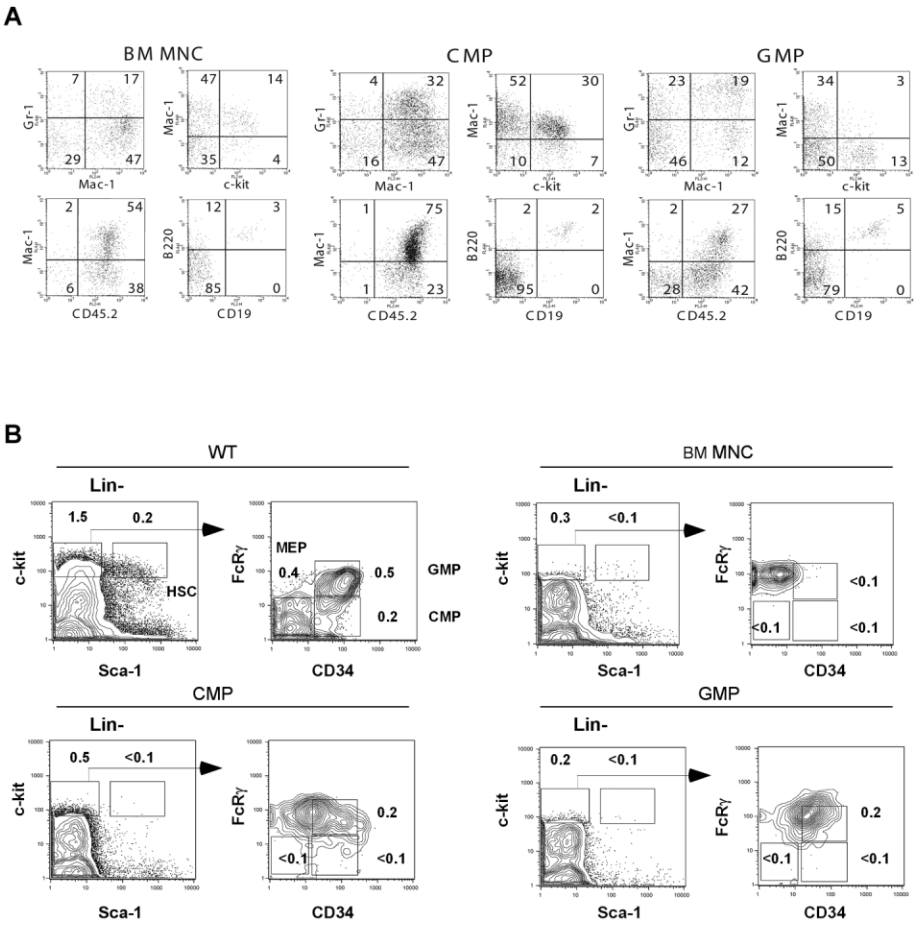

Figure 5. FACS analysis of *MOZ-TIF2*-associated leukemias demonstrates that the compartment with leukemia stem cell properties lies downstream of GMP

A: Similar expression patterns of Gr-1, Mac-1, c-kit, CD45.2, CD19, and B220 are shown for ungated leukemic bone marrow cells from mice transplanted with transduced BM MNC, CMP, and GMP. Relative fluorescence for the markers indicated on the x and y axes are shown on a logarithmic scale. Quadrant markers segregate positive and negative cells, and quadrant percentages are given.

B: Myeloid progenitor analysis of bone marrow from representative animals transplanted with *MOZ-TIF2*-transduced BM MNC, CMP, and GMP. A wild-type animal (WT) is shown for comparison. Relative fluorescence for the markers indicated on the x and y axes are shown on a logarithmic scale. The values shown represent the percentage proportion of total bone marrow cells analyzed. Stem cell and progenitor populations are reduced in leukemic bone marrow of all animals.

demonstrating mild splenomegaly and characteristic submental masses. Microscopically, the pattern of organ infiltration and the histological appearance of the leukemic cells were characteristic of other *MOZ-TIF2*-associated leukemias, as was expression of immunophenotypic markers (Figures 6B and 6C). Analysis of myeloid progenitor populations also showed these populations to be reduced (Figure 6D), again suggesting that the compartment with self-renewal capacity lies downstream of GMP.

AML induced by transduction of myeloid progenitors produces leukemia in secondary recipient mice

The long-term self-renewal properties of leukemic cells derived from *MOZ-TIF2*-transduced CMP or GMP were further tested by transplantation into sublethally irradiated secondary recipients at a range of cell doses from 1×10^6 bone marrow cells down to 1×10^2 cells (Figure 7A). All recipient mice transplanted with $\geq 1 \times 10^5$ cells developed leukemia with latencies of between 32 and 53 days, with an occasional mouse transplanted with 1×10^4 cells also developing disease. Mice below the threshold dosage of 1×10^4 did not develop leukemia with a follow up of 84 days. The leukemias were histologically and immunophenotypically indistinguishable from the primary disease (Figure 7B and data not shown), and Southern blot analysis demonstrated oligoclonal retroviral integration, as did the primary disease (data not shown).

Discussion

CMP and GMP are populations of cells that are irrevocably committed to terminal differentiation and apoptotic cell death. Remarkably, transduction of these committed progenitors with *MOZ-TIF2* confers properties of self-renewal in serial replating assays, and induces acute monocytic leukemia that can be serially transferred into secondary recipients.

The histological and immunological phenotypes of the monocytic *MOZ-TIF2*-associated leukemias are virtually indistinguishable regardless of whether the initially transduced cell population was whole bone marrow mononuclear cells, CMP, GMP, or HSC. The explanation that we favor for this observation is that *MOZ-TIF2* confers a specified phenotype regardless of the transduced cell type among whole bone marrow, CMP, GMP, or HSC populations. The phenotype includes conferring properties of leukemic stem cells, as assessed by serially replating assays and development of leukemia in vivo that can be serially transplanted, and impaired differentiation downstream of GMP in the monocytic lineage. A similar pattern of differentiation has also recently been described for transduction of hematopoietic progenitors with the oncogene *MLL-ENL* (Cozzio et al., 2003).

An important question is whether these results can be explained as a consequence of retroviral insertional mutagenesis. To address this question, we have undertaken two types of control experiments. First, we have retrovirally transduced CMP

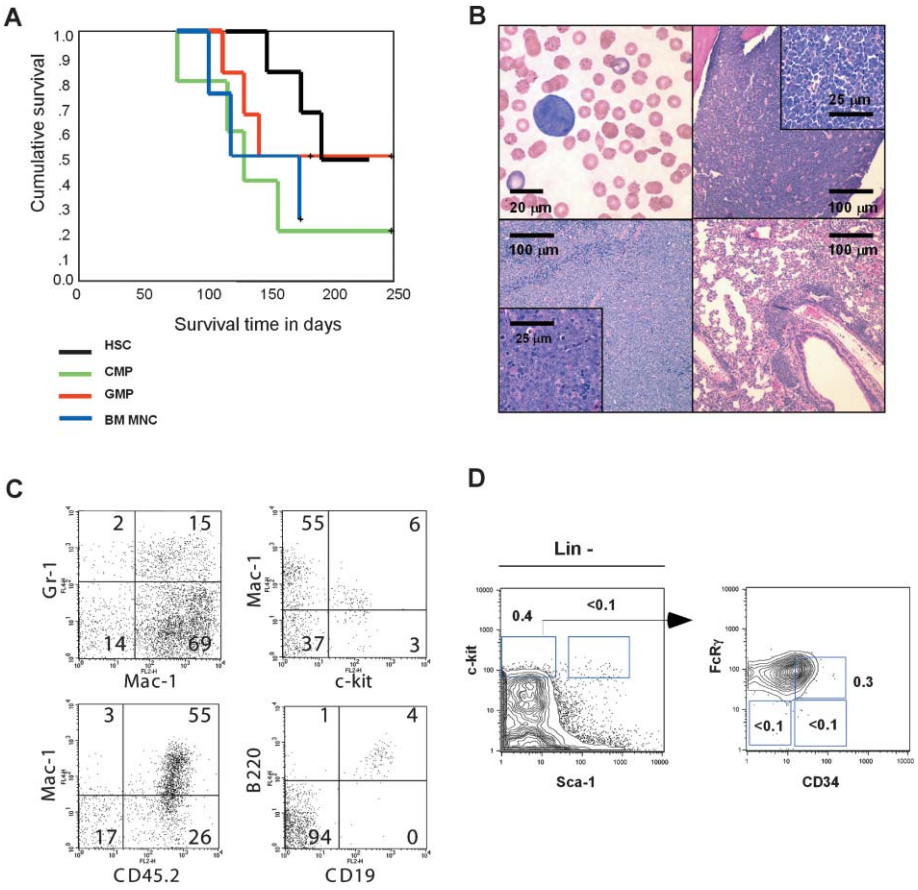

Figure 6. Transduction of HSC with *MOZ-TIF2* also engenders AML in recipient mice

A: Kaplan-Meier graph comparing the latency and incidence of AML in recipients of *MOZ-TIF2*-transduced HSC with transduced CMP, GMP, and BM MNC.

B and C: Representative histology and immunophenotype from a *MOZ-TIF2*-transduced HSC-associated AML demonstrating the similarity to other *MOZ-TIF2*-associated leukemias. Relative fluorescence for the markers indicated on the x and y axes are shown on a logarithmic scale. Quadrant markers segregate positive and negative cells and quadrant percentages are given.

D: Myeloid progenitor analysis, again demonstrating a decrease in myeloid progenitor subsets, suggesting that the compartment with self-renewal potential lies downstream of GMP. Relative fluorescence for the markers indicated on the x and y axes are shown on a logarithmic scale.

and GMP with *MOZ-TIF2* point mutants that do not cause leukemia in the murine BMT system using whole bone marrow. These lack the ability to confer properties of leukemic stem cells to CMP or GMP, indicating that retroviral insertional mutagenesis with nonleukemogenic mutants of *MOZ-TIF2* alone is not sufficient to confer such properties. Second, retroviral transduction of *BCR-ABL* did not confer properties of leukemic stem cells to CMP or GMP. These data demonstrate that retroviral insertional mutagenesis, even in collaboration with a fully active leukemogenic tyrosine kinase, is not sufficient to cause leukemia. Together, these data argue strongly that retroviral insertional mutagenesis alone cannot explain these results. However, we cannot exclude the possibility that an active *MOZ-TIF2*, but not *BCR-ABL*, can collaborate with mutations induced by retroviral mutagenesis to confer properties of leukemic stem cells to committed progenitors. Limiting dilution analysis indicated that the frequency of the leukemia repopulating cell was low in this assay system, similar to data reported in other murine models of leukemia (Lessard and Sauvageau, 2003) and in human leukemic cells transplanted into NOC-SCID recipient mice (Bonnet and Dick, 1997; Hope et al., 2004). Taken together, these data support the existence of a subset of leukemic cells that retain long term self-renewal potential.

What is the nature of the qualitative difference between *BCR-ABL* and *MOZ-TIF2* in conferring properties of self-renewal? This remains an important question. There are various qualitative differences between these two oncogenes. *BCR-ABL* expression in humans, and in mouse models, confers proliferative and/or survival advantage to hematopoietic progenitors without affecting differentiation, and is associated with a chronic myeloproliferative phenotype characterized by leukocytosis and normal differentiation. In contrast, *MOZ-TIF2* is associated with acute monocytic leukemia in humans and in murine models of disease characterized by impaired hematopoietic differentiation and properties of self-renewal. This is illustrated in the proposed model in Figure 8, where *BCR-ABL* confers proliferative and survival signals to the committed myeloid progenitors, which may allow transient expansion of downstream progeny, but does not confer properties of self-renewal. In contrast, *MOZ-TIF2* confers properties of self-renewal while also impairing myeloid differentiation. This allows preleukemic expansion of this compartment and may facilitate the subsequent development of AML. The basis for the difference between *BCR-ABL* and *MOZ-TIF2* in conferring properties of leukemic stem cells to CMP and GMP remains to be discerned, but it is clear that leukemia-associated oncogenes vary in their ability to do so.

In addition, these data may have important clinical implications for treatment of leukemias associated with the *BCR-ABL* oncogene. If, as our data suggests, *BCR-ABL* signals are dispensable for maintenance of self-renewal, then small molecule inhibitors of BCR-ABL may not target the relevant "leukemia stem cell" in this disorder (Graham et al., 2002)—indeed, it appears that imatinib therapy as a single agent, though quite effective in remission induction, is rarely if ever curative in *BCR-ABL*-positive CML (Melo et al., 2003).

It is not certain how many, or what type of, leukemia oncogenes will confer properties of leukemic stem cells to committed progenitors. However, *MLL-ENL* has recently been shown to

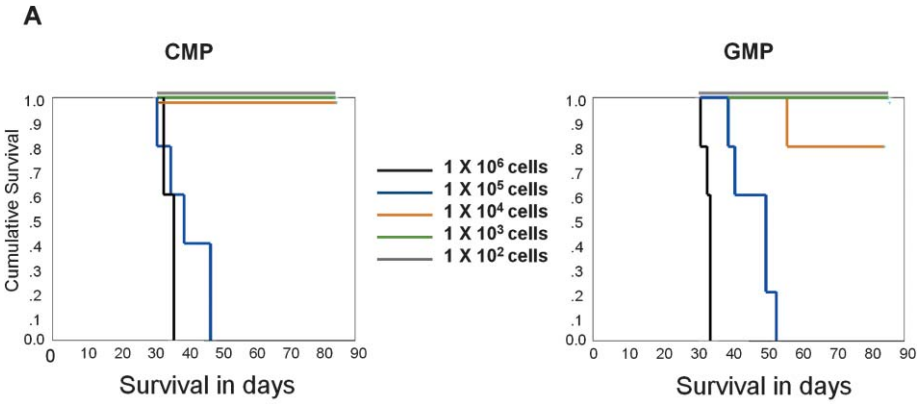

Figure 7. Transplantation of leukemic cells from animals transplanted with *MOZ-TIF2*-transduced CMP and GMP causes identical leukemia in secondary recipients in a cell dose-dependent manner

A: Kaplan-Meier graph of survival for secondary recipients transplanted with leukemic cells from primary mice transplanted with either *MOZ-TIF2*-transduced CMP or GMP. All animals transplanted with greater than 1×10^4 cells developed leukemia, while no animals transplanted with less than 1×10^4 developed disease.

B: Representative histology demonstrating the same morphological appearance and tissue distribution of *MOZ-TIF2* disease in secondary recipients.

confer similar properties of self-renewal and to generate AML in committed myeloid progenitors (Cozzio et al., 2003). We speculate that this ability may be a generic property of certain leukemia-associated fusion genes involving hematopoietic transcription factors. For example, there is evidence for increased self-renewal of *AML1-ETO*-expressing progenitors and HSC, both in murine models and transduced human CD34+ cells (de Guzman et al., 2002; Mulloy et al., 2002, 2003; Higuchi et al., 2002; Rhoades et al., 2000; Tonks et al., 2003), although as yet, no evidence exists for the reestablishment of such properties in progenitors which inherently lacked self-renewal potential. In addition, analysis of gene expression profiles in the U937 hematopoietic precursor cell line stably expressing *AML1-ETO, PML-RARα*, or *PLZF-RARα* demonstrated the induction of genes involved in the maintenance of the stem cell phenotype (Alcalay et al., 2003; Muller-Tidow et al., 2004). This suggests that induction of self-renewal by a number of transcription factor fusions may have a wider relevance in the pathogenesis of AML.

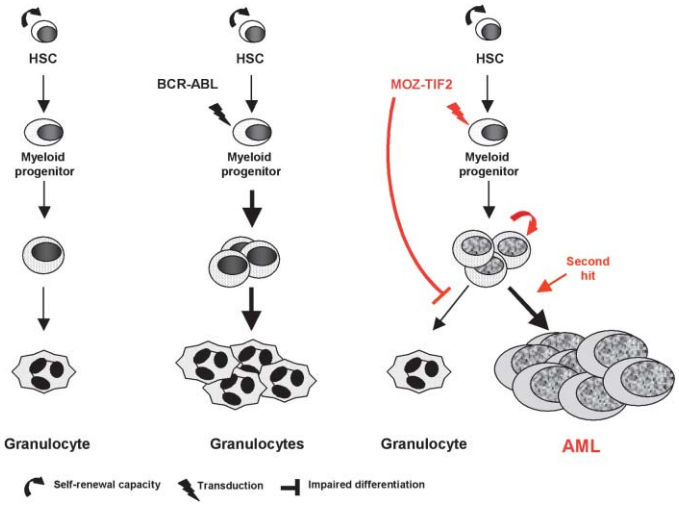

Figure 8. Development of AML is facilitated by the properties of self-renewal conferred to myeloid progenitors by *MOZ-TIF2*

The normal differentiation of an HSC to a granulocyte through specific myeloid progenitor intermediates is shown in the left column. In the center column, transduction of myeloid progenitors with BCR-ABL may provide a proliferation and survival signal to these progenitors without impairing differentiation. This in turn may lead to an expansion of the granulocyte compartment, but without self-renewal is insufficient to generate leukemia in the recipient mice. In contrast, transduction of myeloid progenitors with MOZ-TIF2 (right column) both restores self-renewal to this compartment and impairs its further differentiation. In combination, these properties allow preleukemic expansion of the compartment and may facilitate the development of AML upon the acquisition of further cooperating mutations.

It should be noted that our data do not exclude the possibility that leukemogenic mutations often occur in HSC, as expression of *MOZ-TIF2* in this population also resulted in leukemia. The data only indicate that if such mutations arise in committed progenitors, they are capable of transforming committed progenitors to cells with the properties of leukemia stem cells. However, use of this assay in committed progenitors to compare *BCR-ABL* or inactive *MOZ-TIF2* mutants with *MOZ-TIF2* itself should allow for definition of the transcriptional programs that confer properties of self-renewal to hematopoietic stem cells. These programs may be targets for therapeutic intervention in leukemia, and may also provide strategies for conferring properties of self-renewal to adult somatic cells in other therapeutic contexts, such as tissue regeneration.

Experimental procedures

Cell staining and sorting

Bone marrow mononuclear cells were flushed from the leg bones of C57B6 mice (Taconic, Germantown, NY). For transplants using transduced bone marrow mononuclear cells, the mice were treated with 5FU 150 mg/kg (Sigma, St. Louis, MO) intraperitoneally 6 days prior to sacrifice. The cells were then washed and the red cells lysed on ice with RBC lysis buffer

(Gentra, Minneapolis, MN). HSC, CMP, and GMP populations were sorted and analyzed as previously reported (Na Nakorn et al., 2002).

Retroviral constructs, retroviral transduction, and bone marrow transplant assay

The *MOZ-TIF2*, *MOZ-TIF2* mutants, and *BCR-ABL* MSCV-pgk-Neo and MSCV-IRES-GFP vectors and the production of retroviral supernatants are as previously described (Dash et al., 2002; Deguchi et al., 2003). Bone marrow MNC, HSC, and CMP were all incubated overnight in IMDM (Life Technologies, Rockville, MD) supplemented with murine IL-11 10 μg/ml and SCF 10 μg/ml (both R&D Systems, Minneapolis, MN), and transduced the next day. GMP cells were transduced immediately following sorting. For transduction, cells were plated in IMDM supplemented with IL-11 and SCF in 6-well plates (Becton Dickinson, Franklin Lakes, NJ) coated with 40 μg/ml Fibronectin (Takara, Japan). Retroviral supernatant was added along with Polybrene 4 μg/ml and the cells were incubated overnight at 37°C in 5% CO_2. The cells were then harvested, counted, and resuspended in 0.5 ml Hank's balanced salt solution (Life Technologies, Rockville, MD). For BM MNC, each of 4 mice received 1×10^6 cells. For HSC, CMP, and GMP, 3 experiments each with transplantation of 2 mice for each group were performed. The transplanted cell dose range was 4–12 $\times$ 10^3 for HSC, 3–9 $\times$ 10^4 cells for CMP, 3–8 $\times$ 10^4 for GMP, and 1×10^6 cells for each of the MOZ-TIF2-transduced myeloid cell lines. Transduction efficiencies were measured for each batch of MOZ-TIF2 and BCR-ABL viral supernatants used in these experiments by either GFP expression on FACS analysis or the ratio of colony number in the presence or absence of G418 selection, and were comparable for each progenitor population between constructs. Secondary transplants using bone marrow from primary leukemic animals initially transplanted with either MOZ-TIF2-transduced CMP or GMP were performed at limiting dilution using doses of 1×10^6, 1×10^5, 1×10^4, 1×10^3, or 1×10^2, with 5 mice transplanted for CMP and GMP at each dosage. The bone marrow from CMP- and GMP-induced MOZ-TIF2 leukemias was matched for leukemic infiltration (CMP 45% and GMP 39% of cells GFP-positive). Progenitor cells and cell lines were transplanted along with 0.5×10^6 BM MNC from C57B6.SJL mice (Stock # 002014, Jackson Laboratories, Bar Harbor, ME). Cells were injected into lethally irradiated (650 rads $\times$ 2) C57B6.SJL recipients (Jackson Laboratories, Bar Harbor, ME) via the lateral tail vein. Secondary transplant recipient mice received sublethal irradiation (650 rads).

Serial replating assay and generation of IL-3-dependent myeloid cell lines

Serial replating assays were performed as previously described (Lavau et al., 1997) for 4 platings. Excess cells from the 3rd and 4th platings were amalgamated and each total cell population was expanded in RPMI supplemented with 20% FCS and IL-3 10 μg/ml (R&D systems, Minneapolis, MN). These cells were then counted weekly on an inverted microscope by trypan blue exclusion. Cells were passaged for 8 weeks prior to injection into lethally irradiated recipients.

Histopathology

Murine tissues were fixed for at least 24 hr in 10% neutral buffered formalin (Sigma, St. Louis, MO), dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin on an automated processor (Leica, Bannockburn, IL). Tissue sections (4 μm thick) were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions, and stained with hematoxylin and eosin (H&E).

Immunophenotypic analysis of leukemic cells

Single cell suspensions of bone marrow, spleen, submandibular glands, and lymph nodes, and granulocytic sarcomata if present, were prepared. Red blood cells were lysed in ammonium chloride solution (150 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA [pH 7.4]) for 5 min at room temperature. The cells were washed in PBS with 0.1% NaN_3 and 0.1% bovine serum albumin (BSA; staining buffer). To block nonspecific Fc receptor-mediated binding, the cells were preincubated with supernatant from the 2.4G2 hybridoma line (anti-CD16/CD32; cell line American Type Culture Collection, Rockville, MD) for 20 min on ice. Aliquots of 0.5 to 1.0 $\times$ 10^6 cells were then stained for 20 min on ice with monoclonal antibodies specific for B220 (CD45R), CD19, CD34, CD45.2 (Ly5.2), c-kit, Gr-1, Mac-1, CD4, or CD8 conjugated with fluorescein isothiocyanate (FITC), phycoerthyrin (PE), or biotin. Binding of biotinylated primary antibodies was detected using PE-conjugated streptavidin (Immunotech, Westbrook, ME) or FITC-conjugated avidin (Southern Biotechnology, Birmingham, AL). Cells were washed once in staining buffer followed by two-colored flow cytometric analysis with a FACScan (Becton Dickinson, San Jose, CA). A minimum of 10,000 events was acquired showing FITC and PE fluorescence signals of viable cells gated on the basis of forward and side scatter signals.

Southern blotting for proviral insertion and clonality

Genomic DNA was prepared from single-cell suspensions of tumor cells using a PUREGENE DNA isolation kit according to the manufacturer's protocol (Gentra Systems, Minneapolis, MN). Twenty micrograms of genomic DNA was digested with EcoRI, which cuts once within the proviral sequence and once within the integrated locus, and was subjected to electrophoresis and hybridization according to standard protocols.

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